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Water availability moderates N_2 fixation benefit from elevated [CO_2]: A 2-year FACE study on lentil (*Lens culinaris* MEDIK.) in a water limited agro-ecosystem

Running title: N_2 fixation of lentil under elevated [CO_2]

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ABSTRACT

Increased biomass and yield of plants grown under elevated [CO_2] (e[CO_2]) often corresponds to decreased grain N concentration ([N]), diminishing nutritional quality of crops. Legumes through their symbiotic N_2-fixation may be better able to maintain biomass [N] and grain [N] under e[CO_2], provided N_2-fixation is stimulated by e[CO_2] in line with growth and yield. In Mediterranean type agro-ecosystems, N_2-fixation may be impaired by drought and it is unclear whether e[CO_2] stimulation of N_2-fixation can overcome this impact in dry years. To address this question, we grew lentil under two [CO_2] (ambient ~400 ppm and elevated ~550 ppm) levels in a Free-Air CO_2 Enrichment (FACE) facility over two growing seasons sharply contrasting in rainfall.

Elevated [CO_2] stimulated N_2-fixation through greater nodule number (+27%), mass (+18%) and specific fixation activity (+17%) and this stimulation was greater in the high than the low rainfall/dry season. Elevated [CO_2] depressed grain [N] (-4%) in the dry season. In contrast, grain [N] increased (+3%) in the high rainfall

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season under e[CO_2], as a consequence of greater post-flowering N_2 fixation. Our results suggest that the benefit for N_2-fixation from e[CO_2] is high as long as there is enough soil water to continue N_2-fixation during grain filling.

Key-words: Climate change; legume; N acquisition (fixation vs uptake); nodule; soil water; grain protein.

INTRODUCTION

Atmospheric CO_2 concentration ([CO_2]) has been rising since pre-industrial times and based on current trends will reach ~550 µmol mol^{-1} by 2050 (IPCC 2013). Elevated [CO_2] (e[CO_2]) stimulates photosynthesis, growth and yield of C_3 plants (Ainsworth & Long 2005; Leakey et al. 2009). Increased growth increases the demand for nitrogen (N), yet stimulation of N uptake by e[CO_2] is often less than stimulation of growth (Pleijel & Uddling 2012). Plant tissue N concentration ([N]) commonly decreases under e[CO_2] and this is often related to decreased protein concentrations in vegetative tissues and grains (Cotrufo et al. 1998; Taub & Wang, 2008). Declining grain protein and other important nutrients in grains raise nutritional concerns, especially in developing countries where grains contribute significantly to protein supply (Myers et al. 2014). The reasons for the decline in tissue [N] under e[CO_2] are not fully understood. Limited supply of labile N in soils or changes in root N uptake (Pleijel & Uddling 2012; Shimono & Bunce 2009) or inhibition of plant N acquisition (Feng et al., 2015) due to lower nitrate assimilation and subsequent downregulation of nitrate uptake (Bloom et al. 2014) are among the suggested mechanisms.

Legumes may be better able to overcome limitations to N supply or assimilation in future climates, because e[CO_2] can stimulate the rates of N_2 fixation in their nodule symbionts (Rogers et al. 2006). They can often maintain grain protein under e[CO_2] or grain protein depression is less than in non-legumes (Myers et al. 2014; Taub & Wang 2008). Stimulation of N_2-fixation by e[CO_2] can be driven by increasing nodule size, nodule numbers or stimulating nodule activity (amount of N_2 fixed per unit of nodule mass) or combinations (Rogers et al. 2009). A meta-analysis reported about 38% greater N_2-fixation under e[CO_2], which was attributed to the

e[CO_2]-induced stimulation of nodule number (33%), nodule biomass (39%) and nitrogenase activity (37%) (Lam et al. 2012b). Such stimulation of N_2 fixation by e[CO_2] can result in a greater proportion of total plant N coming from symbiotically fixed N_2, limiting or even reducing soil N uptake (Feng et al. 2015; Guo et al. 2013; Lee et al. 2003; Zanetti et al. 1996).

Both e[CO_2] stimulation of yield and N_2-fixation of legumes depend on environmental factors, most prominently on water availability (Gray et al. 2013; Kimball 2016). Elevated [CO_2] increases water use efficiency (WUE) either through reduction of stomatal conductance or increases in net CO_2 assimilation rate or both (Bernacchi et al. 2007). Such improvement in WUE, especially if it leads to soil water conservation for later in growing season, is particularly important in water limited dryland cropping systems (Kimball 2016). Thus, it has been widely assumed that the relative CO_2-effect is greater under drought, a paradigm that that may not be universally applicable (van der Kooi et al. 2016). Even in relatively high rainfall environments, more severe drought years can constrain rather than amplify the CO_2 stimulation of yield (Gray et al. 2016). Water availability can also determine the ability of legumes to maintain grain [N] by moderating N_2 fixation. For example, in high rainfall conditions, soybeans maintained grain [N] under e[CO_2] (Gray et al. 2013) but experiments in semi-arid environments showed small but significant decreases in grain protein concentration of lentil (Bourgault et al. 2017), field pea (Bourgault et al. 2016) and chickpea (Lam et al. 2012a). Such interactions with environmental conditions highlight the importance of assessing and better understanding N_2-fixation under e[CO_2] in water limited agro-ecosystems.

In addition, N_2-fixation is regulated by the availability of carbon (C) to the bacteroids, which determines nodule activity (Larrainzar et al. 2009). Therefore, C and N metabolism is tightly coupled between bacteroids and host plant. The host plant supplies photoassimilate in form of sucrose through the phloem, providing energy and C skeletons to the bacteroids. The sucrose is then metabolised into malate, supplying ATP and reducing energy for N_2-fixation (Streeter 1987). In return, bacteroids provide NH_4^+ to the host plant which is further metabolised into other N transport forms. Depending on the legume species, either amino acids such as asparagine or ureides are transported through the xylem to meet the host plant's N demand. Water limited conditions commonly result in

declining photosynthetic rates and consequently decreased C supply to the nodules, constraining nodule function (Aranjuelo et al. 2013). Simultaneously, as N demand of shoots decreases, N compounds accumulate in nodules and cause feedback inhibition of N_2-fixation (Gil-Quintana et al. 2013). Stimulation of photosynthesis by e[CO_2] can support greater supply of assimilates to the nodules (Rogers et al. 2006) and may therefore delay the effect of drought on N_2-fixation (Serraj et al. 1998). However, whether such changes in nodule C and N metabolism occur under the combined effect of e[CO_2] and drought in the field and how this is associated with changes in N_2-fixation is not well understood.

Grain [N] in legumes is not only dependent on N acquisition, either by fixation or uptake from soil, but also on N translocation to grains from other organs. Particularly in dryland agro-ecosystems, N_2 fixation and N uptake from soil may be strongly inhibited by seasonal drought during the reproductive phase. Nitrogen, which was previously taken up and assimilated into vegetative biomass, can then be remobilised to the pods and seeds to meet the demand of maturing grains (Schiltz et al. 2005). In soybean, the extent of N remobilisation can be 80-90% depending on genotype (Kinugasa et al. 2012). Previous study suggested that e[CO_2] decreases N remobilisation of soybean due to enhanced N_2-fixation at later reproductive stages (Li et al. 2017). However, maintenance of N_2-fixation may be more difficult under drought conditions. It is therefore important to determine how e[CO_2] changes the relative proportions of grain N derived from fixation, soil N resources or remobilisation processes.

Selection of genotypes that are more responsive to e[CO_2] attracts interest as a potential foundation for crop breeding (Ziska et al. 2012; Tausz et al. 2013). For legumes, intraspecific variability in grain yield response to e[CO_2] has been reported for soybean (Bishop et al. 2015), common bean (Bunce 2008) and cowpea (Ahmed et al. 1993) where increased grain yield under e[CO_2] was associated with greater harvest index, short stature and greater number of pods. However, no clear cultivar difference in response to e[CO_2] of grain yield was observed for six lentil genotypes grown under higher [CO_2] in a semi-arid environment (Bourgault et al. 2017). Apart from growth and yield response, differences in N_2-fixation ability in response to e[CO_2] would be a useful candidate trait for selecting genotypes for a carbon-rich environment. Genotypic variability in terms of N_2-

fixation enhancement and maintaining grain [N] under e[CO_2] has been reported for soybean (Lam et al. 2012c; Li et al. 2017) and for field pea (Bourgault et al. 2016).

