Metabolic responses relating to changes in N_2 fixation of lentil (*Lens culinaris* MEDIK.) under contrasting growing seasons in a Free-Air CO_2 Enrichment (FACE) facility

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Abstract
Elevated CO_2 (e[CO_2]) can stimulate N_2 fixation of legumes via increases in photosynthetic carbon supply to symbionts. N_2 fixation mechanisms are highly sensitive to drought but little is known about changes in nodule metabolism under e[CO_2] and water restriction. To address these challenges, N_2 fixation and changes of carbohydrate and nitrogen metabolic profiles were investigated in lentil (*Lens culinaris* MEDIK.) grown under ambient [CO_2] (~400 ppm) and e[CO_2] (~550 ppm) in the Australian Grain Free Air CO_2 Enrichment (AGFACE) facility over two seasons with strongly contrasting rainfall. Elevated [CO_2] stimulated N_2 fixation to a greater extent in a wet season than in a dry season. The lower stimulation during a dry season was associated with decreased sugars and organic acids but increased concentration of sugar-alcohols and certain amino acids in nodules. Metabolic changes under e[CO_2] may have contributed to mitigating drought impacts on N_2 fixation of lentil.

Keywords: FACE, nodule C-and N- metabolites, N-feedback inhibition, drought

Introduction
Atmospheric [CO_2] has been increasing from ~280 ppm pre-industrial to about 400 ppm currently and is predicted to increase to 550 ppm by 2050 (IPCC, 2014). Because CO_2 is a direct substrate for photosynthesis, elevated [CO_2] stimulates carbohydrate production and growth of C_3 crops . In legumes, e[CO_2] increases N_2 fixation through providing greater carbohydrate supply to root nodule bacteria (Rogers et al., 2006). Sucrose, the main photoassimilate provided by shoots to nodules, enters the glycolytic pathway to provide carbon skeletons, predominantly in the form of malate, feeding bacteroid respiration and maintaining nitrogenase activity (Ladrera et al., 2007). In return, root nodule bacteroids convert N_2 to NH_4^+. Depending on legume species, NH_4^+ is metabolized into either amino-N or ureides and translocated through the xylem to the aerial part of plants (King and Purcell, 2005). Carbon and N metabolism of nodule bacteria and their host plant is key to regulating the N_2 fixation process. The status of host and nodule metabolism can be elucidated through analysing metabolite profiles.

As climate change advances with rising atmospheric [CO_2], droughts are predicted to occur more frequently and to be of greater severity in the future (IPCC, 2014). Up- and down-regulation of nodule metabolites has been observed under drought. For example, concentrations of hexoses (e.g. sucrose, fructose, glucose), minor sugars (e.g. trehalose and mannitol), organic acids (e.g. malate, fumarate, isocitrate) and amino acids (asparagine, GABA, β-alanine, alanine, and proline) increased under water restriction in *Medicago truncatula* (Aranjuelo et al., 2013). Such studies on metabolite profiles have been conducted in controlled environments, and mostly under ambient [CO_2] conditions. It has been suggested that e[CO_2] mitigates the impact of stress on sugar and amino acid metabolism, because [CO_2] may stimulate C-assimilation. As well, e[CO_2] may also lead to better conservation of soil water, and thus maintain the activities of the Calvin cycle, sucrose and amino acid metabolism for longer during drought (De Souza et al., 2015). How e[CO_2] would

modify the response of C- and N-metabolites in relation to N_2 fixation under drought is not well understood. Therefore, we conducted an experiment exposing lentil (*Lens culinaris* MEDIK) to either ambient [CO_2] (~400 ppm) or e[CO_2] (~550 ppm) under two contrasting growing seasons (dry season and wet season) in a Free Air CO_2 Enrichment (FACE) facility.

