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Effect of heat wave on N_2 fixation and N remobilization of lentil (*Lens culinaris* MEDIK) grown under Free Air CO_2 Enrichment in a Mediterranean-type environment

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ABSTRACT

- The stimulatory effect of elevated [CO_2] (e[CO_2]) on crop production in future climates is likely to be cancelled out by predicted increases in average temperatures. This effect may become stronger through more frequent and severe heat waves, which are predicted to increase in most climate change scenarios. Whilst the growth and yield response of some legumes grown under the interactive effect of e[CO_2] and heat waves has been studied, little is known about how N_2 fixation and overall N metabolism is affected by this combination.

- To address these knowledge gaps, two lentil genotypes were grown under ambient [CO_2] (~400 µmol mol^{-1}) and e[CO_2] (~550 µmol mol^{-1}) in the Australian Grains Free Air CO_2 Enrichment facility and exposed to a simulated heat wave (3-day periods of high temperatures ~40°C) at flat pod stage. Nodulation and concentrations of water-soluble carbohydrates (WSC), total free amino acids, N and N_2 fixation were assessed following the imposition of the heat wave until crop maturity.

- Elevated [CO_2] stimulated N_2 fixation so that total N_2 fixation in e[CO_2]-grown plants was always greater than in ambient [CO_2], non-stress control plants. Heat wave triggered a significant decrease in active nodules and WSC concentrations, but e[CO_2] had the opposite effect. Elevated [CO_2] accelerated leaf N remobilization and was associated with increased grain N concentration following heat wave. Interaction between genotype and heat wave was significant for N_2 fixation and grain N yield.

- These results suggested that greater WSC pools and nodulation under e[CO_2] can support post- heat-wave recovery of N_2 fixation.

Keywords: Elevated [CO_2], short-term high temperature, N_2 fixation, nodulation, water soluble carbohydrates, free amino acids, grain nitrogen concentration

INTRODUCTION

Atmospheric CO_2 concentrations ([CO_2]) have increased from 280 µmol mol^{-1} in the pre-industrial era to 409 µmol mol^{-1} in 2019 (NOAA, 2018) and on current trend will continue to rise to at least 550 µmol mol^{-1} by 2050 (IPCC, 2014). On its own, elevated [CO_2] (e[CO_2]) stimulates photosynthesis of C_3 plants and leads to greater crop biomass and yield (Ainsworth and Rogers, 2007). This "CO_2 fertilization effect" is often accompanied by a decrease in tissue N/protein concentration, particularly in cereal grains (Taub et al., 2008). A recent multi-modeling global ensemble study indicates that grain protein concentration of wheat will decrease by 9% by 2050, taking into account e[CO_2] and climate change adaptions that benefit grain yield (Asseng et al., 2019). This decrease affects both nutritional value and product quality of wheat in line with other cereals grown under e[CO_2] (Myers et al., 2014). In legumes, the decrease of tissue N or grain protein concentrations under e[CO_2] is absent or less than in cereals (Taub et al., 2008). Because, e[CO_2] stimulates symbiotic N_2 fixation of legumes in line with biomass growth so that N limitations are overcome (Rogers et al., 2006).

The "CO_2 fertilization effect" on crops, including legumes, may be limited by other climate change factors (Ruiz-Vera et al., 2013), including an increased frequency and severity of heat waves (acute high temperature for short periods) (IPCC, 2014). Particularly in Mediterranean-type climates, episodic heat waves are likely to become more frequent: a 1-in-10-year event is expected to become a 1-in-3-year event by the middle of the 21st Century (IPCC, 2014). Among cool season grain legume crops, lentil is highly sensitive to heat waves during reproductive development, when such heat can result in floret abortion, reduced carbohydrate supply to grain and reduced final grain weight and quality (Sehgal et al., 2017). Heat stress reduces photosynthesis and impairs metabolic pathways, and this corresponds to decreased biomass accumulation (Awasthi et al., 2014).

Direct or indirect effects of high temperature on N_2 fixation have been reported (Hungria and Vargas, 2000). Direct effects are those that respond immediately to high temperature such as decreased pollen fertility and accelerated nodule senescence, whereas indirect effects of high temperature are considered secondary that affects the survival of rhizobia, C supply to nodules, and subsequent reduction of nodule activity (Prasad et al., 2001). The decreased N_2 fixation of alfalfa under high temperature was associated with decreased photosynthesis and reduced C supply to the nodule, resulting in decreased nodule activity (Aranjuelo et al., 2007).

Several studies have assessed the combined effects of e[CO_2] and heat stress on the growth and yield of legumes (Bourgault et al., 2018). Elevated [CO_2], in combination with the high temperature, increased biomass accumulation in legume crops (Wang et al., 2012). Recent studies (Bourgault et al., 2018; Delahunty et al., 2018) reported that e[CO_2] could not fully protect against reduced grain yield from heat waves, but at least moderated the negative effects. This might be associated with greater photosynthetic CO_2 assimilation under e[CO_2] following heat events (Shanmugam et al., 2013), resulting in higher grain yield. Greater soluble carbohydrate pools under e[CO_2] may help nodule formation and activity and thereby, N_2 fixation (Parvin et al., 2019a), but the effects of a heat wave combined with e[CO_2] on N_2 fixation as well as the underlying mechanisms are still unclear.

In environments experiencing terminal drought, C during grain filling comes from either current assimilation or, at least for cereals, from stem reserves stored from pre-anthesis assimilates (Palta et al., 1994). Grain N demand is met by current fixation, uptake by roots or remobilization from vegetative tissues (Tausz et al., 2017b). In legumes, 90-95% of C in grain comes from current assimilation, but N can come from both current fixation or remobilization from vegetative organs (Parvin et al., 2018). As N_2 fixation generally decreases after flowering, the remobilization process accelerates and gains importance (Larmure et al., 2005). Elevated [CO_2] has been reported to accelerate (Agüera and De la Haba, 2018), reduce (Li et al., 2017) or leave unchanged (Tausz et al., 2017b) N remobilization during grain filling phases. The interaction between e[CO_2] and heat waves can alter N acquisition or remobilization patterns and thus may affect grain N yield and concentration.

Traits or genotypes that are better adapted to e[CO_2] can be incorporated into breeding programs. Intraspecific variability in N_2 fixation response under e[CO_2] × drought has been observed (Parvin et al., 2018). As climate change continues to intensify, heat waves will increasingly threaten crop productivity and may limit the biological N_2 fixation process. Identifying genotypes or traits that can maximize N_2 fixation benefits under such conditions are therefore important for adaptation to future climates.

Harvest index (the ratio of grain weight to total plant weight) is an important trait associated with the increases in crop yields (Sinclair, 1998). Genotypic variability in the response to e[CO_2] (Bishop et al., 2015) was found to be associated with differences in HI. Lower HI might be related to sink limitation and lower the response to e[CO_2]. In addition, environmental conditions such as high temperature may also constrain dry matter partitioning to grain, especially in rain-fed areas of the Mediterranean climates where increases in frequency of heat wave are more severe at grain filling (Kobata et al., 2018). Investigating genotypic variation in HI in response to e[CO_2] and heat wave is essential to predict the adaptability of lentil genotypes in future climate.

Consequently, we investigated the mechanisms and to what extent, e[CO_2] can mitigate the heat wave effects on N_2 fixation and grain [N] of lentil and whether this response varies between genotypes. To address these gaps in knowledge, we conducted an experiment in the Australian Grain Free Air CO_2 Enrichment (AGFACE) facility by growing lentil under a[CO_2] or e[CO_2], exposed to a simulated heat wave during the reproductive phase. Two lentil genotypes with contrasting harvest index (ratio of grain yield to total biomass) were chosen in this experiment (Bourgault et al., 2017). We hypothesised that lower harvest index could constrain the capacity to utilize the additional assimilate derived from e[CO_2] and this may compound the heat effects compared to a genotype with higher harvest index. The experimental design and set-up allowed to address the following research questions:

1. Does e[CO_2] mitigate the negative impacts of a short-term heat wave on N_2 fixation by protecting the mechanisms of N_2 fixation process?
2. How does e[CO_2] change N allocation/remobilization patterns from vegetative plant part to the grain after a heat wave to maintain grain [N]?
3. How do genotypes with contrasting in harvest indices differ in N_2 fixation and N remobilization patterns in response to e[CO_2] and a heat wave?