In the current study, we investigated the mechanism of N acquisition via N_2-fixation and uptake from soil and N remobilisation and allocation to grains in two lentil genotypes contrasting in harvest index (Bourgault et al. 2017). These are selected because low harvest index (lower grain yield relative to total biomass) could limit the capacity of plants to utilise the additional assimilates provided by e[CO_2] (Bishop et al. 2015). Among grain legumes, lentil is widely used to meet the protein requirement of poor people with less access to meat and vegetarians throughout the world and are commonly grown in low rainfall environments (Erskine et al. 2011; Daryanto et al. 2015). In the present study, lentil was grown under ambient [CO_2] (a[CO_2], ~400 ppm) and e[CO_2] (~550 ppm) in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility in a semi-arid environment characterised by large season-to-season variability especially in rainfall. Experiments were conducted over two growing seasons with contrasting rainfall: 2015 was very dry (below average) and 2016 had high rainfall (above average). This allowed us to address the following research questions:

1. How does e[CO_2] and a low/high rainfall growing season affect nodule biomass, number and specific N_2-fixation and how are such changes associated with changes in N_2-fixation?
2. How does e[CO_2] and a low/high rainfall growing season change N acquisition (fixation vs uptake) and N allocation patterns in lentil?
3. Are N acquisition and allocation patterns different between a high and a low harvest index lentil genotype and is there any interactive effect with e[CO_2] and low/high rainfall growing season?

MATERIALS AND METHODS

Plant materials and sowing

Two lentil (*Lens culinaris* MEDIK.) genotypes cv. PBA Ace and 05H010L-07HS3010 (shortened to HS3010) were used in this experiment. PBA Ace is a high yielding, vigorous and medium seeded commercial cultivar,

which is well suited to dry areas. The breeding line HS3010 has a smaller harvest index and displays greater biomass accumulation under favourable environments (Bourgault et al. 2017).

Before sowing, seeds were inoculated with Group F® (WSM1455, *Rhizobium leguminosarum*) peat based inoculant (NoduleNTM, NewEdge Microbials Pty Ltd. Albury, NSW, Australia). Inoculated seeds were hand sown on 22 May 2015 and 01 June 2016 with a target sowing density of 150 plants m^{-2} and row spacing of 24.4 cm. The plot size of each genotype was 1.5 m by 4 m length and 1.5 m by 2 m length in 2015 and 2016, respectively. In each year, superphosphate fertiliser was applied just before sowing at the rate of 9 kg P ha^{-1} and 11 kg S ha^{-1} but no N fertilizer was applied. Plots were also treated with pre-emergence herbicide (simazine, dimethenamid-P, trifluralin) prior to sowing to control weeds.

Experimental site and design

The experiment was conducted at the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility in the Agriculture Victoria research farm near Horsham, Victoria, Australia ($36^0$45′ S, $142^0$06′ E, 127m above sea level). The soil is a Murtoa clay consisting of ~35% clay at the surface increasing to 60% in 1.4 m depth classified as a Vertosol according to the Australian Soil Classification (Isbell 2002) and the physiochemical properties were decribed elsewhere in detail (Butterly et al. 2015). A detailed description of the AGFACE site and the CO_2 exposure facility is given by Mollah et al. (2009). Meteorological data during the experimental period were collected from an automatic weather station installed at the AGFACE site (MEA Premium Weather Station 103, Measurement Engineering Australia, Magill, SA, Australia). Evapotranspiration data were obtained from the nearest Horsham Aerodrome Weather Station (ID: 079100), Bureau of Meteorology, Victoria, which is situated ~5.5 km away from the AGFACE site. Data of evapotranspiration (ET) were synchronized with the data of precipitation (P) to calculate daily values of P-ET as described by Gray et al. (2016). Drought index was calculated from the difference of seven days running average P and ET values and plotted against days after sowing (Fig. 1). The total amount of rainfall during the crop growing season was 128.4 mm in 2015 and 334.2 mm in 2016 (Table 1). As the total amount of rainfall in 2015 was well below the average long term growing season amount (274 mm), additional irrigation (32 mm each on 21 September, 08 October and 21 October) was

applied close to the reproductive stages to prevent total crop failure. Even with added irrigation the 2015 season remained well below average ('**dry season**'), whereas 2016 was well above ('**high rainfall season**').

Table 1

Figure 1

In each year, four octagonal areas ('rings') with elevated [CO_2] (e[CO_2]) at ~550 µmol mol^{-1} and four areas with ambient [CO_2] (a[CO_2]) at ~400 µmol mol^{-1} were set up in a new location to avoid 'carry over' effects from previous experiments. In 2015, rings were 12m in diameter and in 2016, ring diameter was 16 m. The horizontal tubes injecting CO_2 were raised as the crop grew to maintain them just above canopy height. For the e[CO_2] rings, pure CO_2 was injected into the upwind side through 0.3 mm holes in the injecting tubes. CO_2 was then mixed quickly with the air as it was blown throughout the plot by prevailing winds. The central ring [CO_2] was targeted at 550 µmol mol^{-1} from sunrise to sunset. In each ring, CO_2 injection started near emergence and continued until maturity. CO_2 concentration was monitored using sensors installed centrally in each ring.

Measurements

Destructive sampling and biomass

Destructive samples were collected at flowering, pod filling and physiological maturity corresponding to the growth stages of full bloom (R2), at early seed (R4) and full maturity (R8) (Erskine et al. 1990). At each sampling time, plants of four middle rows of 30 cm length across the plot (corresponding to 0.29 m^2) were collected for biomass measurements. For metabolomic analysis at flowering stage, just after uprooting the plants in the field, approximately 100 mg freshly collected nodules were immediately frozen in liquid nitrogen and stored in -80°C until analysis. Each time, roots and nodules were collected from the harvested area using soil cores (10 cm diameter) to 40 cm depth, because 90% of the lentil root biomass and nodules is in the top 40 cm soil depth (Gorim & Vandenberg 2017). The cores were kept on ice after excavation (except maturity samples) and brought to the laboratory, then rinsed with tap water and soaked in 0.01M $CaCl_2$ solution for 5 min to remove clay particles and desorb nutrients from the root surface. Nodules were separated from the roots

immediately after washing and counted. At maturity, fully formed pods were separated, counted, threshed and weighed for grain yield. All biomass is reported on dry weight basis (oven dried for 72 hours at 70^0C). At each sampling stage, wheat (*Triticum aestivum* L. cv. Yitpi) grown as a non-N_2 fixing reference adjacent to lentil plots was also sampled.

Soil water profile probes

Soil water content was monitored on a weekly basis with a PR2 profile probe (PR2/6, Delta-T Devices Ltd., Cambridge, UK) having six sensors positioned at 10, 20, 30, 40, 60 and 100 cm depth. 100 cm long thin-walled access tubes (ATS1/ATL1, Delta-T Devices Ltd., Cambridge, UK) were inserted into the soil at the centre of each plot and left *in-situ* for the entire growing season. Measurements were taken by inserting the PR2 probe into the access tubes. The output of the PR2 probe (mV) was converted to volumetric water content (m^3/m^3) with a site-specific calibration curve for each soil depth. Consistent with the depth of roots and nodule sampling, averaged soil water is reported to 40 cm depth. Field capacity and permanent wilting points are indicated as described by Rab et al. (2011).

Gas exchange measurements

Leaf gas exchange was measured at the flowering stage using an infrared gas analyser (IRGA) system (Li- 6400, Li-Cor, Lincoln, NE, USA) with a default clear top window chamber and a maximum measurement area of 6 cm^2 (Li- 6400, Li-Cor, Lincoln, NE, USA) on clear days in full sunshine, above saturating natural light conditions (approximately 2000 µmol m^{-2} s^{-1}). A fully expanded youngest leaf was measured *in-situ* at a block temperature of 20 ºC and an air flow rate through the chamber of 500 µmol s^{-1} with reference [CO_2] adjusted to 400 and 550 µmol mol^{-1} in a[CO_2] and e[CO_2] rings, respectively. The leaf area enclosed inside the cuvette (6 cm^2) was sampled and measured with a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). Gas exchange parameters were recalculated based on this actual leaf area. Light saturated photosynthesis rate (A_{sat}) and stomatal conductance (g_s) are reported.