Materials and methods

A field experiment was conducted over a low rainfall season (2015, 'a dry season') and another in a high rainfall season (2016, 'a wet season') at the Australian Grains FACE (AGFACE) facility in the Agriculture Victoria research farm in Horsham, Victoria, Australia. Total rainfall during the 2015 growing season (May to November) was 128 mm, which was well below the long-term average (~274 mm), whereas in 2016 it was 334 mm, well above the long-term average for the study area. A detailed description of the AGFACE site and the CO_2 exposure facility is given by Mollah et al. (2009). In both seasons, experiments were designed as fully randomized complete block with four replications, including 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}. Seeds of lentil (cv. PBA Ace) were inoculated with Group F® (WSM1455, *Rhizobium leguminosarum*) peat-based inoculant and were hand sown on 22 May 2015 and 01 June 2016 with row spacing of 0.24 m. The plot size was 1.5 m by 4 m length in 2015 and 1.5 m by 2 m length in 2016. Destructive samples were collected at full bloom stage (R2) (Erskine et al., 1990) and nodules were excavated using soil-corer (10 cm diameter) to 40 cm depth. Freshly harvested nodule tissues were immediately frozen in liquid nitrogen and stored at -80°C until analysis. ^{15}N values were measured by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon) to estimate N_2 fixation by the ^{15}N natural abundance method (Parvin et al., 2018). Nodule metabolites were analysed using GC-QqQ-MS and LC-QqQ-MS techniques (Dias et al., 2015). For N_2 fixation, analysis of variance (ANOVA) was performed by linear mixed-effect model fit by REML using R package "nlme" (Pinheiro et al. 2017) considering season and [CO_2] as fixed effect and ring numbers as random effect. Statistical effects were considered significant when $P < 0.05$. Heat map was produced through the web-based, open-source metabolomic data analysis tool MetaboAnalyst version 3.0. Before analysis, metabolite data were checked for data integrity and normalized using MetaboAnalyst normalization protocols (selecting normalization by sum, log transformation, and autoscaling).

Results

Elevated [CO_2] increased N_2 fixation proportionally more in the wet than in the dry season (Table 1). In the dry season, N_2 fixation was 15% higher under e[CO_2] compared with a[CO_2], but in the wet season, this stimulation was 47% greater. Using a GC-QqQ-MS and LC-QqQ-MS metabolomics approach, a total of 65 known metabolites were identified and quantified in nodule tissues. Among them, eight sugars and sugar-alcohols, three organic acids and 11 amino acids were affected significantly by season and [CO_2] treatments (season × [CO_2], P<0.005) (Figure 1). In the dry season, the concentration of sugars and organic acids was lower but the concentration of certain amino acids (asparagine, GABA, proline and lysine) was higher. Contrasting trends were evident in the wet season. In the dry season, the concentrations of mannitol, inositol, glutamine, aspartic acid were slightly increased, whereas the concentrations of sucrose, glucose, maltose, asparagine, GABA and tyrosine were slightly decreased under e[CO_2]. In the wet season, e[CO_2] increased the concentrations of sucrose, malate, succinate, aspartate, tyrosine and valine.

Table 1 N_2 fixation (kg ha^{-1}) of lentil 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 wet (2016) season. Means and standard errors of n=4 replicates.

Dry season		Wet season		P-value		
a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	Season	[CO_2]	Season × [CO_2]
72.6±4.0a	89.8±9.3a	129.7±4.5b	191.7±8.4c	<0.001	0.005	0.001

Discussion

Elevated [CO_2]-induced stimulation of N_2 fixation is lower in the dry than the wet season (Parvin et al., 2018). This response can be explained by the changes in C- and N-metabolite profiling in nodules. In our study, sucrose, glucose and malate concentrations were lower under e[CO_2] during a dry season than a wet season. Sucrose produced in leaves is transported to roots and nodules, where it is then hydrolysed through the glycolytic pathway to form phosphoenol pyruvate (PEP). PEP then combines with respiratory CO_2 to form oxaloacetate and then malate. Malate can be either used as a source of carbon for bacteroid

consumption or enter the mitochondria through the TCA cycle (Aranjuelo et al., 2013). In bacteroid, malate provides ATP and reducing power to the nitrogenase enzymes to convert inert N_2 into ammonia and then synthesizes amino acids (Ladrera et al., 2007). The concentration of malate decreased in nodules in the dry season, indicating decreased C flux towards the TCA cycle for the assimilation of NH_4 into amino acids. This might be associated with decreased N_2 fixation in the dry season.

