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Biochemical and physiological mechanisms of legume nitrogen fixation under higher atmospheric CO_2 concentrations

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ABSTRACT

Atmospheric CO_2 concentration ($[CO_2]$) is expected to rise from a current level of ~400 to 550 µmol mol^{-1} by 2050. It is well established that elevated $[CO_2]$ enhances plant growth and yield. However, the stimulation of plant growth at elevated $[CO_2]$ requires additional nitrogen (N) and prolonged exposure to elevated $[CO_2]$ potentially risks N limitation. Legumes can overcome such limitations by fixing aerial N. Previous studies under Free Air CO_2 Enrichment (FACE) have shown that elevated $[CO_2]$ can stimulate N_2 fixation, but it is unknown to what extent this applies to dryland Mediterranean environments or what impact environmental interactions have. Legumes grown in dryland environments frequently experience terminal drought accompanied by high temperature during reproductive phases. It has been suggested that elevated $[CO_2]$ delays the effect of drought by conserving soil water, maintaining N_2 fixation mechanisms for longer under drought. This thesis addresses this gap by investigating the growth and N economy of three legumes (lentil, field pea and faba bean) in a FACE facility in a semi-arid environment where seasonal and experimentally controlled drought was imposed. In addition to N_2 fixation itself, the supply and translocation of N compounds to the maturing grain is another point of interest, because it is crucial in maintaining grain N concentration. This thesis investigated N_2 fixation, remobilization and grain quality of dryland legumes under predicted future e$[CO_2]$ atmosphere conditions, including interactions with drought, heat waves, and genotypes. Free Air CO_2 Enrichment technology was used to simulate future growing conditions in the field with target $[CO_2]$ as expected by the middle of this century. Elevated $[CO_2]$ stimulated N_2 fixation through increased nodule number, nodule biomass, and nodule activity to a greater extent under unstressed conditions. Soil water savings under elevated $[CO_2]$ were only temporary, so that drought reduced nodule activity due to lower C/sucrose supply and therefore decreased N_2 fixation. Consequently, elevated $[CO_2]$ was found to stimulate N_2 fixation of all three species of legumes, but this effect was smaller under drought or heat stress. The decrease of N_2 fixation under drought caused depletion of grain N concentration under elevated $[CO_2]$. In contrast, when soil water was sufficient, N_2 fixation continued throughout the grain filling period, and grain N concentration was maintained under elevated $[CO_2]$. Traits that allow N_2 fixation for longer throughout the growing season, e. g. by exploiting potential water savings mechanisms under elevated $[CO_2]$, may confer benefits under future climatic conditions. Findings of this study are now available to underpin new strategies for improvement of the N_2 fixation potential of legumes as atmospheric $[CO_2]$ continues to increase in the future.

Declaration

This is to certify that

i. the thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100000 words in length, exclusive of tables, bibliographies, and appendices.

Shahnaj Parvin

Preface

The experiment reported in **Chapter 2** was conducted in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility to evaluate N_2 fixation and grain N response of lentil grown under e[CO_2]. I identified the research gaps and collected samples from the AGFACE site, performed laboratory works, and statistical data analyses, and wrote the chapter with scientific input from my supervisors and other scientists from our team and another collaborating organization (Agriculture Victoria). An edited version of this chapter was published in **Plant, Cell and Environment** with the title "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" (Shahnaj Parvin, Shihab Uddin, Maryse Bourgault, Ute Roessner, Sabine Tausz-Posch, Roger Armstrong, Garry O'Leary, Glenn Fitzgerald and Michael Tausz). Co-author contributions to this publication: Shihab Uddin and Maryse Bourgault supported sample collection and processing. Michael Tausz, Glenn Fitzgerald, Roger Armstrong, Garry O'Leary, and Sabine Tausz-Posch contributed to the design and maintenance of the AGFACE field facility. Ute Roessner, Glenn Fitzgerald, and Michael Tausz as my PhD supervisors gave support on editing and drafting the manuscript.

Linking C-and N-metabolites for enhancing N_2 fixation of lentil, **Chapter 3**, is based on an experiment conducted in the AGFACE in two contrasting growing seasons, which differed in water regimes. In this chapter, metabolite profiles from leaves and nodules were evaluated through gas chromatography and liquid chromatography. Samples were collected from the experiment described in detail in chapter 2. I collected samples, analysed the data and produced graphics using various multivariate statistical software. I wrote the chapter under direct guidance from my supervisors. Based on this chapter, a manuscript is under preparation to be submitted to a journal under the title "Metabolite profiling reveals distinct changes in C-and N-metabolism of lentil (*Lens culinaris* MEDIK.) exposed to seasonal drought under Free-Air CO_2 Enrichment (FACE) facility".

N_2 fixation response of field pea under short-term/periodic terminal drought, **Chapter 4**, is based on an experiment conducted in the Soil Free Air CO_2 Enrichment (SoilFACE) facility, where I designed and conducted my experiment, collected gas exchange measurements, assessed N_2 fixation and carried out all laboratory analyses and statistical data analyses. I also washed the roots systems and examined nodulation attributes. I wrote the chapter with input from my supervisors and other AGFACE project collaborators. An edited version of this chapter was published in **Plant and Soil** as "Free Air CO_2 Enrichment (FACE) improves water use efficiency and moderates drought effect on N_2 fixation of *Pisum sativum* L." (Shahnaj Parvin, Shihab Uddin, Glenn J Fitzgerald, Sabine Tausz-Posch, Roger Armstrong, Michael Tausz). Co-author contributions to this paper: Shihab Uddin contributed to sample collection and processing. Sabine Tausz-Posch and Roger Armstrong provided research funding and valuable input into the conception, design, and operations of the SoilFACE field experiments. Glenn J Fitzgerald and Michael Tausz as my PhD supervisors reviewed and advised on the draft manuscript.

N_2 fixation potentials of faba bean under terminal drought, **Chapter 5**, is based on an experiment conducted in the Soil Free Air CO_2 Enrichment (SoilFACE) facility, which was a sub-facility of the broader AGFACE facility, where I designed the experiment, measured gas exchange parameters, evaluated N_2 fixation and soil N uptake and carried out all laboratory analyses and statistical data analyses. I also did root washing, collected nodules and

prepared samples for ^{15}N analysis. I drafted the chapter with input from my supervisors and other AGFACE project collaborators. An edited version of this chapter was published in **Environmental and Experimental Botany** as "Elevated CO_2 improves yield and N_2 fixation but not grain N concentration of faba bean (*Vicia faba* L.) subjected to terminal drought" (Shahnaj Parvin, Shihab Uddin, Sabine Tausz-Posch, Glenn Fitzgerald, Roger Armstrong, Michael Tausz). Co-author contributions to this paper: Shihab Uddin contributed to sample collection and processing. Sabine Tausz-Posch and Roger Armstrong provided research funding and valuable input into the conception, design, and operations of the SoilFACE field experiments. Glenn J Fitzgerald and Michael Tausz as my PhD supervisors helped in drafting the manuscript.

Interaction of e[CO_2] and heat waves on N_2 fixation of lentil, **Chapter 6**, is based on the experiment conducted in the AGFACE facility where plants were exposed at reproductive stages to a 3-day heat wave using custom-built heat chambers. I collected samples, did gas exchange measurements and performed laboratory analysis. I analysed the data and drafted the manuscript under the close guidance of my PhD supervisors Michael Tausz and Glenn J Fitzgerald. An edited version of this chapter was submitted to a journal as "Effect of heat wave on N_2 fixation and N remobilization of lentil (*Lens culinari*s MEDIK) grown under Free Air CO_2 Enrichment in a Mediterranean-type environment."

Evaluation of carbon sink strengths of nodules and other organs using $^{13}CO_2$ pulse-labeling, **Chapter 7**, is based on a glasshouse experiment conducted with faba bean during two consecutive seasons. I designed and conducted the experiment, built an air-tight plexiglass chamber for $^{13}CO_2$ pulse-labeling, measured photosynthetic response (A/Ci) curves and fitted the curves using R, collected samples and processed them, performed laboratory analysis and produced graphics. I analysed all the data and wrote the chapter with scholastic guidance from my supervisors. Based on this chapter, a manuscript is under preparation to be submitted to a journal under the title "Carbon sink strength of nodules and other organs of faba bean (*Vicia faba* L.) grown under elevated [CO_2] and different water supply". Michael Tausz as my PhD supervisor provided suggestions and guidance on writing the manuscript.

Grain minerals quality of dryland legumes under e[CO_2] and drought, **Chapter 8**, is based on the experiment conducted in the AGFACE facility for lentil (Chapter 2) and SoilFACE facility for faba bean (Chapter 5). I designed the experiment, collected samples and analysed the data. Under direct guidance from Michael Tausz, I wrote the manuscript. An edited version of this chapter is published in **Crop and Pasture Science** as "Grain minerals quality of dryland legumes as affected by elevated CO_2 and drought: A FACE study on lentil (*Lens culinaris*) and faba bean (*Vicia faba*) (Shahnaj Parvin, Shihab Uddin, Sabine Tausz-Posch, Roger Armstrong, Glenn Fitzgerald, Michael Tausz). Co-author-contributions to this paper: Shihab Uddin contributed to sample collection and laboratory analysis. Sabine Tausz-Posch, Roger Armstrong and Glenn J Fitzgerald provided research funding and contributed to the design and conception of the FACE experiments. Glenn J Fitzgerald and Michael Tausz as my PhD supervisors gave feedback on draft manuscripts.

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I am grateful to my supervisors Professor Michael Tausz, Associate Professor Glenn J Fitzgerald, Professor Ute Roessner and Professor Gerd Bossinger for their continuous support during my PhD studies. I express my acknowledgments to Gerd Bossinger for his cordial support and guidance in overcoming numerous obstacles I have been facing throughout my PhD study, Glenn J Fitzgerald for writing a lot of reference letters and Ute Roessner for supervising the metabolomic analysis.

Importantly, I would like to direct my heartfelt gratitude, sincere appreciation and acknowledgments to Michael Tausz for his valuable suggestions, unfailing enthusiasm, patience, understanding my hardship, and scholastic supervision throughout the entire period of my PhD journey. Without your excellent guidance and invaluable supports, it would have been impossible to finish and submit my PhD thesis.

I am grateful to Dr Sabine Tausz-Posch, Professor Dr Roger Armstrong and Dr Garry O'Leary for providing invaluable support for conducting my PhD research and publishing the research work in journals. I am also acknowledging the contributions of Dr. Jason Brand and Dr. Garry Rosewarne for providing quality seeds of lentil, faba bean and pea and advising on lentil agronomy practices. Also, Dr Markus Löw and Dr Alireza Houshmandfar for sharing their extensive knowledge of R and teaching me statistical analysis during my PhD. I am thankful for the support by Dr Maryse Bourgault and the help provided by Sam Henty and fellow students Osmin Torres, Yao Dai, Samuel Martin Sanchez, and Chinthaka Jayasinghe.

Thanks to the Scholarship Office at the University of Melbourne for offering me financial support during the entire period of my study through the Melbourne International Research Scholarship and Melbourne International Fee Remission Scholarship and Postgraduate Writing-Up Grant supported by *The Albert Shimmins Fund.*

My PhD research was supported by the Australian Grains Free Air CO_2 Enrichment (AGFACE) project. AGFACE was jointly run by the University of Melbourne and Agriculture Victoria Research (Department of Economic Development, Jobs, Transport and Resources) with substantial funding from the Grains Research and Development Corporation and the Australian Department of Agriculture and Water Resources. I gratefully acknowledge the contributions of the AGFACE field team lead by Russel Argall, Mel Munn and Roger Perris (all Agriculture Victoria Research) for agronomic management of the experiment and collecting soil water data. Special thanks to Dr Mahabubur Mollah (Agriculture Victoria Research) for operating and maintaining the CO_2 enrichment technology.

I would like to express my gratitude and deep appreciation to my husband/colleague, Dr. Shihab Uddin who tolerated the difficulties of the past four years and made them easier to bear, made the hard times sweeter and taught me so much about scientific research and family life. I dedicate my PhD to my two lovely kids (Ahnaf Shadeed and Sarah Tasnuva) for their kind patience, dedication and sacrifice during this hardest period. Finally, my eternal gratitude to my parents for their well wishes and moral support over the years of my study.

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Chapter 1: Introduction

1.1 Climate change

1.1.1 Increase in atmospheric CO_2 concentration

Prior to the beginning of the Industrial Revolution, atmospheric carbon dioxide concentration ($[CO_2]$) was steady at ~ 270 μmol mol^{-1} for 12,000 years. Twenty-thousand years ago, $[CO_2]$ was even as low as ~ 170 μmol mol^{-1} as gleaned from ice core data (IPCC 2013). Atmospheric $[CO_2]$ has been rising from 315 μmol mol^{-1} in 1959 to a current 400 μmol mol^{-1} (NOAA, 2015) with an annual increase rate of nearly 2 μmol mol^{-1} (Keeling and Whorf, 2005). Most of the IPCC scenarios project that atmospheric $[CO_2]$ will reach 550 μmol mol^{-1} by 2050 and >700 μmol mol^{-1} at the end of the century (IPCC, 2013). Increasing emissions from burning fossil fuels and land use changes are considered the main reasons for the increase in atmospheric $[CO_2]$ (Carter et al., 2007; Peters et al., 2011).

1.1.2 Increasing $[CO_2]$ changes Earth's climate

Rising atmospheric CO_2 is associated with an increase of global average temperature (by 1.4-5.8ºC depending on scenarios: IPCC, 2013), along with alterations of precipitation patterns such as more frequent and severe incidents of drought by the end of this century (IPCC, 2013) with some predicting ten-fold increases in drought events (Kundzewicz et al., 2008). As a result, overall drying will cause a decrease of the Palmer Drought Severity Index of 0.3 and 0.56 globally for the first and the second half of this century, respectively (Burke et al., 2006). Agricultural crop production will be especially affected by extreme weather events such as more severe and frequent incidents of drought and heat, which are created by the changes in global climate along with rising CO_2.

1.1.3 Consequences for crop production (especially in Australia)

Mediterranean-type environments are predominantly characterised by winter rainfall (250-750 mm/year) along with very hot and dry summers. These environments are already water-limited, and crops mature under terminal drought (drought during the final phase of crop development in late spring and early summer). Any further drying of soils or more severe heat waves can, therefore, have negative impacts on crop production. Such Mediterranean environments are prevalent among Australian agro-ecosystems. As a result, Australia has already experienced extensive droughts alongside considerable heat waves (de Oliveira et al., 2013; Tambussi et al., 2007). According to the Commonwealth Bureau of Meteorology, the coastal and inland fringe surrounding Australia (especially north of Darwin) receive > 600 mm while mid-latitude areas (especially Alice Springs) receive < 300 mm of rainfall each year. As a result, Australia has a very low annual average rainfall of 419 mm.

The uncertainty of rainfall and prevalence of drought events create considerable risk with regards to estimating benefits derived from N fertilizer application. For plants to take up N, the N must move with water toward the root — a process called mass flow**.** Even though the soil contains adequate amounts of N, plants may exhibit symptoms of N deficiency if soil water is scarce (Guo et al., 2007). Growing legumes as providers of N in rotation with cereals can improve soil fertility in such dry cropping systems (Unkovich et al., 1997).

1.2 Crop response to elevated CO_2

1.2.1 Growth and yield under elevated [CO_2]

The photosynthesis of C_3 plants is increased under elevated [CO_2] (e[CO_2]), which increases carbohydrate acquisition and consequently, leads to better growth and yield (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; Long et al., 2004). This phenomenon is called the "CO_2 fertilization effect". The degree of photosynthetic stimulation under e[CO_2] has also been reported to vary among species such as C_3 non-legume crops (13%) and legumes (19%) (Leakey et al., 2009). Greater photosynthesis under e[CO_2] stimulated above ground plant biomass by on average 20% for C_3 species grown in six different FACE (Free Air CO_2 Enrichment) studies (Long et al., 2006). Quantitative reviews of many FACE experiments revealed that e[CO_2] (550 μmol mol^{-1}) increased grain yield of C_3 non-legumes, C_3 legumes and C_4 plants by 11–31%, 14–54%, and 18–27%, respectively (Kimball, 1983; Long et al., 2006).

1.2.2 N limitation under e[CO_2]

Long-time exposure of C_3 species to e[CO_2] may cause a decrease of the initial stimulation of photosynthesis, a phenomenon that is known as photosynthetic acclimation (Long et al., 2004). Photosynthetic acclimation under e[CO_2] is commonly assessed by measuring the reduction in maximum carboxylation rates ($V_{c,max}$) of the main photosynthetic enzyme called Rubisco (Riboluse-1, 5 biphosphate) and maximum electron transport rates leading to ribulose-1,5- bisphosphate (RubP) regeneration (J_{max}) (Ainsworth and Rogers, 2007; Leakey et al., 2009; Long et al., 2004; Nowak et al., 2004). A review by Tausz et al. (2013) highlighted two major hypotheses put forward to explain photo-synthetic downward acclimation to e[CO_2]: i) the "Sink limitation" hypothesis and ii) the "Nitrogen limitation" hypothesis. The sink limitation hypothesis suggests that photosynthetic capacity decreases under e[CO_2] because the synthesis of Rubisco (Riboluse-1, 5 biphosphate) is down-regulated. Rubisco consists of two types of protein sub-units, called large chain (L) and small chain (S). The *large-chain* gene (*rbcL*) is part of the chloroplast while the *small chain gene* (rbcS) is in the nucleus of the plant cell and their expression is primarily restricted to leaves (Patel and Berry, 2008). Higher accumulation of carbohydrates in leaves under e[CO_2] suppresses the expression of *rbcL* and *rbcS* genes and decreases photosynthetic efficiency of C_3 plants (Rogers et al., 1998).

The N limitation hypothesis suggests that photosynthetic acclimation is associated with deficiency of N, caused by the failure of N supply to keep up with stimulated biomass. Consequently, higher C uptake under e[CO_2] may dilute N in the total biomass, which limits the Rubisco concentrations in the leaves (Leakey et al., 2009). This hypothesis is consistent with less growth and yield stimulation by e[CO_2] in N deficient compared to N sufficient wheat (Tausz et al., 2013) or with the fact that downward photosynthetic acclimation was observed in a non-nodulating soybean cultivar but not in a nodulating isogenic line (Ainsworth et al., 2004).

Regardless of the exact mechanism, reduction of leaf and grain N content under e[CO_2] is prominent in C_3 non-legumes (10-15%) and less so in C_3 legumes (-1.4%) (Jablonski et al., 2002; Rogers et al., 2009; Taub and Wang, 2008). The decrease of leaf N concentration relative to higher C gains may limit the allocation of N into reproductive tissues and thus deteriorate grain protein concentration (Taub et al., 2008). However, legumes could

overcome such a situation by fixing additional N from the atmosphere and by allocating additional photosynthates to the nodule symbionts (*Rhizobia*) to maintain such fixation.

1.3 Importance of legumes in agro-ecosystems

Legumes belong to the family Fabaceae and the primary feature of legumes is their symbiotic relationship with N_2 fixing bacteria. There are approximate 20000 legume species capable of forming an association with nodule inducing bacteria, collectively called *Rhizobia* (Rogers et al., 2009).

Grain legumes are collectively known as pulse crops and comprise more than sixty different legume species grown across the world. Seeds are rich in proteins and essential amino acids and are known as a "poor man's meat" (Erskine et al., 2011). A large proportion of the global population cannot afford animal products and therefore, relies on legumes for their protein intake. In addition, a growing number of people choose to be vegetarian for health and ecological reasons and depend on pulses to fulfil their daily protein requirements. Pulse seeds also contain special bioactive compounds known as nutraceuticals, which may prevent and cure diseases (Brower, 1998). Frequent intake of legumes has been associated with a reduction in the risk of cardiovascular diseases, diabetes, digestive tract diseases, and obesity (Duranti, 2006). In the foreseeable future, demands for legumes are expected to rise both in developing and developed nations with a focus on healthy diets (Daryanto et al., 2015) and their potential benefits of N_2 fixation from the atmosphere (Zanetti et al., 1996).

In recognition of the importance of pulses in the human diet, 2016 has been declared the International Year of Pulses. For instance, at the global level legume production increased from 150 million tons in the 1980s to 300 million tons in the 2000s (Daryanto et al., 2015; Daryanto et al., 2017). In 2011–2013 (average), pulses accounted for 80.3 million hectares of global arable land area producing 72.3 million metric tons of grain. Australia produced an average of 3.05 million metric tonnes of pulses from more than 1.8 million hectares, which contributed 4.2% of total world pulse production in the year 2011-2013 (Joshi and Rao, 2017). The capability of legumes to fix N through N_2 fixing bacteria (i.e. *Rhizobia*) sets them apart from virtually all other groups of plants. A range of legumes has been introduced into Australian agriculture over the past 20 years (Unkovich et al., 1997). Among several pulses, *Pisum sativum* L., *Lens culinaris* Medik. and *Vicia faba* L. are the most important grain legumes in Australia. Both grain and pasture legumes play key roles in long term-rotation with cereals and other broad-leaved crops in many regions of Australia.

1.4 Mechanisms of nitrogen fixation

Nitrogen fixation is a natural process in which nitrogen gas is reduced to ammonium (NH_4^+) by bacteria with the help of the enzyme nitrogenase (Howard and Rees, 1996). These bacteria are called *Rhizobia* and reside in nodules of the legume roots (Bergerse, 1971). *Rhizobia* contain a special *nif* gene produced on plasmids (called sim plasmids) which regulate N_2 fixation, while the *nod* gene controls nodulation of host plants (Fischer, 1994). Development of root nodules depends on the formation of infection threads between rhizobia and host cells. The symbiotic forms of the genus *Rhizobium* are called bacteroids (containing differentiated rhizobia) and occupy central tissues of root nodules. Inside the root nodule, a bacteroid is isolated from the host cell with a peribacteroid membrane. Leghaemoglobin present in the root nodules gives the characteristic pink colour and regulates the O_2

supply to the cytoplasm (Crawford et al., 2000). The host supplies C (in the form of sucrose or malate) to the bacteroid to fuel N_2 fixation (Rogers et al., 2009). Such organic C is used by the nitrogenase enzyme as a source of energy and provides reducing power to bacteroids for sustaining N_2 fixation. In return, bacteroids provide NH_4^+ to the host plant. NH_4^+ is converted into N rich compounds that are transported through the xylem to the shoots (Olivares et al., 2013). For most temperate legumes, the metabolic products are mainly amides, for tropical legumes such as *Glycine max* L. ureides, while other legumes (such as *Pisum sativum*, *Medicago* spp.) transport amino acids when the plant actively fixes N. Ureides such as allantoin and allantoic acid, or amides and amino acids such as asparagine and glutamine are transported towards the shoots (Tegeder, 2014).

Approximately, 45-50% of newly photosynthesized C is transferred from leaves to the nodules within a 12 hours photoperiod (Lluch et al., 2002; Voisin et al., 2003). Symbiotic nitrogen fixation is regulated by photosynthesis (C supply), soil N availability (N source strength), and N demand (N sink strength) (Aranjuelo et al., 2009). Symbiotic N_2 fixation needs C for nodule function (morphological and structural maintenance as well as reduction of atmospheric N) and the transfer of reduced N as amine products to the shoots (Salon et al., 2001). Therefore, under higher atmospheric CO_2 concentration the C supply to nodules may increase, leading to greater N_2 fixation (Rogers et al., 2009).

1.5 Nitrogen fixation under elevated [CO_2]

Among C_3 crops, legumes have the greatest potential to respond with increased production at higher atmospheric [CO_2] (Rogers et al., 2006) and the hypothesis of greater advantages of legumes over non-legumes under e[CO_2] is supported by some studies (Rogers et al., 2006; Serraj et al., 1998; Soussana and Hartwig, 1995). The acquisition of C and N is tightly linked in N_2-fixing plants and increases under e[CO_2] (Rogers et al., 2009; Ross et al., 2004; Soussana and Hartwig, 1995; Zanetti et al., 1996). Two general mechanisms are expected to stimulate N_2 fixation: (a) a greater C investment in tissues where N_2 fixation occurs, i.e. nodules (increase in number and size) or (b) a greater activity of nitrogenase enzymes, i.e. a greater amount of N_2 fixed per unit nodule biomass over time (Schortemeyer et al., 2002).

Stimulation of photosynthetic C uptake and greater leaf carbohydrate content under e[CO_2] has been confirmed in Free Air CO_2 Enrichment (FACE) studies of *Glycine max* L. and *Trifolium repens* (Ainsworth et al., 2003; Leakey et al., 2009). FACE experiments conducted in northern China reported that e[CO_2] increased the N_2 fixation of *Glycine max* L. by 79% (calculated from the percentage of N derived from the atmosphere, %Ndfa) and the amount of N fixed was 109 kg ha^{-1} higher in e[CO_2] grown *Glycine max* L cv. Zhonghuang 13 (Lam et al., 2012b). As a result, higher biomass production of *Glycine max* L. did not reduce the tissue N concentration, which implies that increased C gain was matched with additional N_2 fixation under e[CO_2] (Lam et al., 2012b; Rogers et al., 2009).

However, the symbiotic nitrogen fixation rate is not constant throughout the growing season, and maximum fixation rate occurs during the flowering stage. Leaf N and amino acid contents were lower under e[CO_2] during the early season in *Glycine max* L. and increased markedly during the middle of the season, which coincided with higher N fixation rate at these growth stages (Rogers et al., 2006).

1.6 Nitrogen acquisition and partitioning under elevated [CO_2]

Nitrogen uptake is strongly determined by the plant's ability to develop a vigorous root system. Increased root biomass of crops grown under e[CO_2] could increase N uptake from soil (Bahrami et al., 2017). Moreover, increases in root length under e[CO_2] may change the spatial patterns of soil exploitation for water and nutrients from different soil layers (Benlloch-Gonzalez et al., 2014; Uddin et al., 2018). Such stimulation of root length under e[CO_2] can benefit dryland crops by improving access to soil N across the soil profile.

N utilisation is associated with the production of biomass and grain yield, and in the context of grain quality, also with mechanisms of remobilising N to grains. C supply generally comes from current photosynthesis whereas reduced N_2 fixation during seed filling (Salon et al., 2001) means that N remobilization from vegetative plant parts plays an important role. Newly assimilated N (derived from soil N and symbiotically fixed nitrogen) is generally not enough to meet seed N requirements. Legumes generally accumulate most N at flowering stages, and this total N content can be used as an indicator for how much N is available for remobilization (Barbottin et al., 2005).

During the grain filling process, deposition of N into seeds is regulated by the N pool available for remobilization. N imported to developing seeds is made available as amino acids produced by proteolysis of proteins (called reserve N) that have been synthesized during vegetative stages (Liu et al., 2008). Rubisco accounts for 50% of the total soluble protein present in C_3 plant leaves. Rubisco together with other photosynthesis-related proteins is the major source of N available for remobilization when leaves begin senescence. The fraction of N stored in the leaf cell wall and in stems is not available for remobilization and is known as structural N (Pask et al., 2012). In the case of legumes, small glycoproteins, called vegetative storage proteins (VSP), accumulate to high levels in the leaves, stems and pod walls, and constitute an additional source of proteins that can be remobilized (Masclaux-Daubresse et al., 2008).

Prospects for enhanced grain legume seed filling include i) optimizing C and N partitioning between nodules and host plants, ii) prolonging symbiotic N_2 fixation during seed development (Salon et al., 2001) and iii) increasing the N pool available for remobilization. Legumes grown under e[CO_2] can have higher C supply to nodules (Aranjuelo et al., 2008; Cabrerizo et al., 2001; Sanchez et al., 2010). This increased C availability under e[CO_2] could extend the duration of N_2 fixation (Peoples et al., 1995). Considering this, higher N_2 fixation could strengthen the N pool for remobilization to the grain (Lee et al., 2003). However, it is not yet clear under which circumstances higher N_2 fixation or changes in N allocation/remobilization pattern can counteract the potential decrease of seed protein concentration of legumes under e[CO_2].

1.7 Effects of environmental factors on N_2 fixation

The physiological state of host plants governs N_2 fixation which is susceptible to various environmental stresses such as drought (Serraj, 2003a; Zahran, 1999), temperature (Aranjuelo et al., 2007; Hungria and Vargas, 2000), salinity etc. (Ben Salah et al., 2009; Ben Salah et al., 2011). Under these conditions, the major processess inhibiting nodule function are: (a) less carbohydrate supply to nodules, (b) accumulation of nitrogenous compounds, (c) reduced O_2 availability to bacteroids and (d) accumulation of reactive oxygen species (Aranjuelo et al., 2014).

1.7.1 Drought response

Nitrogen fixation is sensitive to drought and this has been assessed for a few grain legumes including soybean (*Glycine max* L.), cowpea *(Vigna unguiculata* L.), black gram *(Vigna mungo* L.), chickpea (*Cicer arietinum* L.), common bean *(Phaseolus vulgaris* L.), faba bean (*Vicia foba* L.), lupin *(Lupinus albus* L.), pea *(Pisum sativum* L.), and peanut *(Arachis hypogaea* L.) (Sinclair and Serraj, 1995). Drought has pronounced effects on all processess of nodule functions. It inhibits N_2 fixation (Rogers et al., 2009) and decreases nodule initiation, growth, and activity (Aranjuelo et al., 2014). Leaf level photosynthesis declines sharply under moderate drought stress. The transport of photosynthates and N compounds in the phloem and xylem also drops because of reduced water flux in the drought-stressed plant (Serraj, 2003b). The result is a lower investment of photoassimilates into the bacteroid with a consequent accumulation of N compounds in nodules, which further limits N_2 fixation (Serraj, 2002).

Drought-induced decreases in N_2 fixation have been associated with increases in ureides, amides, and other amino acids in the leaf and root nodules (Rogers et al., 2009; Serraj, 2003a; Serraj et al., 2001). Soybean usually transports more than 80% of the N-compounds in the form of ureides (Serraj et al., 2001). Ureide exporting species were found to be more sensitive to drought than amide exporters (King and Purcell, 2005). However, under severe drought conditions, other metabolites like sugars (sucrose, hexose, trehalose, raffinose etc,), sugar alcohols (galactinol, myo-inositol, pinitol etc.), and organic acids (fumarate, malate) etc. also accumulated in nodules (Aranjuelo et al., 2013b). A characterization of the metabolite composition of nodules also showed high levels of ascorbic acids and proline under severe water stress (Marino et al., 2006).

Drought has deleterious effects on the leaf N status as well as Rubisco activity. Studies conducted in *Medicago sativa* (Aranjuelo et al., 2011) revealed depletion of leaf level Rubisco and amino acid contents in stressed plants. Shoot level N demand is reduced in drought-stressed plants, and this could cause an accumulation of amino acids in nodules. Several compounds such as amino acids and ureide have been considered for such an N feedback mechanism (King and Purcell, 2005; Larrainzar et al., 2009; Serraj et al., 2001; Sulieman and Schulze, 2010). In addition to this, the activity of enzymes (malate dehydrogenase and aspartate aminotransferase) involved in N_2 assimilation was also negatively affected in stressed plants (Aranjuelo et al., 2009; Aranjuelo et al., 2007).

The current knowledge of nodule performance under drought conditions has focused on carbohydrate shortage, accumulation of N compounds and other metabolites. However, little information is available about how shoot N demand may reduce the nodule functions, and the physiological mechanisms underlying such a response remain unknown.

1.7.2 Elevated [CO_2] and drought stress

Nodule primary C and N metabolism are sensitive to water restrictions. A number of metabolites (88) have been identified in *Medicago sativa* under drought (Aranjuelo et al., 2013b). Among them, 32 metabolites were significantly affected by drought. Two major groups of metabolites were categorized i) hexose, pentose, alanine levels increased and ii) sucrose, myo-inositol and organic acid levels decreased under water limitations. In that study, leaves, stems, and roots responded differently to drought. In leaves, drought caused an increase in sucrose

and sucrose derived compounds and a decrease in asparagine and glutamine. Results suggested that transport of N metabolites was restricted under drought. In nodules, export-related N compounds, asparagine, glutamate and glutamine, the most abundant amino acids accumulated significantly as the intensity of drought progressed (Aranjuelo et al., 2013b; Gil-Quintana et al., 2013b). This accumulation of N metabolites has both systemic and local feedback effects for N_2 fixation (Serraj et al., 1999b).

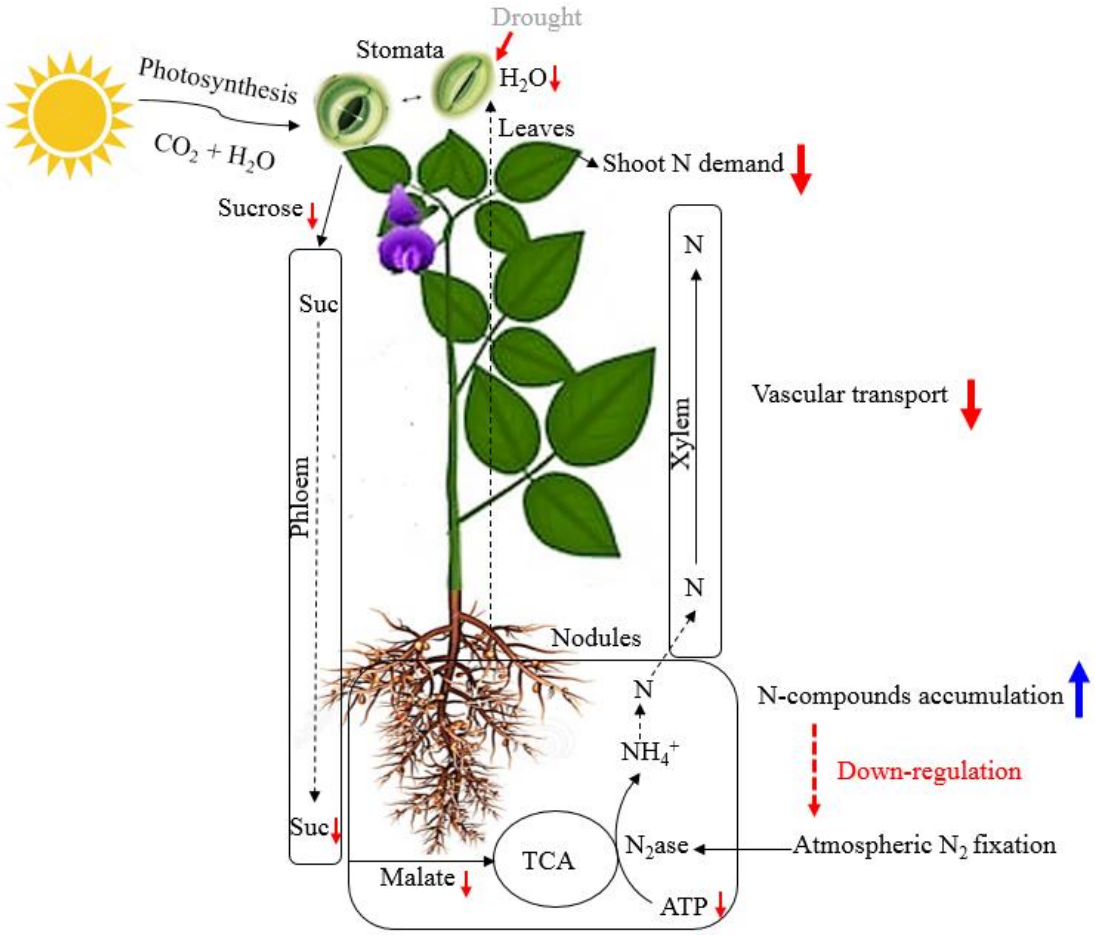


Fig. 1. 1 Mechanism explaining the decrease of N_2 fixation under drought stress through N-feedback inhibition processes, where red arrows indicate a decrease and blue arrows an increase in response to drought (Adapted from Aranjuelo et al. (2014)).

Three possible explanations have been suggested to explain the accumulation of amino acids (Valentine et al., 2010) (Fig. 1.1): a) reduced translocation through the xylem, b) lower shoot N demand and c) alteration of nodule metabolism. Firstly, amino acids may accumulate in nodules due to lower xylem translocation rates as a consequence of decreased transpiration (Pate et al., 1969). The second hypothesis suggests a decline in shoot N demand (Valentine et al., 2010) which was observed in droughted *Glycine max* L. (Gil-Quintana et al., 2013b). When the shoot demand for amino acids is less, the nodules cannot export N-compounds. Such a change in the leaf to nodule N balance strongly suggests that a lower export of nitrogenous compounds to the shoot contributes to their build-up in nodules. Thirdly, decreases in several enzyme activities involved in amino acid syntheses,

such as glutamate synthetase and nitrogenase, were observed (Aranjuelo et al., 2013b). However, how and to what extent e[CO_2] can overcome such N-feedback regulation is unknown.

Accumulation of reactive oxygen species (ROS) under drought also inhibits N_2 fixation process. Decreased O_2 permeability in nodules under drought limits O_2 supply to the bacteroids. Such imbalance in O_2 control is associated with the formation of ROS, which could produce cellular damage (Aranjuelo et al., 2014). Increased C availability under e[CO_2], possibly resulting in increased supply of defense (antioxidant) molecules such as ascorbic acid or glutathione, is often held primarily responsible for improved protection against oxidative damage in elevated [CO_2] (AbdElgawad et al., 2016).

Drought-associated decrease of N_2 fixation has been related to increases in ureids, amides and other amino acids in leaves and nodules. Elevated [CO_2] would provide more C to metabolize foliar amino acids, amides and ureides. This greater N sink at the leaf level could increase the utilization of N metabolites and therefore, reduce their accumulation in nodules. Therefore, e[CO_2] has the potential to ameliorate drought-induced reduction in N_2 fixation by increasing the C supply, lessening the accumulation of N metabolites and maintaining favourable soil moisture surrounding nodules reduces formation (De Luis et al., 1999; Serraj et al., 1999a).

The hypothesis of soil water preservation in crops grown under e[CO_2] has been discussed for some time (Serraj et al., 1999a). There is evidence that e[CO_2] reduces stomatal conductance (g_s) and hence, may also decrease transpiration at the canopy level (Leakey et al., 2009), for example, between 9% and 16% in *Glycine max* (L) in a FACE study (Bernacchi et al., 2007). This results in improved water use efficiency and thereby, conserves soil water later in the season (Bernacchi et al., 2007; Leakey et al., 2009). Plants grown at e[CO_2] deplete soil water more slowly and delay the development of drought stress (De Luis et al., 1999). As a result, the effect of drought on N_2 fixation can be delayed by maintaining favourable soil moisture surrounding the nodules (Rogers et al., 2009). Plants growing under e[CO_2] should, therefore, be able to avoid drought-induced reduction of N_2 fixation by increasing the flow of C supply to nodules through their increased photosynthesis rates (Rogers et al., 2009), as well as by decreasing g_s and conserving soil moisture for a longer period. A multiyear FACE study by Gray et al. (2016) reported that soil water preservation under e[CO_2] grown crops enabled them to transpire more than a[CO_2] grown crops later in the growing season, which led to greater soil water use later in the season (Gray et al. 2016). It is however unclear whether such conservation of soil water under e[CO_2] is sufficient to maintain N_2 fixation under prolonged drought.

Taking published data as a whole, it is widely accepted that changes in nodule metabolism are the key physiological response of legumes to drought (Serraj and Sinclair, 1997). Together with ureides, other amino acids (glutamine, asparagine etc.) have been proposed to act as signal molecules in shoots or nodules under drought depending on the legume species (Larrainzar et al., 2009). An investigation of nodule metabolites under drought was done in *Medicago sativa* L. (Aranjuelo et al., 2013b), *Medicago truncatula* L. (Gil-Quintana et al., 2013a) and the model legume, *Glycine max* L. (Gil-Quintana et al., 2013b). These studies indicated that changes in metabolite patterns between nodules and other plants organ under drought regulate N_2 fixation; metabolism in other organs is likely critical to sustaining nodule metabolism during drought (Aranjuelo et al., 2013b). However,

these studies were conducted in controlled environments and did not investigate the interactive effects of drought and e[CO_2] under field conditions.

The interacting effects of e[CO_2] and drought on N_2 fixation have been considered in previous studies (Aranjuelo et al., 2008; Cabrerizo et al., 2001; De Luis et al., 1999; Serraj, 2003a) but nodule metabolites have not been investigated directly in those studies. If nodule metabolism is affected by e[CO_2] in a way that would alter the ability of nodules to fix nitrogen under drought, these studies may ignore the complexity in a plants' response to e[CO_2] in field conditions. Only one field experiment has been conducted in *Glycine max* L. in the SoyFACE facility which suggested that reduced precipitation and drier soils might cause inhibition of N_2 fixation (reflected by lowest leaf N content) (Gray et al., 2013). This is contrary to previous studies (De Luis et al., 1999; Rogers et al., 2009) which concluded that e[CO_2] might reduce drought effects on N_2 fixation by conserving soil moisture surrounding the nodules. However, the underlying mechanism of N_2 fixation inhibition is not fully understood under the above conditions. The measurement of metabolite composition, including N-rich compounds derived from the nodules, can be used as an indicator of the availability of fixed N and corroboration of N_2 fixation (Rogers et al., 2006).

Although the exchange of C and N metabolites between bacterial symbiont and host plants is considered the key factor controlling the symbiotic N_2 fixation process; no published reports were found that studied this in a FACE facility or in open-top chambers or addressing nodule metabolism directly under e[CO_2]. Also, assimilation and transport of these metabolites are highly sensitive to environmental stresses, such as drought. For example, among several metabolites, sucrose, glutamine, asparagine, and ureide marked down-regulation of nodule activity in *Medicago sativa* and *Glycine max*, respectively (Aranjuelo et al., 2013b; Gil-Quintana et al., 2013b). However, such metabolite profiles may vary from species to species and may respond differently in field conditions, especially in dry environments. It was not fully understood whether e[CO_2] could ameliorate the drought-induced reduction of N_2 fixation of legumes by conserving soil water and regulating the balance supply of C and N metabolites towards nodules and host plants. The characterisation of metabolic responses in relation to N_2 fixation of legumes under drought and e[CO_2] will break new ground in a FACE study.

*1.7.3 Heat respons*e

Legume N_2 fixation is sensitive to increased temperature (Hungria and Vargas, 2000). High temperatures as often defined as an increase of 3-5^{0}C in air temperature over the typical average or average high. Heat stress is typically considered as >8^{0}C above the normal growth temperature (Morison and Lawlor, 1999). High temperatures generally affect above- ground plant parts, and little attention has been paid to below ground parts, such as nodules and their N_2 fixation activities (Aranjuelo et al., 2014). Suitable below ground temperatures for N_2 fixation range from 15-25ºC for temperate legumes and 27-40ºC for tropical legumes. In the case of *Phaseolus vulgaris* L., nodulation was inhibited when grown above 38^{0}C, but N_2 fixation was significantly decreased when grown just above 28^{0}C (Hungria and Franco, 1993). The survival potential of *Rhizobia* decreased under high soil temperatures of 39^{0}C, which hampered the release of *nod*-gene inducer from host plants as well as *nif*-gene expression (Aranjuelo et al., 2007; Hungria and Kaschuk, 2014). The formation of infection thread and root-hairs,

nodule initiation and bacteroid development decreased under high soil temperatures. Nodules formed at high temperature have been shown to function poorly and senescence earlier (Hungria and Franco, 1993).

The exchange of molecular signals between host plants and *rhizobia* is altered by high temperature (Hungria and Vargas, 2000). Synthesis of leghaemoglobin decreases under heat stress and a drop in nitrogenase activity was linked with an inefficient allocation of electrons to N_2 reduction (Hungria and Vargas, 2000). N_2 fixation efficiency of legumes depends on the overall balance between photosynthesis and respiration, which determines carbon availability. Negative effects of heat stress on plants can be caused by increased respiration and decreased photosynthesis, which might lower carbohydrate availability to nodules (Hungria and Vargas, 2000; Wang et al., 2008). N assimilation is also affected by lower synthesis of ureides, amides or amino acids (depending on the plant species). The transport of these N metabolites through the xylem was shown to be inhibited by heat stress (Aranjuelo et al., 2007). Also, activities of enzymes involved in amino acid biosynthesis such as glutamine synthetase and glutamate synthetase decreased under high temperatures. Overall, temperature stress affects plant N content negatively and lowers specific nodule activities (Aranjuelo et al., 2014).