Nodule metabolites

Nodule total sugar, organic acid and amino acid concentrations and their derivatives were analysed using gas chromatography coupled to triple-quadrupole mass spectrometry (GC-QqQ-MS) and liquid chromatography coupled to triple-quadrupole mass spectrometry (LC-QqQ-MS), respectively (Dias et al. 2015). Briefly, 30 mg frozen nodule tissue was weighed into cryomill tubes, then extracted twice with 400µl each of methanol (100%) and water. After each extraction, metabolite extracts were transferred into new reaction tubes followed by centrifugation. Sugars and organic acids were derivatised using *N, O*-bis-trimethylsilyl (TMS) prior to analysis with GC-QqQ-MS. Amino acids and amines were derivatised using 6-aminoquinolyl-N-hydrosysuccinimidyl carbamate (AQC) reagent before being subjected to LC-QqQ-MS analysis. For analyses, external calibration curves were prepared using authentic standards. Approximately, 1µl aliquot was injected into the GC-QqQ-MS and LC-QqQ-MS for both samples and standards. Finally, using the standard calibration curves, the concentration of the metabolites was determined by fitted linear regression curves.

Tissue N concentration and N_2 fixation

Finely ground dry plant tissue samples (leaves, stems, flowers, roots, nodules, chaff, grains and reference wheat) from three sampling stages were analysed for [N] (% of dry weight) and $\delta^{15}N$ values by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon) operating in line with a CHN analyser (Carlo Erba). The nitrogen results (atom % ^{15}N) were expressed as $\delta^{15}N$ values (‰) using atmospheric air (0.3663 % ^{15}N) as the international standard for N, which by definition is given a delta (δ) ^{15}N of 0‰ (Unkovich et al. 1997).

N_2 fixation was measured by ^{15}N natural abundance method based on the difference in $\delta^{15}N$ (‰) signature between atmospheric N_2 and soil N. To confirm the basis of this method, the $\delta^{15}N$ (‰) of the soil must be at least 2-7‰ (Gathumbi et al. 2002). In this present study, soil and reference plant (wheat) $\delta^{15}N$ ‰ were 8-10‰ and 5-7‰, respectively and therefore, ^{15}N discrimination between atmospheric N_2 (0‰) and soil N was sufficient to estimate N_2 fixation by this method (Unkovich et al. 1997).

The percentage of N derived from atmosphere (%Ndfa) was determined by the following formula as described by Unkovich et al. (1994).

% Ndfa = (δ^{15}N reference plant – δ^{15}N legumes) × 100 / (δ^{15}N reference plant – B)

where 'reference plant' refers to a non-N_2 fixing plant selected to match closely to the studied legume in terms of uptake of soil sources of N. In the absence of non-nodulating isolines of the lentil varieties subject to our study, we used wheat grown adjacent to the lentil plots in each ring according to accepted practice for ^{15}N natural abundance method (Lam et al. 2012a, 2012c; Rennie & Dubetz, 1986). The factor B refers to the δ^{15}N value of the effectively nodulated legume grown in media totally lacking N. Nodulated lentil grown in sand were harvested at each growth stage and organ specific B-values (δ^{15}N, ‰) were estimated for %Ndfa calculation (Unkovich & Pate 2000). B-value was corrected for seed N based on Nebiyu et al. (2014).

The amount of total N_2 fixed from atmosphere was calculated as follows:

Total N_2 fixation (kg ha^{-1}) = Total N content × (%Ndfa)/100, where total N content (kg ha^{-1}) was measured as the sum of all organ N contents and expressed as kg ha^{-1}. Organ N contents were calculated as the tissue N concentration multiplied with the biomass of plant organs in each sample based on plot surface area.

Soil N uptake

The remainder of plant N was assumed to be derived from soil uptake and calculated for each organ based on the following:

Soil N uptake (kg ha^{-1}) = Total N content – total N_2 fixation

Post-flowering N_2 fixation and N remobilisation

In both years, post-flowering N_2 fixation was calculated by subtracting the amount of N from N_2 fixation at flowering from the amount of N from N_2 fixed at maturity.

The amount of N remobilized from vegetative tissues was calculated as the difference between N in vegetative organs (leaves and stems) at flowering and N in those same organs plus chaff (inflorescence minus grains) at maturity, assuming the difference has been translocated into the grains (Tausz et al. 2017).

N (vegetative organs) at flowering (kg ha^{-1}) = (Leaf tissue [N] × Leaf biomass) + (Stem tissue [N] × stem biomass)

N (vegetative organs plus chaff) at maturity (kg ha^{-1}) = (Leaf tissue [N] × Leaf biomass) + (Stem tissue [N] × Stem biomass) + (Chaff tissue [N] × Chaff biomass)

N remobilization (kg ha^{-1}) = N (vegetative organs) at flowering - N (vegetative organs plus chaff) at maturity

N allocation/partitioning

N allocation patterns were determined by partitioning the N accumulated (product of biomass and organ [N]) in above (leaves, stems, chaffs and grains) and below ground (roots and nodules) organs as well as differentiating the sources of N either derived from atmospheric N_2 or soil resource. N derived from atmosphere (Ndfa, kg ha^{-1}) was calculated in above and below ground organ contents and the remainder was assumed to be derived from soil uptake. Grain N allocation was determined from the grain N yield by distinguishing the sources of N (either fixed N_2 or soil N) translocated to grains at physiological maturity.

Statistical analysis

The experiment was designed as a split-plot design (year and [CO_2]: main plots, genotypes: sub-plots). Analysis of variance (ANOVA) was performed by linear mixed-effect model fit by REML using R package "nlme" (Pinheiro et al. 2017) considering year, [CO_2] and genotypes as fixed effect and ring numbers as random effect. Repeated measured ANOVA (measurement dates as random effect) was performed for soil water during the entire growing season and for total N content, N_2-fixation and soil N uptake from flowering to maturity (growth stages as random effect). When the main effect of year, [CO_2], genotype, or their interaction were significant, pairwise comparisons of the means were performed by least significant difference (LSD) test to assess significant differences among treatment combinations ($P < 0.05$) using the R package "predictmeans" (Luo et al. 2014). Linear regression model (function "lm()" in R package "stats") was used to evaluate the relationship between grain [N] and fixed N_2 allocation to grains. Levene's test was carried out in the R package "DescTool" (function LeveneTest (Signorell 2016)) to test each variable for the homogeneity of variance across the groups and necessary data transformations was done where applicable. All analyses were done by using statistical

software “R” version 3.4.1 (R Core Team 2017). Statistical effects are regarded significant at $P < 0.05$. P-values between 0.05-0.10 are presented for discussion purposes.

RESULTS

Soil water content

In the dry season, soil water remained very low for much of the growing season, only slightly above the wilting point until after flowering, when the effect of emergency irrigation is detectable, but even then soil water remained low. Soil water was always greater in the high rainfall season and nearly reached field capacity between flowering and pod filling (Fig. 2). These measurements confirmed the characterisation of the seasons as dry versus high rainfall (or wet; for the local environmental conditions). In the dry season, soil water was slightly higher under e[CO_2] until flowering, but became depleted more in the later growth stages. In contrast, in the high rainfall year, soil water content was higher under e[CO_2] throughout the growing season peaking at flowering and pod filling stages.

Figure 2

Photosynthesis and stomatal conductance

Elevated [CO_2] stimulated photosynthesis (A_{sat}) and decreased stomatal conductance (g_s) at flowering, and these differences were diminished in the dry season relative to the high rainfall season. There were complex three-way interactions with genotypes for A_{sat} and g_s, whereby e[CO_2] effects on A_{sat} and g_s were greatest for PBA Ace in the high rainfall season (Tables 2 & 3).

Table 2

Yield and biomass accumulation

Elevated [CO_2] increased stimulated grain yield across both cultivars by 65% in the high rainfall season, but there was no significant stimulatory effect of e[CO_2] in the dry season (Tables 2 & 3).

Aboveground biomass (AGB) and total biomass stimulation by e[CO_2] was greater in the high rainfall season than in the dry season at both flowering and maturity. At maturity, the increase of below ground biomass under e[CO_2] was only detected for PBA Ace in the dry season (Tables 2 & 3). Grain yield response to e[CO_2] was greater than biomass response in the high rainfall season but not in the dry season.