MATERIALS AND METHODS

Site description and experimental design

The experiment was conducted at the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility near Horsham, Victoria, Australia (36°45′70″S, 142°06′52″E, 127m above mean sea level) in 2015. The soil type in the field is Murtoa Clay classified as a Vertosol according to Australian Soil Classification (Isbell, 2002). A detailed description of the AGFACE site and the CO_2 exposure facility is given in Mollah et al. (2009). Meteorological data during the experimental period (May to November 2015) were collected by an onsite weather station described in Mollah et al. (2009) and presented in Fig. 1S.

Four 12 m diameter plots with elevated [CO_2] (centre target concentration of ~550 µmol mol^{-1}) and four control plots with ambient [CO_2] (a[CO_2]) at 400 µmol mol^{-1} were used in this experiment. Elevated [CO_2] plots were surrounded by octagons of stainless-steel pipes (‘FACE rings’), delivering pure [CO_2] into the upwind side of the plots. Delivery was regulated to meet target [CO_2] of 550 µmol mol^{-1} at the plot centre from sunrise to sunset. In each ring, CO_2 injection started when 50% of the crop emerged after sowing and continued till maturity. More details on the description and performance of the CO_2-exposure system are given in Mollah et al. (2009). The present experiment was designed as a split-split–plot with CO_2 as the main plot, split for two lentil genotypes and each split again for two heat treatments.

Plant materials

A widely grown lentil (*Lens culinaris* L.) genotype cv. PBA Ace and breeding line 05H010L-07HS3010 (short HS3010 from here onward) were selected as they have contrasting growth habits. PBA Ace is a modern cultivar that has been shown to perform well under Australian conditions, whereas HS3010 has been shown to have smaller harvest index (HI) than PBA Ace (Bourgault et al., 2018). Inoculated seeds (Group F® *Rhizobium leguminosarum*) were hand sown on 22 May 2015 with a sowing density of 120-150 plants m^{-2} and row spacing of 24.4 cm. The plot size for each genotype was 4 m by 1.5 m in length and width, respectively.

Heat treatments

Heat treatments were imposed at R4 based on the lentil developmental stages described by Erskine et al. (1990) and continued for three days from 6-8 October 2015 (137-140 days after sowing). Custom built heat chambers (0.80 m × 1.2 m × 1.0 m height) were placed on lentil sub-plots (Fig. 3S). Details of chamber design and performance are given in Bourgault et al. (2018). Heat treatments were imposed from 0900 to 1600 hrs local time each day with a targeted air temperature of 40^0C. A hand-held infrared thermometer (EVEREST 100L AGRI-THERM II, ICT International, Armidale, NSW, 2350) was used to record canopy temperature in control and heat plots. Diurnal temperature during the heat wave simulation period from 6-8 October 2015 was as reported by Bourgault et al. (2018). Average canopy temperature in the control and heat-treated plot was ~33°C and 40°C, respectively (Fig. 2S). The heat chamber allowed gas exchange with the outside air as the bottom of the chamber was about 200 mm off the ground and the roof had two 25 mm breathing holes. In addition, CO_2 concentration inside and outside of these chambers at the canopy level were checked by Nuttall J et al. (2012) who found no differences of CO_2 concentration inside and outside of the heat chamber.

Sampling

Destructive plant samples were collected at four growth stages: flat pod (R4, the last day of the heat treatment), early seed (R5, 2 weeks after the heat treatment), full seed (R6, 4 weeks after the heat treatment) and physiological maturity (R8) based on the lentil developmental stages described by Erskine et al. (1990). As a non-N_2 fixing reference plant, wheat grown adjacent to lentil plots was also collected at each harvest. Except for R8, leaves and pods were collected from two randomly selected plants from the plots, immediately frozen in liquid nitrogen and stored in a -80^0C freezer prior to analysis. At maturity, aboveground biomass was hand harvested from 0.3 m^2 of each sub-plot. Biomass was oven dried at 70^0C for 72 h and separated into leaves, stems, and pods and dry weights measured. Grains were separated from pods and weighed. The remainder of the pods (after removing the grains) is reported as chaff.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange measurements were conducted inside the heat chamber on the last day of the heat treatment. An infrared gas analyser (IRGA) was used with a default clear top window chamber and a maximum measurement area of 6 cm^2 (Li- 6400, Li-Cor, Lincoln, NE, USA) under natural light conditions. A randomly chosen fully expanded youngest leaf was measured *in situ* at a leaf temperature range of 30- 40 ºC and an air flow rate through the chamber of 500 $\mu mols^{-1}$. Reference [CO_2] concentrations were adjusted to 400 and 550 $\mu molmol^{-1}$ in ambient and elevated [CO_2] treatments, respectively. All measurements took place on clear sunny days between 1000 to 1200 h. After each measurement, the fraction of leaf inserted into the cuvette was collected and leaf area was measured in the laboratory (LI-3100C, LI-COR, Lincoln, NE, USA). Net CO_2 assimilation rate (A_{net}), stomatal conductance (g_s) and intrinsic transpiration efficiency (ITE=A_{net}/g_s) were adjusted by the actual leaf area. On the last day of the heat treatment, pre-dawn chlorophyll fluorescence measurements were taken using a portable fluorometer (os30P+, Opti-science, Inc. Hudson, NH, USA) and the maximum quantum efficiency of PSII was measured (F_v/F_m).

Nodule attributes

Nodules were collected twice (on the last day of heat treatments and two weeks after treatment at early seed stage R5) with a soil corer with an internal diameter of 7.5 cm. Root systems were excavated to 30 cm depth. Roots were cleaned with tap water, and nodules were separated and counted. Nodules were sectioned longitudinally and classified as active and inactive based on the colour of leghaemoglobin. Active nodules were selected by their reddish to a pink colour indicating intact leghaemoglobin, whereas greenish or brownish and soft nodules were considered inactive nodules (Fischinger et al. 2010). Nodules were oven dried at 70^0C for 72 h and dry weight was recorded.

Plant N calculations

Oven dried biomass samples (leaf, stem, chaff, grain, and reference wheat) from physiological maturity (R8) were finely ground and analysed for total N concentration (% of dry weight) and $\delta^{15}N$ by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon). N_2 fixation, soil N uptake and N remobilization were calculated as described by Parvin et al. (2018). Briefly, 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. The percentage of N derived from atmosphere (%Ndfa) was determined by the following formula as described by Unkovich et al. (1994).

$\% \text{ Ndfa} = (\delta^{15}\text{N reference plant} - \delta^{15}\text{N legumes}) \times 100 / (\delta^{15}\text{N reference plant} - \text{B})$

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 N. In the present study, wheat was selected as reference crop because it had been grown in close vicinity of studied lentil and was exposed to similar heat treatments. Above ground biomass of wheat was collected and organ specific ^{15}N value was used for calculation of %Ndfa. The factor B refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking N. Nodulated lentil grown in sand was harvested at each growth stage and organ-specific B-values ($\delta^{15}N$, ‰) were estimated for %Ndfa calculation (Unkovich and Pate, 2000). B-value was corrected for seed N based on Nebiyu et al. (2014).

N_2 fixation (kg ha^{-1}) = Plant 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}.

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

The amount of N remobilized was calculated as the difference between N in vegetative organs (leave and stems) at R4 and N in those same organs plus chaff (inflorescence minus grains) at R8, assuming the difference has been remobilized into the grains (Tausz et al., 2017a).

N (vegetative organs) at R4 (kg ha^{-1}) = (Leaf tissue [N] × Leaf biomass) + (Stem tissue [N] × Stem biomass)

N (vegetative organs plus chaff) at R8 (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 R4 - N (vegetative organs plus chaff) at R8

Biochemical analyses

Water soluble carbohydrate (WSC) concentration was determined from oven dried and finely ground leaf, stem, chaff and grain tissues with the anthrone method based on Yemm and Willis (1954) modified for use in a plate reader (Tecan Sunrise, Tecan, Austria) (Tausz-Posch et al., 2015). Total free amino acid concentrations (AA) of leaf, chaff and grain tissues were determined from frozen tissue (-80ºC) using the acid ninhydrin method (Yemm and Cocking, 1955) as described by Parvin et al. (2019a).