1.7.4 Elevated [CO_2] and heat stress

Combined effects of e[CO_2] and high temperatures were investigated by Aranjuelo et al. (2008) in a glasshouse experiment in which N_2 fixing *Medicago sativa* L. was exposed to elevated CO_2 (~400 versus ~700 µmol mol^{-1}) with and without elevated temperature (~19 versus ~24◦C). Interaction of e[CO_2] and high temperatures reduced shoot N concentration (reflected by Rubisco depletion). Together with reduced nodule carbohydrate availability (as reflected by lower nodule starch concentration), N_2 fixation was negatively affected. These glasshouse findings, however, may not reflect the legume N_2 fixation under field conditions. In addition, timing and duration of heat waves during plant development is important, because certain damage mechanisms only apply during particularly sensitive growth stages. Legumes are particularly sensitive to high temperature (>30°C) during the reproductive phase, when heat causes pod and flower abortion along with senescence of nodules resulting in a significant reduction in grain yield and quality (Erskine et al., 2011). The interactive effects of e[CO_2] and heat waves on N_2 fixation during the reproductive phases have not investigated in detail.

Apart from high temperature and CO_2 effects on legume N_2 fixation, there are also likely effects on grain filling. Heat stress during the reproductive phases can be more harmful than during vegetative stages because it accelerates the grain filling rate and shortens the grain filling period (Dias and Lidon, 2009). However, faster grain filling rates do not compensate for shorter grain filling duration. Most importantly, the transfer of assimilates into grains decreases due to decreasing photosynthesis and N assimilation (Farooq et al., 2011). Growth under e[CO_2] might contribute to larger assimilate pools (Macabuhay et al., 2018), which may provide greater C to support N_2 fixation during grain filling duration even if photosynthesis is impaired, thereby offsetting the susceptibility to heat stress of grain N/protein content.

1.8 Crop breeding efforts

Selection of genotypes that are more responsive to e[CO_2] attracts interest as a potential foundation for crop breeding. Intra-specific variability in growth and yield response to e[CO_2] was found for wheat, rice, oats, soybean and cowpea (reviewed in Tausz et al., 2013). Genotypic variability in N_2 fixation response under e[CO_2] was also observed in soybean (Lam et al., 2012b; Li et al., 2017). Greater N_2 fixation response under e[CO_2] was associated with the ability to produce greater nodulation, nodule biomass, nodule activity and greater N demand of plant biomass (Lam et al., 2012a). In addition to [CO_2] response, the sensitivity of legume genotypes to either drought (Sall and Sinclair, 1991; Serraj and Sinclair, 1996) or heat stress (Delahunty et al., 2018) has also been documented. Climate change scenarios predict that increased [CO_2] will be accompanied by altered precipitation patterns and high temperature along with greater intensity and frequency of drought and heat events. Therefore, genotypes that can maintain [CO_2] response in interaction with drought and heat need to be investigated.

1.9 New approaches to study N_2 fixation mechanism in a changing climate

Nodule primary C and N metabolism seem to be at the heart of the physiological responses of N_2 fixation to environmental stresses (Aranjuelo et al., 2014). In recent years, several technologies such as gene expression (transcriptome analysis), metabolomic analyses, proteomic study, and isotope tracing have enabled advances to focus on the biochemical and physiological basis of nodule functions. Transcriptomics and proteomics provide an incomplete picture of all the aspects of biological processes in response to abiotic stresses. For example, changes in transcript or protein levels do not always correlate to actual changes of cellular metabolites due to post-transcriptional and post-translational modifications that modulate protein activities, which affects metabolite levels directly (Dias et al., 2015). Therefore, the study of metabolite concentration can reveal the plant metabolic status in response to environmental cues more directly than transcriptome and proteome analysis.

1.9.1 Metabolomics

Metabolites are the products of cellular regulatory processes, and their concentrations represent the response of the organisms to environmental and developmental changes. The set of metabolites synthesised by an organism constitute its metabolome (Fiehn, 2002). Plant metabolomics is the term used to identify and quantify the complex set of many (ideally: all) plant metabolites (Hill and Roessner, 2013). Metabolite profiling aims at a quantitative assessment of a predefined number of target metabolites and provides a snapshot of plant/organ/tissue metabolism at any given moment. Due to the complexity of studying hundreds of thousands of compounds when using an untargeted metabolomics approach, the study of certain groups of metabolites (i.e. metabolite profiling) has been proposed as a methodological approach for understanding plant metabolism to genetic/environmental perturbations (Dias et al., 2015). Metabolomics is regarded as the link between genotypes and phenotypes (Fiehn, 2002), because it signifies the "ultimate phenotype" of gene networks and their complex interaction with the environment (Hill and Roessner, 2013). Metabolic profiling is an effective method to elucidate the multifaceted and diverse nature of metabolites towards abiotic stress tolerance, including heat and drought stress (Yu et al., 2012).

Among primary metabolites, carbohydrates and amino acids are the most important target metabolites because they directly connect C and N metabolism. There is a very high chemical diversity of carbohydrates, often with similar structures which presents a challenge for their accurate analysis. More efficient and effective protocols are required for precise identification and quantification of sugars and organic acids derived from C metabolism (Dias et al., 2015). Several primary metabolites with low molecular weight can be made volatile which makes them amenable to analysis with gas chromatography (GC). Due to the diversity of metabolites, no single methods can accurately detect the complete metabolomes. GC coupled to mass spectrophotometry (MS), and liquid chromatography (LC) coupled to MS are methods commonly used for precise quantification of primary metabolites (carbohydrates, amino acids, organic acids) and secondary metabolites. New triple quadrupole (QqQ) instrumentation coupled to GC and LC provides the opportunity to determine actual concentration of targeted metabolites (Sumner et al., 2015). A new protocol developed by Dias et al. (2015) uses GC coupled to triple quadrupole (QqQ)-MS for quantification of four major classes (sugar, sugar acids, sugar phosphates and organic acids) of metabolites accurately. An established LC-QqQ-MS based technique developed by Boughton et al. (2011) can be used for precise quantification of amino acids and amines.

Only few studies analysed primary metabolites in legumes, and these studies cover the model legumes *Medicago truncatula* (Farag et al., 2008), both model and cultivated legume species of the *Lotus* genus (Suzuki et al., 2008) and *Glycine max* L. (Komatsu et al., 2011). Concerted changes of primary C and N metabolites have been studied in *Medicago sativa* under drought stress in a glasshouse study (Aranjuelo et al., 2013b). Empirical evidence suggests that metabolites linked to stress tolerance, for example, hexoses (e.g. sucrose, fructose, glucose) and minor sugars (e.g. trehalose and mannitol) accumulate under water restriction (Aranjuelo et al., 2013a). For organic acids and amino acids, it has been reported that the abundance of many metabolites, such as asparagine, γ-aminobutyric acid (GABA), β-alanine, alanine, and proline as well as malate, fumarate, isocitrate can be enhanced by drought stress in *M. truncatula*. However, studies investigating these metabolite profiles have been conducted in controlled environments under a[CO_2] conditions where plants were neither exposed to natural gradients nor e[CO_2] environments. In a FACE study, *Glycine max* L. metabolites were determined at the leaf level without investigating the nodule metabolite profiles (Rogers et al., 2006). Metabolite patterns and their coordinated changes between different plant organs such as leaf, root and nodule, especially under free air conditions including factors such as e[CO_2], drought and heat, could provide further insight into regulation and inhibition of N_2 fixation in a changing environment.

1.9.2 Isotope tracing

Different methodologies have been developed to monitor metabolic fluxes into different plant compartments. Use of the isotopic composition of carbon ($\delta^{13}C$) has proved to be a good tool to provide details on the metabolic exchange between nodules and different plant organs (Aranjuelo et al., 2008; Fischinger and Schulze, 2010). Two powerful approaches are possible with isotope labeling: i) assessing the distribution of isotope label over time (dynamic analysis) and ii) identification of metabolic labeling patterns (steady-state analysis). Recent C/N tracer studies have provided essential information about the exchange of newly synthesised C and N between nodules and leaves. The distribution of C and N between plant and symbiont via different amino acid metabolic pathways was identified using this technique (Molero et al., 2011). Furthermore, the dynamics of N metabolites can be

identified using both ^{15}N enrichment and ^{15}N natural abundance techniques. The drawbacks of the first technique include the possibilities that availability of mineral N (if ^{15}N enriched fertiliser is used) can inhibit nodule N_2 fixation, loss of ^{15}N labeled fertilizer by leaching or immobilization, and the high cost of ^{15}N labeled materials (Unkovich and Pate, 2000). Considering this limitation, the ^{15}N natural abundance technique is now widely used to estimate legume N_2 fixation and N dynamics in time and space. This method relies on a small enrichment in the ^{15}N abundance of soil mineral N (e.g., to between 0.368 and 0.373 atoms % ^{15}N) compared with atmospheric N_2 (0.3663 atoms % ^{15}N) (Peoples et al., 1989).

1.10 Effect of elevated [CO_2] and environmental factors on grain quality

Grain yield of C_3 crops is expected to increase under e[CO_2] treatments, but there is a significant trade-off with grain nutritional quality traits such as protein, mineral nutrients and starch (Högy et al., 2009). Among C3 crops, legumes can overcome the decrease of grain protein concentrations (Taub et al., 2008) through stimulation of N_2 fixation (Rogers et al., 2006). In particular, the changes in grain [N] of legumes has been well documented under elevated [CO_2], but less attention has been paid to the changes in other grain mineral elements. A global meta-analysis reported that e[CO_2] decreases the concentration of Fe and Zn in grains of field pea and soybean, too (Myers et al., 2014). Currently, more than 2 billion people suffer from Fe and Zn deficiency worldwide (Kumssa et al., 2015) and the impact of e[CO_2] on nutrition is expected to worsen this potentially increasing the number of people at risk of Zn deficiency by 138 million by 2050 (Myers et al., 2015). Given this prevalence of micronutrient deficiency in human populations and the near certainty of significant further increases in [CO_2] (IPCC, 2014), concerns have raised about potential implications for human health and food security.

Several mechanisms have been suggested to decrease mineral concentration under e[CO_2] such as limited transpiration flow or lower stomatal conductance (Houshmandfar et al., 2015; McGrath and Lobell, 2013). Increased biomass growth under e[CO_2] causes depletion of tissue mineral concentration (Loladze, 2002). This phenomenon is referred to as growth dilution or biomass dilution (Taub and Wang, 2008). In a meta-analysis, Myers et al. (2014) argued that reduction of grain minerals at e[CO_2] could not be fully explained by growth dilution/carbohydrate accumulation or diminished transpiration flow (Houshmandfar et al., 2018). Therefore, the underlying physiological mechanism is still not fully understood.

Legumes, especially those grown in water-limited Mediterranean environments, already experience intermittent drought at some stage in their vegetative growth period, and terminal drought throughout their reproductive period when temperatures are higher, and rainfall is diminishing. More extreme conditions may lead to low grain yields and poor grain quality (Farooq et al., 2017; Sehgal et al., 2017). A better understanding of how grain mineral elements changes under e[CO_2] and their interaction with different growing conditions, is required to underpin adaptation strategies.

1.11 Free-Air CO_2 Enrichment (FACE) and Australian Grain FACE (AGFACE) facilities

Free-Air CO_2 Enrichment facilities are "real-world" approaches which avoid the artifacts of CO_2 response in more controlled and enclosed environments such as glasshouses, growth chambers, open-top chambers etc. (Allen Jr, 1992; Drake et al., 1985). FACE technology allows studies under natural field conditions including the natural

environmental and biotic interactions (Macháčová, 2010). To address crop responses to future higher [CO_2] in a water-limited, low-yielding cropping systems, the Australian Grains FACE (AGFACE) facility was established in 2007 on a 7.5 ha site 7 km west of Horsham, Victoria, Australia (36°45'07"S, 142°06'52"E, 127 m above sea level) (Fitzgerald et al., 2016; Mollah et al., 2009). Another sub-facility, namely SoilFACE (Butterly et al., 2015) was set up within the AGFACE facility in 2009. The design of the CO_2 fumigation systems and performance of FACE rings within the AGFACE facility are described in detail by Mollah et al. (2009) and (Mollah et al., 2011), respectively. Briefly, each FACE ring (Fig. 1.2) was composed of eight horizontal stainless-steel tubes surrounded by the plots, which were positioned about 150 mm above the canopy at any developmental stage. Pure CO_2 was injected into the atmosphere through 0.3 mm laser-drilled holes on the upwind side, which then mixed with air as it blew across each plot by the prevailing wind. In the FACE ring, [CO_2] enrichment was performed from sunrise to sunset with a target of 550 µmol mol^{-1} at the centre of each ring, starting within a few days of 50% seedling emergence until harvest. CO_2 concentrations were monitored and recorded every minute with an infrared gas analyser (IRGA, SBA-4, PP Systems, Amesbury, MA, USA) positioned at the centre of each ring.

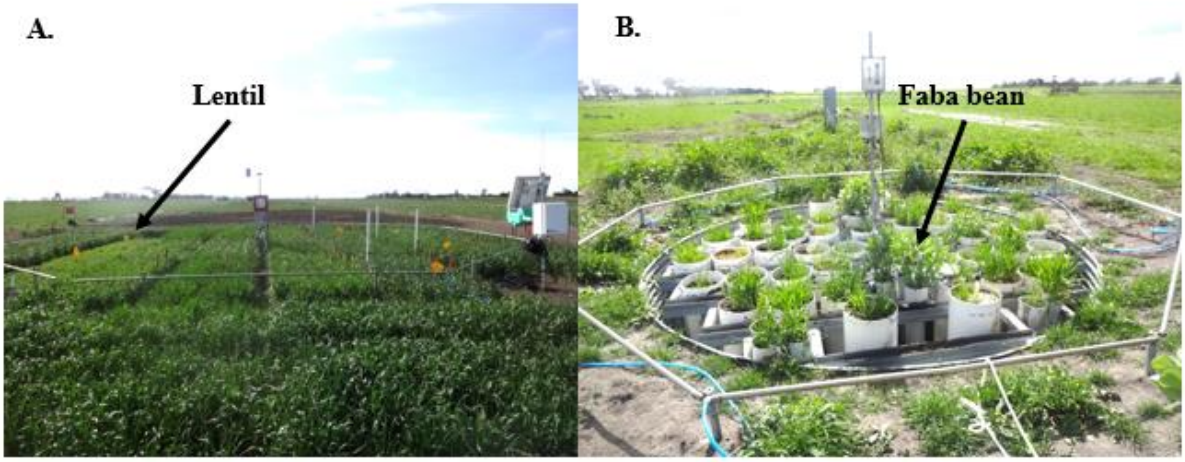


Fig. 1. 2 Free Air CO_2 Enrichment (FACE) ring in the (A) Australian Grains FACE (AGFACE) and (B) Soil FACE (SoilFACE) facility in Horsham, Victoria, Australia.

1.12 Justification of the study

From the literature review, the following research gaps were identified:

1. Among legumes, soybean has been studied extensively in SoyFACE in Illinois, USA (Rogers et al., 2006) which is located in a high rainfall, temperate climatic zone. Since environmental factors strongly influence crop performance, legumes grown under dryland Mediterranean environments (such as typical for Australian cropping systems) were expected to perform differently. From 2010-2012, field peas were studied in the Australian Grain Free Air CO_2 Enrichment (AGFACE) facilities focusing on grain quality and N_2 fixation parameters (Butterly et al., 2015; Myers et al., 2014). To date, only two leguminous food crops (soybean in SoyFACE and field pea in AGFACE) were the subject of a fully open-air CO_2 enrichment experiment. These studies did not fully investigate how e[CO_2] interacts with abiotic stress (drought and heat) to modulate N_2 fixation.

2. Also, at the initiation of this thesis project, it was unclear whether legumes could overcome the e[CO_2]-induced decrease of tissue N/protein concentrations; either by increasing N_2 fixation and or by changing the allocation of the N pool during grain filling.

3. Studies analysing the mechanisms of elevated CO_2-mediated stress mitigation are rare. Accumulation of metabolites i.e., sucrose, asparagine, glutamine and ureides in nodules show that there may be an N feedback mechanism under water restriction. Despite much research in this area, no single report has evaluated the relationship between N_2 fixation and changes in metabolites concentration in leaves and nodules in response to water deficits in a FACE facility.

4. Studies reported that e[CO_2] depletes grain mineral elements of legumes especially Fe and Zn. In addition to e[CO_2], growing conditions under high temperature and or drought may also affect the deposition of minerals into grains through different mechanisms. Therefore, it was not fully understood whether how e[CO_2] interacts with drought/high temperature to change grain mineral composition.

1.13 Thesis outline and research aim

The first chapter of this thesis (**Chapter 1: Introduction**) critically evaluates the available literature investigating the effect of e[CO_2] on N_2 fixation of legumes in agroecosystems. **Chapter 1** also identifies the underlying factors influencing mechanisms of N_2 fixation. Based on the knowledge gap identified, the present study was undertaken to investigate how e[CO_2] interacts with abiotic stress conditions to strengthen or weaken mechanisms of N_2 fixation and maintain grain quality of legumes under dryland conditions.

In addition, several specific objectives were:

- To investigate how e[CO_2] mitigates the impact of abiotic stress (drought and heat) on N_2 fixation of legumes (lentil, field pea, and faba bean) through decreasing stomatal conductance or soil water saving mechanisms and whether inter- or intra-specific variation exists in these responses.

- To investigate whether increased N_2 fixation under e[CO_2] can overcome the phenomenon of decreasing protein concentrations of legumes through changing N allocation and or N remobilization patterns.

- To investigate how e[CO_2] modifies C-supply or changes in C- and N-metabolism to adjust N_2 fixation mechanisms under drought

- To investigate how e[CO_2] changes grain quality attributes especially mineral elements of dryland legumes in interaction with drought

To address these objectives, this thesis includes seven experimental chapters derived from a total of five independent experiments. Among them, four experiments were undertaken under field conditions in the AGFACE facility with a target e[CO_2] of ~550 μmol mol^{-1} and one under glasshouse conditions with a target e[CO_2] of ~700 μmol mol^{-1}.

Chapter 2 presents an experiment that investigated the interactive effect of [CO_2] and seasonal water availability on nodulation attributes as well as N_2 fixation and remobilization of lentil. The aim of this experiment was to investigate whether e[CO_2] maintains N_2 fixation mechanisms under seasonal drought and how these affect grain [N] of lentil. The experiment was conducted under field conditions within the AGFACE facility. Two lentil cultivars (contrasting in harvest index) were grown under two [CO_2] (a[CO_2]; ~400 μmol mol^{-1}) and e[CO_2]; ~550 μmol mol^{-1}) and two contrasting growing seasons sharply differing in rainfall (low rainfall, 2015 and high rainfall, 2016).

Chapter 3 consists of an experiment that investigated the changes in leaf and nodule metabolome subjected to seasonal drought and to relate these shifts in the metabolism with photosynthesis and N_2 fixation responses. Two lentil (PBA Ace and HS3010) genotypes showing different drought sensitivity were grown under ambient [CO_2] (~ 400 ppm) or elevated [CO_2] (~ 550 ppm) conditions in the AGFACE facility during two contrasting growing seasons (dry 2015 vs. wet 2016 growing season). In this experiment, metabolites were analysed from leaf and nodule tissues at the flowering stage from the experiment reported in **Chapter 2**. The objective of the study was to identify possible target metabolites related to the metabolism of soluble sugars, organic acids and amino acids that may be involved in moderating photosynthesis and N_2 fixation under e[CO_2] and drought. Using GC-QqQ-MS and LC-QqQ-MS metabolomic approaches, metabolites involved in TCA, glycolysis, photorespiration and amino acids metabolism were identified.

Chapter 4 describes an experiment that investigated the interactive effect of [CO_2] and terminal drought on N_2 fixation of field pea. The aim of this experiment was to investigate whether e[CO_2] delays the onset of drought on nodule activity and N_2 fixation through increasing water use efficiency and conserving soil water. The experiment was conducted under field conditions within the AGFACE facility in 2015. Field pea was grown under two [CO_2] (a[CO_2]; ~400 μmol mol^{-1}) and e[CO_2]; ~550 μmol mol^{-1}) subjected to terminal drought at reproductive phase.

Chapter 5 reports an experiment that investigated the interactive effect of e[CO_2] and drought on yield, N_2 fixation and grain N response of faba bean in a water-limited environment. The experiment was undertaken in the AGFACE facility under two water regimes (well-watered to at least 80% field capacity, FC and drought, left to dry to about 30% FC and maintained afterward) in 2016. The aim of this experiment was to examine whether decreased g_s under e[CO_2] translates into soil water conservation in a prolonged drought, thereby improve N_2 fixation and seed yield. And if so, whether this stimulation of N_2 fixation could avoid e[CO_2]-induced depletion of seed N concentration.

Chapter 6 is written based on an experiment that investigated N_2 fixation, allocation and remobilization of lentil under e[CO_2] in combination with a heat wave in AGFACE facility. Purpose built heat chambers were used to impose a heat wave (~ 40°C compared to control 30°C) for three consecutive days at the sensitive flat pod stage in 2015. The aim of this experiment was whether e[CO_2] mitigate the negative impacts of a heat wave on N_2 fixation and maintain grain [N] by changing its N remobilization. Two lentil genotypes were grown and genotypic variability in response to e[CO_2] and a heat wave was also investigated.

The experiment reported in **Chapter 7** utilizes $^{13}CO_2$ pulse-labeling approach to quantify directly the C sink strength of nodules and other organs under the interactive effect of e[CO_2] and drought. Experiments were conducted with faba bean in glasshouse chambers either in ambient [CO_2] (~400 ppm) or elevated [CO_2] (~700 ppm) and exposed to one of two watering regimes, a high rainfall scenario or the simulation of a dry season in a typical Mediterranean agro-ecosystem. We chose faba bean, because it has higher nodulation and N_2 fixation potential but also greater drought sensitivity than most other cultivated legumes (Dayoub et al., 2017). The objective of this experiment was to investigate how e[CO_2] maintains C sink strength of nodules and other organs under drought and whether this contributes to overcoming photosynthetic acclimation under drought.

Chapter 8 presents an experiment that investigated changes in minerals composition of grain legumes under e[CO_2] × drought interaction. This experiment reports on two independent but related experiments, one on lentil and one on faba bean, conducted within the AGFACE facility over two contrasting growing seasons sharply differing in seasonal rainfall for lentil (described in **Chapter 2**) or experimentally imposed drought during a growing season for faba bean (described in **Chapter 5**). The objectives of this experiment were to investigate the changes in grain mineral concentrations of lentil and faba bean under e[CO_2] and drought alone or combination. Finally, we tested the dilution hypothesis associated with whether e[CO_2]-induced yield stimulation further dilutes mineral concentration through accumulating excess carbohydrate.

Chapter 9 describes the synthesis results obtained across all experiments and discusses these in line with the main objectives stated in the introduction. Finally, general conclusions and future recommendation are also presented.

1.14 References

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Chapter 2: 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

(ii) Abstract

Increased biomass and yield of plants grown under elevated [CO_2] (e[CO_2]) often correspond to decreased grain N concentration ([N]), diminishing the 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, lentil was grown 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 season under e[CO_2], as a consequence of greater post-flowering N_2 fixation. These 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.

2.1 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, 2014). Elevated [CO_2] (e[CO_2]) stimulates photosynthesis, growth, and yield of C_3 plants (Ainsworth and 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 and 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 and 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 and Uddling, 2012; Shimono and 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 and 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., 2012c). 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 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 of 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, 2003). 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., 2014a). 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 remobilization can be 80-90% depending on genotype (Kinugasa et al., 2012). Previous study suggested that e[CO_2] decreases N remobilization 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 remobilization processes.

Selection of genotypes that are more responsive to e[CO_2] attracts interest as a potential foundation for crop breeding (Tausz et al., 2013; Ziska et al., 2012). 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., 2012b; Li et al., 2017) and for field pea (Bourgault et al., 2016).

The current study investigated the mechanism of N acquisition via N_2-fixation and uptake from soil and N remobilization 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 (Daryanto et al., 2015; Erskine et al., 2011). 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?

2.2 Materials and methods

2.2.1 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.

2.2.2 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 described 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. 2.1). The total amount of rainfall during the crop growing season was 128.4 mm in 2015 and 334.2 mm in 2016 (Table 2.1). As the total amount of rainfall in 2015 was well below the average long-term growing seasonal rainfall (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**').

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.

Table 2. 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 years to ensure optimum seed germination. For grain yield, all experimental plots were averaged in each year. GSR: global solar radiation.

Parameters	**Growing season**	
	2015, low rainfall season	2016, high rainfall season
Average minimum (^{0}C)	5.9	5.8
Average maximum (^{0}C)	20.3	18.4
Average (^{0}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

2.2.3 Measurements

2.2.3.1 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., 2011). 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 and 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 five min to remove clay particles

and desorb nutrients from the root surface. Root washing was followed according to the procedure described by Frasier et al. (2016). Briefly, roots were soaked in a bucket filled up with water. In order to remove roots from soil wash, the samples were washed through a submerged 2 mm sieve with running tap water and then collect the roots retained by- and floating on the sieve with tweezers. All collected roots were recovered by carefully sieving and repeating the procedure several times. 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) and grain mineral composition is reported in Chapter 8. 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.

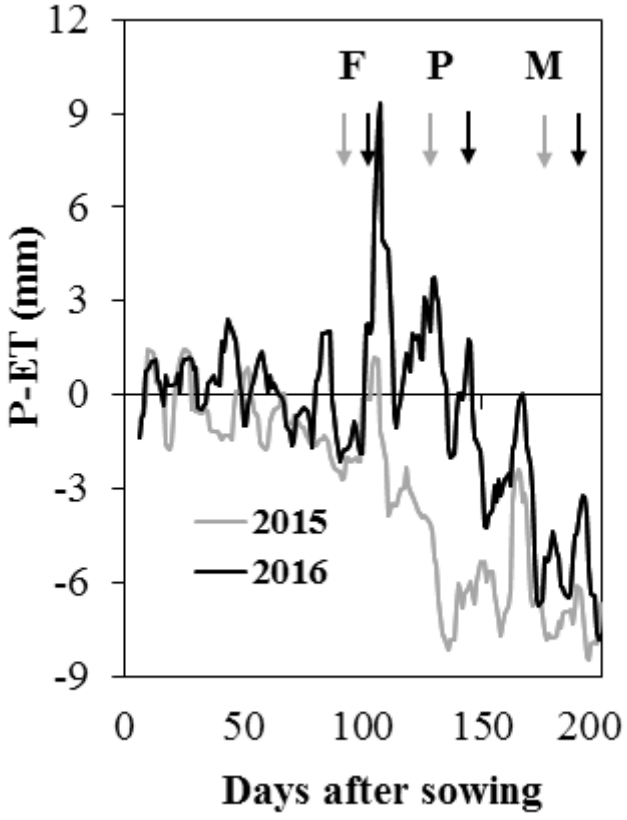


Fig. 2. 1 Drought index calculated as the difference between precipitation (P) and evapotranspiration (ET) in a dry (2015, grey line) and a high rainfall (2016, black line) season. Growth stages (F: flowering, P: pod filling and M: Maturity) are given for lentil growing under ambient [CO_2] (~400 ppm) or elevated [CO_2] (~550 ppm) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia.

2.2.3.2 Soil water profile probes

Soil water content was monitored every week 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).

2.2.3.3 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.

2.2.3.4 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.

2.2.3.5 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 the 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; Lam et al., 2012b; Lam et al., 2012c; Rennie and 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 and 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.

2.2.3.6 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

2.2.3.7 Post-flowering N_2 fixation and N remobilization

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

2.2.3.8 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.

2.2.4 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 et al., 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, 2018). Statistical effects are regarded as significant at $P < 0.05$. P-values between 0.05-0.10 are presented for discussion purposes.

2.3 Results

2.3.1 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. 2.2). 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.

2.3.2 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 2.2 & 2.3).

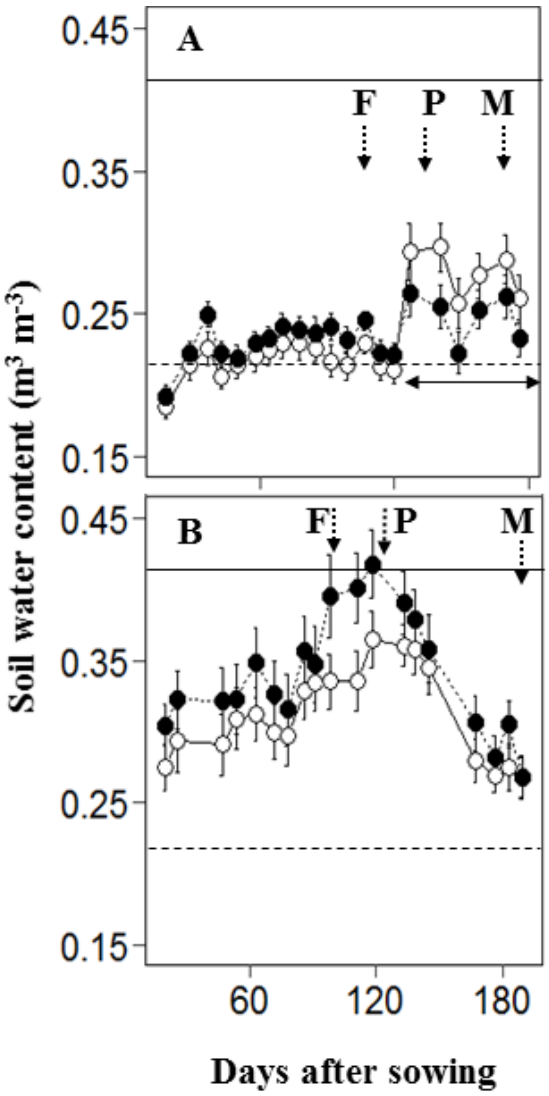


Fig. 2. 2 Volumetric soil water content (m^3 m^{-3}) in lentil plots in a dry (2015, A) and a high rainfall (2016, B) season under ambient [CO_2] (~400 ppm, ○ with continuous lines) or elevated [CO_2] (~550 ppm, ● with broken lines) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia. Horizontal continuous line indicates field capacity and broken line indicates the 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 were significant at $p < 0.001$ ($F = 28.61$).

2.3.3 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 2.2 & 2.3). Aboveground biomass (AGB) and total biomass stimulation by e[CO_2] were 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 2.2 & 2.3). Grain yield response to e[CO_2] was greater than the biomass response in the high rainfall season but not in the dry season.

2.3.4 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 2.2 & 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 2.2 & 2.3). Root [N] increased in the high rainfall season and decreased in the dry season under e[CO_2], but e[CO_2] did not affect 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. 2.3).

Table 2. 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 2.3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

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 2. 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 as 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 2.2 and Figures 2.3 to 2.8. Sample size was four replicates (n = 4) for each combination of year, CO_2 and genotypes. Significant effects are shown in bold type.

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 remobilization	**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)**

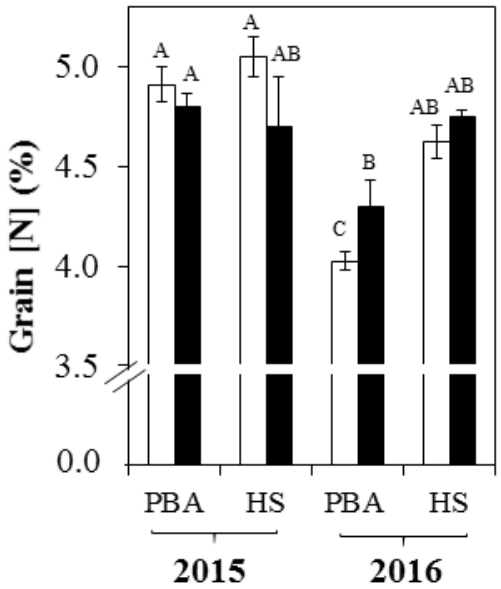


Fig. 2. 3 Grain N concentration ([N], % dry weight) of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" at maturity grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a 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 2.3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

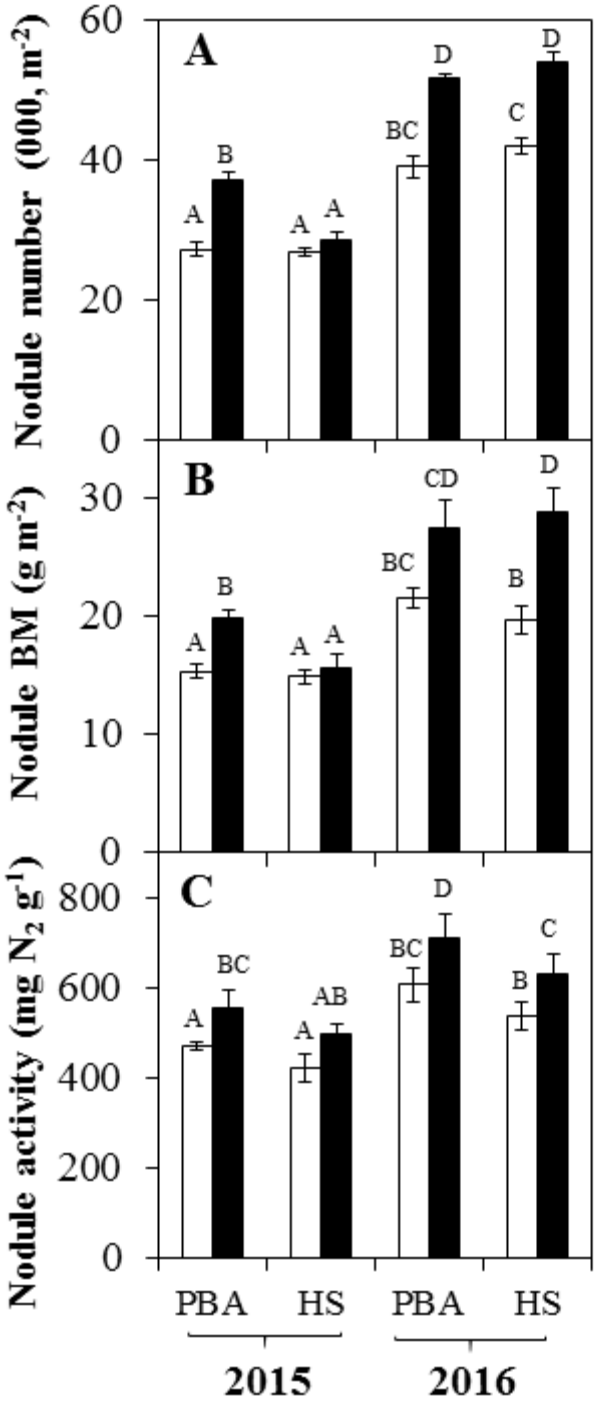


Fig. 2. 4 A. Nodule number (000, m^{-2}), B. Nodule biomass (BM, dry weight basis, gm^{-2}), C. Nodule activity (mg N_2 fixed g^{-1} nodule dry weight) of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) 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 the flowering stage. Means and standard errors of n=4 replicates. Statistics are reported in Table 2.3. Mean values that share no common letters are significantly different from each other ($p < 0.05$)

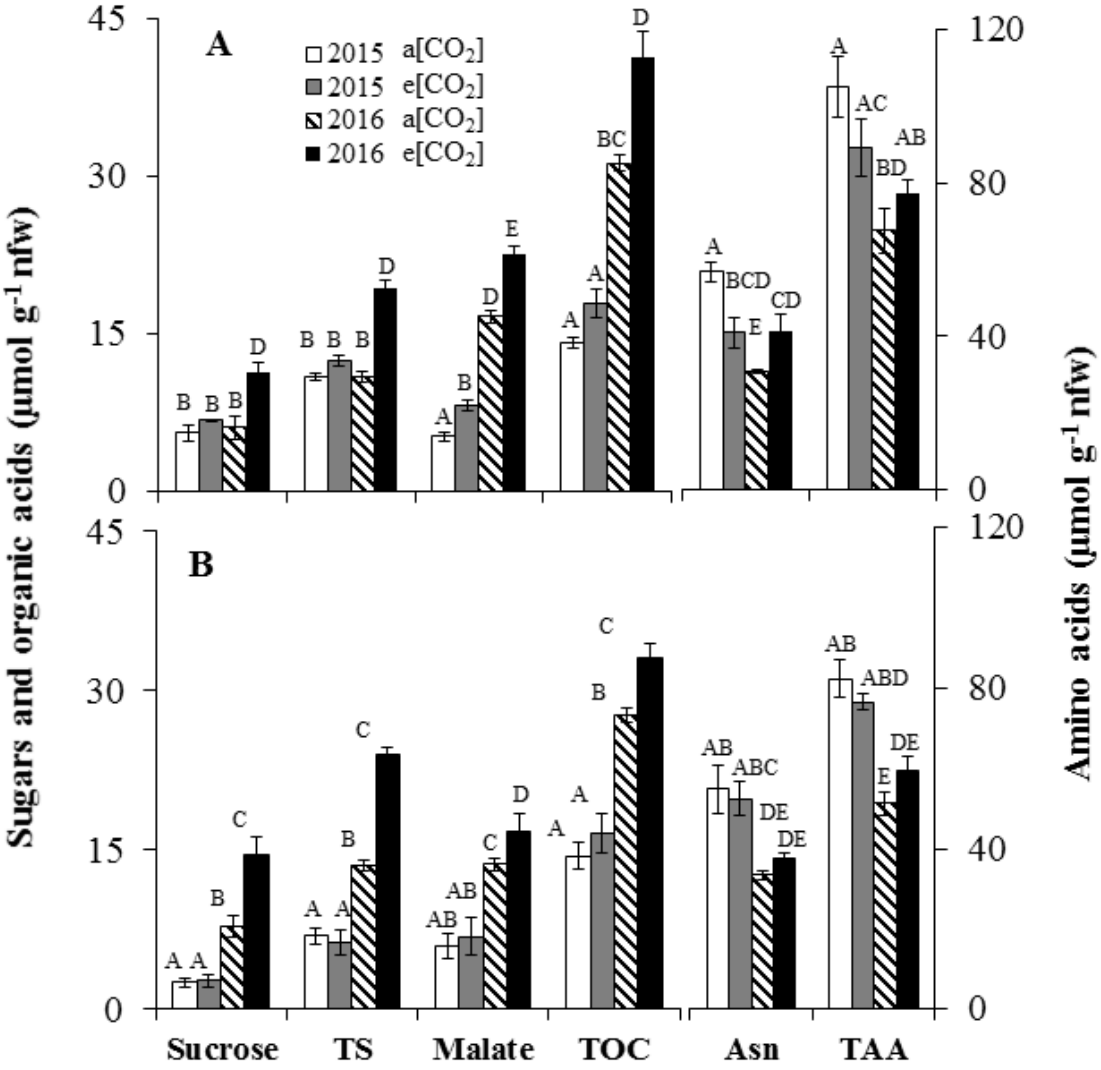


Fig. 2. 5 Nodule sucrose, total sugars (TS), malate, total organic acids (TOC), asparagine (Asn) and total free amino acids (TAA) concentrations of two lentil genotypes "PBA Ace" (A) and "HS3010" (B) grown under ambient [CO_2] (a[CO_2], ~400 ppm) or elevated [CO_2] (e[CO_2], ~550 ppm) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a high rainfall (2016) season. Measurements were taken at the 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 2.3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

2.3.5 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 2.4 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 2.4C). 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. 2.5 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. 2.5 A, B, right panel).

2.3.6 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. 2.6 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. 2.6 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 the atmosphere (%Ndfa) showed similar trends with a significant three-way interaction ($p = 0.017$) (Table 2.2). N uptake from soil decreased under e[CO_2] to a greater extent in the high rainfall season compared to the dry season (Fig. 2.6 E, F).

2.3.8 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. 2.8 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. 2.8 B, middle of the panel). Elevated [CO_2] led to a greater proportion of belowground N coming from fixed N_2.

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. 2.8 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. 2.9).

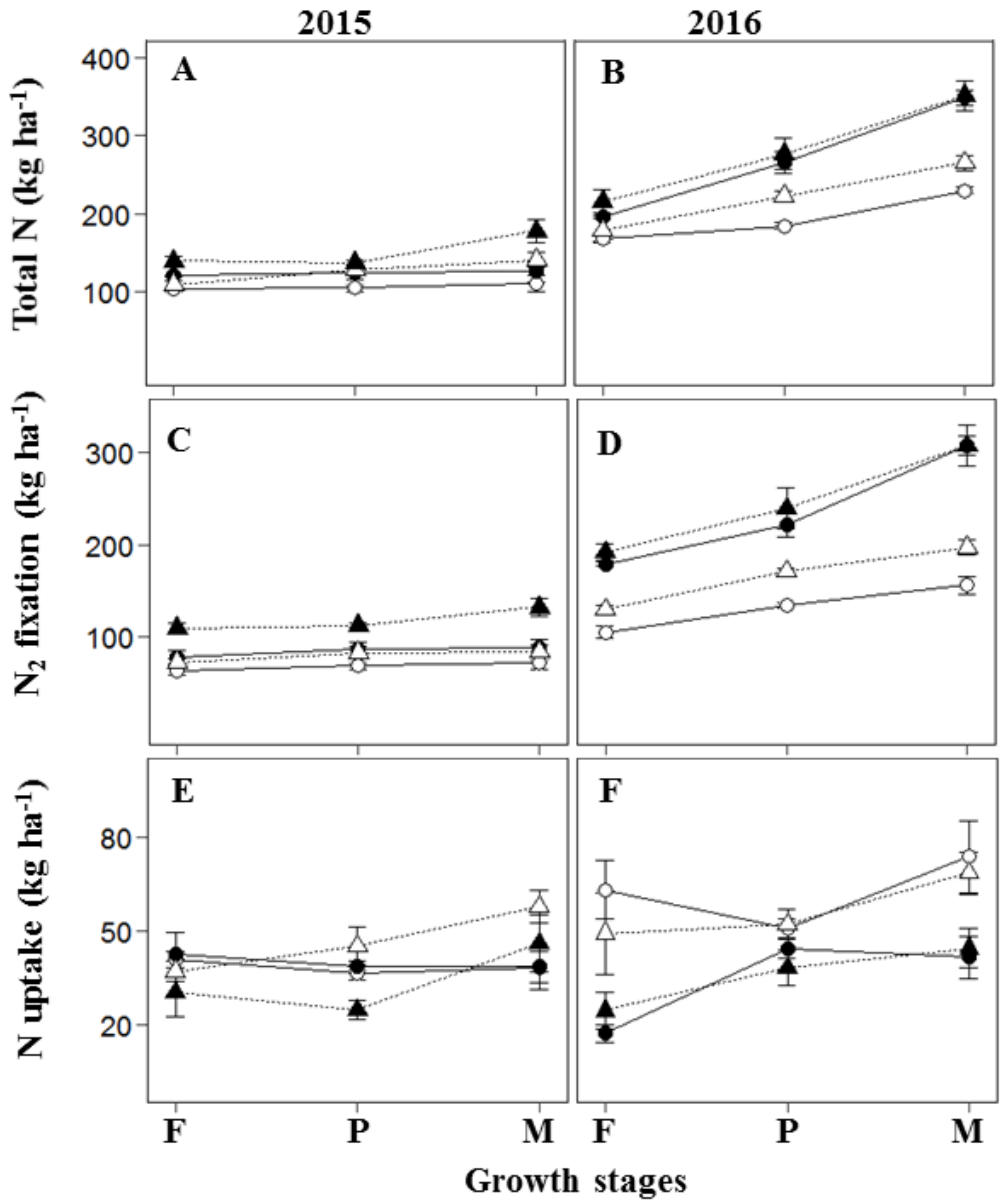


Fig. 2. 6 Total plant N content (A, B), N from N_2-fixation in plant biomass (C, D) and N from soil N uptake in plant biomass (E, F) of two lentil genotypes "PBA Ace" (Δ, ▲ with broken lines) and "HS3010" (○, ● with continuous lines) from flowering to maturity and grown under ambient [CO_2] (~400 ppm, 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 2.3.