Table 3

N concentration and content

At flowering, leaf [N] decreased under e[CO_2] by 6% in the dry season but there was no significant decrease in the high rainfall season (Tables 2 & 3). The magnitude of reduction in the dry season was greater in HS3010 (7%) compared to PBA Ace (5%). Flower [N] in PBA Ace and HS3010 decreased under e[CO_2] in the dry season, whereas in the high rainfall season it was unaffected by e[CO_2] (Tables 2 & 3). Root [N] increased in the high rainfall season and decreased in the dry season under e[CO_2], but e[CO_2] had no effect on stem and nodule [N]. Elevated [CO_2] depressed grain [N] by 4% in the dry season and increased it by 3% in the high rainfall season (significant year × [CO_2] interaction, $p = 0.048$) (Fig. 3).

Figure 3

Nodule number, biomass, activity and metabolite concentrations

Elevated [CO_2] increased nodule number and nodule biomass to a greater extent in the high rainfall season and this applied to both PBA Ace (32%, 27%) and HS3010 (28%, 46%). In the dry season, such e[CO_2]-driven stimulation of nodulation (35%) and mass (29%) was only detected for PBA Ace (Fig 4 A, B). Nodule activity increased under e[CO_2] (17% greater on average) compared to a[CO_2] and the magnitude of this increase was greater in the high rainfall than the dry season (Fig 4C). PBA Ace had greater nodule activity (12%) than HS3010, regardless of growing season and CO_2 exposure. Nodule sucrose, total sugars, malate and total organic acids concentrations increased under e[CO_2], and more so in the high rainfall season compared to the dry season. PBA Ace nodules had greater concentrations for total sugars (5%) and organic acids (13%) compared to HS3010 (Fig. 5 A, B, left panel). In the dry season, e[CO_2] decreased asparagine concentrations in nodule, and

this decrease was greater for PBA Ace (by 38%) than HS3010. In the high rainfall year, in contrast, e[CO_2] increased asparagine concentration in nodules in both genotypes. Nodule had greater concentration of total amino acids (13%) under a[CO_2] than e[CO_2] in the drier season, whereas in the high rainfall season there was even a (non-significant) trend towards increased total amino acid concentration for both genotypes under e[CO_2] (Fig. 5 A, B, right panel).

Figure 4

Figure 5

Total N accumulation, N_2 fixation and uptake

Total N content increased gradually for both genotypes from flowering to maturity in the high rainfall season (Fig. 6 A, B) but to a greater extent under e[CO_2] than a[CO_2]. Elevated [CO_2] significantly increased N_2-fixation from flowering to maturity in the high rainfall season for both genotypes to a similar extent but this effect was more pronounced on PBA Ace in the dry season, in patterns similar to total N content (Fig. 6 C, D). Overall, the amount of fixed N_2 was 1.3-fold lower in the dry season compared to the high rainfall season. The percentage of N derived from atmosphere (%Ndfa) showed similar trends with a significant three-way interaction ($p = 0.017$) (Table 2). N uptake from soil decreased under e[CO_2] to a greater extent in the high rainfall season compared to the dry season (Fig. 6 E, F).

Figure 6

Post-flowering N_2 fixation and N remobilization

Post-flowering N_2 fixation was stimulated by e[CO_2] by 107% in the high rainfall season but no effect was detected in the dry season (Fig. 7A). Total N remobilisation was greater in the high rainfall than the dry season, and greater for PBA Ace than HS3010, but not affected by [CO_2] (Fig. 7B).

Figure 7

N allocation and grain N yield

Above ground N content increased more under e[CO_2] in the high rainfall compared to the dry season, with a greater proportion of N derived from fixed N_2 (Fig. 8 A, top of the panel). Allocation of soil N was significantly lower under e[CO_2] in the above ground biomass.

N allocation to belowground biomass was stimulated by e[CO_2] consistently in PBA Ace in both seasons. HS3010 had more belowground N under a[CO_2] than e[CO_2] in the dry season (Fig. 8 B, middle of the panel). Elevated [CO_2] led to a greater proportion of belowground N coming from fixed N_2.

Figure 8

Grain N yield significantly ($p < 0.001$) increased under e[CO_2] and this increase was much greater in the high rainfall season than the dry season (Fig. 8 C, bottom of the panel). Allocation of fixed N_2 to grains was greater under e[CO_2] and positively correlated ($r = 0.72$, $p < 0.05$) with grain [N] in the high rainfall season (Fig. 9).

Figure 9

DISCUSSION

1. Growth and yield stimulation of lentil by e[CO_2] were greater in the high rainfall than the dry season

Consistent with previous results on wheat from the same site (Houshmandfar et al. 2016; Tausz-Posch et al. 2012), e[CO_2] stimulated net assimilation rates (A_{sat}) and biomass of lentil in our study (by 25 and 30%). Stimulation of grain yield by e[CO_2] was increased more in the high rainfall season (63%) than the dry season (18%), in line with previous results on wheat and lentil in the same experimental facility (O'Leary et al. 2015; Fitzgerald et al. 2016; Houshmandfar et al. 2016; Bourgault et al. 2017), but in contrast to a long-held paradigm that the CO_2 fertilization effect is greater under drier than wetter conditions (Kimball 2016; Leakey et al. 2012; McGrath & Lobell 2013). That paradigm was challenged by a recent meta-analysis (van der Kooi et al. 2016) and also by long term results from a FACE site in a high rainfall agro-ecosystem demonstrating that severe

drought diminished yield stimulation by e[CO_2] to zero (Gray et al. 2016). Consistent with the study of Gray et al. (2016) in soybean, our results indicated that greater soil water availability under e[CO_2] grown lentil in the high rainfall season extended grain filling duration and contributed to greater yield stimulation. In contrast, lack of such soil water conservation along with greater soil water depletion during grain filling under e[CO_2] led to lower yield stimulation of lentil in the dry season.

2. Elevated [CO_2] stimulates N_2 fixation in conjunction with stimulated nodule numbers, nodule biomass, specific nodule activity and C supply to nodules

In our first research question we asked how e[CO_2] affect total N_2 fixation, and how such changes are related to nodule biomass, number and specific N_2 fixation capacity. The strong stimulation of N_2-fixation of lentil by e[CO_2] (57% compared to 30% stimulation of biomass) supports previous assertions that e[CO_2] can sufficiently increase N_2-fixation of some legumes to match the N demand of greater biomass (Rogers et al. 2006). The increase in N_2-fixation of lentil under e[CO_2] was associated with increased nodule number and mass (+27% and 18%, respectively), consistent with previous reports in soybean (Gray et al. 2013; Li et al. 2017), field pea (Butterly et al. 2016; Jin et al. 2012) and chickpea (Lam et al. 2012a). In addition, increases in specific nodule activity can also contribute to increased N_2 fixation under e[CO_2], although this effect is not always found (Cabrerizo et al. 2001). In our study of lentil, the amount of N_2 fixed per unit of nodule mass (an index of nodule activity; Rogers et al. 2009) increased by 27% under e[CO_2]. This suggests that the increase in N_2-fixation of lentil under e[CO_2] appears to be controlled by greater nodule performance i.e. increased nodule number and mass in conjunction with increased specific nodule activity.

Stimulation by e[CO_2] of photoassimilate availability to nodules can increase nodule activity (Tissue et al. 1996). Using $^{13}CO_2$ pulse-labelling approach, Voisin et al. (2003) showed that nodules are a major C sink and there is a close association between nodule activity and nodule carbon metabolism (Aranjuelo et al. 2014b), whereby sugars (particularly sucrose) and malate play vital roles (Aranjuelo et al. 2009; Esfahani et al. 2014; Fischinger et al. 2010; Schulze 2004). Consistent with these studies, concentrations of sucrose, total sugars,

malate as well as total organic acid in nodules were all greater under e[CO_2] marking stimulation of specific nodule N_2-fixation activity by generally greater C-supply.