Statistical analysis

All data were analysed using R version 3.4.1 (R Core Team, 2018). Three-way analysis of variance (ANOVA) was performed using the function "aov" in R statistical software. For all parameters, the split-split-plot design was followed where [CO_2], genotypes and heat considered as main factors and FACE rings as random variables. Levene's test using R package "DescTool" (Signorell et al., 2016) was conducted to check the homogeneity of variances, and data were transformed with natural logarithms where necessary. In all analyses, replicate plots were considered the experimental unit (n=4).

RESULTS

Gas exchange and chlorophyll fluorescence

Net assimilation rate (A_{net}) was greater in plants growing under e[CO_2] compared to a[CO_2] (Fig. 1A) and decreased (by about 12%) in heat stressed lentil compared to non-stressed controls. Lentil grown under e[CO_2] had significantly ($P<0.01$) lower (7%) g_s compared to a[CO_2] (Fig. 1B). Heat stress also decreased gs by 10% compared to control. Increased A_{net} and decreased g_s of lentil under e[CO_2] resulted in greater (20%) ITE (Fig. 1C).

The maximum quantum efficiency of PSII (F_v/F_m, Fig. 1D) increased (by about 5%) under e[CO_2]. HS3010 had significantly ($P<0.001$) lower F_v/F_m under e[CO_2] than PBA Ace. In heat stressed lentil, F_v/F_m decreased by 20% under e[CO_2] and by 26% under a[CO_2] relative to non-stressed controls.

Nodules

The decrease in dry weight of active nodules following the imposition of the heat wave was greater under a[CO_2] than e[CO_2] (Fig. 2). At R5, dry weight of active nodules was increased by e[CO_2], and the magnitude of increase was greater in lentil exposed to the heatwave. At all growth stages, PBA Ace had greater active nodules dry weight than HS3010. Heat stress increased inactive nodule dry weight during and after the heat treatment at R5. Strong positive and significant relationships between active nodule dry mass and N_2 fixation were detected at both stages (Fig. 4A-B).

N_2 fixation, allocation, and remobilization

Nitrogen sources and allocation were evaluated at R8 (Fig. 3 and Table 2S). Elevated [CO_2] increased the proportion of N incorporated into aboveground biomass by N_2 fixation and the total N content of aboveground biomass, but did not significantly affect the amount of N sourced from the soil. PBA Ace had a greater proportion of N in aboveground biomass contributed by atmospheric N_2, a greater total amount of N derived from the atmosphere and greater total N content of aboveground biomass than HS3010. PBA Ace absorbed more soil N than HS3010. Heat wave leads to a lower proportion of N in above ground biomass contributed by atmospheric N_2, the lower total amount of N derived from the atmosphere and lower total N content of aboveground biomass, but the amount of N taken up from the soil increased. An interaction between genotype and heat treatment was significant for the total amount of N_2 derived from the atmosphere and total N content in the above-ground biomass. Reduction of N_2 fixation and total N content by heat was more prominent in HS3010 than PBA Ace.

Total N remobilization and N remobilization from stems were decreased by heat wave (Fig. 5) but this differed between organs. Elevated [CO_2] depressed leaf N remobilization without heat stress but stimulated leaf N remobilization by 35% after heat stress (Fig. 5A). In chaff, more N was remobilized in HS3010 after heat stress than control (Fig. 5C). A significant positive correlation between N_2 fixation and remobilization was observed across all cultivar and CO_2-treatments in heat treated lentil (Fig. 4C).

Water Soluble Carbohydrate Concentration

Under e[CO_2], water-soluble carbohydrate concentration ([WSC]) increased in leaf, stem, chaff, and grain throughout all developmental stages (Fig. 4S and Table 1S). When averaged across all developmental stages, genotypes and heat treatments, e[CO_2] grown plants had 33% greater [WSC] in leaf, 29 % in the stem, 41% in chaff and 24 % in grain compared to those grown in a[CO_2]. Genotypes differed, but the significance across the growth stages depended on plant component.

Heat stress increased grain [WSC] only temporarily at R4 (Fig. 4S, panel D and Table 1S). Plants that experienced heat stress had lower [WSC] in later growth stages. On average across all stages, the decreases in [WSC] after the heat wave were 17, 24 and 23% in leaf, stem and chaff, respectively. The heat wave increased [WSC] in mature grains of PBA Ace but decreased grain [WSC] in HS310.

Total free amino acids concentration

Total free amino acid concentration ([AA]) of leaves increased slightly (5-10%) under e[CO_2] compared to a[CO_2] at R4 but decreased at later growth stages (Fig. 5S and Table 1S). In chaff and grain, [AA] was 2 and 5%, respectively, lower under e[CO_2] at the R4 stage, but this decrease disappeared at later growth stages (Fig. 3 B-C). Significant interactions between CO_2 and cultivar were found for chaff at R5 and grains at R8. HS3010 had 20% higher [AA] in chaff at R5 than PBA Ace under a[CO_2]. Elevated [CO_2] decreased grain [AA] of HS3010 by 20% at R8.

The interaction between CO_2 and the heat wave was significant for [AA] in leaf and chaff at R5 and grains at R6 and R8. Significantly greater [AA] of grains (13%) was observed immediately after the heat treatment (i.e. at flat pod stage), but the concentration of free AA decreased (10 to 40%) in heat treated plants towards the final harvest. Heat treatment decreased [AA] of grains by 40% under a[CO_2] and by 12% under e[CO_2]. The three-way interaction between CO_2, cultivar and heat were only significant for chaff [AA] at R5.

Nitrogen concentration and grain N yield

On average, e[CO_2] decreased nitrogen concentration ([N]) in leaf and stem (Fig. 6S and Table 1S). Leaf [N] was reduced by 15% at R5 and stem [N] by 12% at flat pod under e[CO_2]. Chaff [N] increased (18%) under e[CO_2] at the R5 and decreased by 12% at R8 (Fig. 6S, panel C). Grain [N] was significantly lower (18%) at R4 and greater (9%) at R5 under e[CO_2], but this effect disappeared at R8 (Fig. 6S, panel D). HS3010 had greater (9.4%) chaff [N] compared to PBA Ace under e[CO_2] at R5.

Leaf, stem, and chaff [N] were significantly decreased by heat in all stages, on average by 20 to 30%, compared to controls. Heat stress decreased (4-10%) grain [N] under e[CO_2] at R4 and R5 but increased by 10% at maturity (CO_2 × Heat, P <0.001). Heat stress decreased grain N yield to a greater extent in HS3010 than PBA Ace (Heat × CV significant) (Table 2S).

DISCUSSION

The experimental heat wave applied in this study caused reductions in A_{net} and g_s (Fig. 1A-B). Whilst stomatal closure is a strategy to save water before further damages happen, it also restricts CO_2 input to the leaf resulting in reduced carbon assimilation (Wang et al., 2016). Chlorophyll fluorescence measurements (F_v/F_m) provide useful information on photosystem II processes under stress conditions (Johnson and Maxwell, 2000). In addition, the F_v/F_m value remained above 0.6 in e[CO_2] grown plants even under the heat wave, but was depressed under the heat wave in a[CO_2] grown plants (F_v/F_m values <0.5) (Fig. 1D), indicating that in e[CO_2] grown plants potential damages to photosystem II were less likely (Bauweraerts et al., 2014), which could help to explain the greater WSC accumulation by e[CO_2] grown lentil even under heat wave observed in the present study.

Elevated [CO_2] moderated heat wave effects on N_2 fixation through greater nodulation

Consistent with previously reported trends (Rogers et al., 2006; Parvin et al., 2019a), e[CO_2] increased N_2 fixation of lentil by 30% in the current study (Fig.3). The increased N_2 fixation was associated with greater active/viable nodule biomass, similar to what was reported in soybean (Li et al., 2017). In contrast, the heat wave treatment decreased N_2 fixation by 40%. This decrease was associated with a greater proportion of dried, dead and senescent nodule biomass in heat affected plants as shown by the positive correlation between active nodule dry mass and N_2 fixation at R4 and R5 (Fig. 4 A-B). These results are consistent with previous reports on increased nodule mortality and senescence (Prasad et al., 2002) and/or suppression of nodule formation as a result of lower WSC availability (Sita et al., 2017) under heat stress. In the current study, the reduction of WSC in leaves (Fig. 4S) due to heat stress might have affected carbon supply for nodule formation and activity, a point that needs to be examined further in nodules.