2.3.7 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. 2.7A). Total N remobilization was greater in the high rainfall than the dry season, and greater for PBA Ace than HS3010, but not affected by [CO_2] (Fig. 2.7B).

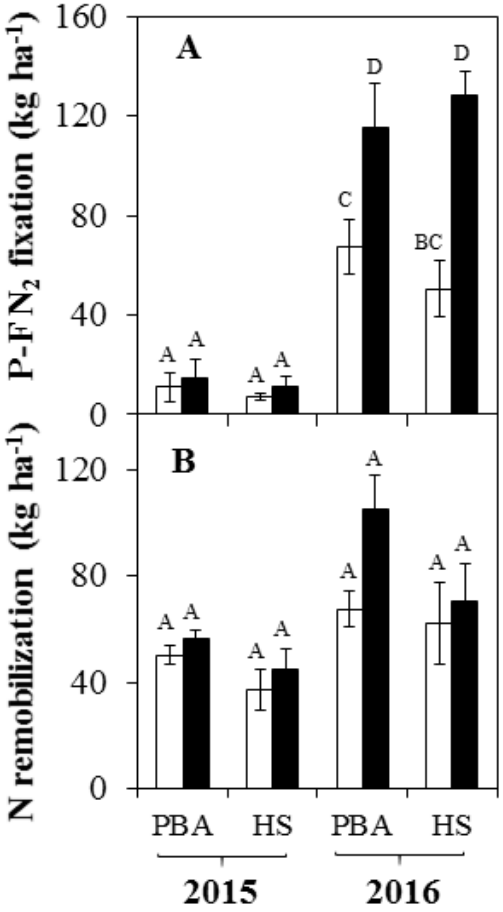


Fig. 2. 7 Post-flowering (P-F) N_2 fixation (A) and N remobilization (B) to grain at maturity of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, black bars) in the Australian Grain Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015, left panel) and a high rainfall (2016, right panel) season. Mean and standard errors of n=4 replicate. Statistics are reported in Table 2.3. Mean values that share no common letters are significantly different from each other ($p < 0.05$).

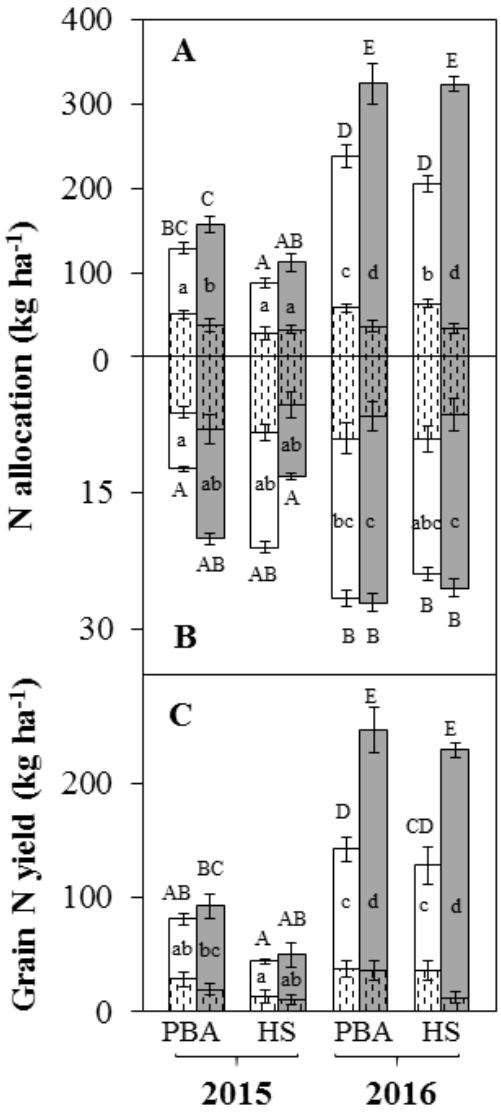


Fig. 2. 8 N allocation in above (A) and below (B) ground biomass and grain N yield (C) at maturity of two lentil genotypes "PBA Ace (PBA)" and "HS3010 (HS)" grown under ambient [CO_2] (~400 ppm, white bars) or elevated [CO_2] (~550 ppm, grey bars) in the Australian Grains Free Air CO_2 Enrichment facility, 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 the atmosphere (%Ndfa) is reported in Table 2. Each bar represents mean values and standard errors of n=4 replicates. Statistics are reported in Table 2.3. 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 ($p < 0.05$).

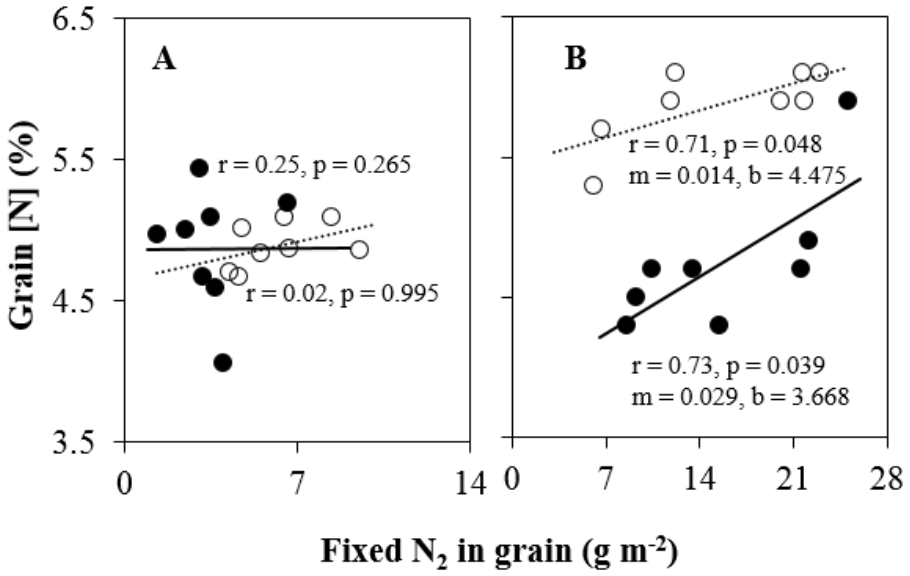


Fig. 2. 9 Relationship between grain N concentration ([N], % dry weight) and N from fixed N_2 in grain (g m^{-2}) at maturity of two lentil genotypes "PBA Ace" (● with continuous lines) and "HS3010" (O with broken lines) grown under ambient [CO_2] (~400 ppm) or elevated [CO_2] (~550 ppm) in the Australian 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.

2.4 Discussion

2.4.1 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 (Bourgault et al., 2016; Fitzgerald et al., 2016; Houshmandfar et al., 2016; O'Leary et al., 2015), 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 and 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, the results of this study 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.

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

In the first research question, this study assessed 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 this study, 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., 1997). Using $^{13}CO_2$ pulse-labeling 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., 2014a), whereby sugars (particularly sucrose) and malate play vital roles (Aranjuelo et al., 2009; Fischinger et al., 2010; Nasr Esfahani et al., 2014; 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.

My question was 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, I 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 (Antolin 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. The decline of nodule mass and functionality by drought are connected with decreased carbohydrate availability to nodules (Aranjuelo et al., 2014a), a notion corroborated by the results this study related to decreases of 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 and 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. 2.5). 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., 2014b), but that it can also be associated with strong stimulation of fixation by e[CO_2] under favourable conditions.

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

In second research question, this study assessed 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. Results demonstrated 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., 2015), 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). 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. The results of this study 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, remobilization 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 and 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 remobilization 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 remobilization, 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 the results of this study showed 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. 2.9). The results of this present study indicate that it is continuing N_2-fixation, but not soil N uptake or N remobilization 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.

2.4.4 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 third research question, this experiment investigated 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 and 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 determined grain [N] depression under rising [CO_2].

2.5 Conclusion

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 fixation and contribute to maintaining grain protein as well as add more N into crop rotation systems.

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Chapter 3: Metabolite profiling reveals distinct changes in C-and N-metabolism of lentil (*Lens culinaris* MEDIK.) in response to seasonal drought and Free-Air CO_2 Enrichment (FACE)

(iii) Abstract

Symbiotic N_2 fixation is finely tuned through significant carbon and energy metabolism by plants. Elevated [CO_2] enhances carbon supply and stimulates N_2 fixation of legumes. Additionally, drought affects both processes and plants modify a series of metabolic processes for adaptation to stress conditions. Currently, there is limited understanding regarding the changes in plant metabolism under elevated [CO_2] grown legumes subjected to drought stress. Therefore, this study investigated the changes in leaf and nodule metabolome subjected to seasonal drought and related these shifts in metabolism to photosynthesis and N_2 fixation responses. Two lentil (PBA Ace and HS3010) genotypes were grown under ambient [CO_2] (~ 400 ppm) or elevated [CO_2] (e[CO_2], ~ 550 ppm) conditions in a Free-Air CO_2 Enrichment (FACE) facility during two contrasting growing seasons (dry vs. wet season).

In the dry season, e[CO_2] showed photosynthetic acclimation and diminished the stimulation of N_2 fixation, and these responses were linked with changes in C- and N-related metabolites. The metabolite profile in leaves revealed that the dry season increased the abundance of metabolites related to carbohydrate metabolites (sucrose, trehalose, mannose, galactose) but reduced the abundance of organic acids (malate, succinate, aspartate) and amino acid (asparagine, glutamine, glycine). Increased sucrose concentration in leaves under e[CO_2] might be associated with decreased photosynthetic stimulation in the dry season. In nodules, the lower abundance of sucrose and malate during the dry season indicated a shortage of C supply into tricarboxylic acid (TCA) cycle. An increased abundance of glutamine and asparagine in nodules indicated feedback inhibition of N_2 fixation, but the accumulation of the later was lower under e[CO_2] grown plants. The synthesis of osmoprotectant (i.e., trehalose, mannitol, inositol, proline) was found to be enhanced under e[CO_2] in the dry season. These results suggest that e[CO_2] induced changes in C-and N-metabolites increased legume tolerance to drought.

Keywords: Free Air CO_2 Enrichment, metabolites, N_2 fixation, carbohydrate, amino acids, metabolic mapping

3.1 Introduction

Atmospheric [CO_2] has been increasing since the Industrial Revolution. Having risen pre-industrial ~280 ppm to currently about 400 ppm, [CO_2] will continue to increase 550 ppm by 2050 (IPCC, 2014). Because CO_2 is a direct substrate for photosynthesis, greater [CO_2] stimulates growth and yield of C_3 crops, which are currently CO_2-limited. Greater photosynthesis under e[CO_2] may lead to increased carbohydrates concentration in the leaves, which may exert feedback on photosynthetic capacity (Long et al. 2004). Therefore, carbohydrate translocation from source leaves to sink organs is crucial to maintaining photosynthetic stimulation (Ainsworth et al., 2004).

Among C_3 crops, legumes may have a competitive advantage over non-leguminous species when grown at e[CO_2] (Rogers et al., 2009). Legumes can trade the extra carbohydrate assimilated under e[CO_2] with N_2 fixing bacteria

(i.e., bacteroids) to stimulate symbiotic N_2 fixation with the help of enzymes, namely nitrogenase. Legume symbiotic N_2 fixation is based on the exchange of C-and N-metabolites between both partners (Streeter, 1993). Nitrogenase activity and consequently N_2 fixation rate is regulated by the supply of C to the bacteroids (Galvez et al., 2005; Gil-Quintana et al., 2013). For instance, sucrose, the main photoassimilate provided by shoots to nodules, enters the glycolytic pathway to provide carbon skeletons, predominantly in the form of malate, feeding for bacteroids respiration and maintaining nitrogenase activity. In return, root nodule bacteria 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). C and N metabolism of nodule bacteria and the host-plant is important to regulate N_2 fixation process (Aranjuelo et al., 2014).

Low water availability is a major limiting factor for plant growth and induces changes in various physiological and metabolic processes (Robredo et al., 2007). As a consequence of increased atmospheric concentrations of CO_2 and resulting climate change, the frequency and severity of droughts are predicted to increase in many regions (IPCC, 2014). Drought-induced stomatal closure reduces photosynthetic C assimilation and also inhibits translocation of assimilated C (Serraj et al., 1999a). As less C is made available to bacteroids, N_2 fixation of legumes is constrained by limited nodule respiration, decreased ATP synthesis and lower nitrogenase activity (Gil-Quintana et al., 2013). Several metabolic changes are associated with the reduction of N_2 fixation under drought, for example, N-compounds such as asparagine, glutamine or ureides have been reported to accumulate in nodules (Aranjuelo et al., 2013; Serraj et al., 1999b). Changes in whole-plant C and N metabolites were also observed in *M. truncatula* under a water-deficit situation (Aranjuelo et al., 2013).

Metabolite analysis is an effective and quantitative method charting the changes and elucidating the mechanisms of abiotic stress tolerance including drought stress. Drought stress can induce changes in many primary metabolites such as organic acids, amino acids, and carbohydrates, which have important functions involved in photosynthesis and respiration (Khan et al., 2019). These metabolites are involved in various functions within the plant, such as regulating plant-water relations, signalling pathways, protein synthesis as well as defense systems against stress (Krasensky and Jonak, 2012). Empirical evidence suggests that metabolites linked to stress tolerance, for example, hexoses (e.g. sucrose, fructose, glucose) and minor sugars (e.g. trehalose and mannitol) accumulate under water restriction (Aranjuelo et al., 2013). For organic acids and amino acids, it has been reported that the abundance of many metabolites, such as asparagine, γ-aminobutyric acid (GABA), β-alanine, alanine, and proline as well as malate, fumarate, isocitrate can be enhanced by drought stress in *M. truncatula*. However, studies investigating these metabolites profiles have been conducted in controlled environments under a[CO_2] conditions where plants were neither exposed to natural gradients nor e[CO_2] environments. How e[CO_2] would modify the response of C- and N- metabolites to drought is not well understood.

It has been suggested that e[CO_2] mitigates the impact of stress on sugar and amino acid metabolism (Zinta et al., 2018), because [CO_2] may stimulate C-assimilation, and also because 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). Among the relatively few studies that have investigated metabolite changes under the interactive effect of e[CO_2] and drought, some reported positive effects of e[CO_2] on N_2 fixation under

drought stress (De Luis et al., 1999), whereas more recent studies reported negative (Gray et al., 2016) or transient effects (Parvin et al., 2018) on N_2 fixation under severe drought. Exchange of C and N metabolites between bacterial symbionts and host plants is key to the symbiotic N_2 fixation process. Better characteristics of metabolic patterns may help to better understand mechanisms controlling photosynthesis and N_2 fixation under drought and this will underpin strategies to enhance legume tolerance to future [CO_2] rich environments.

There is a large variation between organs or even specific tissues in their metabolic response to drought stress (Aranjuelo et al., 2013). Also, field investigations have shown that different genotypes may show distinct N_2 fixation responses under drought stress (Parvin et al., 2018). Therefore, the objective of this study was to characterize responses of primary metabolites including soluble sugars, organic acids, and amino acids to better understand how photosynthesis and N_2 fixation is affected and regulated under e[CO_2] and drought. Using GC-QqQ-MS and LC-QqQ-MS metabolomic approaches, various metabolites involved in TCA, glycolysis, photorespiration and amino acid metabolism were determined quantitatively. To determine the key metabolites that can explain the response of N_2 fixation under seasonal drought, multivariate statistics via principal component analysis was performed. Finally, the changes in key metabolic pathways were mapped which will help us better understand the specific responses of legumes in future climatic changes.

3.2 Materials and methods

3.2.1 Experimental site and plant materials

Two field experiments (also reported in Chapter 2) were conducted during 2015 (low rainfall season) and 2016 (high rainfall season) 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). According to the Australian Soil Classification, the soil type is a cracking clay with a non-dispersive and pedal surface, approximately ~35% clay at the surface increasing to 60% in 1.4 m depth classified as a Vertosol (Isbell 2002). The soil had pH of 7.74 ($CaCl_2$), EC of 0.56 dS m^{-1}, organic C of 0.42 (%), total N of 0.09 (%), ammonium-N of 2.01 mg kg^{-1}, nitrate-N of 1.50 mg kg^{-1}, P (Colwell) of 5.80 mg kg^{-1}, K (Colwell) of 354.58 mg kg^{-1}, S (KCl) of 220.86 mg kg^{-1}, Fe (DTPA) of 16.50 mg kg^{-1}, Cu (DTPA) 1.22 mg kg^{-1}, Mn (DTPA) of 2.68 mg kg^{-1}, Zn (DTPA) of 0.42 mg kg^{-1}, Ca^{2+} (meq/100 g) of 20.71, Mg^{2+} (meq/100 g), K^+ of 0.92 (meq/100 g) and Na^+ of 8.28 (meq/100 g). These values are means of 50 soil samples collected from 0 to 120 cm depth from all plots in the AGFACE site at the beginning of the experiment.

The AGFACE site had a Mediterranean type climate with cooler and wet winters but very hot and dry summers. Long-term (30-year) average annual rainfall of the area is 435 mm (274 mm rainfall during crop growing season). During the crop growing season, long-term average maximum and minimum temperatures are 17.6°C and 5.3°C, respectively, with July being the coldest period (Australian Bureau of Meteorology). The total amount of rainfall during the crop growing season was 128.4 mm in 2015 and 334.2 mm in 2016. Rainfall was extremely low in 2015 and well below the average long-term growing season amount (274 mm), therefore a total of 96 mm irrigation in three splits was applied from September to October to prevent crop failure (all after flowering samplings were collected). Even with added irrigation, the 2015 season remained below average ('dry season'), whereas 2016 was well above ('high rainfall season'). 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 site (MEA Premium Weather Station 103, Measurement Engineering Australia, Magill, SA, Australia) and is presented in Fig. 3.1.

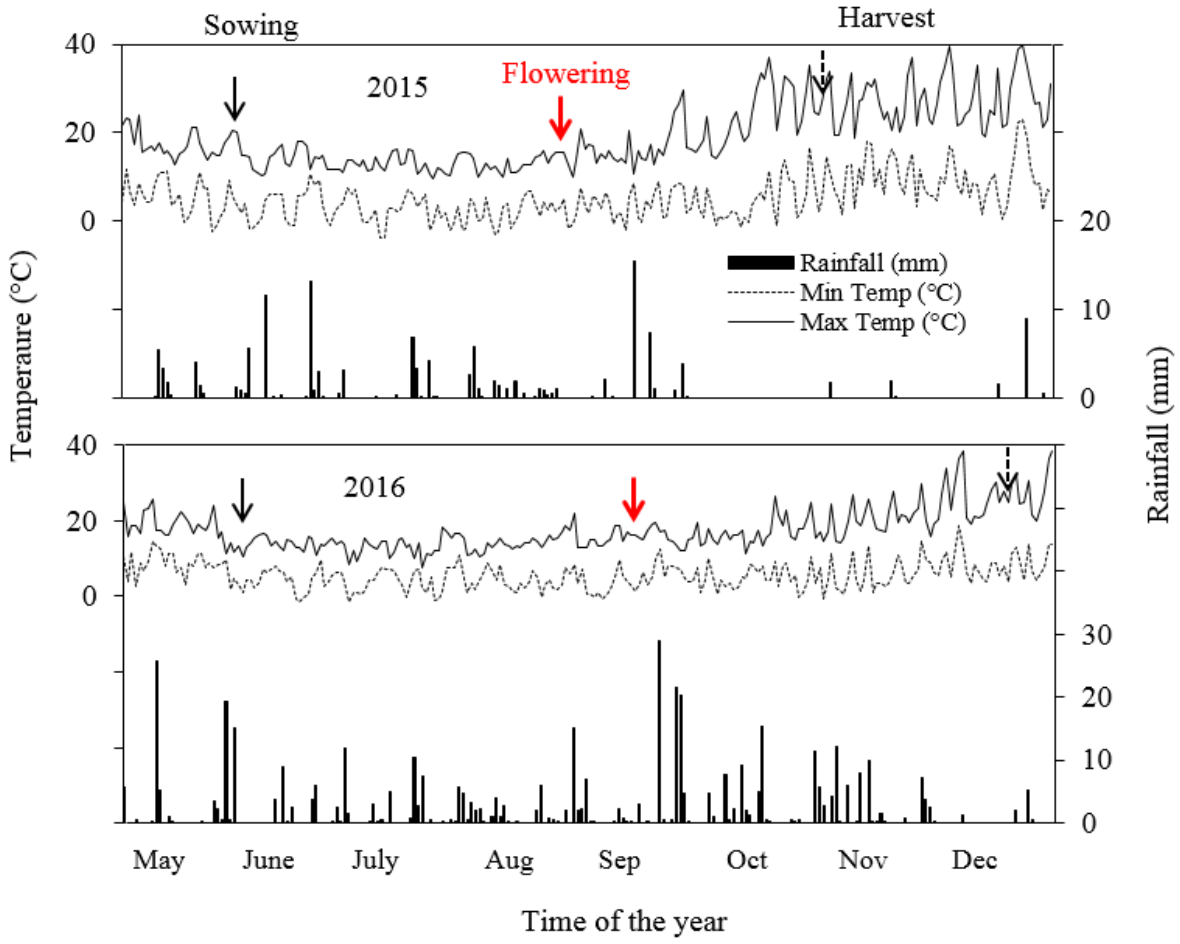


Fig. 3. 1 Seasonal rainfall (black bars, mm), maximum (Max Temp, continuous lines) and minimum (Min Temp, broken lines) temperatures (°C) recorded on-site during a dry (2015) and a wet (2016) growing seasons in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility, Horsham, Victoria, Australia (extracted from Chapter 2). Black solid arrows indicate sowing, red arrows indicate flowering stage (tissue sampling and metabolites were analysed), and broken arrows indicate maturity.

The experimental design was 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}. In 2015, rings were 12m in diameter, whereas in 2016, the ring diameter was 16 m. Each elevated [CO_2] plot was arranged in an octagonal shape and encircled by stainless-steel perforated CO_2-release-tubes. The tubes were adjusted as the crop grew so that the CO_2 was injected about 15 cm above the canopy. At each plot, the centre CO_2 concentration of 550 ppm was maintained for the elevated CO_2 treatment from sunrise to sun-set starting from germination and continued until physiological maturity (Mollah et al., 2009). Average plot central CO_2 concentrations were recorded every minute with an infrared gas analyzer (IRGA, SBA-4, PP Systems, Amesbury, MA, USA) installed at the center of each plot. The performance of the CO_2 exposure system in AGFACE was described by Mollah et al. (2011).

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 is more sensitive to stress conditions (Bourgault et al. 2017). Seeds were inoculated with Group F® (WSM1455, *Rhizobium leguminosarum*) peat-based inoculant (NoduleNTM, NewEdge Microbials Pty Ltd. Albury, NSW, Australia) before sowing. 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 0.24 m. For each genotype, the plot size was 1.5 m by 4 m length in 2015 and 1.5 m by 2 m length in 2016. In each year, superphosphate fertilizer was applied at the rate of 9 kg P ha^{-1} and 11 kg S ha^{-1} just before sowing but no N fertilizer was applied. Pre-emergence herbicide (simazine, dimethenamid-P, trifluralin) was applied before sowing to control weeds.

3.2.2 Sampling

Destructive samples were collected at flowering corresponding to the growth stages of full bloom (R2) (Erskine et al. 1990). Plants of four middle rows of 30 cm length across the plot (corresponding to 0.29 m^2) were collected for biomass measurements (reported in Chapter 2). Wheat was grown as reference adjacent to lentil plots and collected for ^{15}N calculation. For metabolomic analysis, freshly collected leaves from the top shoot were collected and immediately frozen in liquid nitrogen. For nodule collection, intact plants were uprooted in the field. Roots systems were washed with running water very quickly and carefully. After carefully washing and removing all soil particles from the root systems, ~100 mg of freshly separated nodules was immediately frozen in liquid nitrogen and stored in -80°C until analysis.

3.2.3 Leaf gas exchange and chlorophyll content

In both seasons, gas exchange measurements were conducted at the flowering stage using an infrared gas analyzer (IRGA) system (Li- 6400, Li-Cor, Lincoln, NE, USA). The youngest fully expanded leaf was measured *in situ* at a block temperature of 20 ºC and an air flow rate through the chamber of 500 µmols^{-1}. Reference [CO_2] concentrations were adjusted to 400 and 550 µmolmol^{-1} in both ambient and elevated rings to take measurements at growth [CO_2] and also to measure photosynthetic acclimation (Erice et al., 2014). In dry season, relative humidity was within 50-55% and vapour pressure deficit was between 1.8 and 2.5 kPa, whereas in the wet season, these values were between 65-70% and 0.8 to 1.4 KPa for relative humidity and vapour pressure deficit, respectively. But these variables were not significantly different between treatments in each season. In each measurement, the leaf area enclosed inside the cuvette was sampled and measured with a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). Light-saturated photosynthesis rate (A_{sat}) were recalculated based on the actual leaf area. Photosynthetic acclimation index (AI) for e[CO_2] grown plants was calculated from A_{sat} of e[CO_2] grown plant measured at a[CO_2] (~400 ppm) divided by the A_{sat} of ambient-grown plant measured at a[CO_2] (~400 ppm) as described by Jifon and Wolfe (2002). The AI of a[CO_2] grown plants is one and AI values of e[CO_2] grown plants below one is considered as downward photosynthetic acclimation.

Leaf chlorophyll content was measured with a chlorophyll meter (SPAD 502, Konica Minolta, Macquarie Park, NSW, Australia). For each replicate, six readings per plant from the fully expanded topmost leaves were randomly taken and averaged.

3.2.4 N concentration and N_2 fixation

Oven dried plant materials were finely ground and powered in a ball mill (Tissue Lyser, Qiagen). Finely ground powdered tissues (leaves, stems, flowers, roots, nodules, and reference wheat) were analysed for N concentration (% 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). ^{15}N (atom %) values were converted into $\delta^{15}N$ (‰) 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 the ^{15}N natural abundance method based on the difference in $\delta^{15}N$ (‰) signature between atmospheric N_2 and soil N. The details are described in Parvin et al. (2018).

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

Where 'reference plant' refers to a non N_2 fixing plant (wheat in this case) selected to match closely to the studied lentil in terms of uptake of soil sources of N. Factor B refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking N. Nodulated lentil was grown in sand in a glasshouse. Organ-specific B-values ($\delta^{15}N$, ‰) were estimated for %Ndfa calculation (Unkovich & Pate 2000) and B-value for seed N was also adjusted based on Nebiyu et al. (2014).

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 (tissue [N] × biomass) and expressed as kg ha^{-1}.

3.2.5 Metabolite profile analysis

3.2.5.1 Sample preparation and extraction

Frozen leaf and nodule samples were extracted as previously described (Dias et al., 2015). Briefly, 30 mg of frozen leaves and nodules tissues were weighed into *cryomill* tubes (Precellys lysing kit, Bertin Technologies). In each *cryomill* tube, 400 µL of 100% methanol containing 4% internal standard (from a stock solution containing 0.5 mg mL^{-1} $^{13}C_6$- sorbitol and 0.5mg mL $^{13}C_5$–^{15}N valine) was added. Using a Cryomill (Precellys 24, Bertin Technologies), samples were vortexed for the 30s and homogenized at 6400 × g for 60 s at −10°C. The samples were then extracted for 15 min at 70°C in a thermomixer at 850 × g and subsequently centrifuged for 5 min at 4°C and at 13,000 × g. Aliquots of the supernatant of each sample were transferred into new reaction tubes. Water (400 µL) was added into the cryo-mill tubes containing previously ground tissues. These tubes were vortex-mixed for the 30s and centrifuged at 13,000 × g for 10 min at 4°C. Aliquots of the supernatant were then transferred into the reaction tube containing the original supernatant from the previous centrifugation. The supernatants were combined and vortex-mixed for the 30s. For sugars and organic acids analysis, 5 and 125 µL aliquots of the supernatants were transferred into glass vial inserts and dried in *vacuo* for GC–QqQ–MS analysis. Also, a 10 µL aliquot was transferred into glass vial inserts and dried in *vacuo* for LC–QqQ–MS amino acid analysis.

3.2.5.2 Derivatization for GC–MS and LC-MS analysis

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. Metabolites 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. The standard solution for each metabolite was obtained from Sigma Aldrich.

3.2.5.3 Quantification of GC–MS and LC-MS metabolites peak and data analyses

Approximately, 1µl aliquot was injected into a GC–QqQ–MS system comprising of a Gerstel 2.5.2 Autosampler, a 7890A Agilent gas chromatograph and a 7000 Agilent triple-quadrupole MS (Agilent, Santa Clara, USA) with an electron impact (EI) ion source. Similarly, 1µl aliquot of samples or standard was injected into an Agilent 1200 LC-system coupled to an Agilent 6410 Electrospray Ionisation-Triple Quadrupole-MS was used for amide-N quantification. For analyses, external calibration curves were prepared using authentic standards. Finally, using the standard calibration curves, the concentration of the metabolites was determined by fitted linear regression curves. Data were processed using the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for quantitation of all compounds (Dias et al., 2015).

3.2.6 Statistical analysis

All data were analyzed using the statistical software "R" version 3.5.0 (R Core Team, 2018) using a split-plot design (year: main plots, [CO_2]: sub-plots, genotypes: sub-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 the year, [CO_2] and genotypes as fixed effect and ring numbers as random effect. 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 were done where applicable.

Heat map in combination with hierarchical cluster analysis of GC-MS and LC-MS data was performed 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). The changes in the metabolomes with the factors studied (leaves, nodules, genotypes, and season) were analysed by principal component analysis (PCA) and PC scores presented as biplots. The PCAs were performed with the prcomp function of the 'stats' package of R (R Core Team 2018). Metabolic pathway map was constructed using the primary metabolic network of VANTED. Metabolites that significantly differed between growing season, [CO_2] and or genotypes were normalised log-transformed and is presented as heat map in the metabolic mapping.

3.3 Results

3.3.1 Photosynthetic acclimation

In the dry year, A_{net} was significantly lower in e[CO_2] grown lentil than a[CO_2] grown ones, when both were measured at a common ambient [CO_2] (~ 400 ppm) (Fig. 3.2A); the corresponding negative acclimation index suggests photosynthetic downward acclimation in the dry year.

In the wet year, both cultivars had significantly higher A_{net} at 550 ppm CO_2 concentrations irrespective of growth condition (Fig. 3.2B). No significant difference was observed among genotypes. There were significant effects of

CO_2 but not genotype (CV) and their interaction [CO_2] × CV on photosynthetic rates. No photosynthetic acclimation was observed in these measurements (acclimation index values were > 1; Fig. 3.2C).

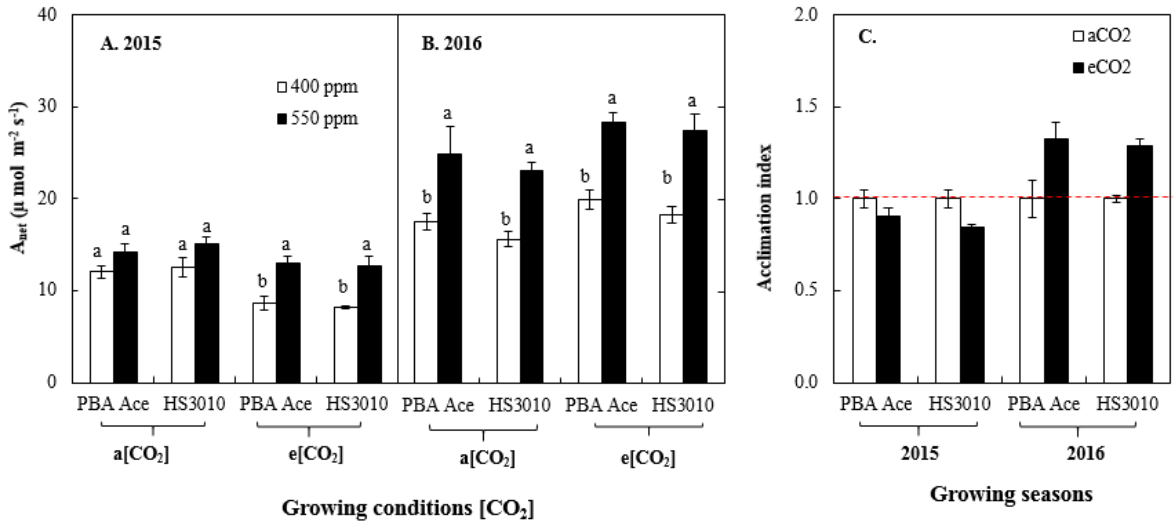


Fig. 3. 2 Photosynthetic net CO_2 assimilation rates (A_{net}) in 2015 (A) and 2016 (B) and photosynthetic acclimation index (C) of two lentil genotypes "PBA Ace" and "HS3010" measured under ambient [CO_2] (~400 ppm, white bars) or measured at elevated [CO_2] (~550 ppm, black bars) at flowering stage in the Australian grains free-air CO2 enrichment facility, Horsham, Australia, during a dry (2015, A) and a high (2016, B) rainfall season. Means and standard errors of n=4 replicates. Mean values followed by different letters are significantly different between [CO_2] treatments (LSD at P<0.05). Parts of the A_{net} data were extracted from Chapter 2.

3.3.2 Chlorophyll content and N concentration

Chlorophyll content was significantly lower in the dry than the wet year, and this difference was greater under e[CO_2] than a[CO_2] (Table 3.1). Consistent with chlorophyll content, leaf N concentration ([N]) was lower under e[CO_2] in the dry season. A significant interaction between e[CO_2] and genotype observed for nodule [N], where e[CO_2] increased nodule [N] of PBA Ace but decreased in HS3010.

3.3.3 N_2 fixation and total N content

Elevated [CO_2] increased N_2 fixation with greater magnitude in the wet than in the dry season (Table 3.1). Under dry season, N_2 fixation of PBA Ace was 13% higher under e[CO_2] compared to a[CO_2], but under wet season, both genotypes showed similar stimulation under e[CO_2] compared to a[CO_2]. Total N content was greater in the wet than in the dry season. Elevated [CO_2] stimulated total biomass N by 1.5 folds over a[CO_2] in the dry and 2-fold in the wet season.

Table 3. 1 Leaf chlorophyll content (SPAD unit), nitrogen concentrations ([N]), N_2 fixation and total N content 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 wet (2016) season. Means and standard errors of n=4 replicates. Unit for [N]: mg g^{-1} dry weight, N_2 fixation: kg ha^{-1}, total N content: kg ha^{-1}

Season	CO_2	CV	Chlorophyll	Leaf [N]	Nodule [N]	N_2 fixation	Total N content
2015	a[CO_2]	PBA Ace	45.7±3.2	43.1±0.5	68.1±3.0	72.6±4.0	109.6±5.1
		HS3010	43.2±2.4	40.1±1.1	65.9±3.7	62.9±3.3	103.6±2.5
	e[CO_2]	PBA Ace	37.2±1.2	43.8±0.5	70.2±4.8	109.8±2.3	140.2±5.1
		HS3010	34.2±1.3	39.2±1.1	66.9±2.1	78.4±7.2	121.0±1.2
2016	a[CO_2]	PBA Ace	58.2±2.5	60.1±1.8	63.3±3.3	129.7±4.5	178.8±15.3
		HS3010	55.3±3.2	48.5±1.1	68.3±1.4	105.5±6.6	168.7±3.0
	e[CO_2]	PBA Ace	57.2±1.2	58.2±2.2	65.2±3.4	191.7±8.4	216.3±14.0
		HS3010	55.2±2.3	45.2±2.1	60.5±2.6	179.0±2.2	196.2±2.2
Statistics							
	Season		0.001	0.001	ns	0.001	<0.001
	[CO_2]		ns	0.045	ns	0.025	0.001
	CV		ns	0.001	ns	0.001	0.004
	Season × [CO_2]		0.045	<0.001	ns	0.025	0.045
	Season × CV		ns	ns	ns	ns	ns
	[CO_2] × CV		ns	ns	0.048	ns	ns
	Season × [CO_2] × CV		ns	ns	ns	0.001	ns

3.3.4 Metabolite profiling

Using a GC-QqQ-MS and LC-QqQ-MS metabolomics approach, a total of 65 known metabolites were identified in leaf and nodule tissues. These metabolites were highly reproducible among the four biological replications across two different growing seasons. The identified metabolites included 16 sugars, 5 sugars-alcohols, 17 organic acids, and 27 amino acids.

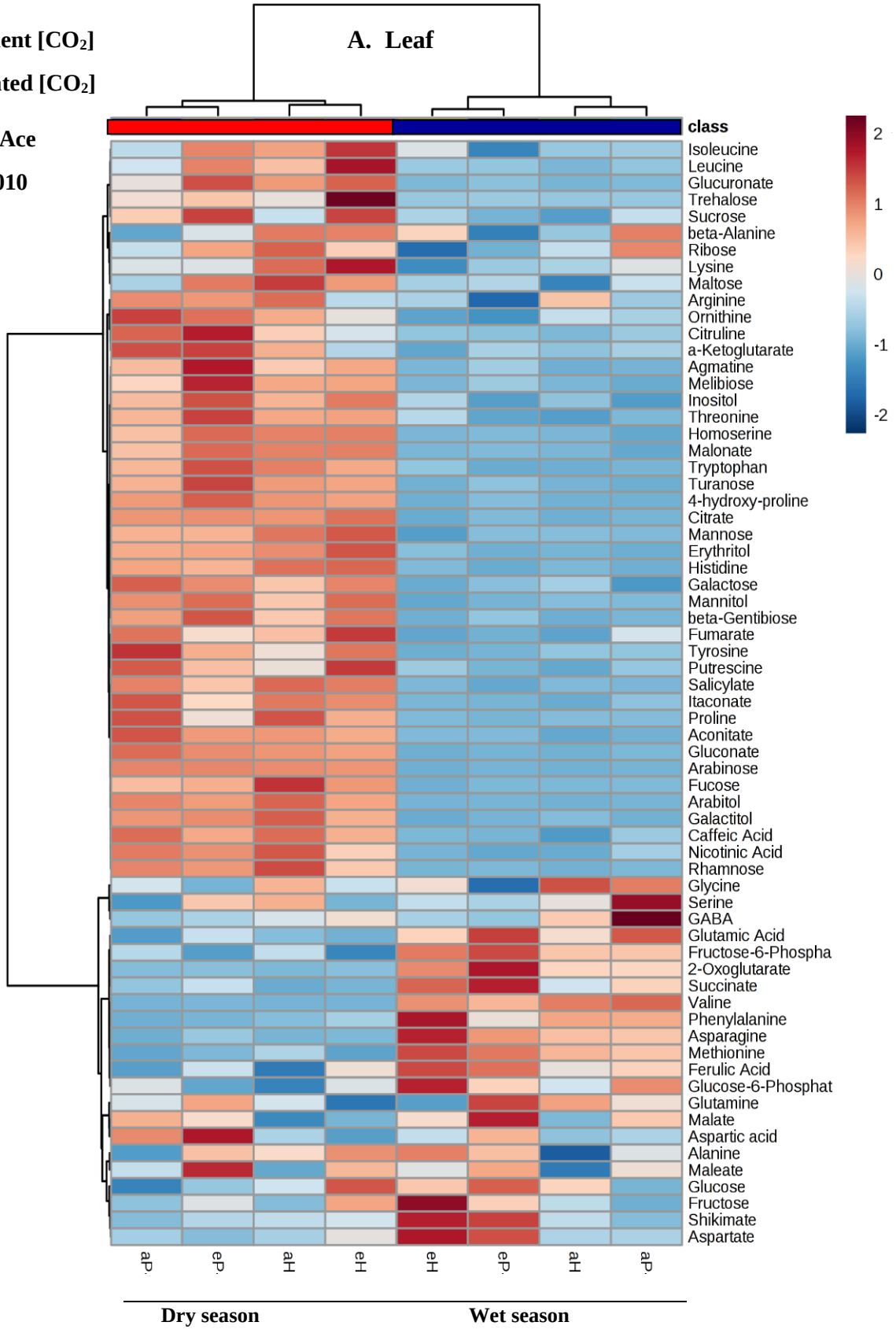
The heat map produced by hierarchical clustering showed that the major difference between metabolites patterns between growing seasons and between leaves and nodules (Fig. 3.3) but the effects of e[CO_2] and genotypes was less pronounced. Two major's metabolite clusters were identified with distinct patterns of relative abundances. In the leaf (Fig. 3.3A), the first cluster consisted of metabolites that were more abundant in the dry season compared with the wet growing season. These metabolites included sucrose, trehalose, proline, sugar alcohols (e.g., arabitol, mannitol, galactitol, erythritol) and organic acids (e.g., fumarate, aconitate, citrulline, citrate, nicotinic acid, caffeic acid). A second cluster consisted of metabolites that were less abundant in the dry season compared with the wet growing season. This second clustered included some organic acids (malate, succinate, shikimate) and amino acids (e.g., asparagine, glutamine, GABA, serine).

a: Ambient [CO_2]

b: Elevated [CO_2]

P: PBA Ace

H: HS3010



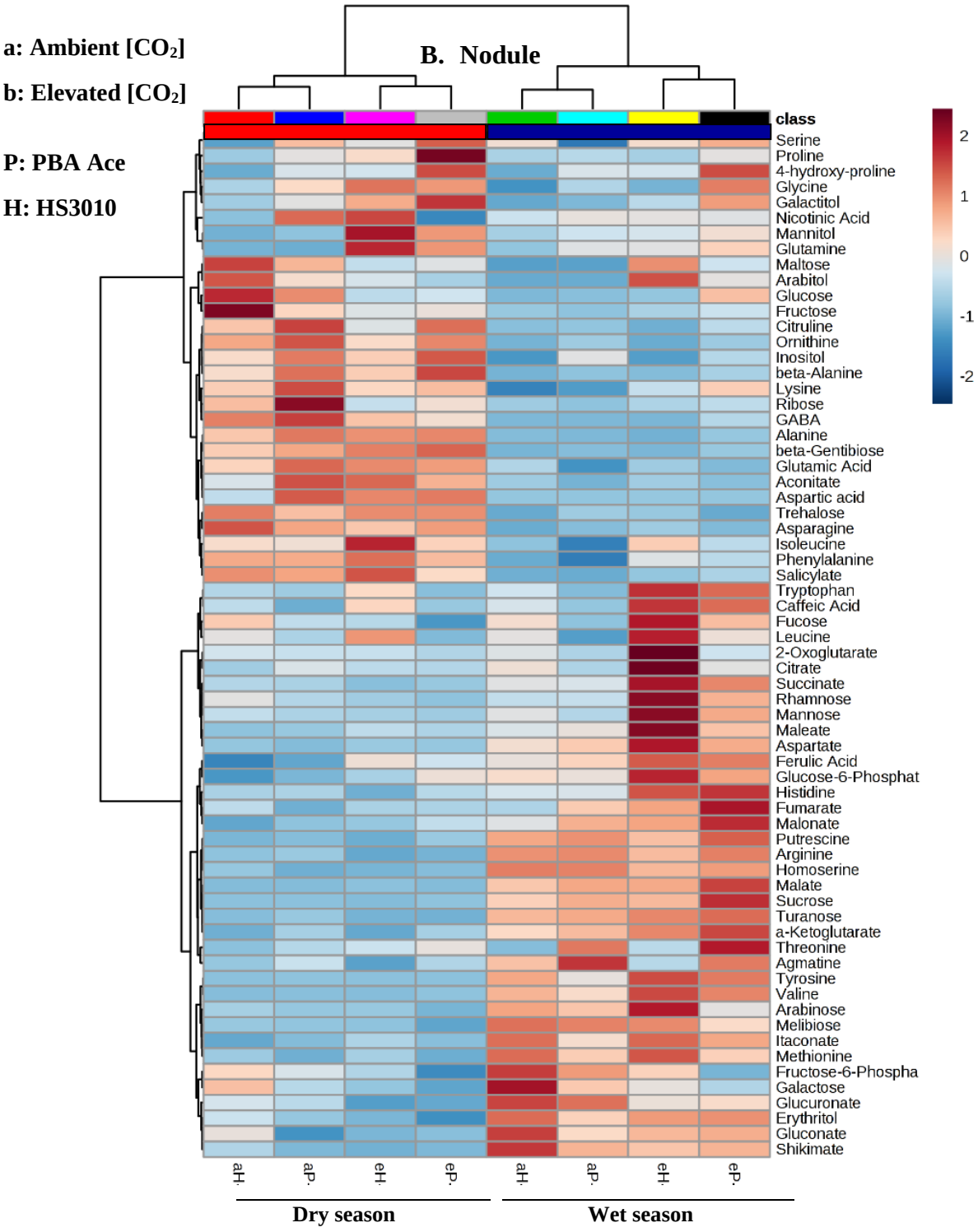


Fig. 3. 3 Hierarchically clustered heat maps showing variation in the concentration (µmol g^{-1} dry weight) of metabolites in leaves (A) and nodules (B) of two lentil genotypes "PBA Ace (P)" and "HS3010 (H)" grown under ambient [CO_2] (a, ~400 ppm) or elevated [CO_2] (e, ~550 ppm) in the Australian 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 column represents four replicates per organ. 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.