Our question also related to how e[CO_2] interacts with between-season differences in regards to N_2-fixation. The two seasons investigated here differed particularly in rainfall, which was linked to the dynamic changes in soil water availability. Decreases in N_2 fixation under dry growing conditions are well documented (Streeter 2003; Zahran 1999). Consistent with these reports, we observed a (61%) reduction of N_2 fixation in the drier season compared to the wetter season. The effects of soil drying on N_2-fixation may be through reduced nodule numbers (Antolín et al. 2010), reduced activity of individual nodules (Sprent 1971) or a combination of both. Our results on lentil indicate a combination of all mechanisms, with lower nodule number (-54%), nodule biomass (-48%) as well as specific nodule activity (-27%) in the drier season. Decline of nodule mass and functionality by drought are connected with decreased carbohydrate availability to nodules (Aranjuelo et al. 2014a), a notion corroborated by our results of decreases in sucrose and malate concentrations in nodules in the drier season.

Elevated [CO_2] has the potential to protect or at least delay reduction in N_2-fixation associated with soil drying either by maintaining greater C supply to nodules or maintaining soil water around the nodules for longer by decreasing stomatal conductance and lowering canopy water use (Bernacchi et al. 2007; Rogers et al. 2009). In our study, there was no significant increase of soil water retention under e[CO_2] in the drier season, despite lower stomatal conductance of e[CO_2] grown lentil. This is comparable with the recent results from a higher rainfall agroecosystem, where e[CO_2] did not conserve soil water under severe drought and dry soil surrounding the nodules decreased N_2-fixation (Gray et al. 2016). In the dry season in this present study, slightly higher soil water under e[CO_2] grown lentil was only observed until flowering, when it was associated with 26% greater rate of N_2-fixation, but post-flowering soil water was depleted even more in e[CO_2] grown crops and N_2-fixation was severely compromised by soil drying (despite deployment of 'emergency' irrigation only designed to keep the crop alive). In contrast, associated with greater soil water content in the wet season e[CO_2] stimulated N_2-fixation until the late grain filling stages. These results suggest that small water savings early in the season under e[CO_2] are not sufficient to maintain N_2-fixation under terminal drought conditions.

The decline in N_2-fixation under soil drying is associated with an accumulation in nodules of N-compounds (King & Purcell 2005), interpreted as a negative feedback mechanism inhibiting nodule activity when there is less demand from shoots (Serraj et al. 2001). In lentil, accumulation of amino acids, predominantly asparagine (as potential candidate compound) appear to mark such a feedback associated with impaired N_2-fixation in the dry season, and this feedback may be slightly less under e[CO_2]. In the high rainfall season, e[CO_2] seemed to stimulate N_2-fixation to such an extent that shoot demand was easily met, or perhaps exceeded, and feedback inhibition was indicated by increased amino acid concentrations in nodules (Fig. 5). Similarly, increases of N-compounds, especially asparagine concentration, in alfalfa nodules grown under e[CO_2] were connected with increased N_2-fixation activity under ample water supply (Fischinger et al. 2010). These data indicate that feedback by N compounds applies particularly under water stress conditions, where it is coupled with downregulation of the enzymes involved in N_2 assimilation (Aranjuelo et al. 2014a), but that it can also be associated with strong stimulation of fixation by e[CO_2] under favourable conditions.

3. Elevated [CO_2] reduces dependency on soil N resources and changes N allocation patterns in lentil

In our second research question, we asked how e[CO_2] and seasonal variation in water availability change the use of soil versus aerial N sources, and how N allocation patterns in lentil might change. Our results demonstrate that N_2-fixation increased in e[CO_2]-grown lentil to such an extent that uptake of soil N decreased in absolute terms, which is an independent confirmation of previous findings on *Medicago trunculata* L. (Guo et al. 2013), the N_2 fixing tree *Robinia pseudoacacia* (Feng et al. 2004), or an earlier FACE experiment on *Trifolium repens* L. (Zanetti et al. 1996) and in line with a meta-analysis on the negative effect of e[CO_2] on plant N acquisition (Feng et al. 2015). Decreases of N uptake under e[CO_2] are generally associated with the decreases in nitrate uptake (Guo et al. 2013). With sufficient assimilate supply, the trade-off between N_2-fixation and nitrate reduction increasingly tips towards fixation, and this shift may even be more pronounced under e[CO_2]. Owing to decreased N uptake and consistent with previous observations (Feng et al. 2015; Guo et

al. 2013; Lee et al. 2003), the proportion of N from N_2-fixation almost doubled under e[CO_2] in the above ground parts, and more than doubled (70%) in grains.

Does e[CO_2] cause differences in N acquisition and allocation in a low and high rainfall season? Some previous FACE investigations on legumes found that that e[CO_2] *per se* does not influence the relative proportions of total plant N coming from N_2-fixation compared to other sources and apparent stimulation tuned with limited soil N supply (Zanetti et al. 1996, Lüscher et al. 2000). In line with these findings, the decrease in absolute allocation of soil N to biomass or grain due to decreased uptake contributed to proportionally increased allocation of fixed N_2 in lentil. Our results indicate that in a high rainfall season, e[CO_2]-grown lentil relied to a greater extent on N_2-fixation, because N uptake from soils was insufficient to meet increased plant N demand, but a very dry season limited these effects in line with the e[CO_2]-effect on growth.

When acquisition of both soil and atmospheric N_2 sources is limited during grain filling, remobilisation of N from the pre-existing N pool in the vegetative tissues can become the major source of N for seeds (Salon et al. 2001; Vikman & Vessey 1992). In the drier season, despite lower absolute amounts of N remobilised, translocated N from vegetative tissues contributed 71% of grain N, compared to only 40% of grain N in the high rainfall season. Under non-water limited conditions, N_2-fixation after flowering can supply up to 92% of seed N demand in lentil (van Kessel 1994). Therefore, grain filling during the late stages of development is more dependent on continuing N_2-fixation than on remobilisation of N from vegetative structures (Bergersen et al. 1992; Polania et al. 2016). Despite the effects on N_2-fixation and soil N uptake, e[CO_2] had no consistent effect on remobilisation, suggesting that e[CO_2] grown legumes satisfy seed N demand mostly through current N_2-fixation and, to a lesser extent, assimilation of soil N, as evident from the high rainfall season in our study.

In legumes, the known decrease in grain [N] under e[CO_2] is less prevalent than in non-legume crops (Myers et al. 2014) and we found that e[CO_2] decreased grain [N] by 4%, but only in the dry season. Decreases in grain [N] under e[CO_2] in a low rainfall environment were previously observed in lentil and field pea (Bourgault et al. 2017; Bourgault et al. 2016). In the high rainfall season, stimulation of N_2-fixation by e[CO_2] was apparently sufficient to maintain grain [N] with higher yield, similar to reports in soybean in high rainfall agro-ecosystems

(Gray et al. 2013) and supported by the positive correlation between grain [N] and N deposition to grain from fixation (Fig. 9). Our results indicate that it is continuing N_2-fixation, but not soil N uptake or N remobilisation that keeps pace with on the greater N-demand of grains under e[CO_2], and the stimulating effect of e[CO_2] on N_2-fixation is constrained by drought. Therefore, e[CO_2]-stimulation of N_2-fixation can optimise N supply to grains only if water supply is sufficient to maintain symbiotic fixation activity during the grain filling period.

4. *There were genotypic differences in response to e[CO_2] and between-season variation in water availability for N_2-fixation and allocation, but not for yield or grain [N]*

In our third research question we asked whether N acquisition and allocation patterns differed between two lentil genotypes, which may provide a mechanism for selecting more CO_2-response cultivars and offering potential targets for improving grain [N] (Bourgault et al. 2016, Li et al. 2017). Elevated [CO_2] stimulated N_2-fixation of both genotypes with a greater increase for "HS3010" than for "PBA Ace" in the high rainfall season (Fig. 6 C, D), whereby the difference was mainly due to increased nodule numbers and mass, in line with previous reports (Rogers et al. 2009). In this season, additional N harvested as a result of growth stimulation under e[CO_2] by both genotypes confirmed to be derived from symbiotic fixation rather than soil N uptake. In contrast, e[CO_2] stimulated N_2 fixation (20%) of PBA Ace in the low rainfall season but had no effect on HS3010, indicating genotypic variation in the sensitivity of N_2-fixation to environmental cues (Vadez et al. 2012; Volk & Körner 2001).