In response to our first research question, our study showed that the limitation on N_2 fixation imposed by a heat wave was less under e[CO_2] (-26%) than a[CO_2] (-37%). This indicated that e[CO_2], to some extent, helped maintain the formation and function of nodules potentially because of greater WSC availability, which may buffer against the reduction of N_2 fixation after heat wave events. As a consequence, e[CO_2] grown lentil exposed to a heat wave tended to fixed greater amounts of N_2 compared to a[CO_2] grown non-stressed plants. This is comparable with N_2 fixation of *Medicago trunculata* grown in temperature gradient tunnels (+4◦C) and e[CO_2] (700 ppm) (Aranjuelo et al., 2008). Some studies reported that the decrease in shoot N demand might inhibit N_2 fixation under stressed conditions (Almeida et al., 2000). According to one theory, N_2 fixation is regulated by a N feedback mechanism, whereby increased concentrations of soluble N compounds in the shoot initiate a signalling chain that leads to increased concentrations of soluble N compounds in the nodules, effecting a downregulation of nodule activity and a decrease in N_2 fixation (King and Purcell, 2005). In soybean, ureides are considered the soluble N signal molecules, but for temperate legumes, which do not produce ureides, amino compounds could play the signalling role (King and Purcell, 2005). In this study, AA concentration actually declined in leaves exposed to both e[CO_2] and a heat wave (Fig. 5S), which according to the model mentioned above would lead relive any inhibition of N_2 fixation. The lack of AA accumulation in leaves under e[CO_2] during the heat wave suggests that shoot N demand was maintained, which potentially avoided the N feedback inhibition (Fig. 6). The lower AA concentration found in e[CO_2] grown leaves may have been linked to enhanced AA demand of the developing grains, as indicated by the transient increase of grain AA concentration following the heat wave.

Elevated [CO_2] maintained grain N concentration under heat wave by stimulating leaf N remobilization

To address our second research question, we investigated how e[CO_2] changes N allocation/remobilization patterns during a heat wave. Elevated [CO_2] increased the proportion of fixed N incorporated into biomass and the uptake of soil N as previously reported for lentil (Parvin et al. 2018) and field pea. (Parvin et al., 2019a). In contrast, the decrease of N_2 fixation in response to the heat wave resulted in greater soil N uptake. This could possibly be associated with an increase in soil N uptake during the grain filling period when the N demand of the developing grain was high and not fully met by the lower N_2 fixation rates. Total N content also decreased in response to the heat wave, and this reduction was matched by a decrease in total biomass (Bourgault et al., 2018).

In the present study, grain [N] in lentil decreased in response to a heat wave (Fig. 6S). Heat stress during grain development reduces the duration of grain filling (Sita et al., 2017) which may result in shortening the time for N deposition into the grain (Sehgal et al., 2017). In agreement with that study, N remobilization significantly declined in lentil in heat stressed plants. This might be related with the inhibition of N_2 fixation following the heat wave which reduced the sources of N available to remobilize into grain. As soil N uptake usually decline during grain filling period, grain N depends to a greater extent on the remobilization process (van Kessel, 1994). The observed correlation between N remobilization and N_2 fixation further supported the view that a major proportion of N remobilized into grain derived from fixation in lentil exposed to heat wave conditions (Fig. 4C). This indicates that remobilization of previously fixed N_2 also plays an important role in grain N regulation under high temperature (Larmure et al., 2005).

Elevated [CO_2] caused a reduction in grain [N] (Parvin et al., 2018), whereas the combination of e[CO_2] and heat wave generally restored grain N concentration to levels above that obtained in a[CO_2] and heat wave conditions. Elevated [CO_2] may result in decreased grain [N] and therefore decreased nutritional quality, but heat stress is likely to offset this adverse effect because of accelerated leaf N remobilization under e[CO_2] (Fig. 6). Increasing canopy temperature of soybean sped up the loss of leaf chlorophyll under e[CO_2] (Long et al., 2006), corresponding to lower AA concentration in leaves under heat wave in our study. These results encompass the possibility that e[CO_2] enhanced faster leaf N translocation into grain when exposed to a heat wave, and this could help to partially improve grain N/protein concentration (Macabuhay et al., 2018).

Genotypic variability in response to N_2 fixation and allocation observed under heat wave

Differences in N_2 fixation among genotypes under e[CO_2] were reported in soybean (Li et al., 2017) and lentil (Parvin et al., 2018). In response to our third question, we investigated how N_2 fixation and allocation varied between two genotypes with contrasting harvest indices. Despite the variation of N_2 fixation between two genotypes, the interaction between CO_2 and genotypes was not significant, perhaps indicating an overriding effect of the relatively harsh growing environments (i.e. terminal drought accompanied with a high temperature in 2015) (Parvin et al., 2019b). However, a significant interaction between genotype and heat wave was observed for N_2 fixation, where the reduction in N_2 fixation was slightly greater for HS3010 (-45%) than for PBA Ace (-30%) (Fig. 2S). The higher number of active nodules in PBA Ace might have mitigated the inhibition of N_2 fixation, which is in accordance with earlier findings on heat-tolerant lentil lines (Sita et al., 2017).

In our study, grain N yield was highly correlated with the total N accumulation in above ground biomass, similar to what was demonstrated in other grain legumes (Anglade et al., 2015; Li et al., 2017). This was associated with genotypic differences because total N accumulation increased only in the genotype with the higher harvest index (PBA Ace). A low harvest index appears to limit both N assimilation and partitioning of N into grains, and this effect may have become even stronger after the heat wave so that grain N yield decreased (Table 2S). Despite the consistent variation of N_2 fixation (Fig. 3) and grain N yield (Table 2S) across the two genotypes, our results provide no strong support for selecting genotypes under the combination of [CO_2] × heat wave environments, because there were no significant [CO_2] × genotype interactions. The lack of [CO_2] × genotype interaction might reflect the limited number of genotypes (2) tested.

CONCLUSION

This study provides evidence that e[CO_2] grown lentil genotypes (cv. PBA Ace and HS3010) that are exposed to acute heat wave conditions during the flat pod phase, can maintain N_2 fixation above that of a[CO_2] non-stress control plants. Increased WSC availability and greater nodulation response under e[CO_2] can reduce the negative impact of a heat wave on N_2 fixation. Elevated [CO_2] accelerated leaf N remobilization in response to a heat wave, which allowed partial maintenance of grain [N]. Two genotypes showed consistent differences in N_2 fixation after a heat wave, whereby the greater fixation in lentil genotype, PBA Ace was associated with increased nodulation. The presence of genotypic variability in response to a heatwave can be used for screening procedures, despite no strong genetic interaction under e[CO_2] observed in this study.

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REFERENCES

Agüera E and De la Haba P. (2018) Leaf senescence in response to elevated atmospheric CO_2 concentration and low nitrogen supply. *Biologia Plantarum*.

Ainsworth EA and Rogers A. (2007) The response of photosynthesis and stomatal conductance to rising CO_2 : mechanisms and environmental interactions. *Plant Cell and Environment* 30: 258-270.

Almeida JPF, Hartwig UA, Frehner M, et al. (2000) Evidence that P deficiency induces N feedback regulation of symbiotic N_2 fixation in white clover (*Trifolium repens* L.). *Journal of Experimental Botany* 51: 1289-1297.

Anglade J, Billen G and Garnier J. (2015) Relationships for estimating N_2 fixation in legumes: incidence for N balance of legume-based cropping systems in Europe. . *Ecosphere* 6.

Aranjuelo I, Irigoyen JJ, Sánchez-Díaz M, et al. (2008) Carbon partitioning in N_2 fixing *Medicago sativa* plants exposed to different CO_2 and temperature conditions. *Functional Plant Biology* 35: 306-317.

Aranjuelo I, Jose Irigoyen J and Sanchez-Diaz M. (2007) Effect of elevated temperature and water availability on CO_2 exchange and nitrogen fixation of nodulated alfalfa plants. *Environmental and Experimental Botany* 59: 99-108.