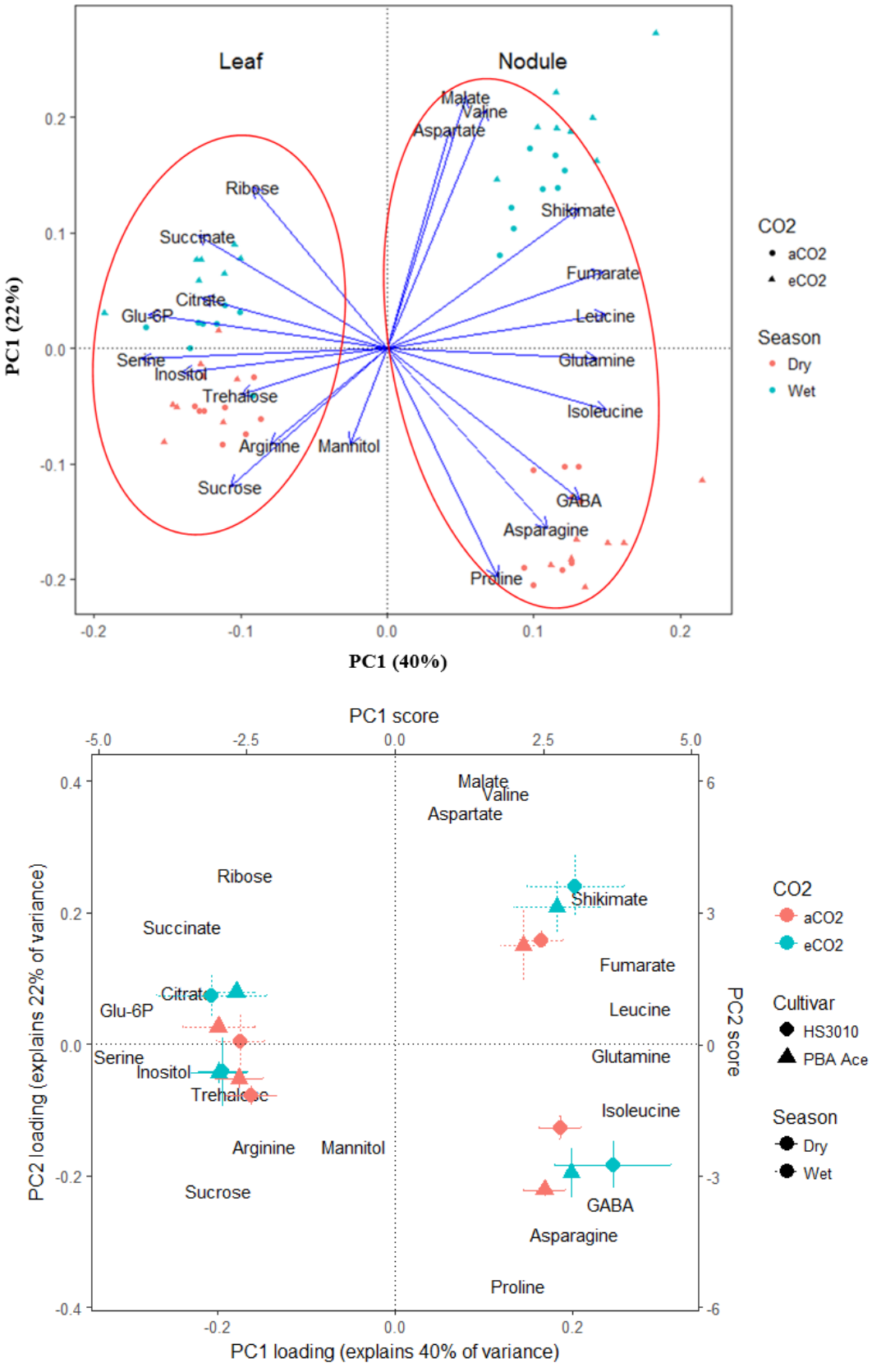


Fig. 3. 4 Principal component analysis (PCA) illustrating the variation of metabolites concentration between leaf and nodule (top panel) and between [CO_2] treatments (bottom panel). Dry and wet seasons are represented as solid standard errors and dotted standard errors, respectively in PC1 vs PC2 axes.

In nodules (Fig. 3.3B), these trends were reversed, whereby greater abundance of amino acids and lower abundance of sugars were observed in the dry season. In both organs, glucose 6-phosphate and fructose 6-phosphate were less abundant during the dry season but was found higher level during the wet season. Comparing two genotypes, serine, GABA, aspartic acid and malate were abundantly found in leaves of PBA Ace during the wet season, whereas trehalose, fumarate, iso-leucine, leucine, and lysine were mostly present in HS3010 under dry condition. In nodules, increased abundance of fructose, ribose, and proline was observed in PBA Ace under dry season, but glucose, mannitol, isoleucine, glutamine, and asparagine abundance were greater in HS3010. The patterns of metabolite clustering clearly indicate the metabolic differences between organs under two contrasting seasons.

To further investigate how the metabolism of each organ was modified by elevated [CO_2], growing season and genotypes, we performed PCA taking into account of biologically important metabolites associated with C- and N_2 fixation process of legumes (Fig. 3.4). The first component (PC1) explained 40% of total variation, whereas the second component (PC2) explained 22% variation across the data set (Fig. 3.4). The bi-plot between PC1 and PC2 revealed two distinct groups associated with the leaf and nodule samples at dry and wet seasons (Figure 4, top panel), suggesting a clear distinction between two growing seasons and organs in the metabolite patterns. In comparison, the difference between varieties and CO_2 were small (Fig. 3.4, bottom panel).

Metabolites that were significantly differed between growing season and [CO_2] and/or genotypes are reported in Table 3.2. In the dry season, sucrose and trehalose were significantly more abundant in the leaves of plants grown under e[CO_2] but oxoglutarate, shikimate, and aspartate were less abundant. In contrast, in the wet season, e[CO_2] decreased the level of sucrose but increased organic acids (i.e. oxo-glutarate, aspartate, succinate, shikimate) in leaves. In nodules, the abundance of trehalose, mannitol, inositol, glutamine, aspartic acid was up-regulated by e[CO_2] during the dry season but down-regulated the level of sucrose, succinate, shikimate, tyrosine, valine, histidine. However, the level of sucrose, malate, and succinate, aspartate in nodules were more abundant under e[CO_2] during the wet season and remained unchanged during the dry season.

In nodules, three-way interactions among growing season, [CO_2] and genotype was significant for sucrose, fructose, maltose, mannose, galactitol, oxoglutarate, malate, asparagine, aspartic acid, glutamic acid, and proline. During the wet season, the accumulation of sucrose and malate under e[CO_2] was substantially higher in nodules of PBA Ace than of HS3010, whereas their accumulation was slightly higher in nodules of PBA Ace during the dry season. In the dry season, the accumulation of proline in nodules of HS3010 was about 4-folds higher under e[CO_2] than a[CO_2] but this accumulation was similar in nodules of PBA Ace under both [CO_2]. In leaves, a three-way interaction was only significant for 2-oxoglutarate. During the wet season, e[CO_2] stimulated the accumulation of 2-oxoglutarate to a greater extent in PBA Ace than in HS3010. Whereas, during the dry season, its accumulation was less in PBA Ace under e[CO_2]. Metabolic mapping (Fig. 3.5) was performed highlighting the metabolites that showed significant two-way (season × [CO_2]) and/or three-way (season × [CO_2] × CV) interactions.

Table 3. 2 Metabolites (μ mol g^{-1} dry weight) that were significantly different between season × $[CO_2]$ and/or season × $[CO_2]$ × CV (indicated by an asterisk) in either leaf or nodule, otherwise as for Table 3.1. Statistics are reported in Table 3.3.

Season	Dry				Wet			
CV	PBA Ace		HS3010		PBA Ace		HS3010	
$[CO_2]$	a$[CO_2]$	e$[CO_2]$	a$[CO_2]$	e$[CO_2]$	a$[CO_2]$	e$[CO_2]$	a$[CO_2]$	e$[CO_2]$
Leaf								
Sucrose	3981.6	5089.2	3241.9	5073.1	3226.8	2625.4	2405.8	3021.5
Fructose	1151.7	1308.7	1133.7	1504.6	1107.7	1415.7	1240.8	1800.9
Trehalose	249.8	329.2	224.1	742.6	59.4	63.2	55.1	58.7
Turanose	349.4	295.4	351.1	477.3	359.5	792.8	367.9	881.3
Melibiose	1.6	2.2	2.3	2.6	1.6	5.2	2.3	5.6
2-Oxuglutarate*	4.4	4.6	4.2	4.6	10.3	18.3	10.4	13.8
Shikimate	1.6	2.2	2.3	2.6	1.6	5.2	2.3	5.6
Aspartate	349.4	295.4	351.1	477.3	359.5	792.9	367.9	881.3
Aspartic acid	159.6	183.7	116.4	99.5	116.5	149.5	110.1	121.4
GABA	8.1	9.1	11.4	13.1	26.1	8.1	14.7	8.9
Proline	232.0	113.2	231.1	167.4	23.4	17.4	23.3	21.3
Nodule								
Sucrose*	298.4	366.3	313.6	214.1	3371.1	5330.5	2706.8	3134.7
Fructose*	16.9	15.2	28.3	14.3	10.2	13.1	10.7	11.5
Fructose-6P	48.9	20.4	59.8	40.8	73.4	30.6	89.7	61.2
Glucose	38.4	26.1	45.5	24.6	20.6	33.9	19.9	21.3
Trehalose	13.9	16.3	17.0	16.4	7.3	5.1	5.1	7.1
Maltose*	61.9	44.8	83.8	38.1	19.8	40.3	19.4	69.7
Mannose*	5.1	3.2	7.8	4.2	5.8	24.9	12.4	46.3
Turanose	175.2	252.6	249.0	281.9	975.4	1159.7	803.8	1877.2
Melibiose	22.2	22.1	30.4	20.6	55.5	55.4	76.1	51.6
Mannitol	10.7	29.2	8.5	40.6	16.4	20.8	12.6	17.1
Inositol	117.5	224.9	111.7	226.3	13.3	16.8	13.3	21.8
Galactitol*	2.1	1.5	2.3	2.1	5.1	3.8	5.8	8.4
Erythritol	4.8	3.9	4.3	4.0	9.8	11.4	9.3	10.7
2-Oxoglurate*	53.1	36.4	63.6	52.4	34.9	57.9	73.4	298.5
Citrate	135.2	118.2	111.6	121.8	116.7	139.6	145.9	249.8
Malate*	558.2	797.9	534.8	563.1	5596.4	8010.6	4686.8	5566.9
Succinate	25.5	20.4	27.3	17.1	38.2	75.7	40.9	104.4
Shikimate	22.2	22.1	30.4	20.6	55.5	55.4	76.1	51.6
Aspartate	175.2	252.6	249.1	281.9	975.4	1159.7	803.8	1877.2
Asparagine*	3771.3	3916.2	4984.8	3182.4	673.7	624.8	344.1	1047.9
Glutamine	157.4	277.4	160.5	329.2	215.3	242.6	172.7	215.9
Aspartic acid*	219.8	443.7	157.1	246.3	178.1	220.1	168.5	164.7
Glutamic acid*	648.3	597.1	529.0	614.8	321.8	373.1	421.9	401.6
GABA	167.2	93.3	141.7	111.5	37.3	59.7	37.5	36.2
Proline*	735.7	792.2	161.2	707.7	13.1	21.7	18.4	28.4
Lysine	38.1	30.9	29.6	28.8	16.9	29.5	14.7	23.6
Tyrosine	0.7	0.6	0.5	0.6	10.9	25.4	20.8	29.9
Valine	3.6	4.5	2.7	3.2	33.5	57.3	46.1	70.0
Histidine	36.7	36.7	25.2	22.9	71.8	99.2	62.9	84.7
Putrescine	9.8	20.1	6.7	9.3	2.1	2.8	1.7	5.5

Table. 3 P-values for the effect of season, [CO_2] and genotype (CV) and their interaction for all identified metabolites

	Season		[CO_2]		CV		Season × [CO_2]		Season × CV		[CO_2] × CV		Season ×[CO_2] × CV	
	Leaf	Nodule	Leaf	Nodule	Leaf	Nodule	Leaf	Nodule	Leaf	Nodule	Leaf	Nodule	Leaf	Nodule
Sugars and sugar-alcohols														
Sucrose	<0.001	<0.001	0.038	0.069	ns	<0.001	ns	**0.045**	ns	ns	ns	0.005	ns	**0.019**
Fructose	0.019	<0.001	0.019	0.085	ns	0.015	ns	**0.009**	ns	0.003	ns	<0.001	ns	**<0.001**
Glucose	ns	0.034	ns	ns	ns	ns	ns	**0.012**	ns	ns	ns	ns	ns	ns
Fructose -6P	<0.001	0.007	ns	<0.001	ns	0.012	**0.004**	ns	ns	ns	ns	ns	ns	ns
Glucose-6P	0.001	0.079	ns	ns	ns	ns	ns	ns	ns	ns	0.003	ns	ns	ns
Trehalose	0.002	<0.001	<0.001	ns	<0.001	ns	**0.002**	ns	<0.001	ns	<0.001	ns	ns	**0.045**
Ribose	ns	0.01	ns	ns	ns	0.022	ns	ns	<0.001	ns	ns	0.021	ns	ns
Maltose	0.004	0.003	ns	ns	ns	0.063	ns	**<0.001**	0.077	ns	ns	ns	0.055	**0.018**
Rhamnose	<0.001	<0.001	ns	ns	ns	0.004	ns	ns	ns	ns	ns	ns	ns	ns
Mannose	<0.001	<0.001	ns	<0.001	ns	<0.001	ns	**<0.001**	ns	<0.001	ns	<0.001	ns	**<0.001**
Galactose	<0.001	ns	ns	0.038	ns	0.005	ns	ns	ns	ns	ns	ns	0.099	ns
Turanose	0.019	<0.001	ns	0.055	ns	ns	**0.025**	0.099	ns	ns	ns	ns	ns	ns
β-Gentibiose	<0.001	ns	ns	0.007	ns	0.003	ns	ns	ns	ns	ns	ns	ns	ns
Fucose	<0.001	ns	0.334	ns	0.013	0.029	ns	0.089	0.005	ns	0.035	ns	0.062	ns
Arabinose	<0.001	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Melibiose	<0.001	<0.001	<0.001	ns	ns	ns	**<0.001**	ns	ns	ns	ns	ns	ns	ns
Inositol	<0.001	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Mannitol	<0.001	0.055	ns	ns	ns	ns	ns	**0.001**	ns	0.047	ns	ns	ns	ns
Inositol	<0.001		ns	ns	ns	0.001	0.094	**0.001**	ns	ns	ns	ns	ns	**0.008**
Galactitol	<0.001	<0.001	ns	ns	ns	0.002	ns	ns	ns	0.017	ns	0.026	ns	**0.046**
Erythritol	<0.001	<0.001	ns	ns	ns	ns	ns	**0.043**	ns	ns	ns	ns	ns	ns
Organic acids														
2-oxoglutarate	<0.001	<0.001	0.005	<0.001	0.112	<0.001	**0.010**	**<0.001**	0.156	<0.001	0.010	<0.001	**0.102**	**<0.001**
Malate	ns	<0.001	ns	<0.001	0.031	0.001	ns	**0.001**	ns	<0.001	ns	0.085	ns	**0.046**
Citrate	<0.001	0.048	ns	ns	ns	0.069	ns	**0.101**	ns	0.021	ns	0.095	ns	ns
Maleate	ns	ns	0.052	0.027	0.019	ns	ns	ns	ns	ns	ns	ns	ns	ns
Malonate	<0.001	<0.001		0.057	ns	0.085	ns	ns	ns	ns	0.021	ns	ns	ns
Succinate	<0.001	<0.001	0.009	0.005	ns	0.005	0.052	**<0.001**	ns	ns	ns	ns	ns	ns
Fumarate	<0.001	0.018	ns	ns	ns	ns	ns	ns	ns	ns	0.035	ns	ns	ns
Aconitate	<0.001	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.079
Nicotinic acid	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.093	ns
Caffeic acid	<0.001	ns	ns	0.041	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Ferulic acid	<0.001	<0.001	<0.001	0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Itaconate	<0.001	<0.001	0.085	0.068	ns	0.016	ns	ns	ns	0.031	ns	ns	ns	ns
Shikimate	<0.001	<0.001	<0.001	ns	ns	ns	**<0.001**	ns	ns	ns	ns	ns	ns	ns
Gluconate	<0.001	<0.001	ns	ns	ns	0.040	ns	0.065	ns	ns	ns	ns	ns	ns
Glucuronate	<0.001	0.067	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Salicylate	<0.001	<0.001	0.061	0.061	0.015	0.014	ns	ns	ns	ns	ns	ns	ns	ns

α-Ketoglutarate	<0.001	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Amino acids														
Aspartate	0.019	<0.001	0.011	0.055	ns	ns	**0.025**	0.048	ns	ns	ns	ns	ns	ns
Asparagine	ns	<0.001	ns	ns	ns	ns	ns	ns	ns	ns		0.046		**<0.001**
Glutamine	ns	ns	ns	0.015	0.003	ns	ns	**0.048**	ns	ns	0.001	ns	ns	ns
Arginine	ns	<0.001	ns	ns	ns	0.002	ns	ns	ns	ns	ns	ns	ns	ns
Glycine	ns	0.067	0.099	0.027	ns	0.047	ns	ns	ns	ns	ns	ns	ns	ns
Serine	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Aspartic acid	ns	ns	ns	0.018	0.006	ns	**0.023**	**0.045**	ns	0.035	0.086	0.048	ns	ns
Glutamic acid	<0.001	ns	ns	ns	0.097	ns	ns	ns	ns	ns	0.028	ns	ns	**0.043**
β-Alanine	ns	<0.001	ns	ns	ns	0.017	ns	ns	ns	0.099	ns	ns	0.057	ns
Alanine	ns	<0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
GABA	ns	ns	0.053	ns	ns	ns	**0.020**	**0.003**	ns	ns	ns	ns	ns	0.072
Proline	<0.001	0.001	ns	0.011	ns	<0.001	**0.026**	**0.017**	ns	<0.001	ns	0.001	ns	**0.001**
Hydroxyproline	<0.001	<0.001	ns	0.008	0.087	0.002	ns	ns	ns	ns	ns	ns	ns	ns
Ornithine	0.002	<0.001	ns	ns	ns	0.006	ns	ns	ns	ns	ns	ns	ns	ns
Lysine	0.008	0.003	ns	ns	ns	ns	ns	**0.027**	0.024	ns	ns	ns	ns	ns
Tyrosine	<0.001	<0.001	ns	ns	ns	ns	ns	**0.048**	ns	ns	0.023	ns	**0.018**	ns
Valine	<0.001	<0.001	ns	0.007	ns	ns	ns	**0.009**	ns	0.093	ns	ns	ns	ns
Isoleucine	0.038	0.007	ns	0.061	ns	0.017	ns	ns	ns	ns	ns	ns	ns	ns
Leucine	0.004		ns	0.06	ns	0.013	ns	ns	ns	ns	ns	ns	ns	ns
Phenylalanine	0.004	0.001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Tryptophan	<0.001	ns	ns	0.060	ns	ns	ns	ns	ns	ns	ns	ns	0.071	ns
Threonine	<0.001	0.082	ns	ns	ns	0.014	ns	ns	ns	0.057	ns	ns	ns	ns
Methionine	<0.001	<0.001	0.012	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Homoserine	<0.001	0.006	0.083	ns	0.063	0.045	ns	ns	ns	ns	ns	ns	ns	ns
Histidine	0.003	<0.001	ns	0.051	0.065	0.043	ns	**0.034**	ns	ns	ns	ns	ns	ns
Citruline	<0.001	<0.002	ns	ns	0.005	0.002	ns	ns	0.011	0.049	ns	ns	ns	ns
Agmatine	<0.001	0.005	ns	ns	ns	ns	ns	ns	ns	ns	0.001	ns	**0.035**	ns
Putrescine	<0.001	<0.001	ns	<0.001	ns	0.002	ns	**<0.001**	ns	0.003	ns	<0.001	ns	**0.001**

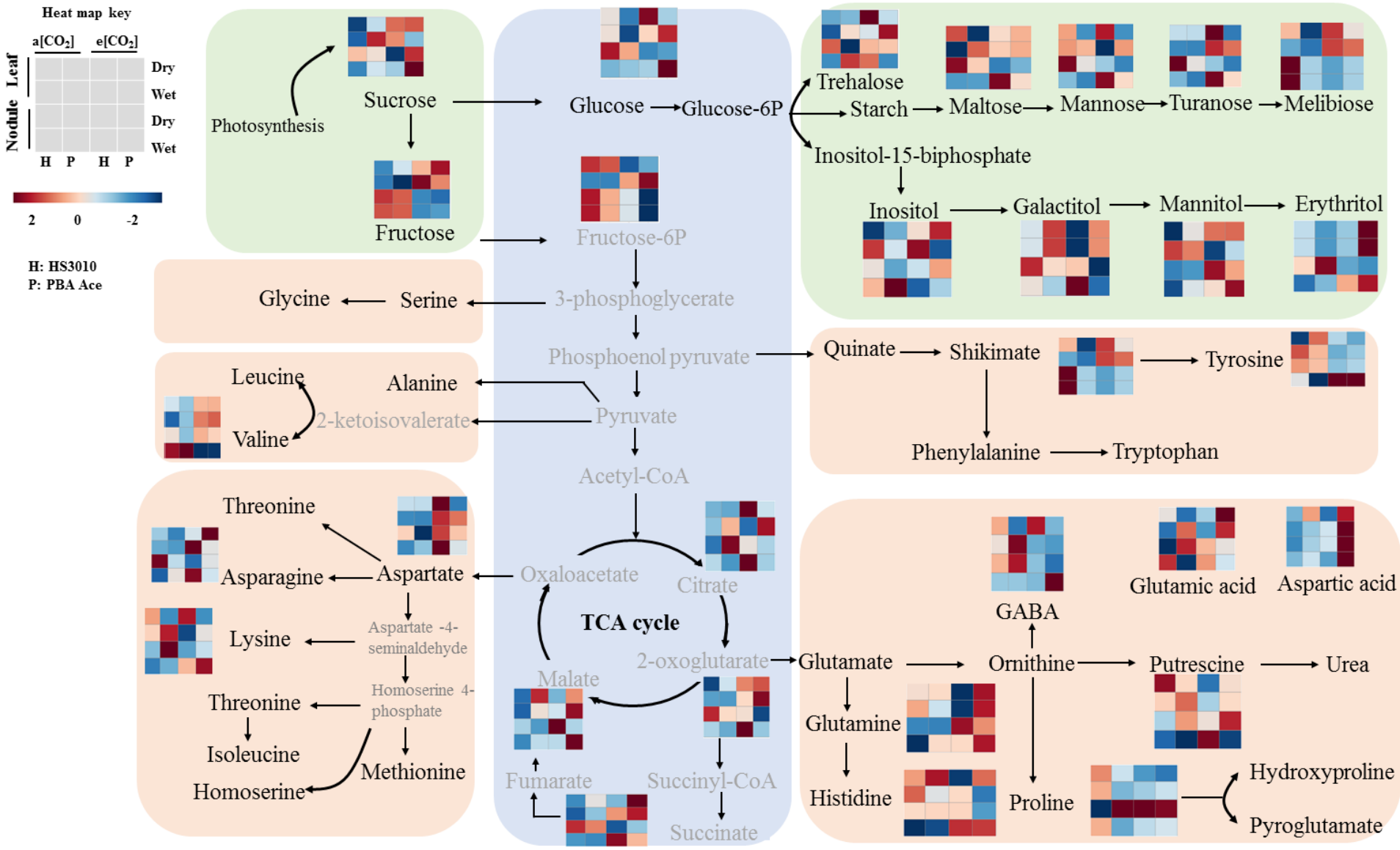


Fig. 3. 5 Pathway map of metabolites in leaves (top two rows in heat map) and nodules (bottom two rows in heat map) of two lentil genotypes (PBA Ace and HS3010) grown under ambient [CO_2] (a[CO_2], left two columns) and elevated [CO_2] (e[CO_2], right two columns) during a dry and a wet season. The 4 × 4 heat maps represent normalized metabolite levels indicating [CO_2] and genotype (vertical) and season and organ (horizontal: dry season-leaf, wet season-leaf, dry season-nodule, wet season-nodule).

3.4 Discussion

Under long term exposure of C_3 plants to e[CO_2], the initial stimulation of photosynthesis may decrease, which is known as photosynthetic acclimation. Based on the acclimation index calculated in this study, such downward acclimation of lentil was observed only in the dry, but not in the wet season. It has been shown earlier that legumes may avoid acclimation, where condition allowed greater biomass growth and increased N_2 fixation under e[CO_2] such as during a wet season (Parvin et al., 2018). Such stimulation of biomass growth and N_2 fixation responses under the interactive effect of e[CO_2] and drought has discussed in Chapter 2.

There is close coupling between photosynthesis and N_2 fixation. In this study, e[CO_2] stimulated photosynthesis and N_2 fixation of lentil to a greater extent in the wet than dry season. This greater N_2 fixation might be associated with greater photosynthetic C supply to N_2 fixing symbionts (Voisin et al., 2003). Moreover, N assimilation is closely linked to C metabolism because photosynthesis provides C skeletons and reducing power for assimilating aerial N_2 into amino acids. Metabolic profiling that captures many of the metabolites involved in these exchanges may help to better understand details of drought and e[CO_2] effects. This FACE experiment was undertaken to investigate the comprehensive metabolites profiling in two lentil genotypes over two contrasting growing seasons. In general, seasonal effects of water availability were prominent on C-and N-metabolites, whereas the effect of [CO_2] treatments was pronounced on both genotypes and organs. These results provide insights into metabolite-type and specific tissue responses and lead to new conclusions that complement my previous work (Parvin et al., 2018).

3.4.1 Changes in sugars and sugar-alcohols

Dry season increased the abundance of sugars (i.e., sucrose, trehalose) in leaves. An increase in leaf sucrose concentration is commonly observed under drought. It has been often interpreted as a means for the plant to osmotically adjust to the lower water potentials (Šircelj et al., 2007). Increased levels of soluble sugars have also been observed in other species under exposure to stress (Hossain et al., 2017), suggesting that this is a more common response in plants. A study by Zinta et al. (2014) showed a decrease in photosynthetic activity in stressed plants, suggesting that sugars accumulation were not the result of extra C-fixation, but instead came from starch breakdown.

In both tissues, the abundance of sugar alcohols (e.g., proline, arabitol, inositol, mannitol, galactitol, erythritol) was greater under dry season (Fig. 3.3). Trehalose together with other sugar alcohols (i.e., inositol, mannitol) acts as an osmoprotectant. Interestingly, e[CO_2] increased the of trehalose level in leaves. Similarly, mannitol and inositol, which are also associated with an osmoregulatory role (Streeter et al., 2001), was increased in nodules of e[CO_2]-grown plants. Such accumulation of mannitol and inositol under e[CO_2]-grown nodules could maintain cell turgidity longer under water limited condition. In addition, in parallel with sucrose, mannitol and inositol are primary photosynthetic products derived from leaves and serve as carbon skeletons and energy sources for the bacteroids (Mechri et al., 2015; Stoop et al., 1996).

The abundance of glycolysis intermediate (glucose 6-phosphate and fructose 6-phosphate) decreased in both leaves and nodules during the dry season. The depletion of these phosphorylated metabolites could indicate photosynthetic inhibition related to the decrease of C fluxes to the TCA cycle. Similarly, in nodules, these metabolites concentration decreased in the dry season, indicating lower energy/ATP supply for atmospheric N_2 or NH_4^+ synthesis (Huang et al., 2008). Drought-induced decreased of glycolysis intermediates also observed in nodules of *Medicago sativa* L (Molero et al., 2019).

In this study, e[CO_2] increased leaf sucrose concentration in the dry season which may involve sugar-mediated feedback control on the photosynthetic enzyme, i.e. Rubisco. Increased levels of sugars produced as a consequence of growth in e[CO_2] has been suggested to repressing the expression of Rubisco gene expression and a subsequent decrease in the photosynthetic capacity (Rogers and Ellsworth, 2002). Because photosynthetic stimulation and growth at e[CO_2] directly depend on the sink ability to utilize additional C/sucrose supply (Aranjuelo et al., 2011; Nebauer et al., 2011). Increased sugar metabolites in leaves under e[CO_2] conditions also reported (Noguchi et al., 2015). A lower concentration of sucrose in nodules under e[CO_2] may be associated with limited transport due to drought, coincided with photosynthetic acclimation of e[CO_2] grown lentil in a dry season.

In the wet season, increased abundance of sucrose was observed in nodules of both genotypes under e[CO_2], indicating greater investment of C not only for N_2 fixation process but also for increasing the nodule biomass (Rogers et al., 2006). Although e[CO_2] increased sucrose concentration for both genotypes during the wet season, this effect was only evident in nodules of PBA Ace during the dry season. This might be associated with greater N_2 fixation and nodule biomass of PBA Ace under e[CO_2] in the dry season (Parvin et al., 2018).

3.4.2 Changes in organic acids and or the tricarboxylic acid (TCA) cycle intermediates

It has been suggested that sucrose produced in leaves is transported to roots and nodules, which is then hydrolysed through the glycolytic pathway to form phosphoenol pyruvate (PEP). PEP then combines with respiratory CO2 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 (Foyer et al., 2003). Also, succinate produced from 2-oxoglutarate is also used as other main respiratory substrates for bacteroids (Naya et al., 2007). The concentration of malate and succinate decreased in nodules in the dry season, indicating decreased C flux towards the TCA cycle for the assimilation of NH_4 into amino acids. The decrease of malate concentration in nodules under water restriction may lead to the inhibition of N_2 fixation (Ladrera et al., 2007), because of the decrease in the respiration rate of bacteroids (Nasr Esfahani et al., 2014).

It has been suggested that e[CO_2] may delay the effect of drought on N_2 fixation by maintaining nodule activity longer (Serraj et al. 1999). In this study, e[CO_2] transiently increased malate level in nodules, which coincided with a small but significant increase of N_2 fixation by the genotype "PBA Ace" during the dry season. This can be explained by greater depletion of 2-oxoglutarate of this genotype, which is converted to malate through succinyl Co-A under stress condition and for the provision of carbon skeletons for amino acid biosynthesis (Vasquez-

Robinet et al., 2008). Increased concentration of malate in nodules of drought-tolerant soybean genotype was also observed (Ladrera et al., 2007).

Dry season decreased the level of other organic acids in nodule, particular, TCA cycle intermediates which have been associated with decreased TCA cycle activity. This may lead to increased investment of C structures for the synthesis of compounds required for coping up with drought stress (Molero et al. 2019). For instance, the precursor for proline/GABA synthesis is glutamate, which is derived from citrate or 2-oxoglutarate, a TCA cycle intermediate. In leaves, an increased level of organic acids such as fumarate, citrate, citrulline, aconitate, caffeic acid, and nicotinic acid was detected in the dry season. Increased levels of some tricarboxylic acid cycle (TCA) intermediates under stress such as aconitate, citrate, and fumarate could be reflective of the plant's mechanisms to withstand water stress by generation more reductant and ATP (Vasquez-Robinet et al., 2008).

3.4.3 Changes in amino acids

The up-regulation of amino acids under drought is associated with the reduction in protein synthesis and or an increase in hydrolysis of proteins, endorsing surge in soluble nitrogen compounds such as free amino acids (Krasensky and Jonak, 2012). The C skeleton from glycolysis is used in the TCA cycle for the synthesis of amino acids through 2-oxoglutarate and oxaloacetate/glyoxalate pathways (Ezquer et al., 2010). Oxaloacetate-derived amino acids include aspartate, lysine, methionine, β-alanine, cysteine, threonine, and isoleucine showed greater in the wet season, whereas 2-oxoglutarate derived amino acids are glutamate, GABA, proline, ornithine, glutamine, and putrescine found in higher concentration in the dry season. The up-regulation of GABA, proline may be related to stress defense and accumulated in both leaves and nodules during the dry season. Both proline and GABA can be synthesized from the same glutamic acid substrate and serves as a signalling molecule to upregulate nitrogenase activity (Aranjuelo et al., 2013; Sulieman, 2011). In this study, both proline and GABA increased in the dry season, but proline showed a higher absolute concentration and percentage (8-fold) than GABA (3-fold). The relatively higher accumulation of proline in nodules of HS3010 under e[CO_2] could, therefore, be an indication that [CO_2] response on proline accumulation was genotype specific. Markedly increased in proline concentration under drought stress have reported by others (Laila et al., 2002). Also, other branched-chain amino acids (e.g., leucine, isoleucine, valine) which are normally produced from pyruvate, known to accumulate under stress (Obata and Fernie, 2012). Similar to these findings, leucine and iso-leucine showed greater abundance in both organs during the dry season.

Changes in amino acids abundance may be related to changes in N-metabolism under stress (Zinta et al., 2018). Accumulation of amino acids has been proposed to play a role in the decline of symbiotic N_2 fixation in legumes under drought (King and Purcell, 2005). The lower shoot N demand of plants subjected to drought could be involved in reduced nodule functioning and exacerbated N_2-feedback inhibition (Aranjuelo et al., 2014). When the shoot N demand decreases, the concentration of N-transporting compounds accumulates in the nodules that negatively affects nodule activity (Serraj et al., 1999a). Among several amino acids, asparagine is considered as candidate molecules for such a feedback process as it constitutes about 70% of the total amino acid pool (Fischinger and Schulze, 2010). In this study, the concentration of asparagine and glutamine increased in nodules during the dry season, suggesting that these amino compounds involved for feedback inhibition (Aranjuelo et al.,

2013). The lower concentration of these amino acids in leaves suggesting that the decline of export to the shoot. This might lead to a decrease in shoot N demand (Gil-Quintana et al., 2013). Serraj et al. (2001) suggested that the accumulation of N compounds in nodules under drought stress is a consequence of decreased xylem transport leading to impaired amino acid export, implying that changes in plant transpiration rates may also affect in this regulation process. Elevated [CO_2] accumulated less asparagine compared to a[CO_2] especially during the dry season, indicating feedback inhibition was apparently lower in e[CO_2]-grown lentil but increased the abundance of glutamine.

Aspartate derived from the TCA cycle intermediate i.e. oxaloacetate, has been suggested to be involved in feedback inhibition (King and Purcell, 2005). In this study, results showed that no changes in aspartate level between a[CO_2] and e[CO_2] during the dry season but e[CO_2] increased aspartate concentration in the wet season. A significant increase of aspartate in e[CO_2] might be correlated with greater biosynthesis of other amino acid derivatives i.e., lycine, threonine, homoserine, β-alanine. However, the lack of significant effect of e[CO_2] among these metabolites suggests that a complex mechanism involves amino acid biosynthesis and degradation process.

In summary, lentil grown under e[CO_2] showed photosynthetic acclimation during the dry growing season, but this acclimatory effect did not detect in the wet season. The acclimatory effect under e[CO_2] was associated with coordinated accumulation of sucrose in leaves and decreased its concentration in nodules. The depletion of TCA cycle intermediates such as malate, succinate and citrate level during the dry season confirmed shortage of C supply for N_2 fixation activity. Decreased N_2 fixation during the dry season associated with increased level of asparagine, and glutamine in the nodules but the accumulation of asparagine was slightly lower under e[CO_2] grown plants. In the dry season, enhanced accumulation of trehalose, inositol, mannitol, proline under e[CO_2] could have offered protection from drought. This study demonstrated that e[CO_2] modify carbohydrate metabolism to increase the abundance of osmolytes. These data provide information and together with further investigation, may help to understand the biochemical pathway underlying drought stress tolerance of lentil grown under e[CO_2].

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Chapter 4: Free Air CO_2 Enrichment (FACE) improves water use efficiency and moderates drought effect on N_2 fixation of *Pisum sativum* L.

(iv) Abstract

Legume N_2 fixation is highly sensitive to drought. Elevated [CO_2] (e[CO_2]) decreases stomatal conductance (gs) and improves water use efficiency (WUE), which may result in soil water conservation and allow N_2 fixation to continue longer under drought. Using a Free-Air CO_2 Enrichment (FACE) approach, this study aimed to elucidate whether e[CO_2] improves N_2 fixation of *Pisum sativum* L. under drought. In a FACE system, plants were grown in ambient [CO_2] (~400 ppm) or e[CO_2] (~550 ppm) and subjected to either terminal drought or well-watered treatments. Measurements were taken of photosynthesis, soil water dynamics, water soluble carbohydrates (WSC), amino acids (AA) and N_2 fixation. Lower g_s under e[CO_2] increased water use efficiency at leaf and plant level, and this translated to slower soil water depletion during drought. Elevated [CO_2] increased WSC and decreased total AA concentrations in nodules, and increased nodule activity under drought. N_2 fixation was stimulated (+51%) by e[CO_2] in proportion to biomass changes. Under e[CO_2] a greater proportion of plant total N was derived from fixed N_2 and a smaller proportion from soil N uptake compared to a[CO_2]. This study suggests that e[CO_2] increased WUE and this resulted in slower soil water depletion, allowing pea plants to maintain greater nodule activity under drought and resulting in appreciable increases in N_2 fixation. Our results suggest that growth under e[CO_2] can mitigate drought effects on N_2 fixation and reduce dependency on soil N resources, especially in water-limited agro-ecosystems.

Keywords FACE; drought; water use efficiency; amino acids; nodule activity; N_2 fixation

4.1 Introduction

Atmospheric [CO_2] has been rising since the Industrial Revolution and is predicted to increase from the current [CO_2] of ~400 ppm to 550 ppm by 2050 (RCP8.5 Scenario IPCC 2014). Rising [CO_2] drives global warming and alters the amount and pattern of precipitation with increased severity and frequency of drought events expected in some regions including Australia (IPCC, 2014; Shrestha et al., 2017). Future climate change may affect crop productivity by limiting acquisition of soil resources such as N and water, especially in Mediterranean-type dry climates in which crops frequently experience terminal drought when entering the reproductive stage (Fitzpatrick, 1970; Leport et al., 1999).

Legumes contribute substantially to human diets and are used as an important crop in rotation with cereal and oil seed production. Nitrogen-fixing bacteria living in the root nodules of legumes have the potential to fix atmospheric N_2 through symbiotic relationships with the host plants. The contribution of legume-rhizobia symbioses to N_2-fixation increases the pool of biologically available N and can partly offset N losses in the form of volatiles (estimated to be up to 2/3 of all fertilizer applied) (Herridge et al., 2008). Legumes also provide 20-30% of the protein in the human as well as animal diet and are an important break crop in rotation systems (Erskine et al., 2011). Higher N_2 fixation could be beneficial for agriculture because it may compensate for N depletion from soil and facilitate N supply to non-legumes in rotation systems. However, the N_2 fixation of legumes can be

restricted by the availability of soil resources (phosphorus, potassium, molybdenum) and abiotic stresses such as salinity, alkalinity or drought (Hungria and Vargas, 2000).

Drought is a major limitation to crop production in semi-arid regions and causes a marked inhibition of N_2 fixation in legumes (Serraj et al., 1999). Elevated [CO_2] (e[CO_2]), on the other hand has been shown to stimulate N_2 fixation of legumes, matching the N requirement of biomass increase (Lam et al., 2012; Rogers et al., 2009; Rogers et al., 2006 and references therein). Elevated [CO_2] enhanced the amount of N_2 fixed primarily via increasing nodule numbers and nodule mass (Schortemeyer et al., 2002) but can also increase the specific nodule N_2 fixation activity (Fischinger et al., 2010; Tissue et al., 1996). A two-year field study on lentil reported that the amount of N_2 fixed from the atmosphere increased by 57% under e[CO_2] (Parvin et al., 2018). Greater biomass accumulation under e[CO_2] increases N demand, which may stimulate an increase in N_2 fixation (Rogers et al., 2006). This increase in N_2 fixation under e[CO_2] may be caused either by greater nodule biomass or by enhanced rate of N_2 fixation (greater amount of N fixed per unit nodule mass) through transferring additional C to the nodules (Aranjuelo et al., 2014) (Aranjuelo et al. 2014). Some studies (De Luis et al., 1999; Rogers et al., 2006; Serraj et al., 1998) have postulated that e[CO_2] may reduce the impact of drought on N_2 fixation by providing sufficient assimilates to nodules and reducing soil water use.

Exposure to e[CO_2] may improve plant water relations through increased photosynthesis and decreased stomatal conductance (Bernacchi et al., 2007), resulting in increased water use efficiency (WUE) at the leaf level (Kimball, 2016). If leaf-level WUE scales to canopy/whole-plant level WUE (ratio of biomass over plant water use), total crop water use may decrease, which can increase soil water availability under e[CO_2] (Bernacchi et al., 2007; Hussain et al., 2013). As a consequence e[CO_2] may also delay the effects of drought on N_2 fixation by maintaining favourable water status in the soil surrounding the nodules later in the season (Rogers et al., 2009). The interaction between e[CO_2] and drought on N_2 fixation has been tested in a few legume crops, most notably soybean (Serraj et al., 1998) and alfalfa (Aranjuelo et al., 2009; De Luis et al., 1999) and these authors suggested that e[CO_2] improved drought tolerance by increasing C assimilation and reducing water use. A recent FACE experiment suggested that soil water depletion was slower in a lentil crop grown under e[CO_2], and this apparently stimulated N_2 fixation in a dry growing season (Parvin et al., 2018). This study compared two growing seasons **(Chapter 2)**, which did not only differ in rainfall, but also other experimental factors, so that side-by-side comparisons of a well-watered and drought treatment under FACE conditions are required to understand better how e[CO_2]-derived increased water use efficiency is associated with increased N_2-fixation.