A genotype with higher harvest index may also allocate more N into the grain and therefore increase N yield (Sinclair 1998). In the higher rainfall season, both genotypes increased the allocation of N from fixed N_2 to grains (PBA Ace +59% and HS3010 +103%) under e[CO_2] and thus improved grain N yield as well as grain [N]. In contrast, the relative advantage in N_2-fixation of PBA Ace under e[CO_2] in the dry season did not translate to greater allocation into grains, resulting in a similar decrease in grain [N] as for HS3010. This shows that growing conditions dominate genotypic expression and any anticipated benefits from e[CO_2] for PBA Ace was less or absent due to terminal drought in the drier season (Jablonski et al. 2002; Jin et al. 2018). Despite

significant genotypic variation in N_2-fixation and its partitioning to grain, there was no interaction with e[CO_2] for grain yield and grain [N]. At least for the two genotypes in this study, environmental growing conditions rather than genotypic characteristics determinated grain [N] depression under rising [CO_2].

CONCLUSIONS

In dryland grown lentil, e[CO_2] stimulated N_2-fixation through a combination of greater nodule numbers, greater nodule mass and greater specific nodule activity. Stimulation of N_2-fixation was marked by increased nodule concentrations of carbohydrates and organic acids, and lower concentrations of free amino acids, consistent with a concept of increasing assimilates feeding fixation and avoiding feedback inhibition by accumulating amino acids. In a relatively high rainfall season, this e[CO_2] stimulation of N_2-fixation was more than sufficient to meet the increased N demand of stimulated biomass growth, so that soil N uptake decreased even in absolute terms. In a dry year however, stimulation of N_2-fixation by e[CO_2] was constrained, feedback inhibition by accumulating amino acids was indicated in nodules, and whilst N_2-fixation was still sufficiently increased to meet (lower) additional demand, soil N uptake remained unaffected. Drought also changed the effect of e[CO_2] on N allocation patterns within the plants, and most importantly, to the grains, so that grain [N] decreased under e[CO_2] in the dry year but not the higher rainfall year. Whilst the e[CO_2]-stimulation of N_2-fixation was different between the two genotypes investigated in this study, this did not translate in differences in yield benefit, and did not moderate the decrease in grain [N]. Sufficient water supply to maintain N_2-fixation well into the grain filling period seems to optimise the benefits from e[CO_2] both in terms of yield and N_2-fixation, and such environmental factors dominated over any genotypic variability, at least with the two genotypes in this highly variable dryland agro-ecosystem. This suggests that climate-adapted management options that maintain soil water later into the growing season in a legume system, would maximise N_2-fxation and contribute to maintaining grain protein as well as add more N into crop rotation systems.

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List of tables and captions

Table 1. Summary of the environmental conditions during two contrasting growing seasons: 2015, low rainfall/dry season and 2016, high rainfall season at the Australian Grains Free Air CO_2 Enrichment facility in Horsham, Australia. Meteorological variables were expressed as total or average during crop growing season from sowing to final harvest. Pre-sowing irrigation was applied in both year to ensure optimum seed germination. For grain yield, all experimental plots were averaged in each year. GSR: global solar radiation.

Table 2. Gas exchange parameters, biomass, nitrogen concentrations ([N]), grain yield and percentage of N derived from atmosphere (%Ndfa) of two lentil genotypes "PBA Ace" and "HS3010" grown under ambient [CO_2] (a[CO_2] ~400 ppm) or elevated [CO_2] (e[CO_2]~550 ppm) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a high rainfall (2016) season. Means and standard errors of n=4 replicates. Unit for A_{sat}: µmol CO_2 m^{-2} s^{-1} and g_s: mol H_2O m^{-2} s^{-1}. AGB: aboveground biomass, BGB: belowground biomass and dwt: dry weight. Statistics are reported in Table 3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

Table 3. Effect of year, [CO_2] and genotype (CV) and their interaction on significance (P) and F-values (in parentheses) for the parameter of interest. Statistical effects are regarded significant at $p < 0.05$. P-values between 0.05-0.10 are presented for discussion purpose. $P \geq 0.1$ is considered not significant (ns). AGB: above ground biomass, BGB: below ground biomass, TOC: total organic acids, TAA: total free amino acids; for parameters and units see Table 2 and Figures 3 to 8. Sample size was four replicates (n = 4) for each combination of year, CO_2 and genotypes. Significant effects are shown in bold type.

Table 1

Parameters	Growing season	
	2015, low rainfall season	2016, high rainfall season
Average minimum (°C)	5.9	5.8
Average maximum (°C)	20.3	18.4
Average (°C)	13.3	12.1
Average evaporation (mm)	3.1	2.4
Average humidity (%)	66.0	76.9
Pre-sowing irrigation (mm)	25.0	50.0
Total growing season rainfall (mm)	128.4	334.2
Rainfall sowing to flowering (mm)	108.8	227.4
Total irrigation (mm) from flowering till maturity	96.0	0.0
Total water inputs (mm)	249.4	384.2
Average GSR (Wm^{-2})	176.9	171.1
Average daylight (h)	11.4	11.4
Growth duration (days)	167	189
Average grain yield ($g\ m^{-2}$)	137.8	488.9

Table 2

Parameters	PBA Ace				HS3010			
	2015		2016		2015		2016	
	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]
Flowering								
Photosynthesis (A_{sat})	8.56±0.67 AB	11.85±1.13 C	20.28±1.08 D	31.49±1.72 F	8.16±0.96 A	11.84±0.94 BC	21.82±2.79 D	28.05±2.37 E
Conductance (g_s)	0.072±0.006 AB	0.06±0.004 B	0.620±0.096 C	0.32±0.045 D	0.073±0.011 A	0.061±0.006 B	0.554±0.057 C	0.363±0.024 D
AGB (g dwt m^{-2})	270.83±5.87 B	329.76±13.63 D	387.42±9.64 EF	485.34±23.99 G	240.55±2.72 A	296.45±5.49 C	371.82±7.35 E	419.88±16.39 F
BGB (g dwt m^{-2})	110.83±21.65 A	152.81±19.86 AB	180.84±5.80 B	214.60±11.83 C	107.42±7.76 A	116.27±9.65 A	154.28±6.57 AB	198.45±9.93 BC
Total biomass (g dwt m^{-2})	381.66±19.80 A	482.57±16.16 AB	568.26±13.53 BC	699.94±29.57 C	347.98±9.21 A	412.72±9.48 A	526.11±11.39 AB	617.82±8.59 BC
Leaf [N] (% dwt)	4.31±0.04 A	4.08±0.05 B	6.0±0.187 A	5.75±0.33 A	4.33±0.09 A	4.01±0.10 B	4.58±0.11 A	4.28±0.21 A
Stem [N] (% dwt)	2.18±0.13 A	2.05±0.07 A	2.43±0.17 A	2.00±0.14 A	2.35±0.09 A	2.33±0.16 A	1.90±0.21 A	1.85±0.13 A
Root [N] (% dwt)	2.17±0.25 AB	2.09±0.13 AB	1.38±0.14 C	2.13±0.14 A	1.98±0.14 A	1.93±0.05 AC	1.90±0.18 A	2.60±0.06 B
Flower [N] (% dwt)	5.17±0.10 A	4.90±0.04 AD	3.85±0.33 C	4.28±0.24 BCD	4.65±0.23 ABCD	4.03±0.18 BC	4.80±0.24 ABD	4.77±0.15 ABD
Nodule [N] (% dwt)	6.40±0.30 A	7.07±0.08 A	6.30±0.33 A	6.58±0.37 A	6.59±0.37 A	6.69±0.18 A	6.83±0.14 A	6.05±0.06 A
Maturity								
AGB (g dwt m^{-2})	574.60±16.05 B	702.21±27.69 C	935.89±23.18 E	1224.31±51.85 G	436.57±25.99 A	582.33±18.53 B	823.58±20.87 D	1104.91±10.29 F
BGB (g dwt m^{-2})	98.99±7.07 C	131.58±13.86 ABC	165.40±8.05 BD	175.74±6.95 D	132.10±12.46 AB	108.58±14.93 AC	145.66±5.28 ABD	168.15±8.98 BD
Total biomass (g dwt m^{-2})	673.59±20.72 B	833.79±31.58 C	1101.29±29.42 E	1400.05±47.10 G	568.67±14.42 A	690.91±45.93 B	969.23±18.13 D	1273.06±17.05 F