Asseng S, Martre P, Maiorano A, et al. (2019) Climate change impact and adaptation for wheat protein. *Global Change Biology* 25: 155-173.

Awasthi R, Kaushal N, Vadez V, et al. (2014) Individual and combined effects of transient drought and heat stress on carbon assimilation and seed filling in chickpea. *Functional Plant Biology* 41: 1148-1167.

Bauweraerts I, Ameye M, Wertin TM, et al. (2014) Acclimation effects of heat waves and elevated [CO_2] on gas exchange and chlorophyll fluorescence of northern red oak (*Quercus rubra* L.) seedlings. *Plant Ecology* 215: 733-746.

Bishop KA, Betzelberger AM, Long SP, et al. (2015) Is there potential to adapt soybean (*Glycine max* Merr.) to future CO_2? An analysis of the yield response of 18 genotypes in free-air CO_2 enrichment. *Plant Cell and Environment* 38: 1765-1774.

Bourgault M, Brand J, Tausz-Posch S, et al. (2017) Yield, growth and grain nitrogen response to elevated CO_2 in six lentil (*Lens culinaris*) cultivars grown under Free Air CO_2 Enrichment (FACE) in a semi-arid environment. *European Journal of Agronomy* 87: 50-58.

Bourgault M, Löw M, Tausz-Posch S, et al. (2018) Effect of a heat wave on lentil grown under Free-Air CO_2 Enrichment (FACE) in a semi-arid environment. *Crop Science* 58: 803-812.

Delahunty A, Nuttall J, Nicolas M, et al. (2018) Response of lentil to high temperature under variable water supply and carbon dioxide enrichment. *Crop and Pasture Science* 69: 1103-1112.

Erskine W, Muehlbauer FJ and Short RW. (1990) Stages of development in lentil. *Experimental Agriculture* 26: 297-302.

Hungria M and Vargas MAT. (2000) Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research* 65: 151-164.

IPCC. (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (Eds Core Writing Team, RK Pachauri, LA Meyer) (IPCC: Geneva).

Isbell RF. (2002) The Australian Soil Classification. *CSIRO Publishing Collingwood, Australia*.

Johnson GN and Maxwell K. (2000) Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* 51: 659-668.

King CA and Purcell LC. (2005) Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. *Plant Physiology* 137: 1389.

Kobata T, Koç M, Barutçular C, et al. (2018) Harvest index is a critical factor influencing the grain yield of diverse wheat species under rain-fed conditions in the Mediterranean zone of southeastern Turkey and northern Syria. *Plant Production Science* 21: 71-82.

Larmure A, Salon C and Munier-Jolain NG. (2005) How does temperature affect C and N allocation to the seeds during the seed-filling period in pea? Effect on seed nitrogen concentration. *Functional Plant Biology* 32: 1009-1017.

Li Y, Yu Z, Liu X, et al. (2017) Elevated CO_2 increases nitrogen fixation at the reproductive phase contributing to various yield responses of soybean cultivars. *Frontiers in Plant Science* 8.

Long SP, Ainsworth EA, Leakey ADB, et al. (2006) Food for thought: Lower-than-expected crop yield stimulation with rising CO_2 concentrations. *Science* 312: 1918-1921.

Macabuhay A, Houshmandfar A, Nuttall J, et al. (2018) Can elevated CO_2 buffer the effects of heat waves on wheat in a dryland cropping system? *Environmental and Experimental Botany* 155: 578-588.

Mollah M, Norton R and Huzzey J. (2009) Australian grains free-air carbon dioxide enrichment (AGFACE) facility: design and performance. *Crop & Pasture Science* 60: 697-707.

Myers SS, Zanobetti A, Kloog I, et al. (2014) Increasing CO_2 threatens human nutrition. *Nature* 510: 139-142.

Nebiyu A, Huygens D, Upadhayay HR, et al. (2014) Importance of correct B value determination to quantify biological N_2 fixation and N balances of faba beans (*Vicia faba* L.) via ^{15}N natural abundance. *Biology and Fertility of Soils* 50: 517-525.

NOAA. (2018) National Oceanic and Atmospheric Administration, https://www.esrl.noaa.gov/gmd/ccgg/trends/global.html visited December 6, 2018.

Nuttall J, Brady S, Brand J, et al. (2012) Heat waves and wheat growth under a future climate. In 'Capturing opportunities and overcoming obstacles in Australian agronomy. Proceedings 16th Australian Agronomy Conference'. Armidale, NSW. (Ed. I Yunusa) (Australian Society of Agronomy Inc.)

Palta JA, Kobata T, Turner NC, et al. (1994) Remobilization of carbon and nitrogen in wheat as influenced by postanthesis water deficits. *Crop Science* 34: 118-124.

Parvin S, Uddin S, Bourgault M, et al. (2018) Water availability moderates N_2 fixation benefit from elevated [CO_2]: A 2-year free-air CO_2 enrichment study on lentil (*Lens culinaris* MEDIK.) in a water limited agroecosystem. *Plant, Cell & Environment* 41: 2418-2434.

Parvin S, Uddin S, Fitzgerald GJ, et al. (2019a) Free air CO_2 enrichment (FACE) improves water use efficiency and moderates drought effect on N_2 fixation of *Pisum sativum* L. *Plant and Soil* 436: 587-606.

Parvin S, Uddin S, Tausz-Posch S, et al. (2019b) Grain mineral quality of dryland legumes as affected by elevated CO_2 and drought: a FACE study on lentil (*Lens culinaris*) and faba bean (*Vicia faba*). *Crop & Pasture Science* 70: 244-253.

Prasad PVV, Boote KJ, Allen LH, et al. (2002) Effects of elevated temperature and carbon dioxide on seed-set and yield of kidney bean (*Phaseolus vulgaris* L.). *Global Change Biology* 8: 710-721.

Prasad PVV, Craufurd PQ and Summerfield RJ. (2001) Response of groundnuts dependent on symbiotic and inorganic nitrogen to high air and soil temperatures. *Journal of Plant Nutrition* 24: 623-637.

R Core Team. (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/.

Rogers A, Gibon Y, Stitt M, et al. (2006) Increased C availability at elevated carbon dioxide concentration improves N assimilation in a legume. *Plant Cell and Environment* 29: 1651-1658.

Ruiz-Vera UM, Siebers M, Gray SB, et al. (2013) Global warming can negate the expected CO_2 stimulation in photosynthesis and productivity for soybean grown in the midwestern united states. *Plant Physiology* 162: 410-423.

Sehgal A, Sita K, Kumar J, et al. (2017) Effects of drought, heat and their interaction on the growth, yield and photosynthetic function of lentil (*Lens culinaris* Medikus) genotypes varying in heat and drought sensitivity. *Frontiers in Plant Science* 8.

Shanmugam S, Kjaer KH, Ottosen C-O, et al. (2013) The alleviating effect of elevated CO_2 on heat stress susceptibility of two wheat (*Triticum aestivum* L.) cultivars. *Journal of Agronomy and Crop Science* 199: 340-350.

Signorell et al. (2016) DescTools: Tools for Descriptive Statistics. R Package Version 0.99.16.

Sinclair TR. (1998) Historical changes in harvest index and crop nitrogen accumulation. *Crop Science* 38: 638-643.

Sita K, Sehgal A, Kumar J, et al. (2017) Identification of high-temperature tolerant lentil (*Lens culinaris* Medik.) genotypes through leaf and pollen traits. *Frontiers in Plant Science* 8.

Taub DR, Miller B and Allen H. (2008) Effects of elevated CO_2 on the protein concentration of food crops: a meta-analysis. *Global Change Biology* 14: 565-575.

Tausz-Posch S, Dempsey RW, Seneweera S, et al. (2015) Does a freely tillering wheat cultivar benefit more from elevated CO_2 than a restricted tillering cultivar in a water-limited environment? *European Journal of Agronomy* 64: 21-28.

Tausz M, Norton RM, Tausz-Posch S, et al. (2017a) Can additional N fertiliser ameliorate the elevated CO_2-induced depression in grain and tissue N concentrations of wheat on a high soil N background? *Journal of Agronomy and Crop Science*: 1–10.