Several mechanisms have been proposed to explain the reduction of N_2 fixation under drought, such as shortage of C supply to nodules, an accumulation of N metabolites in nodules and reduced nodule permeability to O_2 diffusion (Naya et al., 2007; Serraj et al., 1999). A meta-analysis by Rogers et al. (2009) concluded that legumes will be able to capitalize on the benefits of e[CO_2] by reducing the negative impact of drought on N_2 fixation. As a consequence of increased photosynthesis under e[CO_2], C flux to nodules may be increased, which may overcome potential C limitations to N_2 fixation (De Luis et al. 1999; Serraj et al. 1999a). In addition, decreased rates of N_2 fixation in water stressed plants have been directly linked to increases in ureides, amides, and amino acids in leaves and nodules. As drought reduces shoot growth, and therefore demand for N in shoots, reduced

consumption and transport causes amino acid to accumulate in both leaves and nodules, potentially resulting in a systemic negative feedback on N_2 fixation. It has been suggested that several low molecular weight N compounds such as glutamine, asparagine, aspartate and ureides are believed to be involved in a N feedback mechanism (King and Purcell 2005). Plants grown at e[CO_2] may invest more C to metabolize foliar N compounds and this greater N sink would result in greater consumption of low molecular N compounds and therefore, avoid N_2 feedback inhibition on N_2 fixation (Serraj and Sinclair 2003). Drought also causes a decline in the permeability to O_2 diffusion, which leads to a significant reduction in nodule respiration, ATP production, nitrogenase enzyme activity and thus, N_2 fixation (Durand et al., 1987; Minchin et al., 1985). However, soil water saving under e[CO_2] may delay the effect of drought. This would maintain nodule permeability to O_2 via regulation of the O_2 diffusion barrier longer and thus, C related process in nodules i.e. respiration, ATP production and fixation (Purcell and Sinclair, 1994).

Elevated [CO_2] modifies N acquisition patterns of crops, for example through limiting N uptake (McGrath and Lobell, 2013) or through inhibition of NO_3^- assimilation (Bloom et al., 2014). If soil N availability is insufficient to satisfy the increased N demand from stimulated biomass growth or if root N uptake decreases due to limited transpiration driven mass-flow under e[CO_2], plant N uptake will not keep pace with biomass stimulation (Feng et al., 2015; Pleijel and Uddling, 2012). In addition, e[CO_2] reduces photorespiration which limits energy transfer to NO_3^- reduction and thereby NO_3^- assimilation (Bloom et al., 2014). Some studies demonstrated that e[CO_2] grown legumes depend less on soil N resources (Guo et al., 2013; Zanetti et al., 1996). On the other hand, legumes exposed to stress may increase their utilization of soil N to adapt to environmental changes (Guo et al., 2013; Parvin et al., 2018). Interaction of e[CO_2] and drought may alter plant N accumulation by stimulating N_2 fixation or by reducing soil N uptake, but the relative extent of these changes needs to be tested.

In drought prone semi-arid Mediterranean-type agroecosystems, field pea is an important grain and forage crops. The effect of e[CO_2] on the growth, yield and grain [N] of field pea has been investigated at the Australian Grain Free Air CO_2 Enrichment (AGFACE) facility (Bourgault et al., 2016; Butterly et al., 2015), including one study that assessed N_2 fixation under various soil NO_3^- levels under well-watered conditions (Butterly et al., 2016a). Jin et al. (2015) assessed the effect of phosphorus amendment on the interaction of e[CO_2] and drought and reported that the tolerance of field pea to drought was enhanced by e[CO_2] as a result of decreased stomatal conductance, increased WUE and greater carbon assimilation in leaves. Since e[CO_2] improved overall drought tolerance of field pea, it may also help to alleviate the negative impact of drought on N_2 fixation. However, the relative extent of N originating from N_2 fixation and soil under factorial combinations of e[CO_2] and experimental drought has not been quantified yet. Therefore, this present study was conducted to explore the interactive effect of e[CO_2] and drought to test the following hypotheses:

1. Increased WUE under e[CO_2] delays soil drying, allowing nodules to maintain greater activity for longer under terminal drought.
2. Under e[CO_2], drought exposed field pea plants show greater concentrations of carbohydrate compounds and lower concentrations of amino acids in nodules, which is indicative of continuing N consumption and avoidance of feedback inhibition.

3. Increased N_2 fixation under e[CO_2] reduces soil N uptake and decreases the proportion of soil derived N in total plant N.

4.2 Materials and methods

4.2.1 Experimental details

This study was undertaken at the sub-facility of the Australian Grains Free-Air CO_2 Enrichment (AGFACE) facility known as Soil Free-Air CO_2 Enrichment (SoilFACE) at the Agriculture Victoria Research Plant Breeding Centre, Horsham (36°44'57"S, 142°06'50"E; 127 m elevation), Victoria, Australia. SoilFACE consisted of eight round bunkers sunk into the ground (3.7 m diameter; 1.2 m depth). Bunkers were at least 28 m apart and each bunker is treated with either ambient [CO_2] (a[CO_2]) or elevated [CO_2] (e[CO_2]), resulting in four replicates arranged in a completely randomised design (Butterly et al. 2015). The Free Air CO_2 Enrichment (FACE) system used to achieve the e[CO_2] level was similar in design and used the same reticulated CO_2 supply as the facility previously described by Mollah et al. (2009), except that smaller rings (4.0 m diameter instead of 16 m) were used. Octagons of horizontal stainless-steel tubes surrounding the e[CO_2] bunkers were positioned about 150 mm above canopy level at any developmental stage of the crop. CO_2 was injected into the upwind side of the rings through the outward-oriented holes in the stainless-steel tubes. CO_2 enrichment was applied throughout the experimental period from sunrise to sunset. Enrichment target was ~ 550 µmolmol^{-1} and a[CO_2] was ~ 400 µmolmol^{-1}. An infrared gas analyser (IRGA, SBA-4, PP Systems, Amesbury, MA, USA) continuously recorded [CO_2] at the centre of each bunker and the performance of the system against targets is described by Mollah et al. (2011). Average [CO_2] during the crop growing season for ambient and FACE rings is reported in Fig. 3.1S. Temperature (average daily maximum temperature 20.2°C and minimum temperature 4.1°C), rainfall events (total 69 mm) and total irrigation (44 mm) during the experimental period are presented in Fig. 4.1. Meteorological data were obtained from an automatic weather station installed near the SoilFACE site.

The experimental soil was a Vertosol (Isbell, 2002) described in detail elsewhere (Butterly et al., 2015; Jin et al., 2012) and the relevant soil properties during the experiment are as follows: total C: 13.5 g kg^{-1}; total N: 0.95 g kg^{-1}; organic C: 8.3 g kg^{-1}; Colwell P: 6.5 mg kg^{-1}; ammonium-N: 0.85 mg kg^{-1}; nitrate-N, 7.5 mg kg^{-1} and pH (1:5 in 0.01MCaCl2): 7.4; clay 55 %; bulk density: 1.25 g cm^{-3}. Exchangeable mineral elements of the soil were analysed (Ca:5.8 g kg^{-1}; K:498.8 mg kg^{-1}; Mg:2.3 g kg^{-1}; Cu: 1.8 mg kg^{-1}; Fe: 125.7 mg kg^{-1}; Mn:107.9 mg kg^{-1}, Zn:4.2 mg kg^{-1} and Bray extractable PO_4:22.4 mg kg^{-1}). Air-dried soil was sieved through a 4 mm sieve to remove dirt and facilitate root washing at harvest. PVC (polyvinyl chloride) columns (15 cm in diameter × 60 cm deep) were filled with 16 kg of the experimental soil, compressed to original soil bulk density. Columns were placed into the bunkers so that the upper opening of the columns was aligned with the surface of the surrounding field.

4.2.2 Plant growth

Field pea (*Pisum sativum* L.) cv "PBA Twilight" tested as OzP0601 was used in this study. Field pea seeds were inoculated with Rhizobium (Group E® *Rhizobium leguminosarum*) before sowing. Seeds were hand sown in each column at 1.5 cm depth on 01 July 2015. Field pea seedlings were thinned to three healthy plants per column at two weeks after sowing. Two field pea columns were placed in each FACE bunker. To grow shoot vertically in

the column, the round cages (~30 cm diameter) were installed around the column at vegetative growth stages (GS 107) (Fig.3.2Sb).

Plant growth stages were expressed as cumulative growing degree days (GDD) by summing up daily degree days (Darroch and Baker, 1990). Daily degree days were calculated as $T_n = (T_{max} + T_{min})/2 - T_b$, where T_{max} and T_{min} are the maximum and minimum daily temperatures, respectively, and T_b is the base temperature (0°C) (Lecoeur, 2010; Sadras et al., 2012).

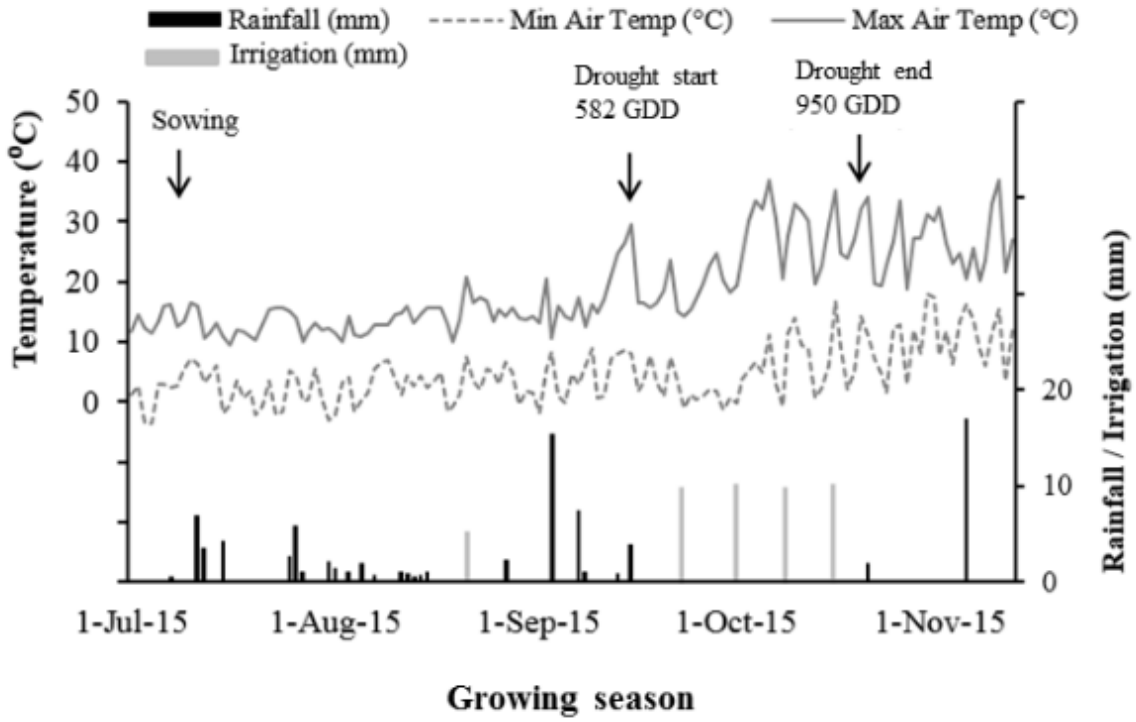


Fig. 4. 1 Daily maximum (Max Air Temp, solid line) and minimum (Min Air Temp, dotted line) temperature, rainfall (black bars) and irrigation (grey bars) near the experimental site at crop growing season including the drought period from 25 September 2015 to 25 October 2015 is presented. Sowing time, starting of drought at 582 growing degree days (GDD) corresponding to 85 days after sowing (DAS) and ending of drought/harvest at 950 GDD (114 DAS) are also indicated by arrows.

4.2.3 Drought treatment

In dryland Mediterranean Environments, legumes frequently experience terminal drought during reproductive phase. To better represent the natural field conditions, plants were subject to terminal drought at reproductive phase especially at the onset of flowering period (GS201). In one of the field pea columns in each replicate, terminal drought was imposed by withholding all water (rain or irrigation) from 582 GDD (correspond to 85 days after sowing, DAS) at reproduction phase (GS201:very small flower bud enclosed at the terminal shoot). This treatment was continued until the permanent wilting point (PWP) reached at 951GDD (114 DAS) corresponding to GS203 (the first flower opens at first reproductive node) according to developmental stages of field pea described by Knott (1987). Well-watered columns were maintained at field capacity (80% ± 5% field capacity (FC), ~33 volumetric soil water) (Li et al., 2017; Rab et al., 2011) by watering to weight every week. Drought

treated columns were allowed to reach nearly PWP (41% ± 5% FC, 16-18% volumetric water) (Jin et al. 2012). Soil water content (volumetric %) was monitored at 20, 40 and 60 cm depth by time domain reflectometry (Theta Probe, ML3, Delta-T Devices, Cambridge, UK) throughout the growing period, every two weeks before drought treatment started and every week during drought treatments. Average soil water within the soil column is reported in Fig. 3.2.

4.2.4 Plant sampling

All columns were harvested at flowering stage (GS203) after four weeks of drought treatment at 951 GDD. Before harvesting, small tissue samples of leaves, stems, floral buds, roots, and nodules were immediately frozen in liquid N and stored in a -80°C freezer for amino acid analysis. Field pea and wheat plants were cut at ground level and leaves, stems and flowers were separated. Fresh leaf area was measured using a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA) and specific leaf area was calculated from the ratio of leaf area to leaf dry weight. Each column was opened vertically by detaching the PVC end caps at the base. Roots were washed with distilled water after soaking in 0.01M $CaCl_2$ solution for 5 minutes to desorb all nutrients. Root washing was followed according to the procedure described by Frasier et al. (2016) and described in details in Chapter 2. Immediately after washing the roots, nodules were separated from the root system and counted. All plant organs were oven dried at 70°C for 72 hrs and weighed to calculate the total biomass corrected for small sub-samples taken previously.

4.2.5 Gas exchange measurements and water use efficiency

Gas exchange measurements with an infrared gas analyser (IRGA) system (Li- 6400, Li-Cor, Lincoln, NE, USA) were conducted every week during the drought event until harvest. Measurements were made between 9.00 to 12.00 hours each time. Reference [CO_2] concentrations were adjusted to 400 and 550 µmol mol^{-1} in bunkers under ambient and elevated [CO_2], respectively. A fully expanded youngest leaflet (Fig. 3.2S) was placed in the measurement cuvette (Jin et al., 2015) and allowed to reach steady state at their growth [CO_2] with the default clear top window chamber at saturating light intensities at or above ~ 1500 µmol m^{-2} s^{-1} photosynthetic photon flux density (clear sunny days) and an air flow rate of 500 µmols^{-1}. Once gas exchanges rates were stable (generally within 60-90 s), three measurements were taken at 5 s intervals and averaged. This protocol allowed water vapour and [CO_2] in the cuvette to reach steady state but did not allow stomata to adjust to cuvette conditions (Uddin et al. 2018b). Before starting the measurements, chambers climatic conditions (temperature, water vapour and humidity) were set close to outside air and continuously adjusted through scrubbing water vapour in incoming air and using coolers. Depending on measurement dates, the temperature was 20-25°C, relative humidity was within 45-55% and vapour pressure deficit was between 1 and 2 kPa but within each measurement date these variables were not significantly different between water treatments and [CO_2]. Across all measurements leaf transpiration was between 2.10 to 5.22 mmol H_2O m^{-2} s^{-1} and neither of these were different between treatments on each measurement date. Light-saturated net CO_2 assimilation rate (A_{sat}) was measured and stomatal conductance (g_s) was estimated and intrinsic water use efficiency (WUEi) was calculated from A_{net}/g_s (Houshmandfar et al., 2016). Water-use efficiency for biomass ($WUE_{biomass}$) was calculated as the total dry weight divided by total crop water use, where crop water use was estimated as the amount of water added to the columns (rainfall and irrigation) and the difference of the column water mass at the beginning and the end of the experiment (Jin et al., 2012).

4.2.6 Relative water content (RWC)

RWC was measured from the same leaves used for gas exchange measurement (Conroy et al., 1988; Jin et al., 2015). Fresh leaves were weighed and floated on distilled water for five hours at room temperature. Turgid weight of the leaves was recorded, and these leaves were oven dried (72 h at 70°C). RWC was measured from the formula:

$$\text{RWC} = \frac{\text{Fresh weight} - \text{dry weight}}{\text{Turgid weight} - \text{dry weight}} \times 100$$

4.2.6 Measurement plant N

4.2.6.1 Tissue N concentration and content

Oven dried biomass samples (leaf, stem, root, nodule, flower and references wheat of each component) were finely ground and analysed for total N concentration (mg g^{-1} dry weight) by an elemental analyzer used in an isotope ratio mass spectrometry (IRMS) setup (Hydra 20–20, SerCon, UK). N content (mg N $plant^{-1}$) was calculated for each organ from N concentration multiplied by biomass and total plant N content by adding up all organ-specific N contents.

4.2.6.2 N_2 fixation and nodule activity

The percentage of N derived from the atmosphere (%Ndfa) was determined by ^{15}N natural abundance method. Organ-specific ^{15}N atom (%) was measured by IRMS. ^{15}N atom (%) was converted to $\delta^{15}N$ values (‰) using atmospheric air (0.3663 at % ^{15}N) as the standard (^{15}N of 0‰) (Unkovich et al. 1997). $\delta^{15}N$ values of each organ are reported in Table 3.1S. Experimental soil and reference plant (wheat) $\delta^{15}N$ were 8-10‰ and 5-7‰, respectively, ^{15}N was therefore sufficiently different between aerial N_2 and soil N to estimate N_2 fixation. %Ndfa was determined according to (Unkovich et al., 1994):

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

where 'reference plant' is a non-N_2 fixing plant, wheat (*Triticum aestivum* L. cv. "Yipti") was grown in this experiment (Butterly et al., 2016a; Lam et al., 2012). The factor B refers to the $\delta^{15}N$ value of the effectively nodulated legume grown in media totally lacking N. To estimate B ($\delta^{15}N$, ‰), nodulated field pea was sand grown and harvested at flowering in glasshouse chambers under a[CO_2] and e[CO_2]. B-values were corrected for seed N (Nebiyu et al., 2014) and are reported in Table 3.1S. Total N_2 fixation was estimated as follows:

N_2 fixation (mg $plant^{-1}$) = Total N content × (%Ndfa/100)

Specific nodule activity (mg N_2 fixed g^{-1} nodule dry weight day^{-1}) was estimated as total Ndfa divided by integrated nodule dry mass (Naudin et al., 2011). Integrated nodule dry mass was calculated as follows (Naudin et al., 2011; Voisin et al., 2007)

$$\int_{ta}^{tb} DWnodule.dt = \sum_{i=a}^{b}((DWnodule\ (i) + DWnodule\ (i+1)) \times (t(i+1) - t(i))/2$$

Where, t_a and t_b are the nodule dry weight at 14 DAS and at final harvest (114 DAS), respectively.

4.2.6.3 Soil N uptake

The remainder of total plant N was assumed to be derived from soil N uptake (%Nds).

Soil N uptake (mg plant^{-1}) = Total N content - N_2 fixation

4*.2.6.4 N allocation*

Total N as well as the sources of N (either fixed N_2 or soil N) were calculated for above and below ground biomass (Parvin et al., 2018). Leaf, stem and flower N content were combined for above ground N and root and nodule N content for below ground N. Both above ground and below ground N contents were summed up for total plant N content.

4.2.7 Measurement of soil N

Soil samples were analysed for NH_4^+ and NO_3^--N before sowing and at harvest. Approximately, 25 g of soils were tumbled with 1M KCl for 1 hour at 25°C employing a soil: solution ratio of 1:5, then centrifuged (at 2000 g for 10 minute) and filtered (Whatman # 1 filter paper). Extracts were analysed for soil NH_4^+ and NO_3^- N simultaneously using a Lachat Flow Injection Analyser (Lachat Instruments, USA). The concentration of NH_4^+-N was measured calorimetrically at 420 nm using the indo-phenol blue reaction. Soil NO_3^- was reduced to nitrite through a copperized-cadmium column and was measured calorimetrically at 520nm. Soil NH_4^+ concentration was very low and did not differ among treatments, therefore, only soil NO_3^- data are presented. Total NO_3^--N of the soil column was calculated by multiplying the concentration (mg NO_3^- kg^{-1} soil) with soil mass and bulk density.

4.2.8 Biochemical analysis

Water soluble carbohydrate concentration (WSC) was determined from oven dried and finely ground leaves, stems, roots, nodules and floral 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). Absorbance was measured at 600 nm wavelength and D-fructose was used as the standard. WSC concentration was calculated as fructose equivalent and expressed as mg g^{-1} of dry matter.

Total free amino acid concentration of leaves, stems, roots, nodules and flower tissues were determined from frozen tissue (-80ºC) using the acid ninhydrin method (Yemm and Cocking, 1955) adapted for the plate reader. Briefly, 50 mg frozen tissue was powdered in a mortar and pestle with liquid nitrogen by addition of 80% methanol. The supernatant was collected through centrifuged at 10,000 g at 4^{0}C for 15 min and incubated at 100^{0}C for 15 minutes after addition of ninhydrin solution. Absorbance (at 570 nm wavelength) was quantified in a plate reader (Tecan Sunrise, Tecan, Austria) using a mixed amino acid (Amino Acid Standard AAS18, Sigma-Aldrich). The concentration of total free amino acids was calculated as amino acid standard equivalent and expressed as µmol g^{-1} dry weight.

4.2.9 Statistical analysis

All data were analysed using R version 3.4.1 (R Core Team, 2018). The experiment was designed as a split-plot with 2 CO_2 concentrations (main-plots, bunkers) × 2 water regimes (sub-plot, columns within bunkers) × 4 replicates (bunkers), for a total of 16 columns. This fully factorial design allowed us to perform statistical analysis using analysis of variance (ANOVA) (StatSoft, 2013). ANOVA was performed with a Generalized Linear Mixed Model (GLMM) effect using package "nlme" as described by Pinheiro et al. (2017). CO_2 concentrations and water regimes were considered as fixed effects and bunkers as random effects. Repeated measure ANOVA was performed for soil moisture and gas exchange parameters during the drought period considering measurement dates as random effects. All data were normally distributed and tested by Shapiro-Wilk Normality test in R function "shapiro.test" using package "dplyr". To check homogeneity of variances, Levene's tests were conducted and where necessary data transformations were done. Relationship between fixed N_2 and nodule attributes were tested by linear regression (function lm() using "stat package" in R). Where interactions were significant, the least significant difference (LSD) was used to assess significant difference between the treatments using the R package "predictmeans" (Luo et al., 2014). Effects were accepted as significant at $P<0.05$.

4.3 Results

4.3.1 Soil water content

During the drought treatment, e[CO_2] grown plants had greater soil water compared to a[CO_2] and this trend was maintained from the onset of drought treatment until reaching permanent wilting point (interaction significant, [CO_2] × W). Under well-watered conditions, both a[CO_2] and e[CO_2] treated columns had similar moisture content (Fig. 4.2).

4.3.2 Growth parameters and nodule attributes

Elevated [CO_2] significantly increased biomass of all organs resulting in greater total biomass production compared to a[CO_2] (Table 4.1). Drought decreased biomass of most plant organs and total biomass compared to well-watered treatments. Significant interactions between e[CO_2] and drought were detected for stem and flower biomass. Root: shoot ratio increased under drought but was not affected by e[CO_2]. Plants grown under e[CO_2] produced 33% more nodules than under a[CO_2]. Elevated [CO_2] increased specific nodule activity by 35% under drought compared to only 10% under well-watered condition (interaction [CO_2] × W significant). Total leaf area was stimulated (+ 22%) by e[CO_2] and reduced by drought (Table 4.1).

4.3.3 Carbon assimilation and water use efficiency

Field pea grown under e[CO_2] had greater net CO_2 assimilation rate (A_{sat}) than under a[CO_2] and this trend was maintained throughout the drought period for both well-watered and drought-treated plants (Fig. 4.3a). Stomatal conductance (g_s) was reduced by e[CO_2] and drought (Fig. 4.3b). Greater A_{sat} and lower g_s resulted in increased intrinsic WUE (µmol CO_2 mol^{-1} H_2O) under e[CO_2] and at later stages, this increase was more prominent under drought (37% vs 56%, [CO_2] × W, Fig. 4.3d). Elevated [CO_2] resulted in greater intercellular CO_2 concentration (C_i) under both well-watered and drought conditions (Fig. 4.3c).

Relative water content (RWC) was increased by e[CO_2] but decreased under drought by 28% (Fig. 4.4). Water use efficiency of biomass production (g biomass produced kg^{-1} water use) increased under drought with a proportionally greater response observed under e[CO_2] than a[CO_2] (50% vs 100%, [CO_2] × W, Fig. 4.3S).

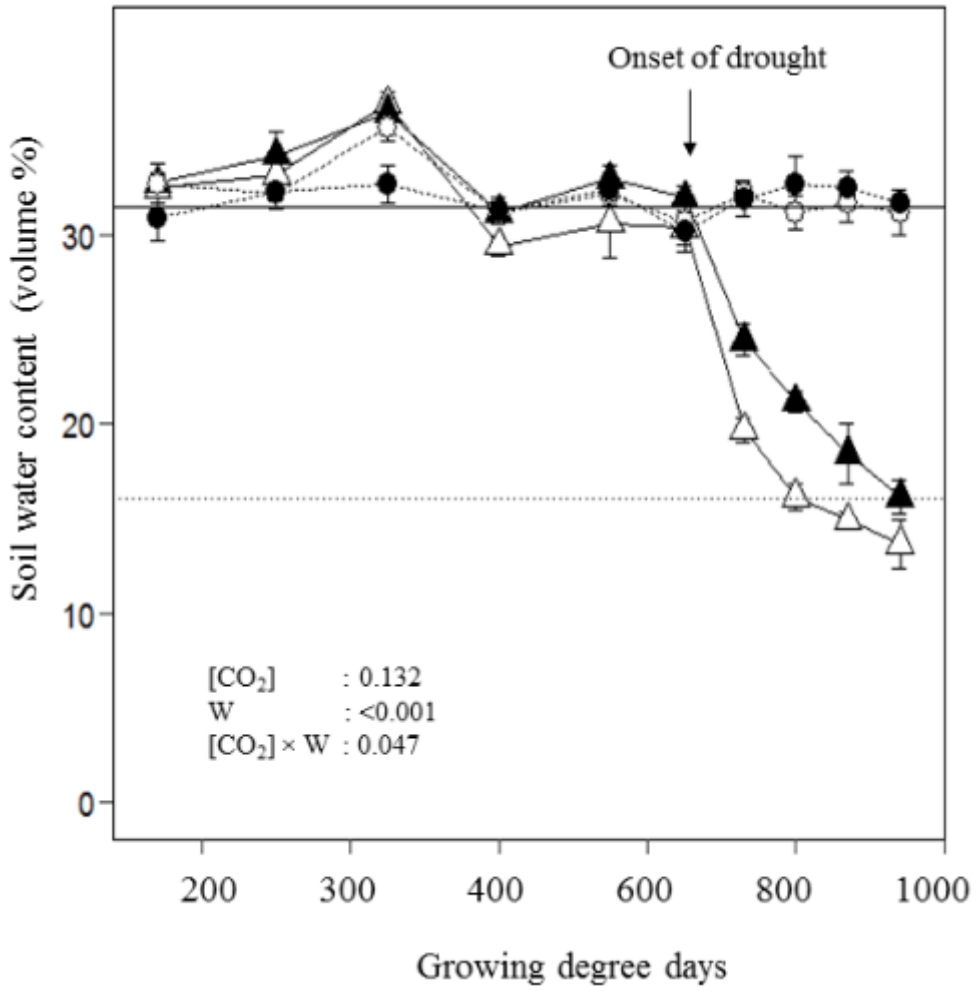


Fig. 4. 2 Soil water content (volume %) during the experiment period of field pea grown under two [CO_2] (ambient [CO_2], ~400 µmol mol^{-1}; symbols O, Δ and elevated [CO_2], ~550 µmol mol^{-1}; symbols ●, ▲) and two water (W) regimes (well-watered, symbols O, ● with dotted lines and drought, symbols Δ, ▲with continuous lines). The arrow represents the onset of the drought treatment. Each point represents mean with ± SE of 4 replicates. The continuous horizontal line indicates field capacity and dotted line permanent wilting point. P-values of two-way ANOVA results are shown.

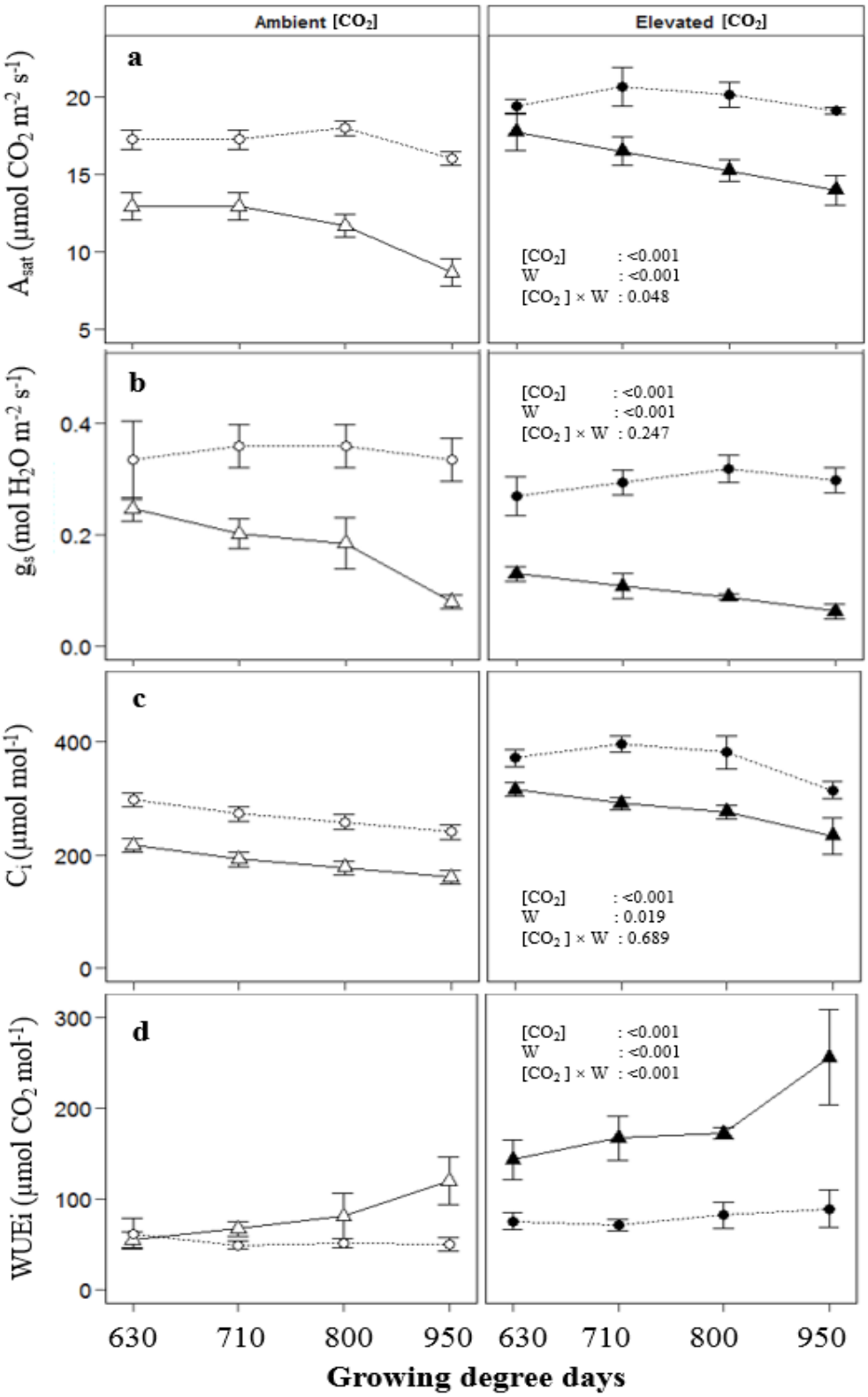


Fig. 4. 3 (a) Light saturated net CO_2 assimilation rate (A_{sat}), (b) stomatal conductance (g_s), (c) internal CO_2 concentration (C_i) and (d) intrinsic water use efficiency (WUEi) of field pea grown under ambient [CO_2] (~400 µmol mol^{-1}, symbols O, Δ) or elevated [CO_2] (~550 µmol mol^{-1}, symbols ●, ▲) and subjected to two water (W) regimes either well-watered (symbols O, ● with dotted lines) or terminal drought (symbols Δ, ▲ with continuous lines). Each value represents mean with ± SE of 4 replicates. P-values of two-way ANOVA results are shown.

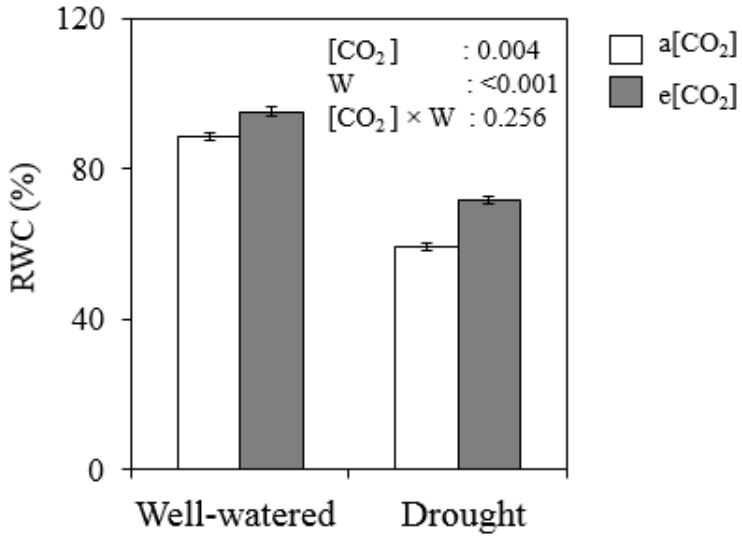


Fig. 4. 4 Relative water content (RWC) in leaves of field pea grown under ambient [CO_2] (~400 µmol mol^{-1}, white bars) or elevated [CO_2] (~550 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) at harvest. Each bar represents mean with ± SE of 4 replicates. P-values of two-way ANOVA results are shown. Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other ($P<0.05$).

4.3.4 Carbohydrate and amino acid concentrations

Elevated [CO_2] significantly increased water-soluble carbohydrate concentration (WSC) in all plant organs (Fig. 4.4Sa). Imposition of drought significantly decreased the accumulation of WSC in all tissues except for flowers. Even under drought, WSC concentration in nodules was greater in e[CO_2] grown plants compared to a[CO_2] grown ones ([CO_2] × W).

Total free amino acids (AA) concentration of leaves and flowers was slightly but significantly lower under e[CO_2] (Fig. 4.4Sb). Drought led to accumulation of amino acids in all plant organs. In comparison to a[CO_2], e[CO_2] increased AA concentration in nodules when plants were well-watered, but decreased nodule AA concentration in drought treated plants (interaction significant). In leaves, AA concentration under e[CO_2] was consistently lower than under a[CO_2] and showed greater reduction under drought than well-watered conditions under a[CO_2].

Table 4. 1 Growth parameters of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and subjected to two water (W) regimes either well-watered or terminal drought at GS201 and harvested at GS203. Each value represents mean with ± SE of 4 replicates and two-way ANOVA results are shown (significant results in bold, P<0.05). Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other (P<0.05). Unit for SLA: cm^2 $g^{-1}DW_{leaf}$ and SNA: mg N_2 fixed g^{-1} nodule dry weight day^{-1}.

Growth parameters	Ambient [CO_2]		Elevated [CO_2]		P-value		
	Well-watered	Drought	Well-watered	Drought	[CO_2]	W	[CO_2] × W
Leaf biomass (g $plant^{-1}$)	4.43±0.55	3.51±0.16	6.75±0.38	5.47±0.48	**0.002**	**0.038**	0.684
Stem biomass (g $plant^{-1}$)	3.94±0.47 B	2.24±0.22 A	5.54±0.41 C	4.91±0.30 C	**0.004**	**<0.001**	**0.026**
Root biomass (g $plant^{-1}$)	3.41±0.46	2.70±0.39	4.29±0.24	4.08±0.24	**0.043**	0.068	0.286
Nodule biomass (g $plant^{-1}$)	0.23±0.03	0.18±0.01	0.38±0.02	0.28±0.01	**<0.001**	**0.005**	0.245
Flower biomass (g $plant^{-1}$)	0.43±0.01 B	0.33±0.03 A	0.86±0.06 D	0.61±0.04 C	**<0.001**	**<0.001**	**0.017**
Total biomass (g $plant^{-1}$)	12.44±1.32	8.95±0.61	17.82±0.85	15.33±0.62	**0.001**	**0.007**	0.535
Nodule number ($plant^{-1}$)	370±10.35	306±15.72	497±16.26	407±26.89	**0.002**	**0.001**	0.380
Root: shoot ratio	0.43±0.04	0.46±0.05	0.37±0.02	0.41±0.02	0.157	**0.026**	0.675
Leaf area (cm^2 $plant^{-1}$)	323.06±4.75	280.50±10.96	416.64±30.35	351.18±11.04	**0.002**	**0.020**	0.530
Specific leaf area (SLA)	76.76±10.20	80.64±5.20	62.42±6.16	65.41±4.62	0.075	0.636	0.950
Specific nodule activity (SNA)	11.27±0.23 B	10.06±0.27 A	12.43±0.42 C	13.65±0.44 D	**<0.001**	0.987	**0.013**

4.3.5 N concentration and content

Elevated [CO_2] had no effect on tissue [N] except that flower [N] increased by ~7 % under drought, whereby it decreased in a[CO_2] grown drought treated plants ([CO_2] × W, P<0.05) (Table 4.2). Drought significantly decreased [N] of leaves and stems. Elevated [CO_2] decreased flowers [N] under well-watered condition but increased it under drought.

Total N content (mg N $plant^{-1}$) of all plant organs and total plant N content were significantly greater under e[CO_2] due to greater biomass production (Table 4.3). Drought stress decreased total N content in all organs except leaves and the extent of this decrease was greater under a[CO_2] than e[CO_2] in stems, flowers and nodules (significant interaction, [CO_2] × W).

Table 4. 2 N concentration ([N], mg N g^{-1} dry weight) in all organs of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and subjected to two water (W) regimes either well-watered or terminal drought at GS201 and harvested at GS203. Each value represents mean with ± SE of 4 replicates and two-way ANOVA results are shown (significant results in bold, P<0.05). Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other (P<0.05).

N concentration (mg N g^{-1} dry weight)	**Ambient [CO_2]**		**Elevated [CO_2]**		**P-value**		
	Well-watered	Drought	Well-watered	Drought	[CO_2]	W	[CO_2] × W
Leaf [N]	40.00±0.91	38.80±0.54	39.95±0.79	37.03±1.07	0.215	**0.041**	0.814
Stem [N]	19.22±1.26	20.94±0.94	17.89±0.71	20.74±1.58	0.313	**0.031**	0.553
Root [N]	19.15±2.54	18.96±2.26	18.28±1.31	17.95±0.5	0.612	0.078	0.519
Nodule [N]	61.25±1.19	69.40±2.35	62.40±1.60	65.55±0.94	0.538	0.612	0.701
Flower [N]	49.16±1.04 B	45.08±1.46 A	46.41±0.43 A	48.62±0.76 B	0.901	0.281	**0.028**

Table 4. 3 Plant N content (mg N plant^{-1}) and N allocation in all organs of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and subjected to two water (W) regimes either well-watered or terminal drought at GS201 and harvested at GS203. Each value represents mean with ± SE of 4 replicates and two-way ANOVA results are shown (significant results in bold, P<0.05). Amount of N_2 derived from atmosphere: Ndfa (mg N plant^{-1}), amount of N derived from soil: Nds (mg N plant^{-1}). Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other (P<0.05).

N content (mg N plant^{-1})		**Ambient [CO_2]**		**Elevated [CO_2]**		**p-value**		
		Well-watered	Drought	Well-watered	Drought	CO_2	W	[CO_2] × W
Leaf	Ndfa	133.61±11.83	115.97±8.61	250.58±12.90	204.43±19.48	**<0.001**	**0.036**	0.276
	Nds	58.56±10.71	40.01±8.80	19.60±3.30	23.66±3.10	**0.003**	0.629	0.159
	Total	**192.17±21.98**	**155.98±9.01**	**270.18±12.16**	**228.09±22.14**	**0.004**	0.065	0.870
Stem	Ndfa	49.48±15.27	20.00±0.93	72.72±8.00	66.34±7.88	**0.017**	0.071	0.207
	Nds	34.67±6.90	32.41±3.31	35.39±10.40	42.47±1.84	0.448	0.728	0.508
	Total	**84.15±12.27 B**	**52.41±3.65 A**	**108.10±8.77 C**	**108.82±7.84 C**	**0.011**	**0.025**	**0.021**
Flower	Ndfa	18.44±1.16	10.27±1.15	39.65±3.30	25.85±1.78	**<0.001**	**<0.001**	0.054
	Nds	3.78±0.84	6.54±1.12	2.38±0.67	3.19±0.90	**0.038**	0.094	0.321
	Total	**22.22±0.95 A**	**16.81±1.96 A**	**42.04±2.77 C**	**29.04±2.62 B**	**0.001**	**<0.001**	**0.036**
Root	Ndfa	49.96±6.89	34.44±3.89	84.10±7.94	67.98±3.07	**<0.001**	**0.004**	0.863
	Nds	20.77±5.12	15.80±1.79	7.21±2.62	11.52±3.59	0.053	0.870	0.270
	Total	**70.73±9.21**	**50.24±4.97**	**91.31±6.51**	**79.50±2.80**	0.012	**0.005**	0.378
Nodule	Ndfa	13.10±1.51	10.40±0.88	25.73±1.15	17.00±0.51	<0.001	**0.001**	**0.031**
	Nds	1.51±0.49	1.64±0.26	0.98±0.18	1.37±0.40	0.307	0.491	0.727
	Total	**14.60±1.72**	**12.04±1.28**	**60.60±3.81**	**18.37±0.29**	**<0.001**	**0.003**	**0.045**
Fixed N_2 (ndfa)		258.22±30.59	191.58±7.83	470.14±25.91	375.84±19.89	**<0.001**	**0.003**	0.530
Soil N uptake		119.28±1.45 C	96.40±10.65 B	65.57±8.44 A	82.22±4.59 B	**0.003**	0.131	**0.033**
Plant N content		377.50±32.04 B	287.98±12.67 A	535.71±21.09 D	458.06±25.81 C	**<0.001**	**0.005**	**0.048**

4.3.6 N_2 fixation and N allocation

The total N budget for field pea indicated significantly greater amounts of fixed N under e[CO_2] in both aboveground and belowground plant N organs, irrespective of water treatment (Fig. 4.5). Elevated [CO_2] decreased total amount of soil N uptake more under well-watered than under drought conditions (significant interaction, [CO_2] × W), whereby total N uptake was lower under e[CO_2] compared to a[CO_2] under all conditions (Fig. 4.5, Table 4.3). Under a[CO_2] soil N contributed ~40% of the total plant N while under e[CO_2] this contribution was ~ 20% for both well-watered and drought-treated plants.

The amount of N derived from the atmosphere (Ndfa) was significantly greater under e[CO_2] compared to a[CO_2], irrespective of water treatment (Table 4.3) for all plant organs. Drought reduced Ndfa in all organs compared to well-watered plants. Nitrogen allocation patterns in stems, flowers and nodules of field peas showed that e[CO_2] grown plants increased N allocation to these organs relatively more under drought (significant interaction, [CO_2] × W).

Total N_2 fixation was 51 % greater in e[CO_2] than a[CO_2] grown field pea when averaged across water regimes (Table 4.3). Although drought reduced overall N_2 fixation regardless of CO_2 exposure, drought treated plants grown under e[CO_2] plants still recorded greater amounts of fixed N_2 than well-watered plants under a[CO_2]. Increased N_2 fixation under e[CO_2] was positively correlated with greater nodule number, mass and specific nodule activity (Fig. 4.6 a, b, c) but negatively associated with amino acid concentration in nodules (Fig. 4.6d).

4.3.7 Soil NO_3^- content

Amount of soil NO_3^- at sowing was similar for all treatments. At harvest, NO_3^- content was greater under e[CO_2] than a[CO_2] and this difference was smaller under drought (Fig. 4.5S).