Grain yield (g dwt m^{-2})	164.58±15.99 BC	189.05±26.48 C	353.77±35.13 E	549.67±28.71 F	88.26±14.51 A	108.44±27.97 AB	277.59±13.53 D	432.55±17.23 F
% Ndfa (total)	51±3.61 A	67±2.45 CD	72±2.11 D	86±2.68 E	55±3.40 AB	61±2.28 BC	66±4.59 CD	85±1.17 E
% Ndfa in grain	66±4.81 A	80±3.17 ABC	74±2.98 ABC	90±2.80 BC	70±6.18 AB	77±5.17 ABC	70±8.47 AB	94±1.89 C

Table 3

Parameters	Year	$[CO_2]$	CV	Year × $[CO_2]$	Year × CV	$[CO_2]$ × CV	Year × $[CO_2]$ × CV
At flowering							
Photosynthesis	**<0.001 (467.02)**	**<0.001 (106.25)**	**<0.001 (2.08)**	**<0.001 (21.85)**	ns (0.35)	0.098 (3.21)	0.046 (4.91)
Conductance	**<0.001 (1468.91)**	**<0.001 (50.85)**	ns (0.05)	**0.016 (7.69)**	ns (1.36)	ns (0.09)	**0.017 (7.54)**
AGB	**<0.001 (329.29)**	**<0.001 (80.74)**	**<0.001 (40.66)**	**0.030 (5.97)**	**0.069 (3.96)**	ns (0.13)	ns (3.11)
BGB	**<0.001 (37.89)**	**0.010 (9.23)**	**0.015 (7.87)**	ns (0.41)	ns (0.01)	ns (0.60)	ns (2.19)
Total biomass	**<0.001 (221.71)**	**<0.001 (53.24)**	**<0.001 (43.95)**	ns (0.13)	ns (0.01)	0.076 (3.73)	ns (0.02)
Leaf [N]	**<0.001 (67.31)**	**0.045 (5.49)**	**<0.001 (38.82)**	**<0.001 (8.75)**	ns (37.42)	ns (0.09)	ns (0.01)
Stem [N]	ns (2.93)	ns (2.15)	ns (0.33)	ns (0.56)	**0.011 (8.83)**	ns (1.64)	ns (0.48)
Root [N]	ns (0.11)	**0.019 (7.21)**	**0.083 (3.58)**	**0.007 (10.54)**	**0.001 (15.57)**	ns (0.01)	ns (0.06)
Flower [N]	**0.094 (3.27)**	ns (0.74)	ns (0.01)	0.046 (4.90)	<0.001 (23.42)	ns (1.88)	ns (0.03)
Nodule [N]	ns (1.85)	ns (0.14)	ns (0.06)	ns (3.00)	ns (0.06)	0.048 (4.83)	ns (0.44)
Nodule number	**<0.001 (414.55)**	**<0.001 (120.26)**	ns (1.27)	**0.001 (16.21)**	**0.001 (18.41)**	**0.019 (7.31)**	**0.042 (5.15)**
Nodule biomass	**<0.001 (84.76)**	**<0.001 (26.02)**	**0.045 (4.73)**	ns (0.88)	ns (2.63)	ns (0.41)	**0.053 (5.17)**
Nodule activity	**<0.001 (26.91)**	**0.005 (11.81)**	**0.039 (6.47)**	ns (0.13)	ns (0.14)	ns (0.05)	ns (0.01)
Sucrose	**<0.001 (90.20)**	**<0.001 (36.16)**	ns (0.77)	**<0.001 (29.30)**	**<0.001 (27.80)**	ns (0.00)	ns (1.60)
Total sugars	**<0.001 (90.20)**	**<0.001 (36.16)**	ns (0.77)	**<0.001 (29.20)**	**<0.001 (27.80)**	ns (0.00)	ns (1.60)
Malate	**<0.001 (382.76)**	**<0.001 (33.97)**	**0.001 (17.94)**	**0.040 (5.24)**	**0.003 (13.39)**	ns (4.71)	ns (0.09)
TOC	**<0.001 (303.92)**	**<0.001 (27.96)**	**0.008 (10.03)**	**0.035 (5.61)**	**0.021 (6.98)**	ns (2.43)	ns (0.57)

Asparagine	**<0.001 (45.71)**	ns (0.04)	ns (36.87)	**0.003 (4.84)**	ns (0.01)	ns (0.55)	**0.048 (1.07)**
TAA	**<0.001 (28.72)**	ns (0.04)	**<0.001 (36.87)**	**0.048 (4.84)**	ns (0.01)	ns (0.55)	ns (1.07)
At maturity							
AGB	**<0.001 (450.14)**	**0.003 (99.54)**	**<0.001 (53.42)**	**0.004 (12.30)**	ns (0.153)	ns (0.027)	ns (0.142)
BGB	**<0.001 (29.91)**	**0.086 (1.55)**	ns (0.53)	ns (0.50)	ns (0.52)	ns (3.48)	**0.013 (8.39)**
Total biomass	**<0.001 (478.91)**	**<0.001 (96.01)**	**<0.001 (38.14)**	**0.004 (12.56)**	ns (0.02)	ns (1.61)	ns (0.28)
Grain yield	**<0.001 (276.13)**	**<0.001 (44.22)**	**<0.001 (20.09)**	**<0.001 (28.27)**	ns (0.04)	ns (0.01	ns (0.040)
Grain [N]	**0.001 (26.01)**	ns (0.01)	**0.012 (8.14)**	**0.048 (4.83)**	**0.011 (9.01)**	ns (1.66)	ns (0.20)
Total N accumulation							
Total N	**0.009 (21.95)**	**<0.001 (82.42)**	**<0.001 (32.26)**	**0.041 (4.48)**	**0.049 (4.09)**	ns (0.29)	ns (1.14)
N_2 fixation	**0.004 (32.22)**	**<0.001 (220.03)**	**<0.001 (54.73)**	**0.001 (11.09)**	**0.031(4.95)**	ns (0.00)	**<0.001 (13.11)**
Soil N uptake	**<0.001 (1.60)**	ns (24.94)	ns (0.08)	**0.026 (9.66)**	ns (0.46)	**0.093 (0.55)**	**0.021 (5.17)**
P-F N_2 fixation	**<0.001 (108.61)**	**0.003 (24.83)**	ns (0.01)	**0.001 (15.75)**	ns (0.03)	ns (0.61)	ns (1.82)
N remobilisation	**0.040 (5.25)**	ns (3.15)	**0.044 (5.04)**	ns (0.89)	ns (0.34)	ns (1.04)	ns (1.14)
% Ndfa	**<0.001 (54.87)**	**<0.001 (28.20)**	ns (1.03)	ns (0.87)	ns (0.45)	ns (0.25)	**0.017 (7.58)**
N allocation							
Above ground							
N content	**<0.001 (309.47)**	**<0.001 (55.41)**	**0.004 (12.04)**	**<0.001 (19.22)**	ns (2.19)	ns (0.61)	ns (1.17)
Fixed N_2	**<0.001 (294.13)**	**<0.001 (94.43)**	**0.013 (8.44)**	**<0.001 (34.58)**	ns (0.44)	ns (0.29)	**0.081 (3.62)**
Soil N	ns (2.97)	**0.035 (5.60)**	ns (2.93)	ns (2.53)	**0.062 (4.22)**	ns (0.43)	ns (3.07)
Below ground							
N content	**<0.001 (30.04)**	ns (0.11)	ns (0.13)	ns (0.09)	ns (0.88)	**0.048 (4.82)**	**0.026 (6.40)**
Fixed N_2	**<0.001 (23.03)**	ns (1.60)	ns (0.08)	ns (0.74)	ns (1.09)	ns (2.20)	**0.077 (3.72)**
Soil N	ns (0.59)	ns (2.16)	ns (0.01)	ns (0.76)	ns (0.01)	ns (1.37)	ns (1.09)
Grain							
Grain N yield	**<0.001 (165.26)**	**<0.001 (33.09)**	**0.011 (9.01)**	**<0.001 (23.07)**	**0.078 (3.71)**	ns (0.02)	ns (0.026)
Fixed N_2	**<0.001 (152.30)**	**<0.001 (55.63)**	**0.064 (4.14)**	**<0.001 (33.47)**	ns (2.59)	ns (0.04)	ns (0.092)
Soil N	**0.093 (3.32)**	**0.018 (7.35)**	**0.042 (5.17)**	ns (2.23)	ns (0.66)	ns (0.01)	ns (0.84)
% Ndfa in grain	**0.062 (4.23)**	**0.004 (12.83)**	ns (0.19)	ns (1.55)	ns (0.42)	ns (0.01)	**0.097 (2.42)**

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Figure 1. Drought index calculated as the difference between precipitation (P) and evapotranspiration (ET) in a dry (2015, grey line) and a high rainfall (2016, black line) season. Growth stages (F: flowering, P: pod filling and M: Maturity) are given for lentil growing under ambient [CO_2] (~400 ppm) or elevated [CO_2] (~550 ppm) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia.