Tausz M, Norton RM, Tausz-Posch S, et al. (2017b) Can additional N fertiliser ameliorate the elevated CO_2-induced depression in grain and tissue N concentrations of wheat on a high soil N background? *Journal of Agronomy and Crop Science* 203: 574-583.

Unkovich M, Pate J, Sanford P, et al. (1994) Potential precision of the $\delta^{15}N$ natural abundance method in field estimates of nitrogen fixation by crop and pasture legumes in south-west Australia. *Australian Journal of Agricultural Research* 45: 119-132.

Unkovich MJ and Pate JS. (2000) An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. *Field Crops Research* 65: 211-228.

van Kessel C. (1994) Seasonal accumulation and partitioning of nitrogen by lentil. *Plant and Soil* 164: 69-76.

Wang D, Heckathorn SA, Mainali K, et al. (2016) Timing effects of heat-stress on plant ecophysiological characteristics and growth. *Frontiers in Plant Science* 7.

Wang D, Heckathorn SA, Wang X, et al. (2012) A meta-analysis of plant physiological and growth responses to temperature and elevated CO_2. *Oecologia* 169: 1-13.

Yemm EW and Cocking EC. (1955) The determination of amino-acids with ninhydrin. *Analyst* 80: 209-213.

Yemm EW and Willis AJ. (1954) The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal* 57: 508-514.

List of figures and captions

Fig. 1. A. Net CO_2 assimilation rate (A_{net}), B. stomatal conductance (g_s), C. intrinsic transpiration efficiency (ITE) and D. chlorophyll fluorescence (Fv/Fm) of two lentil genotypes (PBA Ace and HS3010) grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and exposed to an acute heat wave during the flat pod stage. Each bar represents mean values and standard errors of 4 replicates. P values indicate the significance of the effect of [CO_2], heat, genotypes (CV) as well as their interaction. Only effects $P<0.100$ are shown.

Fig. 2. Active nodule dry mass (A, B) and inactive nodule dry mass (C, D) of two lentil genotypes measured at flat pod (R4) and early seed (R5) stages grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and exposed to an acute heat wave during the flat pod stage. Each bar represents mean values and standard errors of n=4 replicates. P values indicate the significance of the effect of [CO_2], heat, genotypes (CV) as well as their interaction. Only effects $P<0.100$ are shown.

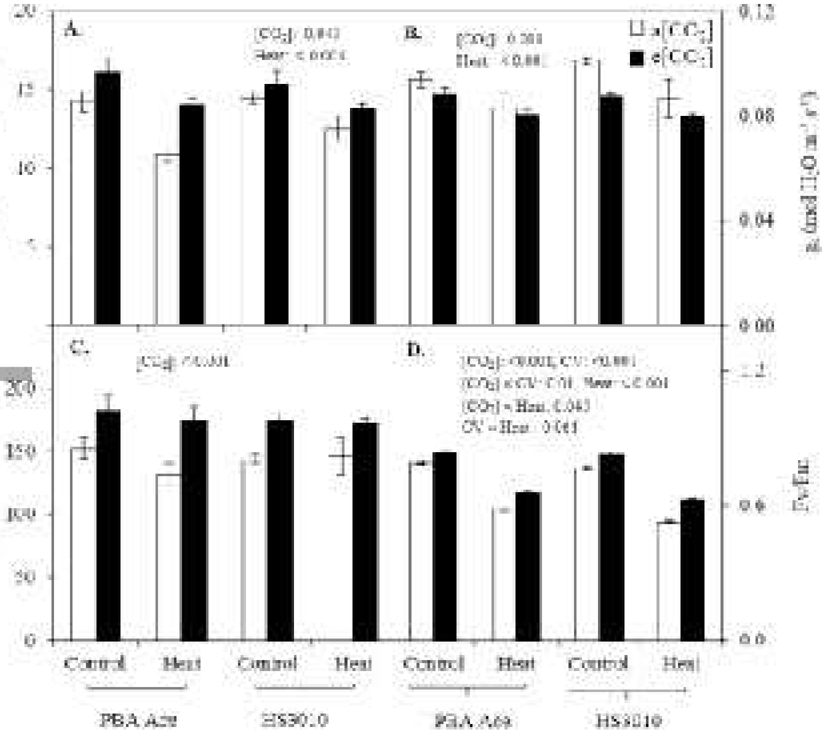
Fig. 3. N content (kg ha^{-1}), N_2 fixation (kg ha^{-1}) and soil N uptake (kg ha^{-1}) of two lentil genotypes measured at physiological maturity (R8) grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}). Data points represent mean values and standard errors of n=4 replicates. P values indicate the significance of the effect of [CO_2], heat, genotypes (CV) as well as their interaction. Only effects $P<0.100$ are shown.

Fig. 4. N_2 fixation as a function of active nodule biomass at R4 (A) and at R5 (B), and relationship between N_2 fixation and remobilization (C) of lentil grown under ambient [CO_2] (~550 µmol mol^{-1}) or elevated [CO_2] (~550

μmol mol^{-1}) and subjected to heat wave during R4. Significant relations are shown at $P < 0.05$. Data points represent mean values and standard errors of n=4 replicates. For 4C, dotted line and equation in grey font colour represent linear relation under control condition, while solid line with equation in black font colour indicates relation under heat wave condition.

Fig. 5. N remobilization (kg ha^{-1}) in leaf (A), stem (B), chaff (C) and total (D) of two lentil genotypes measured at physiological maturity (R8) stage when grown under ambient [CO_2] (a[CO_2], ~400 μmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 μmol mol^{-1}) and exposed to an acute heat wave during the flat pod stage. Each bar represents mean values and standard errors of n=4 replicates. P values indicate the significance of the effect of [CO_2], heat, genotypes (CV) as well as their interaction. Only effects P<0.100 are shown.

Fig. 6. Under heat wave, elevated [CO_2] increased chlorophyll florescence (Fv/Fm) and photosynthesis, thus provided higher carbohydrate supply to ensure greater nodulation and N_2 fixation process. In addition, faster leaf N remobilization under elevated [CO_2] was associated with lower amino acids accumulation in leaves/nodules, thus avoided feedback inhibition of N_2 fixation and maintained grain N concentration following heat wave. Red negative sign (-) refer to the decrease in relative response in elevated [CO_2] compared to ambient [CO_2], while blue positive sign (+) show an increase in relative response. Solid arrows represent direct effects, while dotted arrows show indirect effects.



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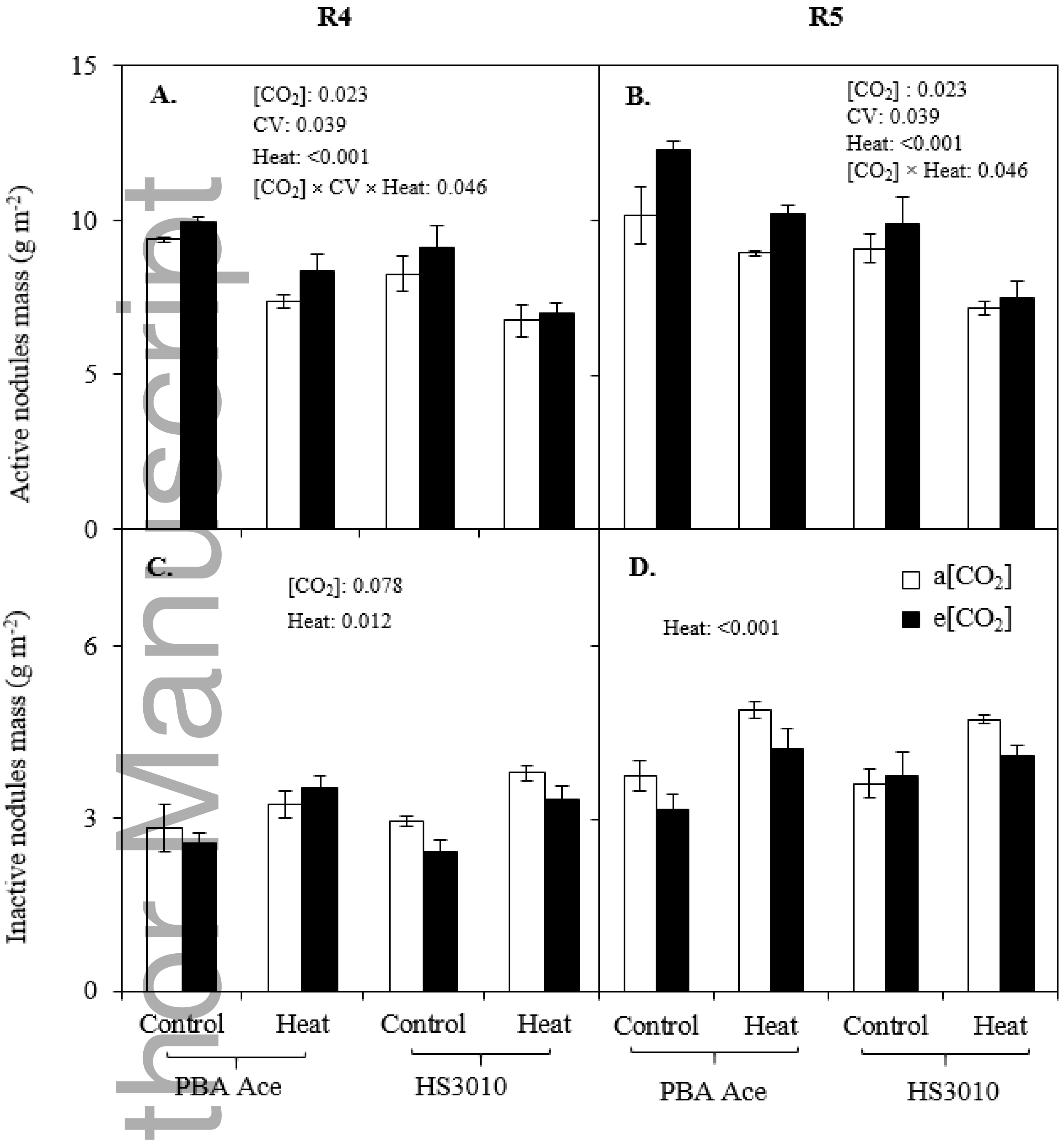