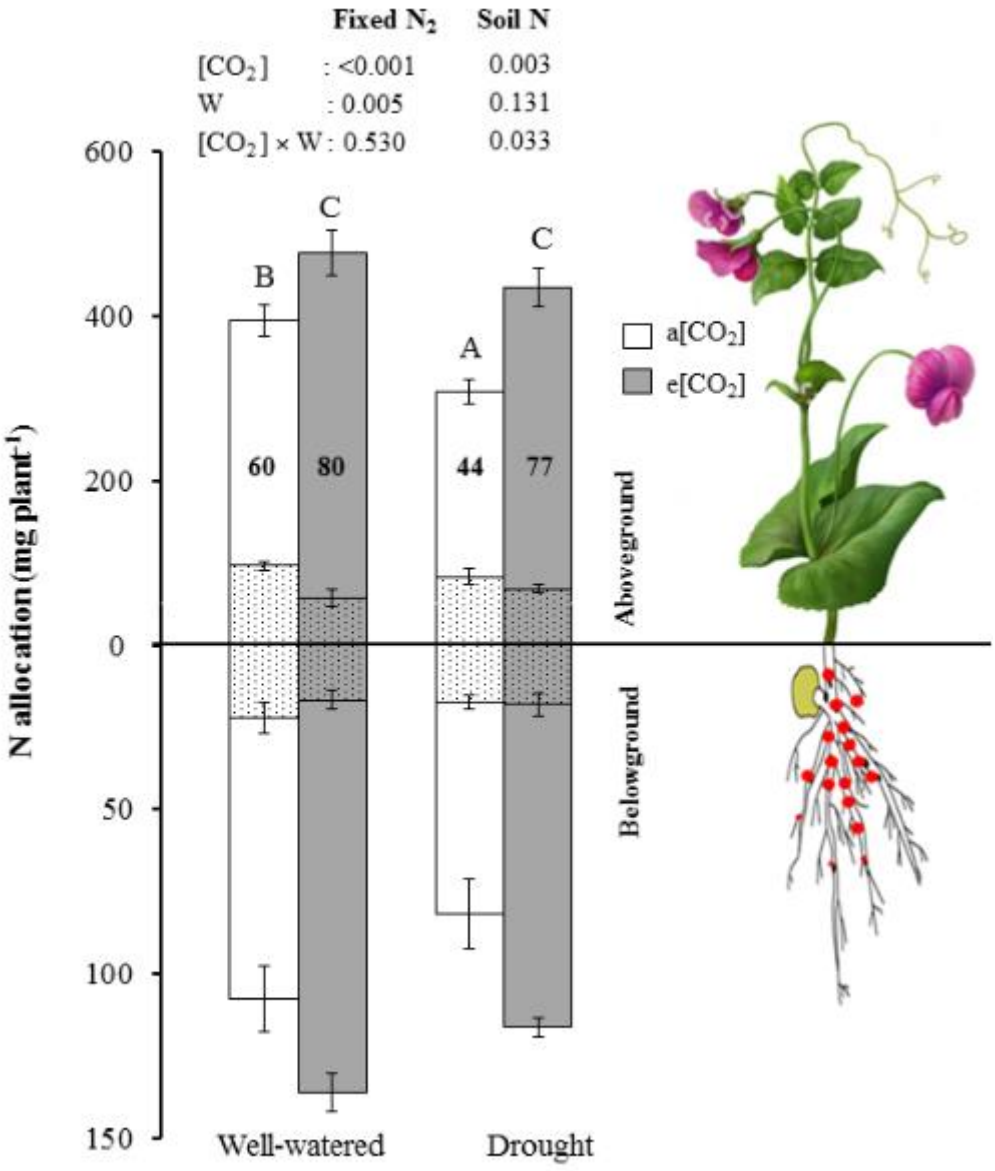


Fig. 4. 5 Total N allocation of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}, white bars) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}, grey bars) and subjected to two water (W) regimes either well-watered or terminal drought. Number in each bar refers to the percentages of total N derived from the atmosphere (%Ndfa). Soil N uptake is indicated as dotted bars for both a[CO_2] and e[CO_2] treatments. Each bar represents mean with ± SE of 4 replicates. P-values of two-way ANOVA results are shown.

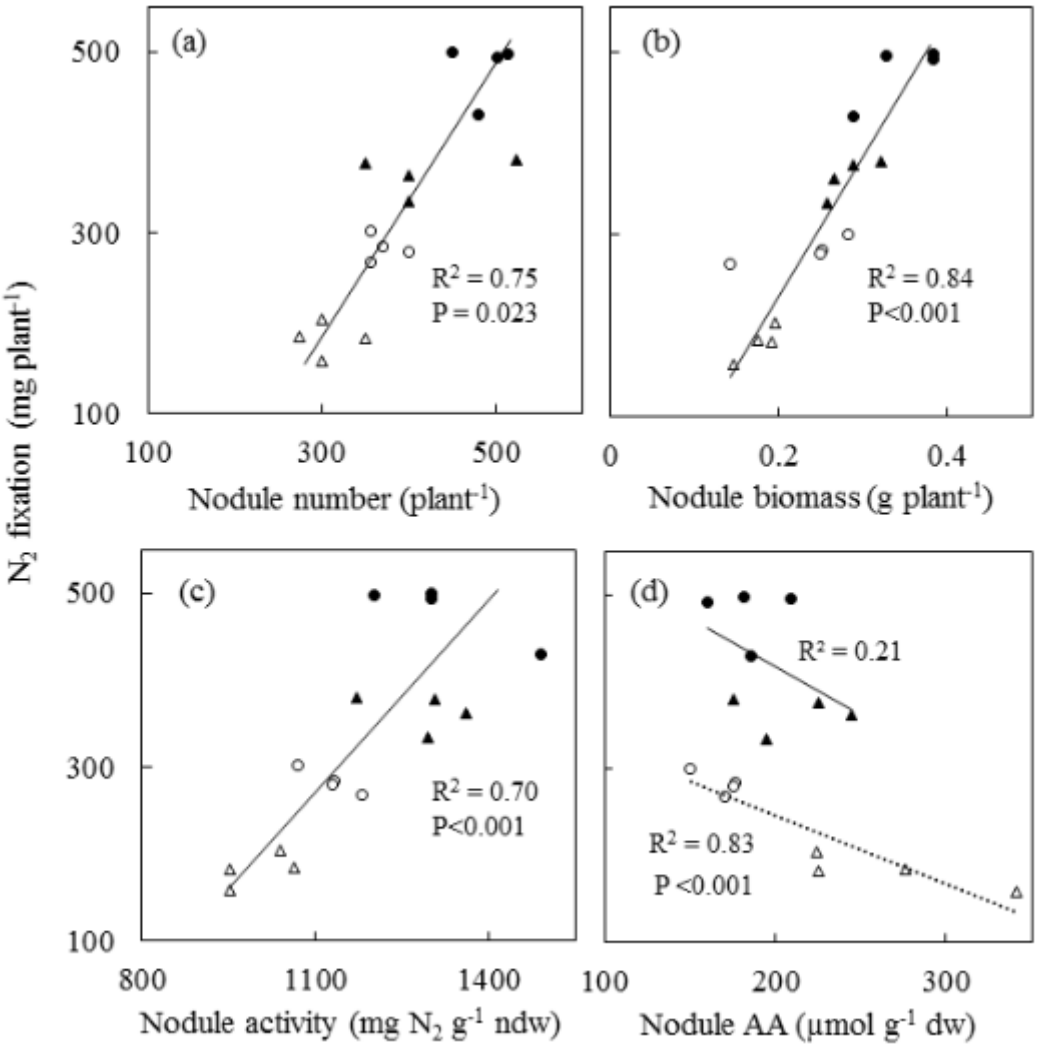


Fig. 4. 6 N_2 fixation as a function of nodule number (a), nodule biomass (b), nodule activity (c) and nodule amino acids (AA) accumulation (d) of field pea grown under ambient [CO_2] (~400 µmol mol^{-1}, symbols O, Δ) or elevated [CO_2] (~550 µmol mol^{-1}, symbols ●, ▲) and subjected to two water (W) regimes either well-watered (symbols O, ●) or terminal drought (symbols Δ, ▲). Unit for nodule activity: mg N_2 fixed g^{-1} nodule dry weight (ndw). dw: dry weight. Significant relations are shown at P<0.05.

4.4 Discussion

4.4.1 Effect on phenological developments

Plants growing in a Mediterranean environment often experience low seasonal rainfall during reproductive phases resulting in terminal drought (Leport et al., 1999). The severity and frequency of such terminal drought are predicted to be intensified even more under future climate change scenarios (IPCC, 2014). Importantly, drought effects on plant fitness depend on the timing and the intensity of drought. In this study, the drought treatment started at the beginning of the early bud stage and continued for four weeks. This did not change development or flowering ontogeny, which is in line with earlier studies on relatively short drought treatments (Lopez et al., 1996; Mouhouche et al., 1998). Elevated [CO_2] itself may accelerate, delay or leave unchanged the reproductive

development (Gray and Brady, 2016; Jagadish et al., 2016). I did not detect any visible effect of [CO_2] on phenological development. This result is corroborated by previous FACE studies on wheat and field pea on the same site (Bourgault et al., 2016; Fitzgerald et al., 2016; Uddin et al., 2018).

4.4.2 Elevated [CO_2] increases water use efficiency, reduces soil water use, and maintains C supply to sustain nodule activity later in the season

This study demonstrated that e[CO_2] increased WUEi of field pea as a result of higher CO_2 assimilation and lower stomatal conductance (g_s) and this may reduce crop water use under moderate drought stress. The net response of water use to e[CO_2] also depends on biomass growth, more specifically on the transpiring leaf area (Gray et al., 2016). Leaf area stimulation by e[CO_2] may offset the effect of water savings through reduced g_s and therefore, may intensify drought effects. Results of this study showed that e[CO_2] decreased gs by 44% but increased leaf area by only 24% under drought, and, consistent with other FACE studies, increased WUEi under e[CO_2] was not offset by greater leaf area and resulted in slower soil water depletion under drought (Manderscheid et al., 2014). Other previous studies have shown that e[CO_2] grown soybean (Erice et al., 2006), barley (Robredo et al., 2007) and alfalfa (Li et al., 2013) utilized water more efficiently and were more tolerant to drought than under a[CO_2]. Both WUEi and $WUE_{biomass}$ of field pea increased by 56% and 79%, respectively under drought and this increase was relatively greater under e[CO_2]. These results corroborated previous notions that increases in WUE under e[CO_2] range from 30-50% in well-watered plants with values up to 180% under water deficit (De Luis et al., 1999).

In the first hypothesis, this study tested whether improved WUE allowed plants to maintain nodule activity and more generally, whether N_2 fixation is maintained under drought. In nitrogen-fixing legumes grown under e[CO_2], additional assimilates acquired through greater C assimilation were shown to feed the symbionts to satisfy energy demand for N_2 fixation (Rogers et al., 2009). Shortage of C supply to nodules due to drought would be associated with a decrease of nitrogenase activity (Serraj et al., 1999). Decreased WSC concentration in nodules under drought resulted in reduced specific nodule activity, similar to that observed in field pea by Prudent et al. (2016). Alternatively, assimilate partitioning towards root growth/elongation rather than the nodule symbiont is prioritized under drought stress to capture soil water from deeper in the soil profile, nodule activity may also be suppressed (Prudent et al., 2016).

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 this 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).

Elevated [CO_2] has the potential to reduce the effect of drought on N_2 fixation mechanism through reducing soil water use and lessening g_s (Bernacchi et al., 2007; Rogers et al., 2009). This may help to maintain nodule N_2 fixation process longer under drought by providing favorable soil water surrounding the nodules. In our study, lower g_s under e[CO2] grown plants resulted in slower rate of soil water depletion, thereby greater soil water was maintained throughout the drought period. Similarly, e[CO_2] grown field pea had greater assimilation rates throughout the drought treatment, and as a result of better C supply, also exhibited greater WSC concentration in nodules than under a[CO_2]. This difference was nearly maintained under drought, resulted in greater nodule activity. This result is in agreement with a greenhouse study conducted under well-watered conditions, where e[CO_2] grown field pea plants were better able to meet nodule C demand (Aranjuelo et al., 2009) and suggest that maintenance of C supply to nodules is one of the mechanisms protecting N_2 fixation from drought under e[CO_2]. Apart from meeting C demand, soil water savings under e[CO_2] might also help to maintain O_2 permeability in the nodules longer during the drought which is considered essential for nodule respiration, nitrogenase enzyme activity and fixation process (Durand et al., 1987).

4.4.3 Elevated [CO_2] avoids N_2-feedback inhibition under drought and modifies N acquisition of field pea

In this experiment, e[CO_2] alleviated the drought-induced increase in nodule amino acid concentration. Increased accumulation of amino acids is indicative of feedback inhibition of N_2 fixation under conditions of reduced shoot N demand (Sinclair and Serraj, 1995). In soybean, ureides are signal molecules involved in such feedback inhibition (Gil-Quintana et al., 2013; Serraj et al., 1999; Streeter, 1993), but temperate legumes such as field pea do not produce ureides, free amino acids could assume the signal role (Cabrerizo et al., 2001). Elevated [CO_2] decreased the accumulation of ureide in soybean nodules (Serraj et al., 1998) and this coincided with greater N_2 fixation. One effect of drought is a decrease in growth so that N demand of the plants decreases, and in this experiment, this growth inhibition was less under e[CO_2]. In support of the second hypothesis, the decrease in total amino acids in nodules under e[CO_2] suggests that the plant N demands was greater, and in contrast to a[CO_2], nodules were not affected by feedback inhibition under drought (Fig. 4.6D).

The prevailing notion is that e[CO_2] grown plants have increased N requirement, but the relative increase in N acquisition from soil is not sufficient relative to the biomass stimulation (Feng et al., 2015; Luo et al., 2006). This concept was further supported in this study showing 50% greater biomass growth under e[CO_2] grown field pea compared to a[CO_2] and maintained this trend even under drought. Such higher biomass growth under e[CO_2] grown plants demanded greater N acquisition either from N_2 fixation or uptake from soil.

From an "energy" perspective, although N_2 fixation is most costly in terms of energy and resources, an average 3g of C is required to fix 1g of N_2 and support respiration of field pea nodules (Schulze et al., 1999). The results of this study showed that photosynthesis rate and WSC concentration was 40-50% greater under e[CO_2] compared to a[CO_2]. This extra carbohydrate derived from photosynthesis under e[CO_2] grown plant can be traded with N_2 fixing bacteria to stimulate N_2 fixation. Nodules are distinguished as 'indeterminate' and 'determinate' based on their mode of development (Puppo et al., 2005). Indeterminate nodules such as those of pea and clover are expected to benefit more from e[CO_2] than legumes with determinate type nodules which may potentially limit the C sink capacity. Owing to the high C consumption required for N_2 fixation, this may help to avoid carbohydrate

build up in leaves, translating to greater photosynthetic capacity and nodule activity (Ainsworth et al., 2004). It would be interesting to compare the effects of e[CO_2] on determinate and indeterminate type nodules growth and associated C cost for N_2 fixation in legumes under a changing climate.

In the third hypothesis, this experiment tested whether increased N_2 fixation under e[CO_2] allowed to uptake less soil N and modified overall N acquisition. In this study, e[CO_2] caused no significant reduction of leaf, flower, and other tissues N concentrations, indicating N_2 fixation increased in line with biomass growth. In non-legumes, reduction of tissue N concentration under e[CO_2] is commonly observed when the greater N demand caused by growth stimulation is not matched by increased uptake (Feng et al., 2015; Taub and Wang, 2008). The results of this study support previous FACE studies on soybean (Rogers et al., 2006), where e[CO_2]-induced reduction of leaf [N] disappeared in the middle of the season, once N_2 fixation was stimulated in tune with biomass gain. This response to e[CO_2] is consistent with other findings of field grown field pea of the same cultivar (PBA Twilight) in AGFACE (Bourgault et al., 2016; Butterly et al., 2016a; Jin et al., 2012). In this current study, increased number and biomass of nodules as well as greater specific nodule activity of field pea was linked to enhanced N_2 fixation under e[CO_2] (Fig. 4.5). Previously, increased N_2 fixation has been largely associated with increases in nodule biomass and number (Butterly et al., 2016a; Rogers et al., 2009; Schortemeyer et al., 2002). Those studies did not assess nodule activity and were not conducted under drought conditions. Increased nodule activity is also a driver of greater N_2 fixation, similar to observations in lentil grown in a semi-arid environment (Parvin et al., 2018).

Symbiotic N_2 fixation is highly sensitive to drought, which results in decreased N accumulation and yield of legume crops (Serraj et al., 1999). The depression of leaf [N] under drought was directly related to decreased N_2 fixation in our study, as also evident in soybean under drought (Streeter, 2003). However, flower [N] increased under e[CO_2] by allocating as a greater proportion of fixed N to flowers under drought conditions. A similar increase in biomass allocation to reproductive organs was reported by Wang et al. (2015), suggesting that e[CO_2] could ameliorate the adverse effect of drought at least on the initial stages of seed production in field pea. In this study, e[CO_2] improved stem and floral N reserves and may have increased the extent of N storage and remobilization potential to grains.

Insufficient soil N uptake has been considered to be one of the reasons for the increased contribution of N_2 fixation under e[CO_2] (Zanetti et al., 1996). In many dryland agro-ecosystems, both soil N and water availability are limiting when the crops reach reproductive phases (Angus and van Herwaarden, 2001). In this study, plants grown under e[CO_2] depended less on soil N sources due to higher N_2 fixation, which is an independent confirmation of previous finding on *Medicago trunculata* L. (Guo et al., 2013) and one from an earlier FACE experiment on *Trifolium repens* L. (Zanetti et al., 1996). Under drought, the proportion of soil N (Nds) in total plant N may increase or decrease depending on CO_2 exposure. In this present study, the total amount of soil N absorbed was less in e[CO_2] grown plants compared to a[CO_2] but this difference was lower under drought. This decrease in soil N uptake, most probably in the form of NO_3^- is consistent with the hypothesis that NO_3- assimilation is inhibited under e[CO_2] (Bloom et al., 2014). Experiments have shown that a greater proportion of unassimilated NO_3^- remains in leaves under e[CO_2], and this might also down-regulate NO_3^- uptake ((Bahrami et al., 2017; Bloom et al., 2014). Several physiological mechanisms have been suggested for e[CO_2]-induced inhibition of leaf

NO_3^- assimilation (Bloom et al., 2014), for example, i) e[CO_2] decreases photorespiration and thereby, the amount of reductant available for NO_3^- reduction ii) e[CO_2] inhibits NO_2^- influx into chloroplasts and iii) e[CO_2] competes for reduced ferredoxin as NADPH formation is the first priority rather than NO_3^- reduction. In support of this hypothesis, soil NO_3^- data revealed that more NO_3^- remained in the soil under e[CO_2]-grown pea, even under drought. In addition, decreased activity of the enzymes (NO_3^- reductase and glutamine synthetase) involved in NO_3^- assimilation may further accentuate slower NO_3^- uptake under drought (Nagar et al., 2015).

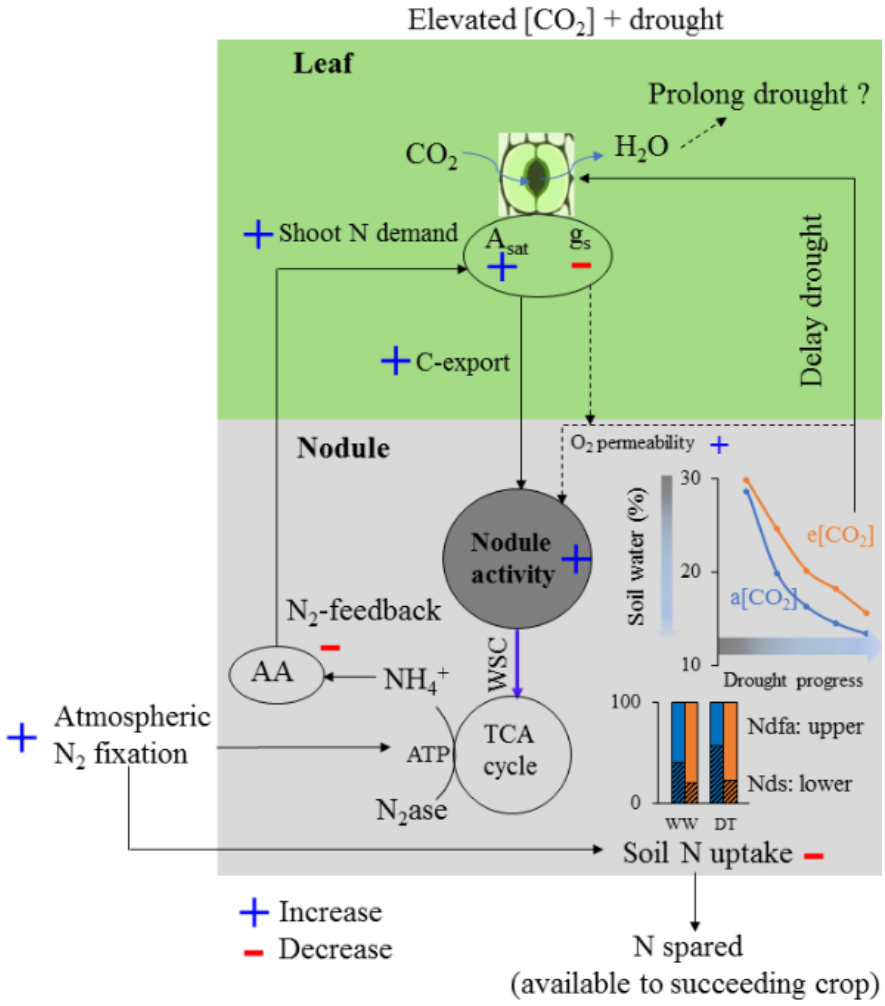


Fig. 4. 7 A summary of the interactive effect of elevated [CO_2] and drought to slowed down soil water depletion by lowering stomatal water loss (g_s) and maintain photosynthesis (A_{net}) and sustain N_2 fixation longer during drought period. Greater A_{sat} provides steady water-soluble carbohydrate (WSC) supply to nodules and lower g_s conserves soil water within the soil profile during dry-down period, which helps to regulate nodule O_2 permeability longer. Overall mechanisms help to continue N_2 fixation and maintain shoot N demand, leading to avoidance of N_2-feedback inhibition by slowing down accumulation of AA in nodules. Increased N_2 fixation (%Ndfa) reduces demand on soil N uptake (%Nds), which can be used by the succeeding crop. Red negative sign (-) refer to the decrease in relative response under elevated [CO_2] compared to ambient [CO_2], while blue positive sign (+) show an increase in relative response. Solid arrows represent direct effect, while dotted arrows show indirect effect. Legends: ambient [CO_2]: a[CO_2], elevated [CO_2]: e[CO_2], AA: amino acids, WW: well-watered, DT: drought.

Studies reported that increased efflux of labile C through rhizodeposition into soil under e[CO_2] may stimulate microbial degradation of soil organic matter, an effect known as "priming," due to microbial mining of soil organic matter for nutrients (Carney et al., 2007), also potentially changing N (and other nutrients) forms / availability in the soil (Drigo et al., 2013). In this study, greater availability of soil NO^{3-}-N under e[CO_2] may improve the amount and quality of substrates available to soil microorganisms, resulting in increased soil microbial abundance and activity (Butterly et al., 2016b). Investigation of microbial activity and associated changes in rhizosphere biogeochemistry under e[CO_2] in response to drought may improve overall understanding on N dynamics and productivity of legumes crop under climate change (Pausch and Kuzyakov, 2018).

The different mechanisms for how e[CO_2] maintained N_2 fixation under drought is summarised in Fig. 4.7. Overall, the effect of e[CO_2] on soil water conservation has been described before, with many studies demonstrating that drier conditions improve WUE at leaf level (Burkart et al., 2011; Hussain et al., 2013; Leakey et al., 2009), albeit not necessarily under all conditions (Gray et al., 2016). In this present study, lower g_s under e[CO_2] lead to slower depleted of soil water slowly, thus maintaining higher soil water within the soil profile during the dry-down period. Sustained photosynthesis in conjunction with a maintained supply of C to nodules were the likely drivers for maintaining nodule growth and activity, which may in turn prolonged N_2 fixation under water deficit. Increased shoot N demand indirectly avoided amino compound accumulation in nodules and relieved feedback inhibition of N_2 fixation. Finally, proportionally more N was supplied from fixed aerial N_2 and less soil N was absorbed (spared) under e[CO_2], which suggests an improved potential of legumes to contribute to agro-ecosystem N balance under e[CO_2].

It has been suggested that small pots may decrease or diminish plant performance under e[CO_2] due to restriction of root growth (McConnaughay et al., 1993). In addition to provide less physical space available for roots growth, small pots also provide less nutrients. Decrease in nutrient availability may eliminate growth stimulation under e[CO_2] (Poorter et al., 2012). Recent study by Bourgault et al. (2017) reported that pot size affected the response to e[CO_2] and the growth restriction associated with growing plants in containers was greater in small pots compared to column grown plants. In the present study, plants were grown in 60cm long columns which provided enough soil volume to grow roots vertically downward. As per visual observation, lack of root curling and bending at the bottom of the column further indicated that the restriction of root growth in this study was not evident. However, considering the spatial and temporal distribution of water and nutrients are highly variable and competition for these resources is often intense due to small soil volumes, therefore future research in a natural field condition is warranted.

4.5 Conclusion

In this study, terminal drought was imposed during the reproductive stage of field pea plants grown either a[CO_2] or e[CO_2] conditions. Results showed that e[CO_2] decreased g_s to a greater extent under drought and increased WUE compared to a[CO_2]. As a consequence, the rate of soil water use was slowed sufficiently under e[CO_2] to enable the maintenance of photosynthesis and nodule activity longer during this drought period. Lower AA concentration in nodules under e[CO_2] grown plant potentially avoided N_2-feedback inhibition and maintained N_2

fixation, thereby reducing dependency on soil N uptake. Consistent with other studies, field pea grown under rising [CO_2] counteracted the negative effect of terminal drought on N_2 fixation and improved N acquisition through incorporating a greater portion of biologically fixed N_2 to plant biomass. Presumably, the increase of biomass growth (also related to increased nodulation, N_2 fixation) and soil water savings through reduced g_s under e[CO_2] during early growth is likely to enhance drought tolerance during reproductive stages (Bernacchi et al., 2007; Kimball, 2016). As the terminal drought will become increasingly more frequent and severe in the coming decades in CO_2 rich environments, e[CO_2]-derived soil water savings and the associated feedback on nodule functionality may support greater N_2 fixation and thus, would help partially modify the N-economy of dryland cropping systems.

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Chapter 4: Supplementary materials

Table 4.1S $\delta^{15}N$ (‰) values of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) and subjected to two water (W) regimes either well-watered or drought and harvested at maximum flowering stage (GS203). Data are presented as mean with ±SE of 4 replicates.

$\delta^{15}N$ (‰)	**Well-watered**		**Drought**	
	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]
Leaf	1.99±0.21	0.52±0.15	1.62±0.23	0.95±0.18
Stem	2.82±0.25	1.92±0.85	4.12±0.12	2.62±0.36
Flower	2.01±0.21	0.95±0.22	1.62±0.14	1.02±0.12
Root	2.11±0.23	1.25±0.12	3.02±0.24	1.89±0.11
Nodule	0.81±0.12	0.04±0.02	1.22±0.23	0.05±0.01
Reference wheat	6.78±0.25	6.69±1.36	6.65±2.45	7.02±1.25
B-value	0.22±0.11	0.19±0.12	0.22±0.11	0.21±0.08

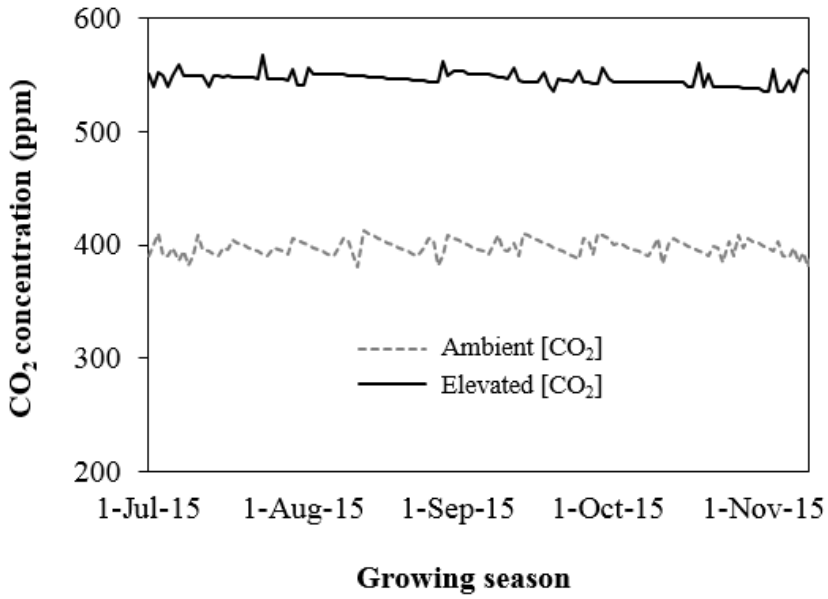


Fig. 4.1S Averaged diurnal CO_2 concentration in ambient [CO_2] (400±15) rings and elevated [CO_2] (550±18) rings during the growing season 2015.



Fig. 4.2S Shoots of field pea cv. PBA Twilight (a: GS107, b: GS201) showing the fully expanded leaflet used for gas exchange measurements. Round cases used to support field plant to grow vertically (b).

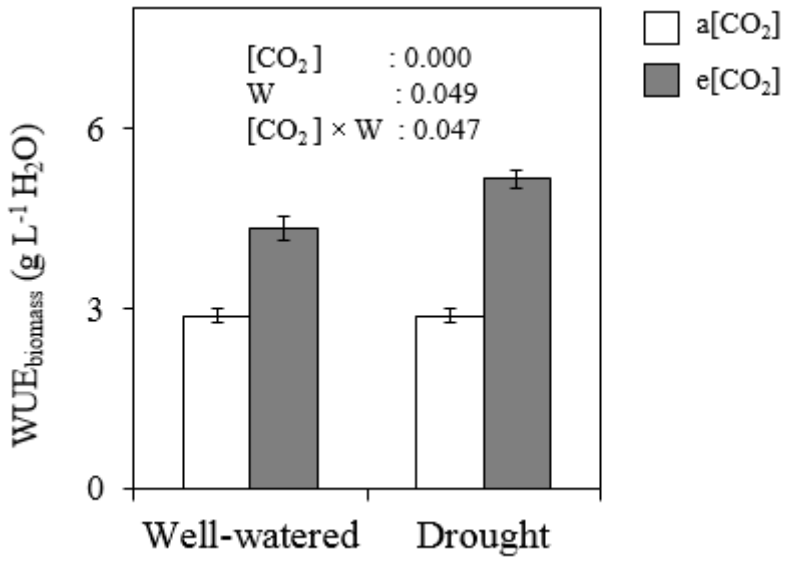


Fig. 4.3S Plant total water use efficiency based on biomass ($WUE_{biomass}$) of field pea grown under ambient [CO_2] (~400 µmol mol^{-1}, white bars) or elevated [CO_2] (~550 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) at harvest. Each bar represents mean with ± SE of 4 replicates. P-values of two-way ANOVA results are shown. Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other ($P<0.05$).

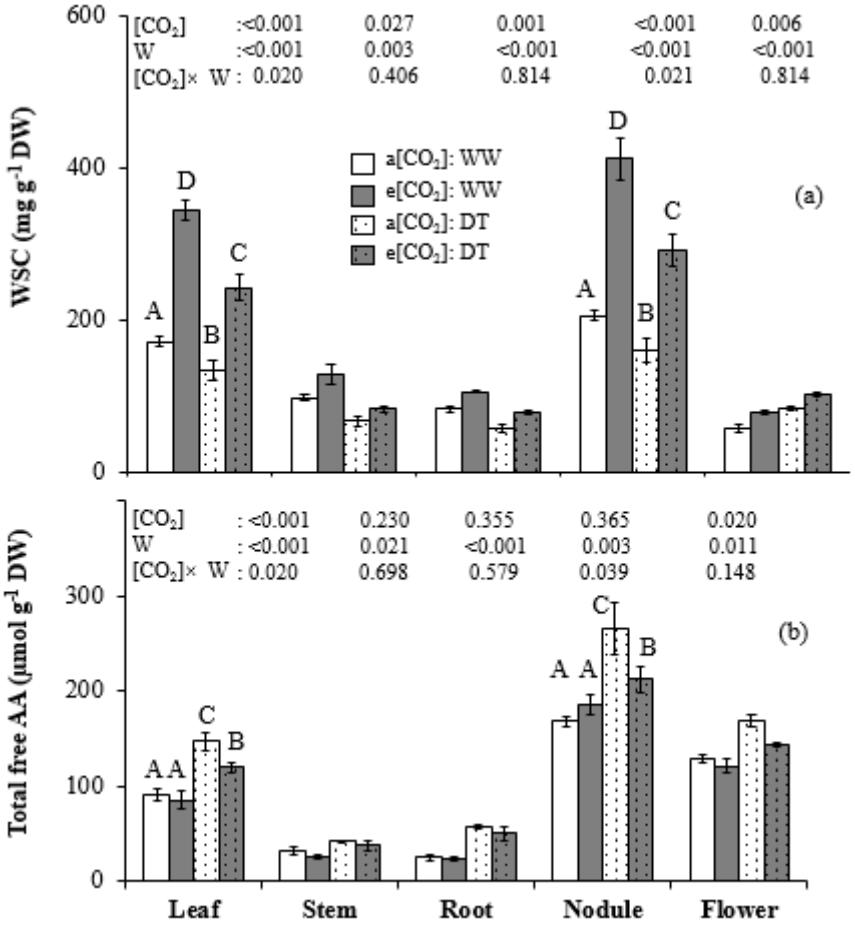


Fig. 4.4S (a) Water soluble carbohydrate (WSC) and (b) total free amino acids (AA) concentration in different organs of field pea grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) CO_2 concentrations and two water (W) regimes (well-watered, WW and drought, DT) at harvest. Each value represents mean with ± SE of 4 replicates. P-values of two-way ANOVA results are shown. Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other ($P<0.05$). Legends: WW under a[CO_2]: white bars, WW under e[CO_2]: grey bars, DD under a[CO_2]: dotted white bars, DD under e[CO_2]: dotted grey bars.

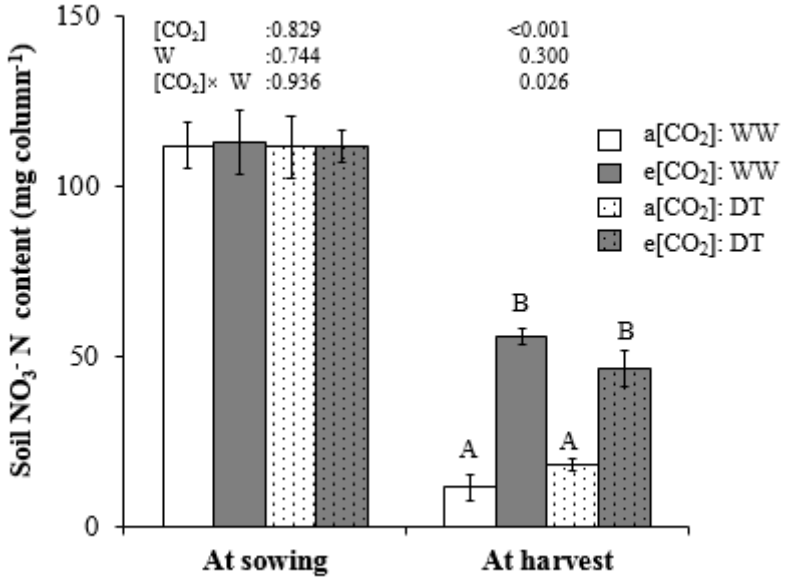


Fig. 4.5S Soil NO_3^- N content (mg column^{-1}) at sowing (left panel) and at harvest (right panel) of field pea grown under ambient [CO_2] (~400 µmol mol^{-1}, white bars) or elevated [CO_2] (~550 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered, WW and drought, DT). Each value represents mean with ± SE of 4 replicates. Legends: WW under a[CO_2]: white bars, WW under e[CO_2]: grey bars, DT under a[CO_2]: dotted white bars, DT under e[CO_2]: dotted grey bars. P-values of two-way ANOVA results are shown. Multiple comparisons are made for significant interaction effects: Mean values that share no common letters are significantly different from each other ($P<0.05$).

Chapter 5: Elevated CO_2 improves yield and N_2 fixation but not grain N concentration of faba bean (*Vicia faba* L.) subjected to terminal drought

(v) Abstract

Legumes grown in Mediterranean environments frequently experience terminal drought which reduces yield and N_2 fixation processes. Decreased N_2 fixation during reproductive phases may constrain seed nitrogen concentrations ([N]), reducing protein concentration of grain legumes. Plants grown under elevated atmospheric CO_2 concentrations ($[CO_2]$) have greater water use efficiency. This may result in reduced use of conserved/stored soil water, potentially helping to reduce soil water deficits later during grain filling. The extent that this process applies to drought sensitive grain legumes, which are extensively cultivated in Mediterranean environments is unclear. The objectives of this study were to investigate yield, N_2 fixation and seed N response of faba bean (*Vicia faba* L. cv. 'Fiesta') grown in a dryland Mediterranean-type environment under elevated $[CO_2]$. Plants were grown in soil columns under ambient $[CO_2]$ (~400 ppm) or elevated $[CO_2]$ (e$[CO_2]$, ~550 ppm) in a Free-Air CO_2 Enrichment (FACE) facility in the field. One sub-group was continuously well-watered (80% field capacity, FC), whereas a second sub-group was exposed to a drought treatment (water was withheld until 30% FC was reached, which was then maintained during the reproductive phases). Biomass, gas exchange, ^{13}C isotopic discrimination, N_2 fixation by the natural abundance ^{15}N method, nodulation and soil water content were assessed throughout the crop developmental stages.

Initially, plants grown under elevated $[CO_2]$ depleted soil water more slowly in the drought treatment than those under ambient $[CO_2]$, but as plants grown under elevated $[CO_2]$ produced more biomass they used soil water more rapidly, especially towards the critical pod-filling phase. Water savings during the first phase of the drought treatment, through flowering up to the start of pod-filling, were associated with increased yield (+25%) and N_2 fixation (+15%) under drought. Elevated $[CO_2]$-induced stimulation of nodulation and nodule density helped maintain N_2 fixation under drought, even though nodule activity decreased under the combined effect of e$[CO_2]$ and drought from pod-filling onwards. This later stage decrease was associated with decreased carbohydrate and increased amino acid concentrations in nodules, indicating a down-regulation of N_2 fixation. Associated with the decrease of N_2 fixation during pod-filling, seed N concentration was lower under the combination of e$[CO_2]$ and drought. We propose a conceptual model to explain the importance of N_2 fixation during the grain filling stage to maintain seed N concentration under e$[CO_2]$. These findings suggest that e$[CO_2]$-induced savings in soil water may mitigate negative effects of drought on yield and N_2 fixation of faba bean, without fully compensating the effect of prolonged drought on seed N concentration.

Key words: Free Air CO_2 Enrichment; drought; *Vicia faba* L.; yield, nitrogen fixation; seed N concentration

5.1. Introduction

Global atmospheric CO_2 concentration ($[CO_2]$) is likely to surpass 550 µmol mol^{-1} later in this century (IPCC, 2014), up from current concentrations of just above 400 µmol mol^{-1} resulting in a greater rate of global climate change. Many climate change scenarios suggest global warming and altered precipitation patterns are anticipated to cause severe drought events in some regions. Mediterranean-type environments are likely to experience more

severe end-of-season water deficits ("terminal drought") during crop reproductive development, reducing crop yield significantly (Leport et al., 1999). Global climate model predicts increased frequency and intensity of such drought episodes in a water-limited dry environment will pose a significant challenge on crop production in future.

Elevated [CO_2] (e[CO_2]) by itself stimulates growth and yield of C_3 crops through increased photosynthetic rate (Ainsworth and Rogers, 2007), which together with decreasing stomatal conductance (g_s) leads to greater leaf-level water use efficiency (WUE) (Bernacchi et al., 2007; Kimball, 2016). If responses of leaf-level WUE (ratio of photosynthetic rate over stomatal conductance) scale to canopy/whole-plant level WUE (ratio of biomass over plant water use), total crop water use may decrease, resulting in conservation of soil water under e[CO_2] (Bernacchi et al., 2007). As e[CO_2] improves plant WUE, the relative increase in grain yields under e[CO_2] may be greater with decreasing water availability (Kimball, 2016). Therefore, e[CO_2] may provide a particular yield advantage in water-limited environments by reducing water use.

Elevated [CO_2] can change the timing of soil water depletion patterns by altering crop water use (Gray et al., 2016; Hussain et al., 2013). However, e[CO_2]-induced reduction of g_s largely depends on the growing conditions and reported to be lower under drier than wetter conditions (Garcia et al., 1998; Uddin et al., 2018b). Therefore, the effect of e[CO_2] on crop water use and hence soil water depletion patterns in dryland conditions might be different from high rainfall irrigated agroecosystem (Uddin et al., 2018a). A recent study by (Parvin et al., 2019) under dryland condition showed that soil water content remained greater under e[CO_2] during short-term drought treatment (till flowering) **(Chapter 4)**. Such water savings were associated with increased leaf-level WUE. However, drought commonly intensifies during the later crop growth stages, when the high temperature increases evaporative demand during the peak grain filling period (Leport et al., 1999). As Parvin et al. (2019) only investigated developmental stages up to flowering, the likely effects of late-season drought were not taken into account in that study. Stimulation of early biomass and leaf area by e[CO_2], in combination with increased canopy temperatures during terminal drought, may cancel any soil water savings (Manea and Leishman, 2014). However, it remains to be determined whether plants can conserve soil water under e[CO_2] and terminal drought for long enough to allow greater yields and N acquisition of crops grown under Mediterranean environments.

Among C_3 crops, grain legumes play a critical role in cropping systems because of their potential to improve soil fertility and cheap source of protein. Grain legume production in rainfed environments is mostly affected by the terminal drought (Pang et al., 2017). A recent global synthesis of legume yield variability under water deficit showed that during reproductive stages (i.e. from flowering to maturity) drought resulted in a similar yield reduction (43.4%) to that observed when drought occurred throughout the entire growing season (42.1%) (Daryanto et al., 2017). Increased CO_2 assimilation rate and reduced water use under e[CO_2] have been proposed as mechanisms to increase crop yields in such dry environments where reduced water availability limits biomass and yield (Kimball, 2016).

Increased biomass and yield under e[CO_2] are often associated with decreases in tissue [N] and grain protein concentration, leading to concerns about the nutritional quality of food crops, especially cereals (Myers et al., 2014). Several mechanisms may explain the decrease of [N] under e[CO_2] (Tausz-Posch et al., 2014); for example, the dilution hypothesis suggests that soil N supply or N uptake fail to keep up with the increased N demand of e[CO_2]-stimulated biomass growth (Feng et al., 2015; Taub and Wang, 2008). In legumes, this decrease in seed protein concentration under e[CO_2], however, is less than in cereals or even absent (Myers et al., 2014), because e[CO_2]-induced stimulation of N_2 fixation may reduce the dependence of legumes on soil N resources (Rogers et al., 2006; Zanetti et al., 1996). Evidence from high rainfall agro-ecosystems suggested that soybean maintains seed [N] under e[CO_2] when N_2 fixation is stimulated in line with biomass production (Gray et al., 2013).

The sensitivity of N_2 fixation to soil drying is a significant constraint for legumes grown in a semi-arid environment (Abdelhamid et al., 2011). If e[CO_2] can help maintain higher soil water contents through increased WUE, then it has the potential to help protect N_2 fixation mechanisms from moderate drought (Rogers et al., 2009). Controlled environment experiments also suggested that e[CO_2] ameliorates drought-induced reductions in N_2 fixation by increasing the exchange of both C and N in nodules at a given soil moisture content (De Luis et al., 1999; Serraj et al., 1998). In contrast, N_2 fixation of field grown soybean and lentil decreased under e[CO_2] combined with lower soil water availability (Gray et al., 2013; Parvin et al., 2018). Data on the interactive effects of e[CO_2] and soil water on N_2 fixation are often complex (Parvin et al., 2018) and it is still unclear whether and to what extent increased leaf-level WUE under e[CO_2] can translate into soil water conservation, and if so, whether this water conservation will support higher nodulation, nodule functionality and greater N_2 fixation under conditions of limited water supply.