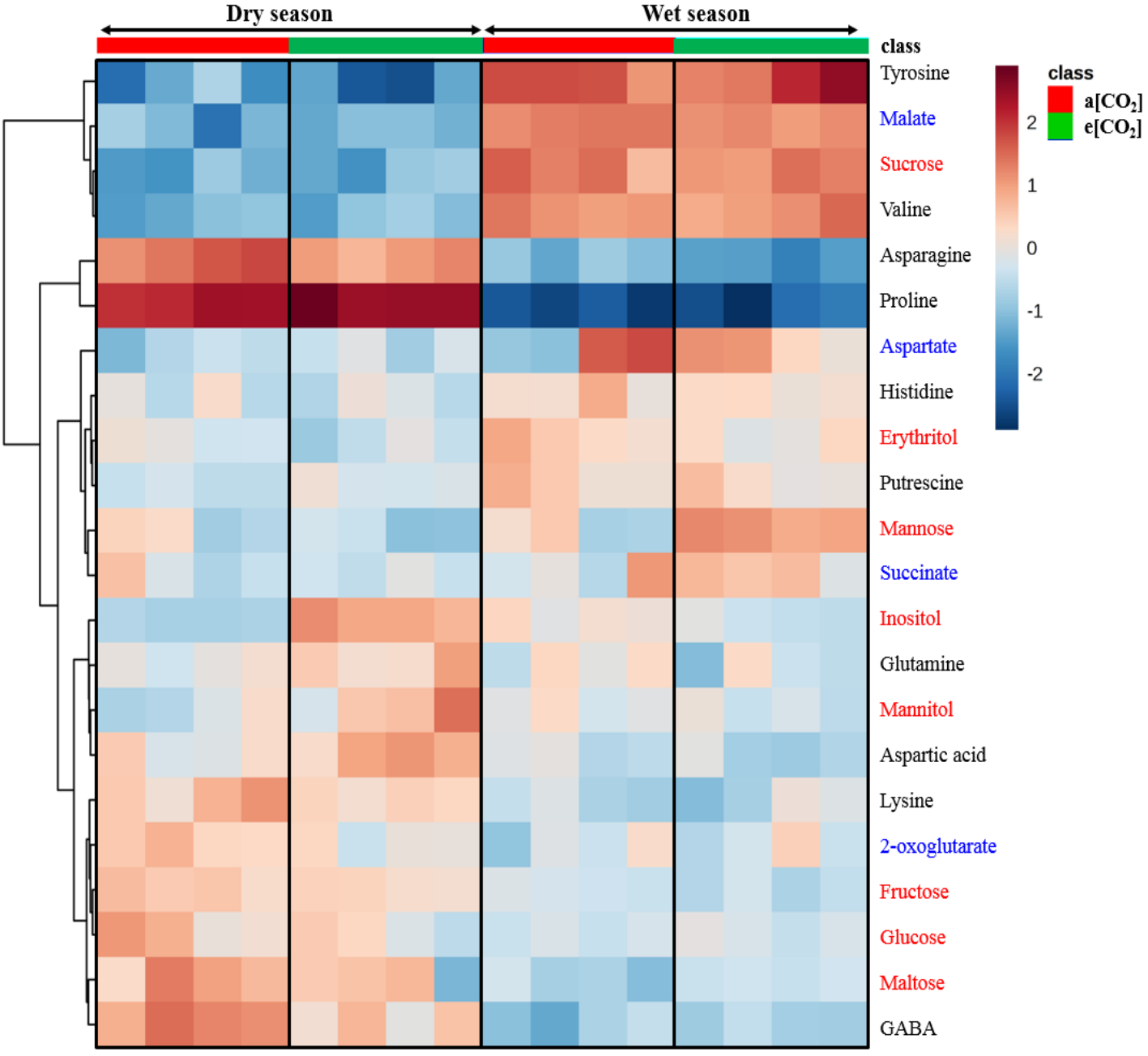

Figure 1 Hierarchically clustered heat maps of nodule metabolites that were found to be statistically significant between season × $[CO_2]$ interactions. Lentil was grown under ambient $[CO_2]$ (aCO_2) or elevated $[CO_2]$ (eCO_2) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a wet (2016) season. The heat map was generated using Pearson and Ward for distance measure and clustering algorithm, respectively using MetaboAnalyst (see text). Each cell represents one replicate. The intensity of red and blue indicates an increase and decrease response relative to the mean according to the colour scale to the right of the heat map. Groups of metabolites are indicated by changes in font colour (sugars and sugar-alcohols: red colour, organic acids: blue colour and amino acids: black colour).

The accumulation of transport-related amino acids, mainly asparagine, in nodules under water restriction may lead to the inhibition of N_2 fixation. In this study, asparagine concentrations were significantly lower under e[CO_2] in the dry season, suggesting that e[CO_2] partially avoided N_2-feedback inhibition. As asparagine is synthesized by the transfer of an amino group from glutamine to aspartate, the synthesis of asparagine is likely to be dependent on the availability of glutamine and aspartate (Carter and Tegeder, 2016). We observed a lower concentration of aspartate but slightly higher abundance of glutamine under e[CO_2], indicating e[CO_2] down-regulated asparagine synthesis to escape N_2-feedback inhibition. In addition, increased concentrations of osmo-protectants such as mannitol, inositol, and proline under e[CO_2] in the dry season might be associated with greater drought tolerance (Obata and Fernie, 2012).

Conclusion

Our findings suggest that e[CO_2] partially offset the effect of drought on N_2 fixation through up-or downregulating the pools of metabolites or changing their biochemical pathways related to drought tolerance.

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