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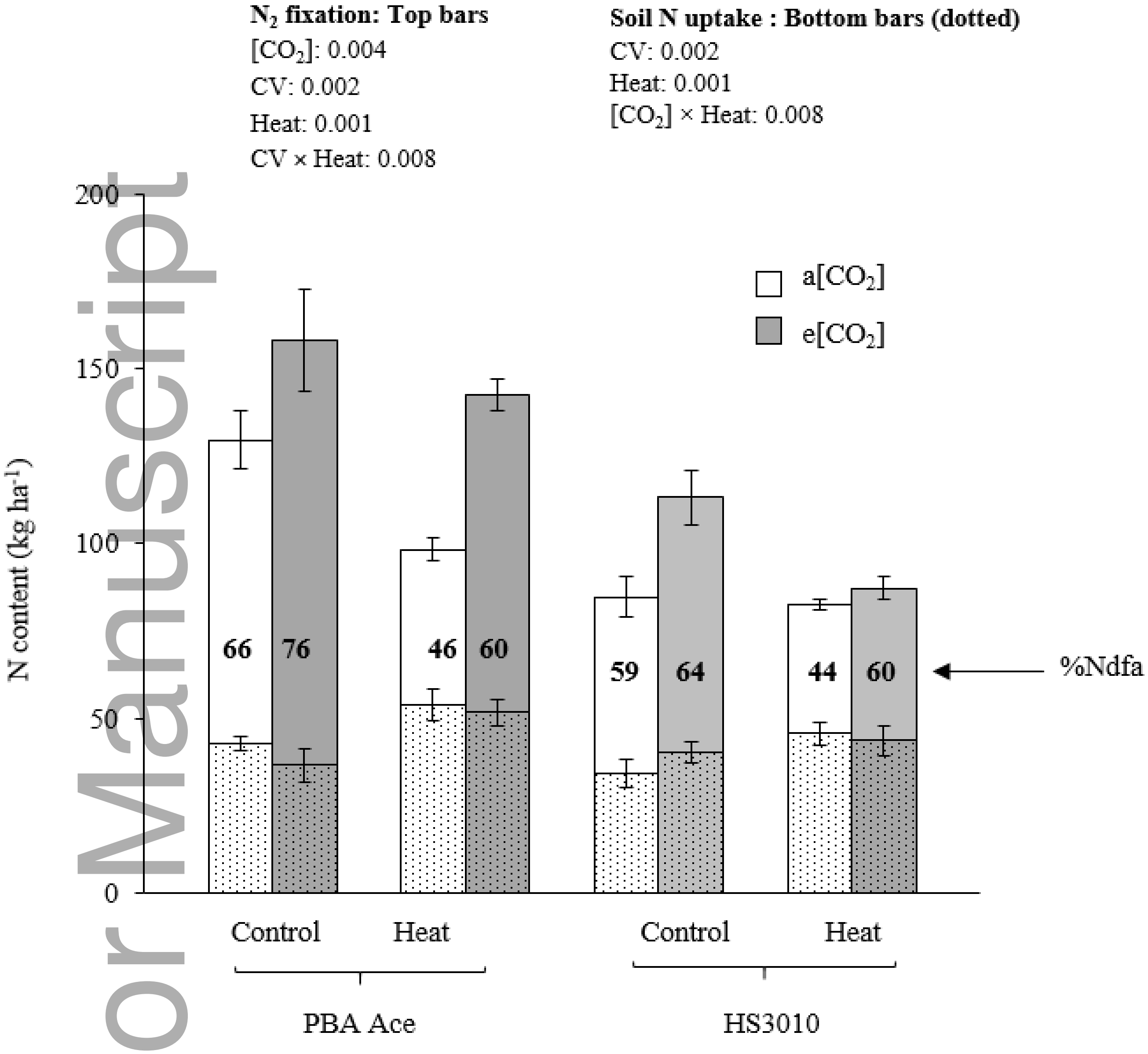

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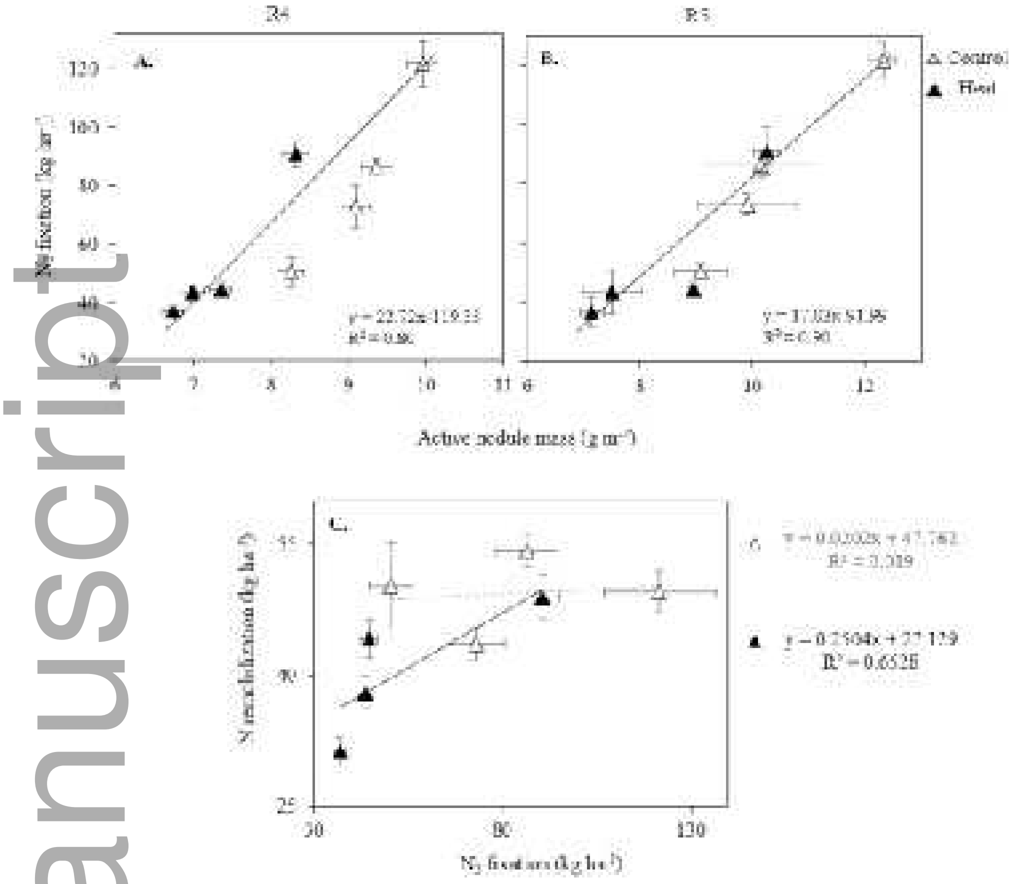