When N_2 fixation and N uptake are constrained, seed N in legumes becomes dependent on the remobilization of previously assimilated N from vegetative tissues. However, remobilization of N to the grains has been shown to be modified by environmental factors, such as drought and e[CO_2] (Barbottin et al., 2005). Also, moderate water deficits during the grain-filling period stimulate the transport of assimilates from the vegetative organs to the developing seeds (Zhang et al., 2008). Elevated [CO_2] has been reported to increase (Agüera and De la Haba, 2018), decrease (Li et al., 2017) or leave unchanged (Tausz et al., 2017) N remobilization during reproductive phases. The interaction between e[CO_2] and drought can alter both plant N acquisition and remobilization pattern and therefore seed [N].

Faba bean (*Vicia faba* L., broad bean, horse bean) is a winter-growing pulse or food legume crop. Apart from providing food and animal feed rich in protein (Crépon et al., 2010), it contributes positively to the N economy in Mediterranean dryland agricultural systems. Faba bean is grown as a rotational crop in cereal-based farming systems because it can fix more than 80% of its own nitrogen needs under a wide range of conditions (Denton et al., 2017). However, because it is highly sensitive to drought compared to some other legumes, its cultivation particularly, in arid and semi-arid regions, raises concern for the future (Ammar et al., 2015; Siddiqui et al., 2015).

To investigate the interactive effect of e[CO_2] and terminal drought imposed during reproductive phases on yield, N_2 fixation and seed [N] of faba bean in a water-limited environment, a Free-Air CO_2 Enrichment (FACE) experiment was conducted under two water regimes (well-watered to at least 80% field capacity, FC and drought, left to dry to about 30% FC and maintained afterward). The experimental design of growing crops in individual soil cores in a field installation allowed testing of the following hypotheses:

1. Elevated [CO_2]-stimulation of early biomass growth will cancel out the effect of lower gs on soil water savings during prolonged terminal drought
2. Elevated [CO2]-stimulation of yield and biomass will be greater under drought than well-watered conditions
3. Soil water savings under e[CO_2] will stimulate nodulation and nodule activity, thereby maintaining N_2 fixation under prolonged drought during later growth stages
4. This stimulation of N_2 fixation will avoid e[CO_2]-induced depletion of seed [N]

5.2. Materials and methods

5.2.1. Experimental site

The experiment was conducted at the sub-facility of Australian Grain Free-Air CO_2 Enrichment (AGFACE) facilities known as Soil Free-Air CO_2 Enrichment (SoilFACE) at the Agriculture Victoria Research Plant Breeding Centre, Horsham (36°44S, 142°06E; 127 m elevation), Victoria, Australia in 2016. SoilFACE consisted of eight round bunkers sunk into the ground (3.7 m diameter; 1.2 m depth). Bunkers were at least 28 m apart and each bunker was treated with either ambient CO_2 (a[CO_2]) or elevated CO_2 (e[CO_2]), resulting in four replicates arranged in a completely randomised design (Butterly et al., 2015). The FACE system used to achieve the e[CO_2] level was similar in design and used the same reticulated CO_2 supply as the facility previously described by Mollah et al. (2009), except that smaller rings (4.0 m diameter instead of 16 m) were used. At the e[CO_2] bunkers, octagons ('rings') of horizontal stainless-steel tubes were positioned about 150 mm above canopy level at any developmental stage of the crop. CO_2 was injected into the upwind side through holes on the outside of the stainless-steel tubes. CO_2 enrichment was applied throughout the experimental period from sunrise to sunset with the target [CO_2] at the centre of elevated bunkers 550 µmolmol^{-1}, whereas [CO_2] of the ambient bunkers was close to 400 µmolmol^{-1}. An infrared gas analyser (IRGA, SBA-4, PP Systems, Amesbury, MA, USA) continuously recorded [CO_2] at the centre of each bunker. The performance of the system with 4m rings against target [CO_2] is described by Mollah et al. (2011) and elsewhere (Butterly et al., 2015; Butterly et al., 2016a; Butterly et al., 2016b; Dong et al., 2018; Jin et al., 2015; Jin et al., 2013; Parvin et al., 2019; Uddin et al., 2019; Xu et al., 2019). Temperature (average daily maximum temperature 20.2°C and minimum temperature 4.1°C), rainfall and cumulative water input (well-watered: 509 mm and drought: 260 mm) during the experimental duration are presented in Fig. 5.1. Meteorological data were obtained from an automatic weather station installed near the SoilFACE site.

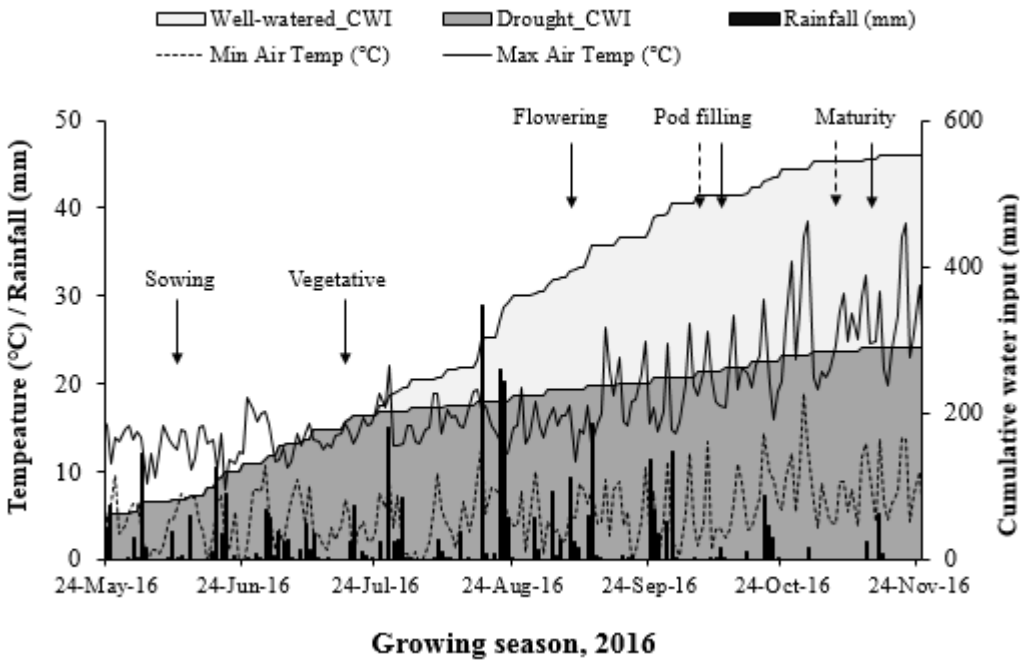


Fig. 5. 1 Daily maximum (solid line) and minimum (dotted line) air temperatures (°C) and daily rainfall (mm) at the experimental site at crop growing season including averaged cumulative water input (CWI) of faba bean grown under well-watered and drought conditions. Sowing time and sampling at each growth stages are indicated by arrows. Dotted arrows in the figure indicate an advanced plant phenology according to drought imposed under ambient [CO_2] grown plants.

Plants were grown in polyvinyl chloride (PVC) columns (60 cm long, 15 cm in diameter) each filled with 14 kg of soil and placed into the bunkers so that the surface was level with the surrounding soil. The soil was a Vertosol (Isbell, 2002) and was collected adjacent to the site and described in detail elsewhere (Butterly et al., 2015; Jin et al., 2015). There were eight columns per bunker with four columns per treatment for four harvest time points. Columns were positioned so that their upper edge was level with the surrounding paddock and other soil columns (carrying crops not used in this experiment) filled the bunkers to create a near continuous canopy.

5.2.2. Plant material

Faba bean (*Vicia faba* L. cv. 'Fiesta') seeds were inoculated with commercial Group F peat-based inoculum (*Rhizobium leguminosarum*, NoduleN, New Edge Microbials Pty Ltd, Albury, NSM, Australia) before sowing. Inoculated seeds were hand sown on 24 May 2016 at a depth of 2 cm (4 seeds per column). Seedlings were thinned to the two most vigorous plants at 15 days after sowing (DAS). To measure the amount of N derived from atmosphere using ^{15}N natural abundance, wheat was grown as a non-fixing reference plant in an additional column in each bunker.

5.2.3. Water treatments

All soil columns were kept well-watered until 60 days after sowing (DAS). Well-watered columns were irrigated weekly to maintain soil water status close to field capacity (80% ± 5% FC, ~33 % volumetric water content) (Parvin et al., 2019). After 60 DAS, half of the columns in each ring were randomly assigned to the drought treatment. Columns assigned to the drought treatment were left to dry down to 30% FC, then irrigated weekly to maintain soil water status close to 30% FC (30% ± 5% FC, ~21 % volumetric water). In drought treatments, rainwater was excluded by covering the soil surface of each column with polyethylene film firmly around the stem base (Jin et al., 2015). Columns were watered to weight every week throughout the drought period to replenish the amount of water lost through evapotranspiration. Volumetric soil water content (%) was measured by time domain reflectometry (Theta Probe, ML3, Delta-T Devices, Cambridge, UK) every week in the middle of the rewatering cycle (3 days after rewatering). Soil water measurements were taken at three depths (20, 40 and 60 cm) from the soil column assigned for final harvest (Pazzagli et al., 2016). Averaged soil water content throughout the profile is reported in Fig. 5.2. The total amount of irrigation applied during the treatment period was recorded.

5.2.4. Sampling

Destructive sampling was conducted at four phenological growth stages such as vegetative, flowering, pod filling and physiological maturity (Figure 5.1 & Table 5.1S). Sampling at flowering and pod filling stages took place when 50% flowers were bloomed and 50% of pods had well-developed seeds notable from the outside of the pod wall. Flowering sampling was done at 100 DAS for all treatment, whereas pod filling and physiological maturity of a[CO_2] grown drought treated plants were advanced by one and two weeks, respectively. Final harvest was performed at physiological maturity when all the pods turned dark brown and dry. Except at maturity, leaves and nodules tissue was frozen in liquid N_2 immediately after collecting in the field and stored at -80^{0}C for total free amino acid analysis. At each sampling event, plants were separated into different organs. Columns were opened vertically and washed thoroughly with tap water. Roots were recovered; nodules were separated and counted. At maturity, dried pods were counted, seeds were separated, counted and weighed to estimate seed yield. Leaf area was measured using a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). All biomass was dried at 65^{0}C for 72 hours and weighed for dry matter estimation. Harvest index (HI) and nitrogen HI were calculated as the ratio of seed yield to total biomass and seed N content to total N content, respectively. Grain mineral composition is reported in Chapter 8.

5.2.5. Root length

At flowering, each soil column was opened vertically and separated into three soil layers, 0–20, 20–40 and 40–60 cm. The root system was rinsed with tap water and then soaked in 0.01M $CaCl_2$ solution for 5 min to desorb nutrients and clay particles from the root surface. Root washing was followed according to the procedure described by Frasier et al. (2016) and described in details in Chapter 2. Roots in each layer were recovered by sieving with a 2mm sieve. Root length in each layer was determined using the WinRhizo Pro version 2003b program (Régent Instruments Inc., Québec, Canada). Total root length was calculated by summed up the root length of three depths.

5.2.6. Leaf gas exchange measurements

Leaf gas exchange was measured at three growth stages using an infrared gas analyser (IRGA) system (LI- 6400, LICOR, Lincoln, NE, USA). Measurement at vegetative and flowering stages were conducted at 60 DAS and 100 DAS for all treatments, whereas measurement at pod filling stage was done one week earlier (128 DAS) for a[CO_2]-grown drought treated plants and rest of the treatments were measured at 135 DAS. A fully expanded youngest leaf was measured with the default clear top window chamber at light intensities around 2000 µmol m^{-2} s^{-1} photosynthetic photon flux density (clear skies) and an air flow rate of 500 µmol s^{-1}. Reference [CO_2] was adjusted to growth [CO_2] of 400 and 550 µmol mol^{-1} for ambient and elevated [CO_2] rings, respectively. Once the steady state condition was reached in each [CO_2] level, light saturated CO_2 assimilation rate (A_{sat}) and stomatal conductance (g_s) were measured and intrinsic water use efficiency (iWUE) was calculated from A_{sat}/g_s. Across all measurements leaf transpiration was between 2.25 to 5.58 mmol H_2O m^{-2} s^{-1}, leaf temperature 21.2 to 26.6 °C, air temperature 20.25 to 26.89°C, leaf-air-vapour pressure deficits 0.78 to 2.14 kPa and neither of these were different between treatments on each measurement date.

5.2.7. Carbon isotope discrimination

For carbon isotope analysis, leaflets of youngest fully developed leaves from four plants per treatment were sampled at noon. Samples were taken at three growth stages: vegetative, flowering and pod filling. Leaf tissues were oven-dried at 65°C and finely ground to powder in a ball mill. Approximately, 2-3 mg of ground material were placed into tin-capsules and the stable isotopic composition was then determined with an isotope ratio mass spectrometer (IRMS) (Hydra 20–20, SerCon) coupled to an elemental analyser (Carlo Erba).

Isotopic composition of a total of 16 air samples taken in a[CO_2] and e[CO_2] rings twice during the growing season was measured by GasBench-IRMS (UC Davis, Stable Isotope Facility, CA, USA). As $\delta^{13}C$ signatures were statistically indistinguishable between both [CO_2] (average of -8.09±0.11‰ for a[CO_2] and -8.03±0.09‰ for e[CO_2]), $\Delta^{13}C$ (^{13}C isotope discrimination of plant materials) is a useful surrogate for WUE in our experiment.

Carbon isotope discrimination (CID) of leaf dry matter was expressed as Δ and calculated from the following equation as described by Farquhar et al. (1989).

Δ (‰) = $[(\delta a - \delta p)/(1 + \delta p)] \times 1000$ (per thousand, ‰)

Where δa and δp are the isotopic compositions of growth [CO_2] and leaf tissues, respectively, expressed relative to Pee Dee Belemnite (PDB) ($\delta = 0$). DL-α-alanine with a δ value of −25.43 was used as a working standard.

5.2.8. Biochemical analyses

Water soluble carbohydrate (WSC) concentration was determined from oven dried and finely ground leaves and nodules with the anthrone method (Yemm and Willis, 1954) modified for use in a plate reader (Tecan Sunrise, Tecan, Austria) (Tausz-Posch et al., 2015). Absorbance was recorded at 600 nm using D-fructose as standard and WSC concentration expressed as mg g^{-1} dry weight.

Total free amino acid concentration (AA) of leaves and nodules were determined from frozen tissues (-80°C) using acid ninhydrin method (Yemm and Cocking, 1955). Absorbance (at 570 nm wavelength) was measured in a plate reader (Tecan Sunrise, Tecan, Austria). The concentration of AA was calculated as amino acid standard equivalent (mixed amino acid standard AAS18, Sigma-Aldrich) and expressed as µmol g^{-1} dry weight.

5.2.9. Tissue N and symbiotic N_2 fixation

Finely ground dry plant tissue samples (leaves, stems, flowers, roots, nodules, chaffs, grains and reference plant) from four sampling stages were analysed for N concentration ([N]) (%) and $\delta^{15}N$ values by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon) coupled to an elemental analyser (Carlo Erba). The results (atom % ^{15}N) were expressed as $\delta^{15}N$ values (‰) using atmospheric air (0.3663 atom %) as the standard (Unkovich et al., 1997). The $\delta^{15}N$ value of soil collected from our experimental field was 8-10‰, which is sufficiently different from air to allow measurement of N_2 fixation by natural abundance method.
The percentage of N_2 fixed from atmosphere (%Ndfa) was determined by ^{15}N natural abundance method according to Unkovich et al. (1994).

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

where 'reference plant' refers to a non-N_2 fixing plant, which should match closely the studied faba bean in terms of soil N utilization (Fan et al., 2006; Nebiyu et al., 2014; Zapata et al., 1987). We used wheat grown in adjacent soil cores to faba bean in each bunker under similar treatment combinations: well-watered and drought, respectively. Factor B refers to the $\delta^{15}N$ value of the effectively nodulated faba bean (cv. Fiesta) grown in media lacking N. To determine B, faba bean was grown in sand using similar [CO_2] and water treatment in a glasshouse.

The B-value was also corrected for seed N (Nebiyu et al., 2014). Symbiotic N_2 fixation in each harvest was calculated as follows:

N_2 fixation (g $plant^{-1}$) = Total N content × %Ndfa/100

where total N content was estimated as the sum of N accumulated in each organ (the product of organ biomass and tissue N concentration) and expressed as g $plant^{-1}$.

Soil N uptake

The remainder of total plant N was assumed to be derived from soil N uptake (%Nds) and calculated based on the following:

%Nds = 100 - % Ndfa

Soil N uptake (g $plant^{-1}$) = Total N content -N_2 fixation

5.2.10 The model

The proportional changes in the percentages of N_2 derived from the atmosphere (%Ndfa) and soil N uptake (%Nds) at four growth stages (vegetative, flowering, pod filling and maturity) of faba bean were estimated as described above.

Seed N during reproductive stages was apportioned to uptake during reproductive phases, fixation during reproductive phases or remobilization as follows:

Seed N (g plant^{-1}) = N_2 fixation + soil N uptake + N remobilization

N_2 fixation during reproductive phases was calculated by subtracting the amount of N_2 fixed at flowering from the amount of N_2 fixed at maturity. Soil N uptake during reproductive phases was estimated by subtracting the amount of soil N taken up at flowering from total soil N taken up at maturity. Remobilization was estimated as the reduction of N from leaf, stem and chaff tissues from flowering to maturity and it was assumed that all remobilized N was allocated to seeds (Tausz et al., 2017). The amount of remobilized N derived from fixation was estimated as the reduction of ^{15}N content (Ndfa content) from vegetative tissues (leaf, stem, and chaff) between flowering to maturity. The rest of the remobilized N was assumed to be derived from soil N uptake.

The percentage contribution to total seed N from each N source was estimated as:

% seed N from fixation = (N_2 derived from fixation/seed N) × 100

% seed N from soil uptake = (N derived from soil uptake/seed N) × 100

% seed N from remobilization (fixation) = (Remobilized fixed N_2/seed N) × 100

% seed N from remobilization (uptake) = (Remobilized soil N/seed N) × 100

5.2.11. Statistical analysis

The experiment was designed as a split-plot with [CO_2] as main-plots (bunkers) and water regimes as sub-plot (columns within bunkers). [CO_2] and water regimes were considered fixed effects and bunkers/rings as random effect. Analysis of variance (ANOVA) was performed with a linear mixed-effect model fit by REML using the package "nlme" as described by Pinheiro et al. (2017). Repeated measure ANOVA was performed for soil water content considering measurement dates (DAS in this case) as a random effect. As this does not allow for testing of the interaction between DAS and treatments, each growth stage was also analysed separately. To explicitly investigate the interactive effects of growth stage, [CO_2] and water treatment on soil water content, this parameter was further analysed considering growth stage as an additional fixed factor. To check the homogeneity of variances, Levene's tests were conducted and where necessary data transformations were done. When the interaction effects were significant, pairwise comparisons of the means were performed by least significant difference (LSD) test to identify significant differences among treatment combinations ($P < 0.05$) using the R package "predictmeans" (Luo et al., 2014). Data were analysed using R Studio version 3.4.1 (R Core Team, 2018). Graphics were produced using "ggplot2" of R software (Wickham, 2009).

5. 3. Results

5.3.1. Soil water status

Soil water content differed significantly between [CO_2] and water treatments (Fig. 5.2) and these effects varied between growth stages (Stage × [CO_2] × W; $P < 0.05$). Significant interactions between [CO_2] and watering regime were observed at flowering and pod filling ($P < 0.05$). Columns under e[CO_2] had greater soil water content up to pod filling stage and the difference was greater under drought, particularly during the dry-down period. The effect diminished at later crop growth stages when there was slightly greater depletion of soil water under e[CO_2] than

a[CO_2], measured halfway between rewatering events. Drought-induced faster senescence of a[CO_2] grown leaf apparently led to a faster decrease in water use and translated to an increased soil water content between rewatering events to the weight target, whereas the greater green leaf biomass/area in e[CO_2] grown plants was associated with greater soil water extraction.

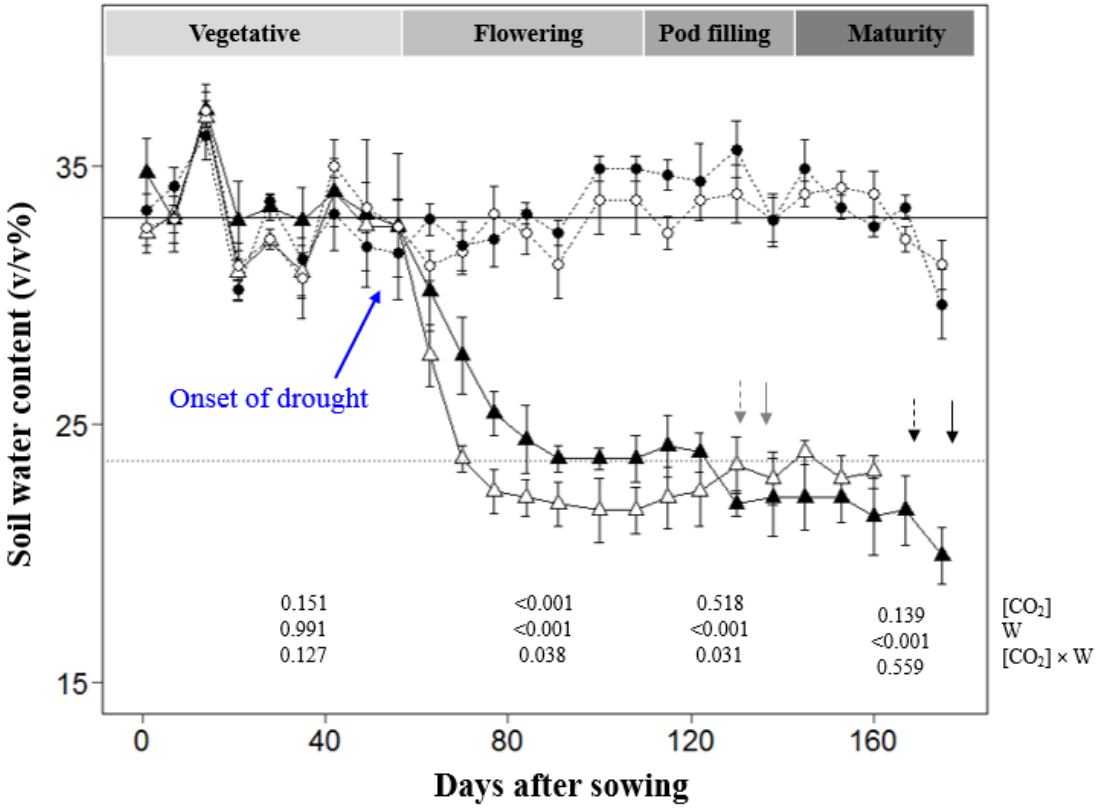


Fig. 5. 2 Soil water content (v/v, volume %) during the growing period of faba bean under two CO_2 concentrations i.e. ambient [CO_2] (~400 µmol mol^{-1}; open symbols O, Δ) and elevated [CO_2] (~550 µmol mol^{-1}; closed symbols ●, ▲) and two water (W) regimes (well-watered; broken lines with circles and drought; continuous lines with triangles). Blue arrow indicates the onset of the drought treatment and dotted arrows in the figure indicate an advanced plant phenology according to drought imposed under ambient [CO_2] grown plants compared to other treatments (indicated by solid arrows). Data are means ± 1 SE of 4 replicates. The continuous horizontal line indicates field capacity ~ 80% under well-watered condition and broken line field capacity ~ 30% under drought condition, the targeted values for weekly rewatering. Once targets were reached after drying, measurements were taken halfway between rewatering. P-values of the effect of [CO_2], water regime (W) and their interactions are shown for each growth stage.

5.3.2. Yield and growth attributes

Stimulation of seed yield by e[CO_2] was greater in the well-watered (58%) than drought treatment (23%) (significant interaction [CO_2] × W; Table 5.1). The number of pods and seeds increased under e[CO_2] but drought reduced both parameters by 28% and 31%, respectively. Elevated [CO_2] increased the harvest index (HI) by 18% compared to a[CO_2]. HI of the drought treated plant was 18% lower than the well-watered plant. Drought decreased nitrogen harvest index (NHI) to a greater extent under e[CO_2] (-18%) than under a[CO_2] (-9%).

At flowering, growth under e[CO_2] increased shoot biomass and total biomass by 50%. At maturity, e[CO_2] stimulation of shoot biomass was greater under well-watered (+28%) than drought (+7%). Elevated [CO_2] increased root biomass and total root length to a greater extent under drought at flowering. Similarly, e[CO_2] increased root:shoot ratios and this increase was greater under drought (57%) than in well-watered conditions (9%) and this trend was observed at both flowering and maturity. Total leaf area was greater under e[CO_2] and was decreased (-23%) by drought at flowering. At pod filling stage, e[CO_2]-grown plants had greater green leaf area and green leaf biomass compared to a[CO_2] grown ones and maintained this trend even under drought conditions. Compared to well-watered plants, drought increased dried/senescence leaf biomass with greater extent under a[CO_2] (-45%) than under e[CO_2] (+16%) (all Table 5.1 & 5.2).

Table 5. 1 Growth and yield parameters of faba bean grown under two CO_2 concentrations either ambient (a[CO_2], ~400 µmol mol^{-1}) or elevated (e[CO_2], ~550 µmol mol^{-1}) and two water (W) regimes (well-watered and drought) and harvested at maturity. Means ±SE (n=4) and two-way ANOVA results are shown. AGB: Above ground biomass, BGB: belowground biomass, %Ndfa: Percentage of N derived from the atmosphere. Significant effects are shown in bold (P<0.05). Different letters within a row indicate significant differences at P<0.05.

At Maturity	Well-watered		Drought		P-value		
	a[CO_2]	e[CO_2]	a [CO_2]	e[CO_2]	[CO_2]	W	[CO_2] × W
Yield and yield components							
Seed yield (g plant^{-1})	18.36±1.77b	29.19±2.25c	13.80±0.93a	17.06±0.67b	**<0.001**	**<0.001**	**0.001**
Pods number (plant^{-1})	17.63±2.74b	25.38±1.80c	11.88±2.06a	17.50±2.16b	**0.038**	**0.010**	0.587
Seeds number (pod^{-1})	29.88±3.42b	41.75±2.06c	21.25±3.25a	29.75±6.01b	**0.035**	**0.020**	0.457
Harvest index (HI)	0.34±0.02b	0.42±0.02c	0.307±0.01a	0.34±0.02b	**0.009**	**0.009**	0.148
Nitrogen harvest index (NHI)	0.55±0.03b	0.64±0.02c	0.50±0.03a	0.52±0.02b	0.065	**0.028**	**0.011**
Biomass and tissue N							
Shoot biomass (g plant^{-1})	45.67±2.60b	58.47±1.67b	36.82±1.65a	38.74±3.23a	**0.030**	**<0.001**	**0.018**
Root biomass (g plant^{-1})	7.83±0.88a	10.41±0.96b	7.70±1.25a	12.88±0.31b	**0.045**	0.378	0.324
Total biomass (g plant^{-1})	53.50±2.41b	68.88±2.42c	44.52±2.58a	51.62±2.65b	**0.007**	**<0.001**	0.130
Root:shoot	0.17±0.02a	0.18±0.02a	0.21±0.03b	0.34±0.03b	**0.026**	**0.007**	**0.039**
Leaf [N]	2.90±0.13b	3.08±0.19b	2.76±0.07a	2.59±0.09a	0.967	**0.012**	0.123
Stem [N]	0.72±0.04ab	0.77±0.11b	0.61±0.07a	0.74±0.12b	0.342	0.480	0.652
Root [N]	2.50±0.17a	2.56±0.33a	2.63±0.23a	2.71±0.07a	0.751	0.552	0.965
Seed [N]	4.80±0.13b	4.88±0.10b	4.64±0.15b	4.24±0.13a	0.363	**0.007**	**0.043**
Total N content (g plant^{-1})	1.62±0.06c	2.25±0.12d	1.23±0.10a	1.46±0.05b	**0.005**	**<0.001**	**0.023**
Grain (%Ndfa)	70±2.32b	86±5.5c	62±3.25a	70±6.54b	**0.036**	**0.045**	0.125
Grain (%Nds)	30.42±4.25b	16.25±5.25a	40.25±8.36b	32.35±6.35b	**0.045**	0.125	0.325
Total remobilized N (%)	47.63±1.83a	40.35±4.13a	56.00±5.89b	54.10±2.68b	0.417	**0.045**	0.249
Remobilized N from fixation (%)	32.14±2.35a	32.56±4.25a	42.12±1.45b	43.85±3.54b	0.125	0.029	0.348
Remobilized N from uptake (%)	15.75±3.45b	8.76±1.25a	13.45±3.25b	10.75±2.86a	0.235	0.354	0.125

Table 5. 2 Growth attributes of faba bean at flowering and pod filling stage grown under two CO_2 (ambient [CO_2], ~400 µmol mol^{-1} and elevated [CO_2], ~550 µmol mol^{-1}) and two water (W) regimes (well-watered and drought). Means ±SE (n=4) and two-way ANOVA results are shown. Significant effects are shown in bold (P<0.05). Different letters within a row indicate significant differences at P<0.05. LA: leaf area; LR: root length

Parameters	Well-watered		Drought		P-value		
	a[CO_2]	e[CO_2]	a [CO_2]	e[CO_2]	[CO_2]	W	[CO_2] × W
At flowering							
Shoot biomass (g plant^{-1})	20.87±0.37a	29.39±1.20b	19.71±1.97a	26.68±0.83b	**<0.001**	0.132	0.441
Root biomass (g plant^{-1})	6.27±0.51a	9.91±0.42b	5.39±0.68a	11.73±0.35b	**<0.001**	0.389	**0.037**
Total biomass (g plant^{-1})	27.12±1.68a	39.30±2.40b	25.09±1.85a	38.40±0.82b	**<0.001**	0.243	0.818
Root:shoot	0.30±0.02a	0.34±0.01a	0.29±0.05a	0.45±0.02b	**0.026**	0.097	**0.043**
Leaf [N]	4.88±0.19a	4.74±.014a	4.51±0.08b	4.38±0.04b	0.334	**0.02**	0.962
Stem [N]	1.50±0.15a	1.53±0.16a	1.32±0.06a	1.43±0.07a	0.605	0.258	0.712
Root [N]	2.96±0.12a	2.67±0.19a	2.90±0.12a	2.75±0.15a	0.293	0.921	0.483
Flower [N]	5.03±0.11a	5.04±0.28a	4.99±0.29a	5.31±0.18a	0.494	0.635	0.525
Total N content (g plant^{-1})	1.40±0.7c	1.61±0.12c	0.85±0.08a	1.27±0.03b	**0.003**	0.127	0.722
Leaf area (cm^2 plant^{-1})	866.89±78.30a	1245.52±33.66c	632.45±45.75a	986.94±66.19b	**<0.001**	**0.005**	0.831
Total RL (m)	40.92±2.85b	54.49±2.11c	32.83±1.33a	52.92±2.96c	**0.001**	**0.008**	**0.040**
Nodule density (nodules m^{-1} RL)	5.25±0.82b	5.64±0.33b	3.41±0.36a	6.59±0.38b	**0.014**	**0.041**	**0.001**
At pod filling							
Green leaf area (cm^2 plant^{-1})	647.29±23.16b	848.69±21.29c	466.57±29.14a	617.15±21.70b	**0.001**	**<0.001**	**0.038**
Green leaf biomass (g plant^{-1})	14.97±0.94b	21.36±2.14c	9.93±0.84a	14.24±0.50b	**0.009**	**0.003**	0.369
Dried leaf biomass (g plant^{-1})	6.40±0.24a	6.27±0.38a	9.31±0.51b	7.33±0.41a	**0.064**	**<0.001**	0.024

5.3.3. Gas exchange parameters

Light-saturated net assimilation rate (A_{sat}) was greater for e[CO_2] grown faba bean from the vegetative to pod filling stages (Fig. 5.3A). This stimulation by e[CO_2] was greater under well-watered conditions at flowering (50%). The increase of A_{sat} and decrease of stomatal conductance (g_s) contributed to increased iWUE. Intrinsic WUE was increased by e[CO_2] and drought; this increase was amplified under this combination at flowering but not pod filling (Fig. 5.3C). Drought treated plants had lower $\Delta^{13}C$ at all growth stages. At flowering $\Delta^{13}C$ was lower for e[CO_2]-grown plants than a[CO_2] ones (Fig. 5.3D).

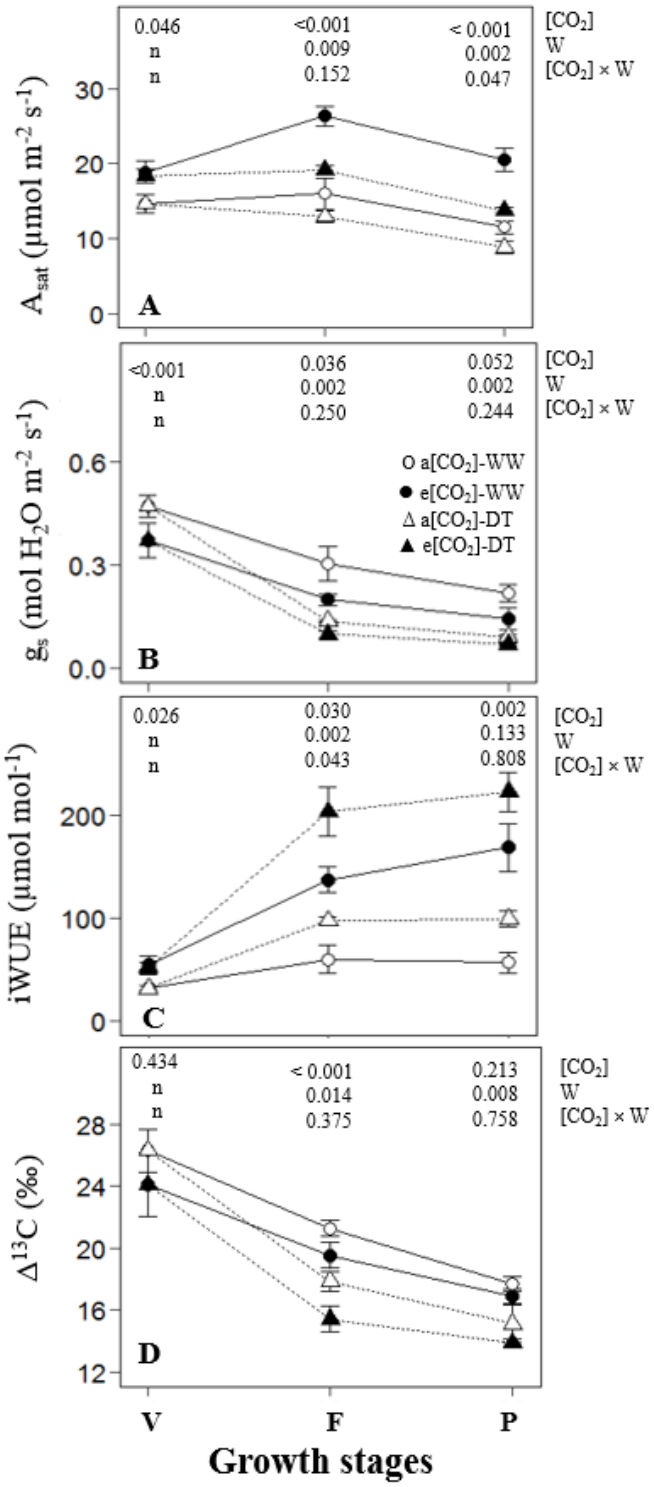


Fig. 5. 3 Light saturated CO_2 assimilation rate (A_{sat}, A), stomatal conductance (g_s, B) and intrinsic water use efficiency (iWUE, C) and ^{13}C discrimination ($\Delta^{13}C$, D) in leaves of faba bean grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}; white bars) and elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}; grey bars) and two water (W) regimes (WW: well-watered and DT: drought) and measured at vegetative (V), flowering (F) and pod filling (P) stages. Means and ±SE (n=4) in each bar. P-values of the effect of [CO_2], water regime (W) and their interactions are shown.

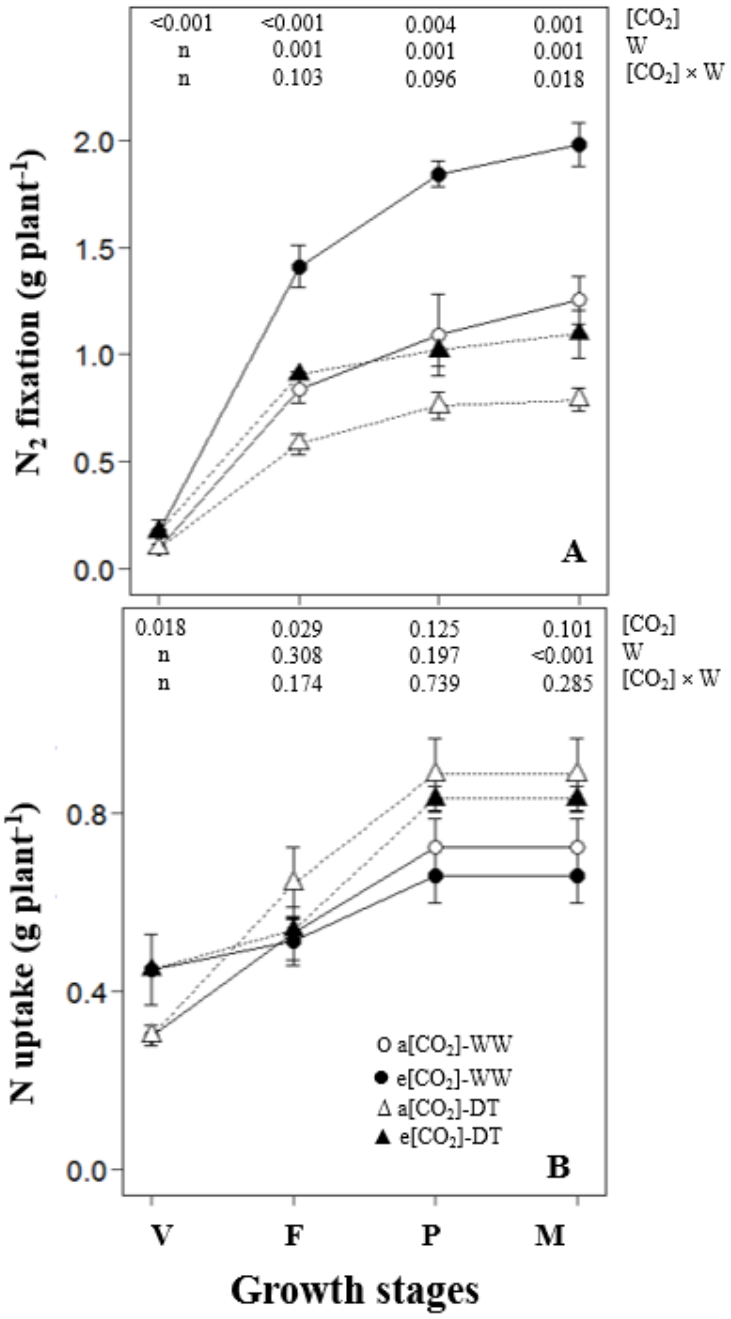


Fig. 5. 4 N_2 fixation (A) and soil N uptake (B) of faba bean grown under ambient [CO_2] (~400 µmol mol^{-1}; open symbols O, Δ) and elevated [CO_2] (~550 µmol mol^{-1}; closed symbols ●, ▲) and two water (W) regimes (well-watered, solid lines and drought, broken) and measured at vegetative (V), flowering (F), pod filling (P) and maturity (M) stages. Means and ±SE (n=4) in each bar. P-values of the effect of [CO_2], water regime (W) and their interactions are shown.

5.3.4. N_2 fixation and uptake

The amount of N derived from N_2 fixation was greater under e[CO_2] at all growth stages with these differences greatest at flowering (Fig. 5.4A). There was no significant N_2 fixation recorded between flowering and maturity under drought. In contrast, well watered plants acquired approximately one-third of the total amount of their total N content from fixation during the post-flowering stages. Despite terminal drought inhibited N_2 fixation, at maturity the total amount of fixed N_2 was greater in e[CO_2] grown plants than a[CO_2] grown ones.

Soil N uptake was higher under e[CO_2] at the vegetative stage in well-watered plants and was consistently lower at later growth stages (Fig. 5.4B). Drought treated plants obtained more N from soil uptake than well-watered ones but there was no significant effect of [CO_2].

5.3.5. Nodulation

Elevated [CO_2] significantly altered the nodule characteristics of faba bean from vegetative to maturity stages (Fig. 5.5A-B). Stimulation of nodule number by e[CO_2] was even greater under drought at flowering, but this interaction was not observed at later stages. Overall, nodules were smaller under drought resulting in a lower stimulation of nodule biomass by e[CO_2] under drought than well-watered conditions. Elevated [CO_2] stimulated nodule density (number of nodules per unit of root length; Table 5.2) to a greater extent under drought (+153%) than in well-watered plants (+7%). Elevated [CO_2] increased nodule activity under well-watered conditions throughout the growth period peaking at pod filling stage. Drought decreased overall nodule activity with a steady decline in activity starting at flowering in e[CO_2] and lasting until maturity (Fig. 5.5C)

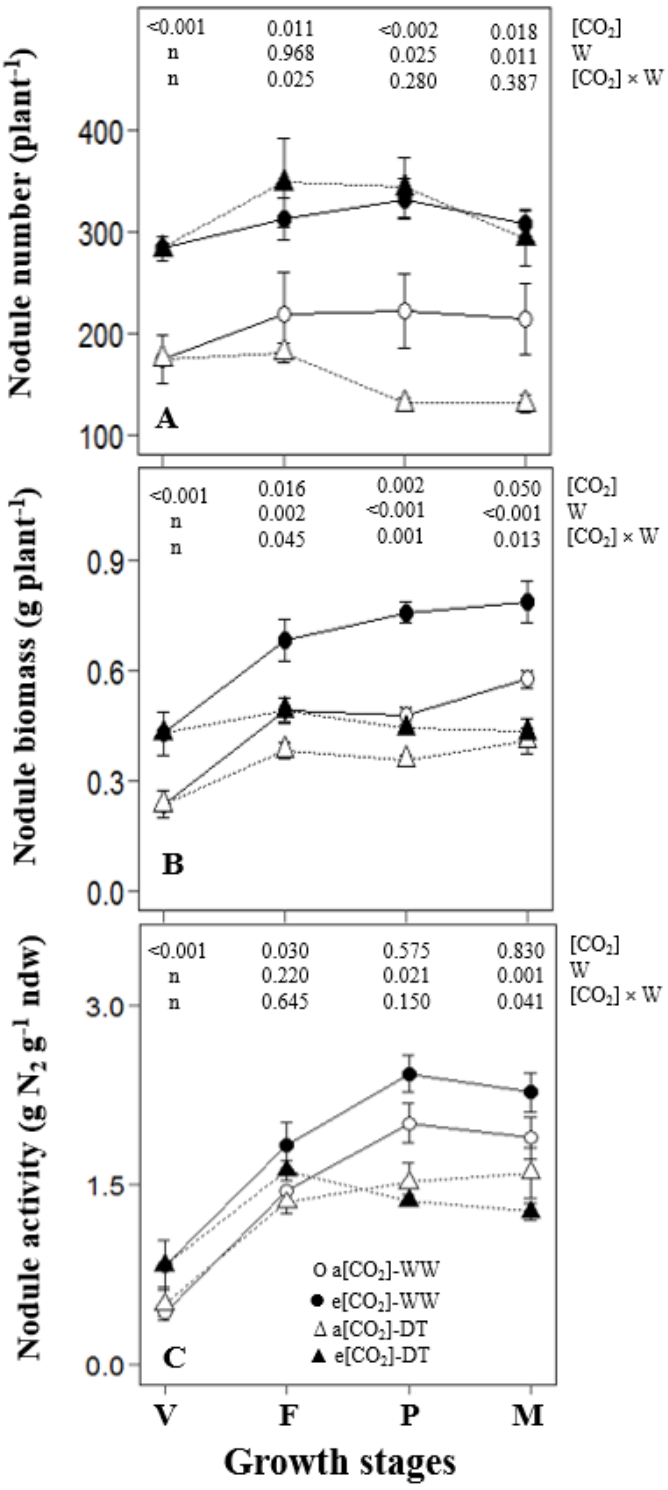


Fig. 5. 5 Nodule number (A), nodule biomass (B) and nodule activity (C) of faba bean grown under ambient [CO_2] (~400 µmol mol^{-1}; open symbols O, Δ) and elevated [CO_2] (~550 µmol mol^{-1}; closed symbols ●, ▲) and two water (W) regimes (well-watered, solid lines and drought, broken lines) and measured at vegetative (V), flowering (F), pod filling (P) and maturity (M) stages. Means and ±SE (n=4) in each bar. P-values of the effect of [CO_2], water regime (W) and their interactions are shown. ndw: nodule dry weight.