Figure 2. Volumetric soil water content (m^3 m^{-3}) in lentil plots in a dry (2015, A) and a high rainfall (2016, B) season under ambient [CO_2] (~400 ppm, ○ with continuous lines) or elevated [CO_2] (~550 ppm, ● with broken

lines) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia. Horizontal continuous line indicates field capacity and broken line indicates permanent wilting point. Soil water content averaged for the top 40 cm depth. Each point represents means and standard deviations of n=8 plots (two lentil genotype sub-plots in each of 4 replicate plots per [CO_2]). Dotted arrows represent growth stages of lentil indicating flowering (F), pod filling (P) and maturity (M). Double sided arrow in the top panel (A) indicates the period receiving additional irrigation (94 mm) after flowering. Year, CO_2 and their interaction was significant at $p < 0.001$ ($F = 28.61$).

Figure 3. Grain N concentration ([N], % dry weight) of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" at maturity grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, left half) and a high rainfall (2016, right half) season. Means and standard errors of n=4 replicates. Statistics are reported in Table 3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

Figure 4. A. Nodule number (000, m^{-2}), B. Nodule biomass (BM, dry weight basis, g m^{-2}), C. Nodule activity (mg N_2 fixed g^{-1} nodule dry weight) of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a high rainfall (2016) season. Measurements were taken at flowering stage. Means and standard errors of n=4 replicates. Statistics are reported in Table 3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

Figure 5. Nodule sucrose, total sugars (TS), malate, total organic acids (TOC), asparagine (Asn) and total free amino acids (TAA) concentrations of two lentil genotypes "PBA Ace" (A) and "HS3010" (B) grown under ambient [CO_2] (a[CO_2], ~400 ppm) or elevated [CO_2] (e[CO_2], ~550 ppm) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a high rainfall (2016) season.

Measurements were taken at flowering stage. Means and standard errors of n=4 replicates. Legends: In 2015 ambient [CO_2] (white bars) and elevated [CO_2] (grey bars), while in 2016 ambient [CO_2] (diagonal bars) and elevated [CO_2] (black bars). nfw: nodule fresh weight. Statistics are reported in Table 3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

Figure 6. Total plant N content (A, B), N from N_2-fixation in plant biomass (C, D) and N from soil N uptake in plant biomass (E, F) of two lentil genotypes "PBA Ace" (Δ, ▲ with broken lines) and "HS3010" (○, ● with continuous lines) from flowering to maturity and grown under ambient [CO_2] (~400 ppm, open symbols Δ ○) or elevated [CO_2] (~550 ppm, filled symbols ▲, ●) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, left panel) and a high rainfall (2016, right panel) season. Measurements were done at flowering (F), pod filling (P) and maturity (M) stages. Means and standard errors of n=4 replicates. Statistics are reported in Table 3.

Figure 7. Post-flowering (P-F) N_2 fixation (A) and N remobilization (B) to grain at maturity of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, left panel) and a high rainfall (2016, right panel) season. Mean and standard errors of n=4 replicates. Statistics are reported in Table 3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

Figure 8. N allocation in above (A) and below (B) ground biomass and grain N yield (C) at maturity of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, grey bars) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, left half-panels) and a high rainfall (2016, right half-panels) season. Dotted

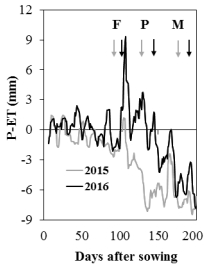
portions refer to soil N uptake. The percentage of N derived from atmosphere (%Ndfa) is reported in Table 2. Each bar represents mean values and standard errors of n=4 replicates. Statistics are reported in Table 3. Mean values that share no common letters (upper-case for total N allocation in above, below or grain; lower-case for fixed N_2 in above, below or grain) are significantly different from each other ($p < 0.05$).

Figure 9. Relationship between grain N concentration ([N], % dry weight) and N from fixed N_2 in grain (g m^{-2}) at maturity of two lentil genotypes "PBA Ace" (● with continuous lines) and "HS3010" (O with broken lines) grown under ambient [CO_2] (~400 ppm) or elevated [CO_2] (~550 ppm) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, A) and a high rainfall (2016, B) season. For significant relationships ($p < 0.05$), slope (m) and Y-intercept (b) are shown.

Water availability moderates N_2 fixation benefit from elevated [CO_2]: A 2-year FACE study on lentil (*Lens culinaris* MEDIK.) in a water limited agro-ecosystem

Brief summary statement highlighting the importance of the work:

Using a Free Air CO_2 Enrichment facility, this study found that elevated [CO_2] stimulated N_2-fixation in lentil through increasing number, mass and specific activity of root nodules, but the effect was different between low and high rainfall year. Only in the high rainfall year, N_2-fixation continued until late in the season and grain N concentration was maintained under e[CO_2]. These findings suggest that e[CO_2]-stimulation of N_2-fixation can optimise N supply to legume grains, but only if sufficient water maintains symbiotic fixation activity during the grain filling period.



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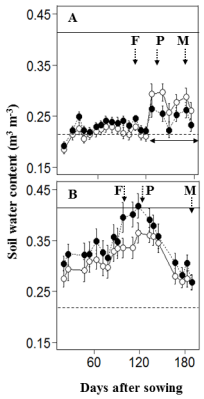

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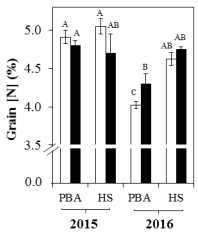

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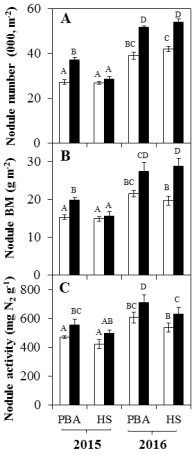

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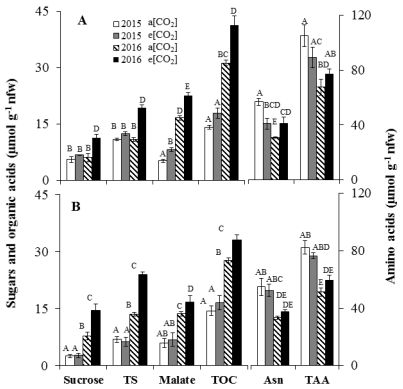

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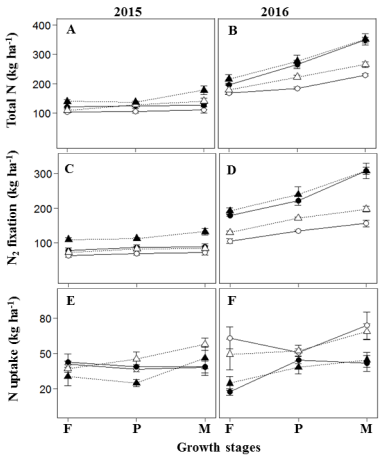

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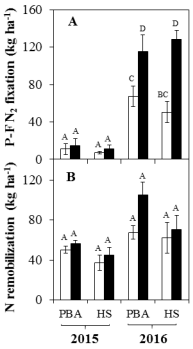

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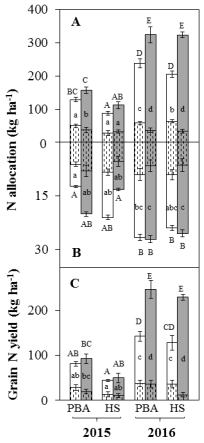

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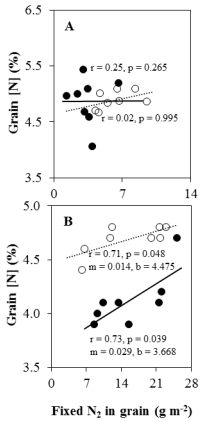

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