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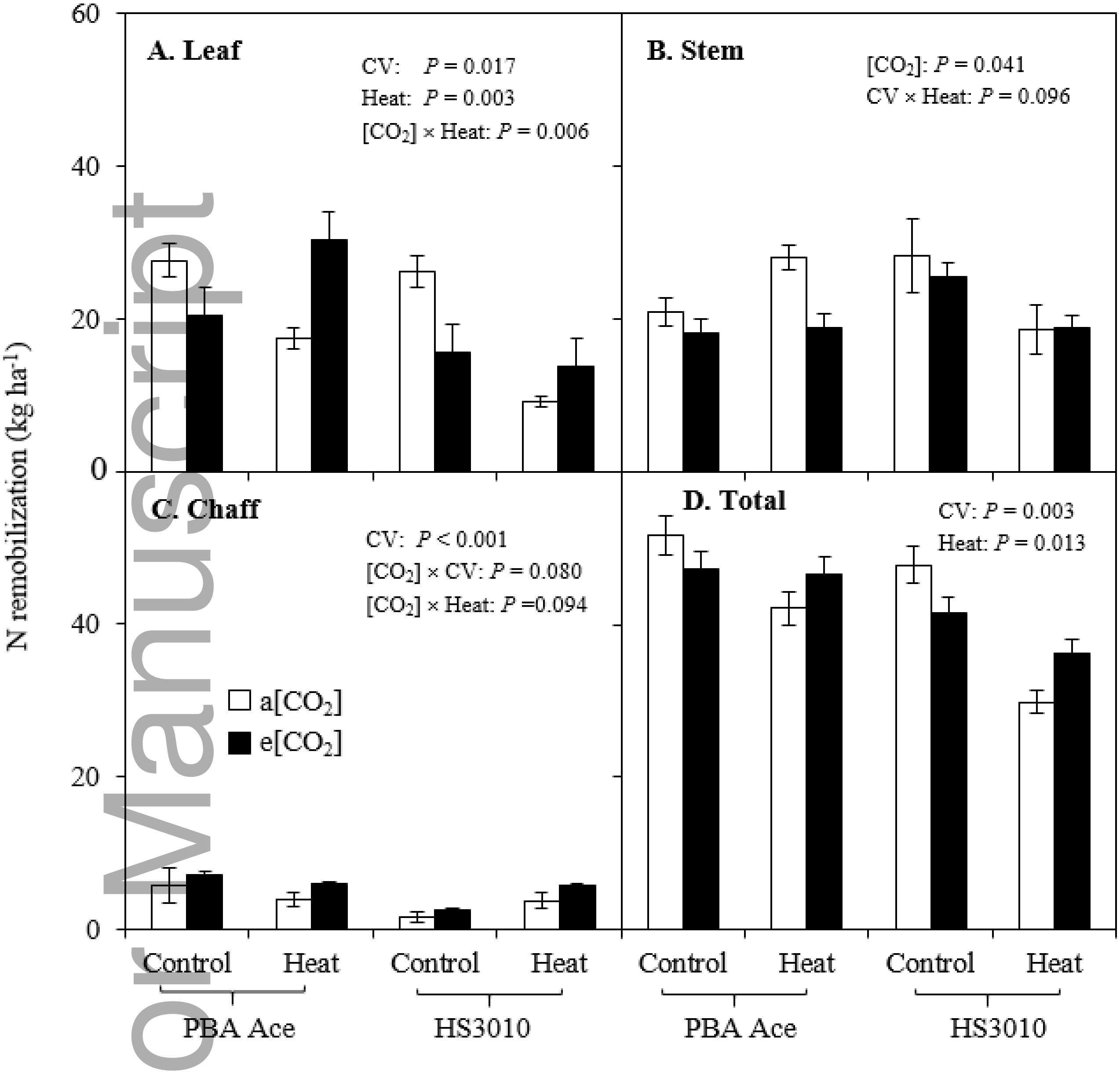

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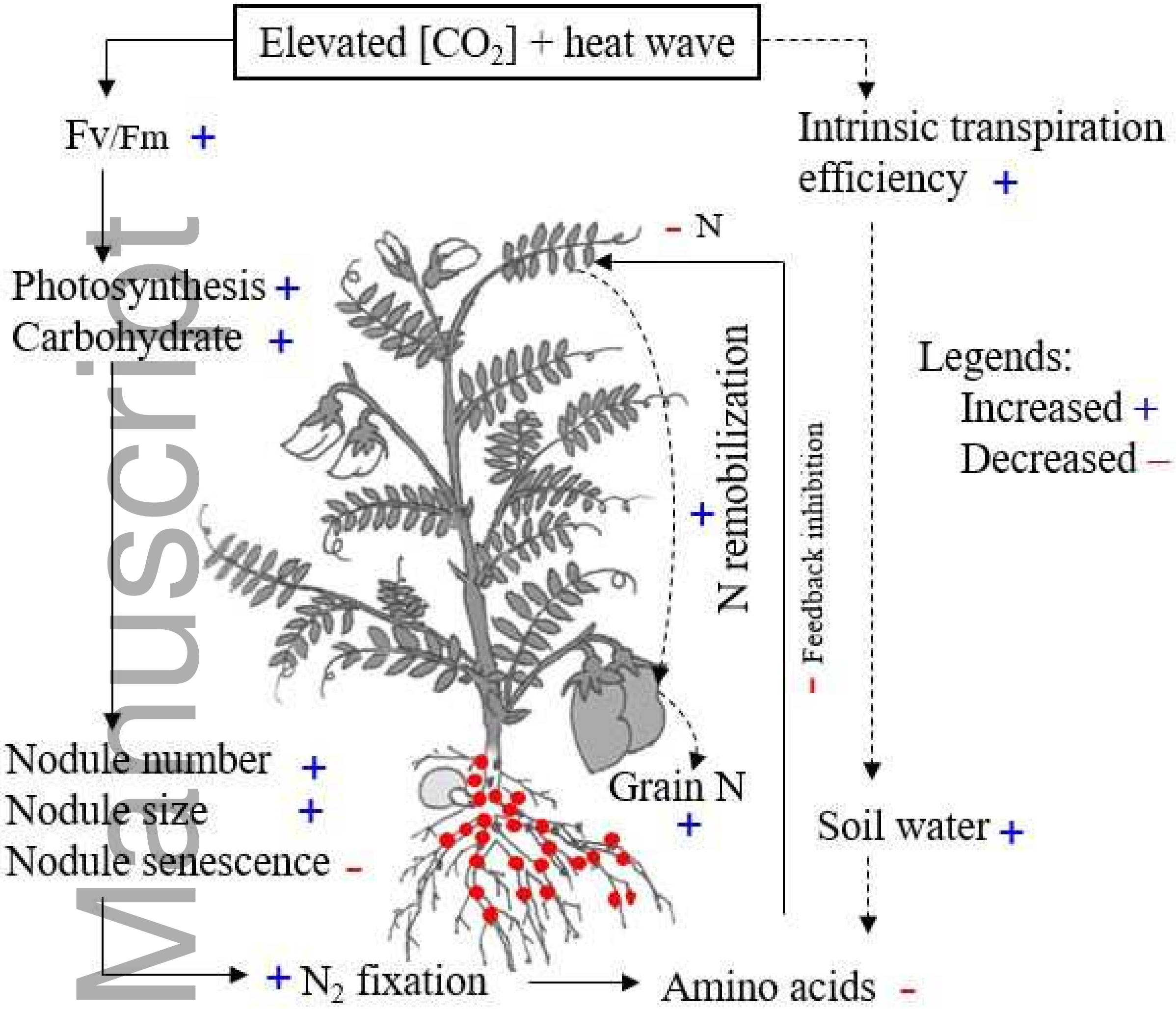

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