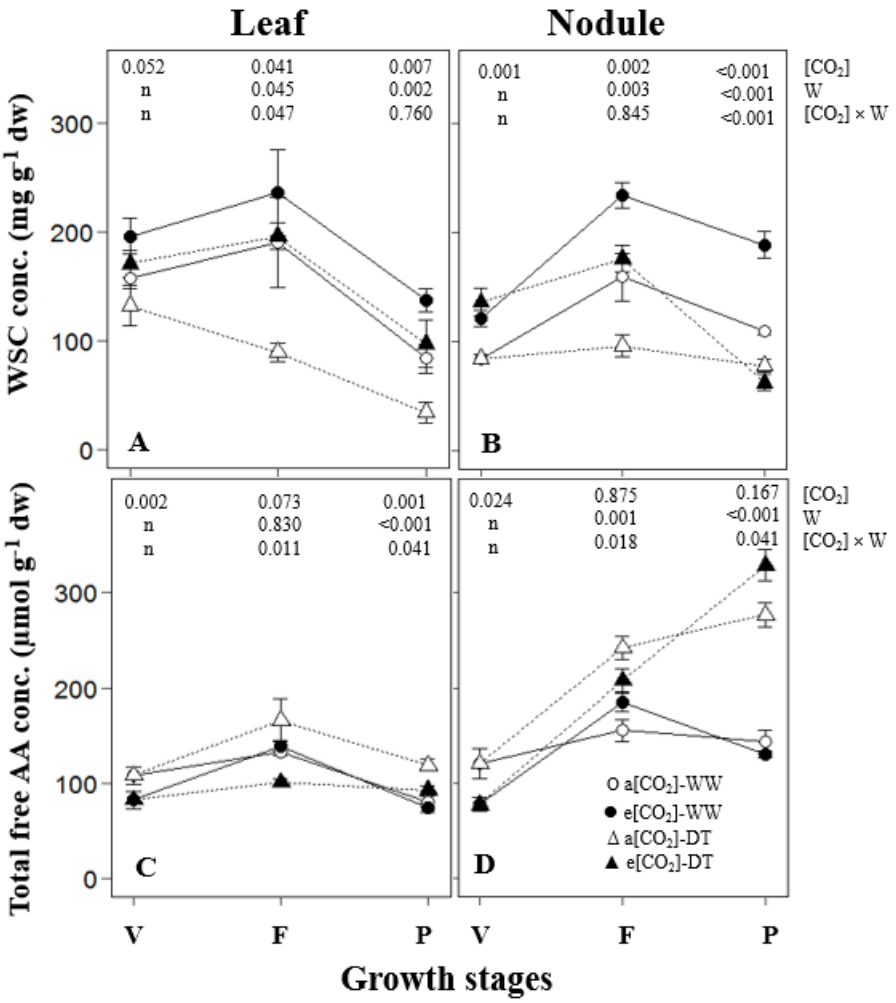


Fig. 5. 6 Water soluble carbohydrate (WSC) concentrations (A, B) and total free amino acid (AA) concentrations (C, D) in leaves (A, C) and nodules (B, D) of faba bean grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}; white bars) and elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}; grey bars) and two water (W) regimes (WW: well-watered and DT: drought) and measured at vegetative (V), flowering (F) and pod filling (P) stages. Means and ±SE (n=4) in each bar. P-values of the effect of [CO_2], water regime (W) and their interactions are shown.

5.3.6. WSC and total free amino acid concentration

Water soluble carbohydrate (WSC) concentration in leaves increased under e[CO_2] throughout the growth period and this difference was maintained under drought (Fig. 5.6A). In nodules, e[CO_2] consistently maintained higher WSC concentration under well-watered conditions, but less or not at all under drought at pod filling (Fig. 5.6B).

In leaves, total free amino acid concentration (AA) was lower under e[CO_2] at the vegetative stage and slightly increased or unchanged at later growth stages. Drought increased AA concentration in leaves under a[CO_2] but decreased AA concentrations under e[CO_2]. This trend was similar at both flowering and pod filling stages (Fig. 5.6C). In nodules, e[CO_2] grown plants had lower AA concentrations at the vegetative stage. Drought increased AA concentration in nodules to a greater extent under e[CO_2] than a[CO_2] at pod filling (Fig. 5.6D).

5.3.7. N concentration and content

In drought treated plants, e[CO_2] significantly decreased seed [N] by 8% compared to a[CO_2], but there was no such change under well-watered conditions (Table 5.1; significant interaction [CO_2] × W). Leaf [N] decreased (-6%) under drought at both flowering and maturity stages but was not affected by [CO_2]. Stem, root and flower [N] were unaffected by either e[CO_2] or drought (Table 5.2).

Plant total N content was greater under e[CO_2] at both flowering (+34%) and maturity (+17%) due to larger total biomass (Table 5.1 & 5.2). At maturity, drought stress significantly decreased (-31%) total N content and the extent of this decrease was greater under e[CO_2] (-37%) than under a[CO_2] (-27%).

5.3.8. Meeting seed N demand

The contribution of N_2 fixation, uptake and remobilization to seed N demand is presented in Fig. 5.7 (right panel). A greater proportion of seed N was derived from fixation and this effect was enhanced under e[CO_2]. Drought increased the relative importance of soil N uptake and remobilization which contributed ~20% and ~60% of seed N, respectively (Table 5.1). A major proportion of remobilized N incorporated into seed was derived from fixation.

5.4 Discussion

5.4.1 Effect on phenological developments

Elevated [CO_2], temperature and drought are important environmental factors affecting the rate of plant growth and development. Elevated [CO_2] itself may hasten, delay or leave unchanged the reproductive growth and development (reviewed in (Gray and Brady, 2016; Jagadish et al., 2016). During early reproductive stages, no obvious shifts in phenology was observed as per visual observations, which is in line with earlier AGFACE studies that reported no visible effect of e[CO_2] on plant phenology (Fitzgerald et al., 2016; Parvin et al., 2019; Uddin et al., 2018a). Low seasonal rainfall in a Mediterranean environment results in terminal drought during the reproductive phases (Leport et al., 1999). The effects of low water availability on the phenological developments are variables and depend on the duration and intensity of the water stress (De Souza et al., 1997). The drought stress we applied in this study was moderate, as judged by the small decreases in stomatal conductance (which remained at or above 0.10 µmol m^{-2} s^{-1}) measured at the later growth stage (Flexas et al., 2006). Such moderate drought stress did not change the onset of flowering, which is in line with earlier studies (Lopez et al., 1996; Mouhouche et al., 1998).

"Stay-green" a phenotypic trait extending the duration of green leaf area may favour yield under stress conditions (Sadras and Richards, 2014). Accelerated and or delayed leaf senescence in response to e[CO_2] and/or drought has been reported in grain legumes (Gray and Brady, 2016). In the present study, e[CO_2] delayed leaf senescence and e[CO_2]-grown plants had greater green leaf area and decreased senescence/dried leaf biomass when exposed to prolonged drought during the sensitive pod filling phase. If e[CO_2] phenotypically triggers a stay green trait,

this may extend the period of carbon capture and nutrient acquisition to the growing seeds, contributing to increased seed yield of plants grown in e[CO_2].

5.4.2 Soil water in elevated [CO_2]-plants decreased slowly, delaying the on-set of drought during early reproductive phases but this effect disappeared at later growth stages

Elevated [CO_2] can affect the dynamics of soil water depletion, because plants grown under e[CO_2] may use less soil water, at least under certain conditions (Gray et al., 2016; Parvin et al., 2018; Uddin et al., 2018b). The slower rate of soil drying due to lower g_s in e[CO_2] grown plants helped to conserve soil water during the key reproductive period (i.e. flowering), thereby slowing down the rate of stress development without subjecting the plants to the same degree of drought as a[CO_2] grown plants (De Luis et al., 1999; Robredo et al., 2007). In contrast, increased g_s immediately after the onset of drought in a[CO_2] grown plants resulted in rapid depletion of soil water in our study.

Previous observations on field pea showed that during short-term drought, more soil water was retained under e[CO_2]-grown plants until flowering, but the effect during later growth stages was not investigated (Parvin et al., 2019). Consistent with these results on field pea, more soil water was retained under e[CO_2]-grown faba bean plants until flowering. In contrast, at the onset of pod filling, soil water in the drought treatments was depleted faster between rewatering under e[CO_2], associated with the increasing transpiring leaf area/biomass. In support with our first hypothesis, our results confirm that soil water savings are highly dependent on the timing and extent of growth stimulation, and increases in transpiring leaf area under e[CO_2] offset the effect of reduced g_s in the longer term. Whilst the watering regime and possible limitations imposed by the column size on soil volume (Poorter et al., 2012) in the current study might have affected the results, they are corroborated by observations in field grown lentil (Parvin et al., 2018) and soybean (Gray et al., 2016). Although soil water savings were not sustained for the entire growing period, the slower rate of soil water depletion that enabled maintenance of water availability during the critical reproductive period under e[CO_2] may be an important factor contributing to yield and N_2 fixation benefits in variable and dynamic terminal drought environments.

5.4.3. Elevated [CO_2] increased seed yield even under drought, while the relative yield stimulation was greater under well-watered conditions

In this study, e[CO_2] increased seed yield of faba bean in line with other studies including wheat, lentil and field pea from this same research site (Bourgault et al., 2017a; Bourgault et al., 2016; Fitzgerald et al., 2016). Increased pod numbers, seed numbers per pod and HI contributed to the seed yield response to e[CO_2]. This result supports a previous observation on faba bean, whereby yield response under e[CO_2] largely resulted from increased pod number, seed number and HI (Wu and Wang, 2000).

Because of the positive effect of e[CO_2] on WUE and the decrease in g_s, it is often suggested that growth and yield benefits from e[CO_2] are greatest under drought (Kimball, 2016). However, in this study, e[CO_2] stimulation of yield was greater (53%) under well-watered conditions than under drought stress (23%). Such greater yield stimulation under e[CO_2] can be explained by greater leaf area, increased CO_2 assimilation rate (A_{net}) and vigorous root growth, which probably enhanced assimilate supply to the developing seeds (Sadras and Richards, 2014). A multi-year evaluation of soybean growing under e[CO_2] showed that there was no yield stimulation under severe drought (Gray et al., 2016). In the present study, slower depletion of soil water and increased root biomass/root length under e[CO_2] improved water extraction throughout the pod filling stage when water was limited, contributing to increased seed yield even under drought. Similar to the seed yield response, e[CO_2] increased shoot biomass by 28% under well-watered and by 7% under drought conditions. Up to flowering, there was no effect of drought on shoot biomass combined with a strong response to e[CO_2]. Such conditions might increase the risk of "haying-off", whereby an early stimulation of vegetative growth leads to depletion of soil moisture at later growth stages, limiting grain yield (van Herwaarden et al., 1998). Furthermore, e[CO_2]-induced lower magnitude of yield response under drought might be related to the limitation imposed by the growing media as discussed below.

Studies reported that small pots might reduce or eliminate plant responses to enriched CO_2 atmospheres due to root restriction (Arp, 1991; McConnaughay et al., 1993). Even though the columns used in this experiment are considered better suited than regular pots (Bourgault et al., 2017b), from about flowering onwards the dry matter to rooting volume ratio exceeded the rule-of-thumb values given in (Poorter et al., 2012). In the absence of such restrictions field grown plants can extract water from deeper soil layers, especially under e[CO_2] conditions (Uddin et al., 2018a). Pot size limitations would limit water availability especially, and this would affect larger plants more, therefore compounding the probability of "haying off" under a prolonged drought. Under real field conditions this risk may be somewhat smaller as a range of different soil physicochemical constraints can effectively limit rooting depth in these environments, but experiments with plants in the field have confirmed the changing interplay between water availability and plant size earlier and later in the growing season (Gray et al., 2016; Parvin et al., 2018; Uddin et al., 2018b).

5.4.4. Increased nodulation but decreased nodule activity under drought limited e[CO_2]-induced stimulation of N_2 fixation with signs of N-feedback regulation

Our study demonstrated that e[CO_2] increased total N_2 fixation of faba bean and that stimulation was associated with increased nodule numbers, nodule mass and nodule activity. These results are consistent with previous reports (Parvin et al., 2018; Rogers et al., 2006; Serraj et al., 1998). In contrast, the decrease of N_2 fixation under drought was linked with the decrease of above-mentioned nodule attributes. The ability to maintain N_2 fixation for longer under terminal drought is a potential candidate for improving crop productivity in water-limited environments.

In testing our third hypothesis, we assessed whether e[CO_2] can maintain N_2 fixation through greater nodulation and nodule activity under drought. In our experiment, e[CO_2] interacted with drought to increase nodulation and nodule density, and this resulted in greater N_2 fixation (+15%) than under a[CO_2]. At later growth stages, once

soils were equally dried down under e[CO_2] and a[CO_2], these additional nodules were in contact with soil of the same or even slightly lower soil water than under a[CO_2] and as a result, had low N_2 fixation activity (King and Purcell, 2005). Pot size needs to be taken into consideration, too and may have limited the ability to fully express the potential activity of such increased nodulation, and the effect would have been greatest for larger plants under a prolonged drought. Despite such potential experimental limitations, results of this present study are corroborated by a recent study that compared effects of e[CO_2] on yield and N_2 fixation in lentil between a dry and a wet season (Parvin et al., 2018). That study was conducted on plants grown in the field without pots.

In the later growth stage, the decrease of nodule activity under the interactive effect of e[CO_2] and drought can be explained by a decline of C availability to nodules, due to assimilation limitations imposed by drought (Serraj et al., 1998). In nodules, greater A_{net} under e[CO_2] provided greater WSC supply until flowering, but then WSC declined rapidly at pod filling under drought. This may indicate that under prolonged drought, plants exposed to e[CO_2] had limited C flux to nodules, either causing or responding to a decline in N_2 fixation (Galvez et al., 2005).

In parallel with the shortage of C, the reduction in nodule activity under water limitation may also be associated with an accumulation of N-compounds, which could provide feedback inhibition of N_2 fixation. It is probable that drought-induced growth limitation decreases shoot N demand and consequently decreases the transport of N-compounds to the shoot (King and Purcell, 2005). In this study, similar to what was observed in other drought sensitive legumes (Erice et al., 2014), amino acids accumulated in nodules under e[CO_2], which may be a signal for downregulation of N_2 fixation. The present study showed that a combination of two mechanisms above is likely responsible for the decline of nodule functionality under prolonged drought. However, e[CO_2]-induced increased nodulation partially maintained total N_2 fixation above a[CO_2] level.

5.4.5. Elevated [CO_2]-stimulation of N_2 fixation during reproductive phases-maintained seed [N] but this depended on water availability

Legume N_2 fixation may maintain seed protein concentration under e[CO_2] (Myers et al., 2014; Rogers et al., 2006) and in the fourth hypothesis, we tested whether this also holds under terminal drought conditions. With ample water, seed [N] was maintained under [CO_2] through increased N_2 fixation, consistent with lentil in a high rainfall season (Parvin et al., 2018). However under drought, seed [N] decreased as in previous studies in semi-arid environments, which showed small but significant decreases in seed [N] under e[CO_2] in legumes (Bourgault et al., 2017a; Bourgault et al., 2016). N_2 fixation contributed the greatest proportion of seed N, which is in line with reports on soybean and clover under e[CO_2], because e[CO_2] did not affect soil N uptake (Guo et al., 2013; Li et al., 2017). When drought constraints both fixation and uptake, seed N demand is supplied by translocation of N previously accumulated in vegetative biomass (Farooq et al., 2017). Drought increased remobilization of N taken up or fixed until flowering, and this remobilization provided ~80% (vs. 50% under well-watered) of seed N (major portion derived from fixation), consistent with wheat N remobilization at post-anthesis under water-limited environments (Palta et al., 1994). Whilst it's important to maintain seed N under drought, remobilization was unaffected by e[CO_2].

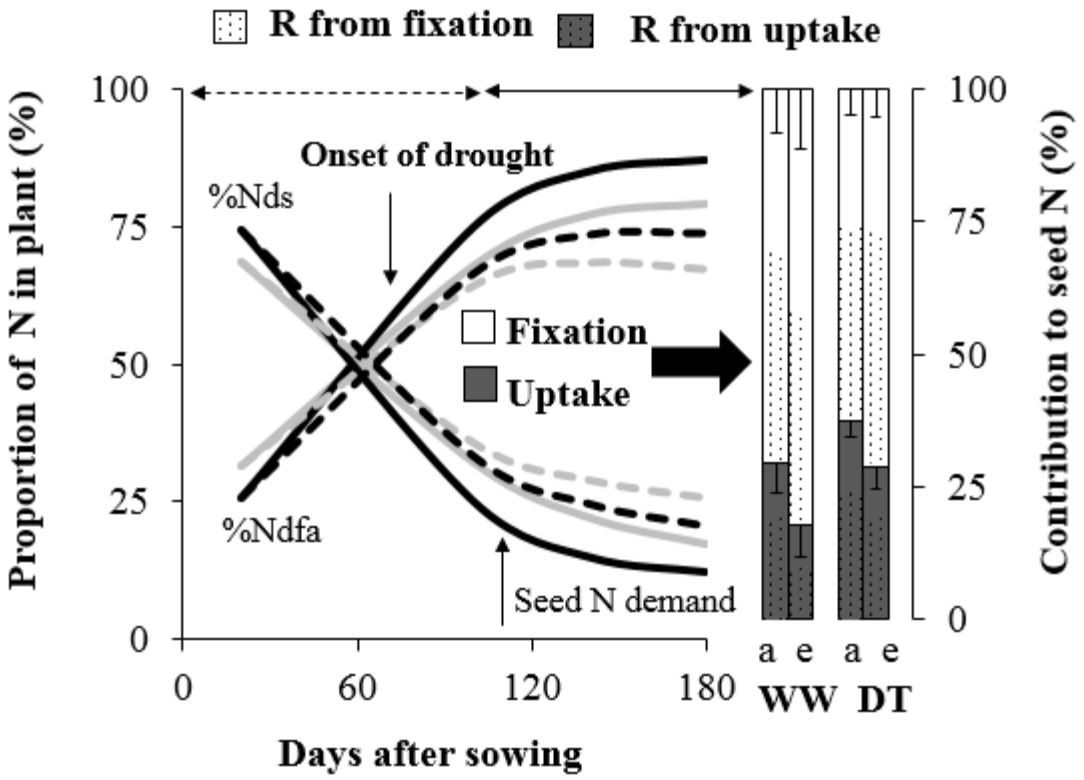


Fig. 5. 7 A conceptual model describing changes in the percentage of atmospheric N (%Ndfa, upward direction) and soil derived N (%Nds, downward direction) in faba bean from vegetative (double-sided broken arrow) to reproductive phases (double sided solid arrow) when grown under ambient [CO_2] (~400 ppm, grey lines) and elevated [CO_2] (~550 ppm, black lines) with two watering (W) regimes (well-watered, solid lines and drought, broken lines). White bars refer to the proportion of seed N derived from N_2 fixation (top portion) and grey bars indicate the proportion from soil N uptake (bottom portion). The dotted portion of the white bars refers to the proportion of seed N contributed by remobilized N previously fixed from N_2 into vegetative parts, whereas the dotted portion of the grey bars refers to remobilized N previously taken up from the soil. During reproductive phases, soil N uptake is decreasing but N_2 fixation is increasing. The contribution of three N sources to seed N is presented in the right panel. The proportional contribution of remobilized N (~35% from fixation) and soil N increased under drought ($P<0.05$) but were unaffected by e[CO_2] (Table 5.1). In well-watered plants, N_2 fixation during reproductive phases contributed ~ 50% to seed N demand under elevated [CO_2] and seed N concentration was maintained (Table 5.1). Drought decreased N_2 fixation during reproductive phases and seed N concentration decreased. N_2 fixation and uptake are shown from flowering to maturity assuming that newly acquired N is directly allocated to seed (in the right panel). a: ambient [CO_2], e: elevated [CO_2], WW: well-watered, DT: drought.

Figure 5.7 shows a conceptual model to illustrate how N acquisition patterns of faba bean change from vegetative to reproductive phases. Soil N uptake generally diminished with advancing growth stage due to limited soil resources and this stimulated N_2 fixation. In well-watered plants, N_2 fixation during the reproductive phase became a major contributor to seed N (~50%) under e[CO_2]. This enhanced N_2 fixation under e[CO_2] might also lead to the decrease in remobilization of N from vegetative organs, maintaining N for photosynthetic capacity and thus, maintain assimilate feeding to the fixation process. Under drought, N_2 fixation was not stimulated in line with seed N demand due to suppression of nodule activity, which depressed seed [N] under e[CO_2]. As neither uptake nor remobilization was modified by e[CO_2], continuing N_2 fixation seems crucial to adjust seed [N] under rising [CO_2].

5.5 Conclusion

In the drought treatment of this experiment, soil water was depleted faster under e[CO_2] in the period between pod filling and maturity. However, greater soil water content under e[CO_2]-grown plants during the earlier period of the treatment, from onset of drought imposition to flowering, contributed to higher yield (+23%) and N_2 fixation (+15%) compared to a[CO_2]-grown plants exposed to drought. Drought-induced increases in nodulation under e[CO_2] sustained N_2 fixation, although nodule activity showed higher sensitivity to prolonged drought and decreased sharply from flowering onwards. As a consequence, drought resulted in decreased N_2 fixation activity during later growth stages, whereby decreased WSC concentrations indicated decreased C-supply to nodules and increased free AA concentrations in nodules indicated a slowing export rate of the fixation products. N_2 fixation during the later reproductive phases appeared to be the key to maintaining seed [N] under e[CO_2], as both N uptake from soil and N remobilization remained unchanged. Interruption of N_2-fixation under drought was associated with decreased seed [N] under e[CO_2]. This study implies that decreased g_s under e[CO_2] can translate to soil water savings during the onset of drought which helps to minimize the drought effects on yield and N_2 fixation. However, the e[CO_2] depression of seed [N] under drought suggests that sufficient soil water during the later reproductive phases is necessary to stimulate N_2 fixation in line with seed N demand under future rising [CO_2] environments.

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Chapter 5: Supplementary materials

Table 5.1S Phenological changes from flowering to maturity of faba bean grown under two CO_2 concentrations either ambient (a[CO_2], ~400 µmol mol^{-1}) or elevated (e[CO_2], ~550 µmol mol^{-1}) and two water (W) regimes (well-watered and drought).

	Well-watered		**Drought**	
	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]
Time of 50% flowering (DAS)	100	100	100	100
Time to 50% podding (DAS)	135	135	128	135
Time to physiological maturity (DAS)	180	180	165	180

Chapter 6: 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

(vi) Abstract

Rising atmospheric CO_2 concentration ($[CO_2]$) drives the increase in global mean temperature and is predicted to increase the frequency and severity of heat waves in many regions. 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 such a combination. To assess the impact of a heat wave on N_2 fixation and the potential of $e[CO_2]$ to mitigate negative effects under dry-land field conditions, two lentil genotypes (PBA Ace-higher harvest index and HS3010-lower harvest index) were grown under ambient $[CO_2]$ (~400 ppm) and elevated $[CO_2]$ (~500 ppm) in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility. Using custom built chambers, heat waves (3-day periods of high temperatures ~ 40°C) were experimentally imposed at flat pod stage (R4). Nodulation, and concentrations of water-soluble carbohydrates (WSC), total free amino acids (AA) and N were monitored in all organs after imposing the heat wave. N_2 fixation, N allocation and N remobilization were measured at 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 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 an increased grain [N] under heat wave. Higher harvest index genotype PBA Ace had greater N_2 fixation and grain N yield than low harvest index one HS3010 and this trend was maintained even under a heat wave. These results have implications for understanding post heat-wave recovery of N_2 fixation via nodulation by incorporating greater WSC pools available under higher $[CO_2]$ environments.

Keywords: Climate change, heat wave, N_2 fixation, nodule attributes, carbohydrate, amino acid, grain protein

6.1 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 rise to at least 550 µmol mol^{-1} by 2050 (IPCC, 2014). Increasing $[CO_2]$ is the major driver of global warming and related climate change phenomena, but also affects all plants and ecosystems directly. 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; Kimball et al., 2002). This "CO_2 fertilization effect" may be constrained by limitations to plant nitrogen (N) nutrition (Feng et al., 2015; Tausz-Posch et al., 2014). If N availability in soils is inadequate or uptake by roots is insufficient to match faster growth under $e[CO_2]$, tissue N concentration ([N]) decreases (Taub and Wang, 2008). Decrease of [N] relative to higher C gain, may not only constrain growth responses (Zanetti et al., 1997), but also limit the allocation of N into reproductive tissues and thus deteriorate grain protein concentration, a significant concern for nutritional and product quality of cereals grown under $e[CO_2]$ (Myers et al., 2014; Taub et al., 2008).

In legumes, the decrease of tissue N or grain protein concentrations under e[CO_2] is absent or less (Taub et al., 2008). Elevated [CO_2] may stimulate symbiotic N_2 fixation of legumes in line with biomass growth so that N limitations are overcome (Rogers et al., 2006; Zanetti et al., 1996). The symbiotic association with N_2-fixing bacteria in root nodules provides legumes with an extra sink for any additional C derived from e[CO_2]. Additional carbohydrates derived from greater photosynthesis rates can be exchanged with the bacterial symbiont to enhance N_2 fixation (Aranjuelo et al., 2014; Rogers et al., 2006; Udvardi and Day, 1997). An increase in N_2 fixation under e[CO_2] could be derived from an increase in the number, size and/or activity of root nodules (Lam et al., 2012; Parvin et al., 2018). Increased photosynthetic rate under e[CO_2] could supply greater carbohydrate to nodules and increase nodule activity. Studies reported that legume N_2 fixation or nodule functionality is highly sensitive to environmental stress such as heat, drought, salinity and others (Aranjuelo et al., 2014) and any mechanisms that impair carbohydrate supply to nodules would directly inhibit N_2 fixation (Galvez et al., 2005).

The "CO_2 fertilization effect" on crops, including legumes, may be undone by other climate change factors (Ruiz-vera et al. 2013), such as increased frequency and severity of heat waves (abrupt rise in temperature for short periods) (IPCC, 2014). Particularly in Mediterranean environments, such episodes of heat wave events are likely to become more frequent and more erratic: a 1-in-10-years event is expected to become a 1-in-3-years event by the middle of the 21st Century (IPCC, 2014). Heat wave effects (effects of abrupt rises in temperature) are more detrimental than high temperatures for an extended period during grain filling (Mahrookashani et al., 2017). Legumes are frequently grown in Mediterranean environments and experienced high temperature, often combined with terminal drought at the reproductive stages, especially when plants are in full bloom (Leport et al., 1999). Among cool season legume crops, lentil is highly sensitive to heat waves during the reproductive development, when heat waves can result in floret abortion, reduced carbohydrate supply to grain and reduced grain weight (~ 87%) 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, 2000a). Decreased nodule number and mass, and accelerated nodule senescence were direct inhibitory effects caused by high temperature, whereas indirect inhibition related to reduced survival of rhizobia, reduced C supply to nodules, and subsequent decrease of nodule activity (Prasad et al., 2001). In one study, the decrease of 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). Heat shock effect revealed that two thermal shocks of 40°C/8 h/day at flowering time drastically decreased nitrogenase activity and nodule relative efficiency of *Phaseolus vulgaris* L. compared to plants grown at 28°C. Nitrogenase activity recovered after seven days when new nodules formed (Hungria and Franco, 1993).

Combined effects of e[CO_2] and heat stress have been reported on growth and yield of legumes (Bourgault et al., 2018; Delahunty et al., 2018; Ruiz-Vera et al., 2013). Elevated [CO_2] in combination with 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 grain yield reduction from heat waves, but at least moderates the negative effects. This might be associated with greater photosynthetic CO_2 assimilation under

e[CO_2] during heat stress, resulting in higher grain yield (Fitzgerald et al., 2016; Macabuhay et al., 2018). Although greater soluble carbohydrate pools under e[CO_2] may help nodule formation and activity and thereby, N_2 fixation in exchange of soluble carbohydrate with root nodules bacteria, little is known about the effects of a heat wave and e[CO_2] on N_2 fixation.

In terminal drought environments, carbon 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 or root uptake or remobilization from vegetative tissues (Tausz et al., 2017). Heat waves during grain filling inhibit C assimilation and further reduce soil N uptake, making remobilization of stored reserves from vegetative tissue, the major contribution of grain C and N (Buchner et al., 2015b; Plaut et al., 2004). Heat stress can accelerate remobilization of water-soluble carbohydrates and N to grains in wheat (Dias and Lidon, 2009; Macabuhay et al. 2018). 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; Salon et al., 2001). As leaf senescence mostly occurs during grain filling period, the breakdown of proteins by proteinases provide a large pool of cellular N for remobilization (Liu et al., 2008). Chloroplast proteins, which account: for >70% of all leaf proteins, are though~ to be a major source of N for mobilization. This protein is further degraded into amino acid and may be exported to the growing seeds via the phloem (Martin 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., 2017) 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.

Selection of genotypes that are better adapted to e[CO_2] can be incorporated into breeding programs (Ainsworth et al., 2008; Ziska et al., 2012; Tausz et al., 2013). Although lentil genotypes did not differ in their responses to e[CO_2] for grain yield (Bourgault et al., 2017), intraspecific variability in N_2 fixation response under e[CO_2] × drought has been observed (Parvin et al., 2018). Also, intraspecific differences in response to heat stress are reported (Delahunty et al., 2018). As climate change continues to intensify, heat wave episodes will increasingly threaten crop productivity and may limit 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.

It is therefore great interest to investigate whether and, if yes, by which mechanisms and to what extent, e[CO_2] can mitigate heat wave effects on N_2 fixation and grain [N] of lentil and whether this response varies between genotypes. To close this knowledge gap, the experiment was conducted in the Australian Grain Free Air CO_2 Enrichment (AGFACE) facility. Purpose built heat chambers were used to impose a heat wave (~ 40°C compared to control 30°C) for the three consecutive days at the sensitive flat pod stage. Two lentil genotypes with contrasting harvest index (ratio of grain yield to total biomass) were chosen in this experiment (Bourgault et al., 2017). 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 higher harvest index one. The experimental design and set-up allowed to answer the following research questions:

1. Does e[CO_2] mitigate the negative impacts of a heat wave on N_2 fixation through protecting the mechanisms of N_2 fixation process?
2. How does e[CO_2] change N allocation patterns under heat waves?
3. Does e[CO_2] maintain grain [N] under a heat wave by changing N remobilization patterns?
4. How do genotypes contrasting in harvest index differ in N_2 fixation and remobilization in response to e[CO_2] and a heat wave?

6.2 Materials and methods

6.2.1 Site description and experimental design

The experiment was conducted at the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility near Horsham (7.5 ha), Victoria, Australia (36°45′70″S, 142°06′52″E, 127m above sea level). The soil type in the field is Murtoa Clay consisting of ~35% clay at the surface and 60% in 1.4 m depth. The soil is classified as Vertosol according to Australian Soil Classification (Isbell, 2002). A detailed description of the AGFACE site and the CO_2 exposure facility is given in Fitzgerald et al. (2016). Meteorological data during the experimental period (May to November 2015) were collected by an onsite weather station described in Mollah et al. (2009). The region has a Mediterranean climate with dry and hot summers and cool winters. Long-term (30-year) average seasonal rainfall of the area is 274 mm, average maximum and minimum temperatures are 19.5◦C and 9.2◦C, respectively (Australian Bureau of Meteorology). In 2015, rainfall was extremely low about 128 mm and therefore, three irrigation was applied in three instalments (total 96 mm) from September to October to prevent crop failure.

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 all combinations of two lentil genotypes and two heat treatments as sub-plot treatments. Therefore, the experiment consists of 2[CO_2] × 2 genotypes × 2 heat treatments (control and heat) × 4 replicates = 32 plots

6.2.2 Plant materials

A commercially-available lentil (*Lens culinaris* L.) genotype cv. PBA ACE and breeding line 05H010L-07HS3010 (shortened 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). 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. 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.

6.2.3 Heat treatments

Heat treatments were imposed at the flat pod stage (137 days after sowing, DAS) and lasted for three days (137-140 DAS, 6-8 October 2015). Custom built heat chambers (0.80 m × 1.2 m × 1.0 m height) were placed on lentil sub-plots. Details of chamber design and performance are given in Bourgault et al. (2018), Delahunty et al. (2018) and Nuttall et al. (2012). Heat treatments were imposed from 9.00 to 16.00 local time each day with a targeted air temperature of 40^0C. Air temperature and relative humidity were logged using MiniDataloggers (Microlite-PRO-RH, Fourtec Fourier Technologies Ltd.). 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. Canopy temperature of heat-treated plots was significantly (P<0.05) greater (40^0C) compared to control (31^0C) plots (Fig. 6.1S).

6.2.4 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 each sampling date, 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. At harvest (R8), grains were separated from pods and weighed. The remainder of the pods (after removing the grains) is reported as chaff. Biomass was expressed on a plot surface area basis (m^{-2}).

6.2.5 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 m^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 µmols^{-1}. Reference [CO_2] concentrations were adjusted to 400 and 550 µmolmol^{-1} in ambient and elevated [CO_2] treatments, respectively. During measurement, the leaf temperature was 30-35°C, relative humidity was within 55-65% and vapour pressure deficit was between 1.2 and 1.8 kPa but these variables were not significantly different between treatments. 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).

6.2.6 Nodule attributes

Three plants from both control and heat plots were randomly selected and nodules were collected twice (i.e. at last day of heat treatments and two weeks after treatment at early seed stage R5). Plants were excavated carefully, and roots were cleaned with tap water. Nodules were separated from the roots 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.

6.2.7 Plant N and grain protein

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 δ^{15}N by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon). N content was calculated from N concentration (%N *10) multiplied by biomass and expressed as kg N ha^{-1}.

For estimating N_2 fixation, δ^{15}N values (‰) were calculated from ^{15}N atom % using atmospheric air (0.3663 at % ^{15}N) as the international standard for nitrogen, which by definition is given a delta (δ) ^{15}N of 0‰ (Unkovich et al., 1997). The percentage of N derived from the atmosphere (%Ndfa) was determined by ^{15}N natural abundance method (Unkovich et al., 1994):

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

where 'reference plant' refers to a non-N_2 fixing plant (in this case: wheat grown adjacent to the lentil plots) selected to match the legumes closely in terms of uptake of soil N sources. Factor B refers to the δ^{15}N value of the effectively nodulated legume grown in media totally lacking N. To estimate B, nodulated lentil was grown in white-washed sand under glasshouse conditions and harvested at maturity. B-values (δ^{15}N, ‰) for each organ were measured (Unkovich and Pate, 2000). The whole plant value includes a correction for seed N (Nebiyu et al., 2014).

N_2 fixation, soil N uptake and N remobilization was calculated (Parvin et al., 2018) as follows:

Total N_2 fixation = Total N content × %Ndfa

Soil N uptake = Total N content - N_2 fixation

N remobilization = N content in each organ at flat pod stage – N content in these organs at maturity

Where, total N content was obtained by multiplying the N concentration with biomass and expressed as kg ha^{-1}.

6.2.8 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). Absorbance was measured at 600nm wavelength and D-fructose was used as the standard. The concentration was calculated as fructose equivalent and expressed as mg g^{-1} of dry matter.

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) and described details in Parvin et al. (2019). Absorbance (at 570 nm wavelength) was quantified by a plate reader spectrophotometer (Tecan Sunrise, Tecan, Austria) using amino acid as standard. Finally, the concentration of AA was calculated as amino acid standard equivalent and expressed as µ mol g^{-1} dry weight.

6.2.9 Statistical analysis

Effects of CO_2, heat, cultivar, and their interactions were evaluated using the ANOVA function (aov) in R statistical software (R Core Team, 2018). ANOVA was done using a split-split-plot design where CO_2 was the main plot, heat the sub-plot and cultivars in the sub-sub-plot (variance ~ CO_2 × Cultivar × Treatment + Error (Ring/CO_2/Cultivar)). Levene's test was conducted to check the homogeneity of variances, and data transformation was done using natural logarithms where necessary. In all analyses, replicate plots were considered the experimental unit (n=4). (Payne, 2009)

6.3 Results

6.3.1 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. 6.1A) and decreased (by about 12%) in heat stressed lentil compared to non-stressed control. Lentil grown under e[CO_2] had significantly ($P<0.01$) lower (7%) g_s compared to a[CO_2] (Fig. 6.1B). Heat 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. 6.1C).

The maximum quantum efficiency of PSII (F_v/F_m, Fig. 6.1D) 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.

6.3.2 Nodule

The decrease in dry weight of active nodules following the heat wave was greater under a[CO_2] than e[CO_2] compared to control (Table 6.1). At the R5 stage, the dry weight of active nodules was increased by e[CO_2], and this increase was greater in lentil exposed to the heat wave. At all stages, PBA Ace had greater active nodules dry weight than HS3010. Inactive nodule dry weight was increased by a heat wave during and after the heat events.

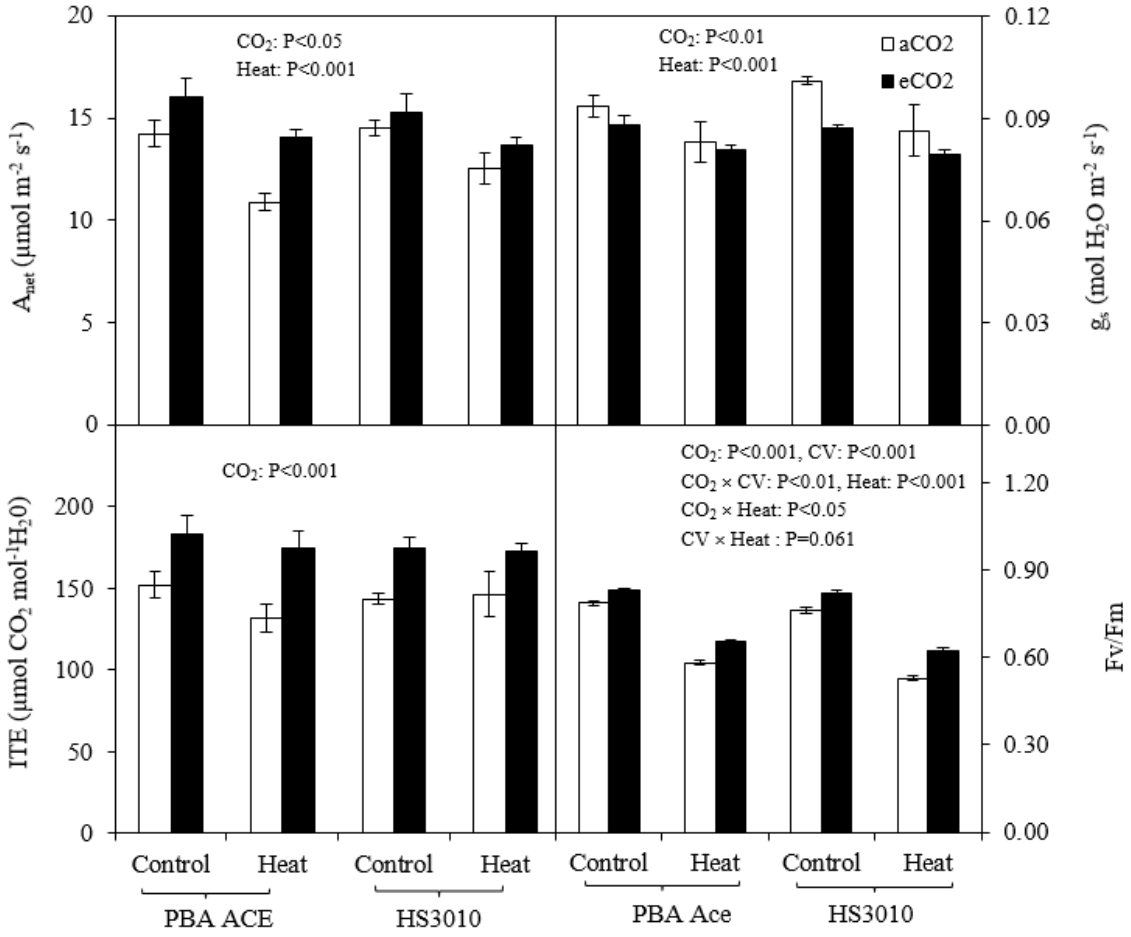


Fig. 6. 1 A. Net CO_2 assimilation rate (A_{net}), B. Stomatal conductance (g_s), C. Intrinsic transpiration efficiency (ITE) and D. Chlorophyll fluorescence (Fv/Fm) of two lentil genotypes (PBA Ace and HS3010) during heat treatment grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Ambient and elevated grown plants were measured at 400 ppm and 550 ppm of CO_2 concentrations, respectively. 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 significant (P<0.05) effects are shown.

Table 6. 1 Dry weight of nodules (mg plant^{-1}) recorded from control and heat plots at last day of heat treatment and two weeks after heat treatment at early seed stage (R5) from two lentil genotypes grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility, Horsham, Australia. Data represent mean values and standard errors of 4 replicate with results for two plants averaged for each replicate.

Parameters			During heat treatment		Two weeks after heat treatment at R5	
[CO_2]	CV	Treatment	Active nodules (mg plant^{-1})	Inactive nodules (mg plant^{-1})	Active nodules (mg plant^{-1})	Inactive nodules (mg plant^{-1})
a[CO_2]	PBA Ace	Control	62.4±0.4	18.8±2.7	67.7±6.2	24.9±1.6
		Heat	49.1±1.6	21.2±1.6	49.6±0.5	32.5±2.9
	HS3010	Control	55.1 ± 3.9	19.6±0.2	60.5±3.1	24.1±1.6
		Heat	44.9 ±3.4	25.2±1.8	47.6±1.4	31.4±1.4
e[CO_2]	PBA Ace	Control	66.3±1.0	17.1±1.3	72.13±4.6	21.1±1.7
		Heat	55.4±3.8	23.5±1.5	68.32±5.2	28.1±2.2
	HS3010	Control	60.6±4.7	16.1±1.5	66.06±5.8	24.9±2.7
		Heat	46.6±2.0	22.1±1.6	50.05±3.4	27.3±1.1
Statistics						
		[CO_2]	0.023	0.078	0.021	ns
		CV	0.039	ns	0.001	ns
		Heat	<0.001	0.012	<0.001	<0.001
		[CO_2] × CV	ns	ns	ns	ns
		[CO_2] × Heat	ns	ns	0.038	ns
		CV × Heat	ns	ns	ns	ns
		[CO_2] × CV × Heat	0.046	ns	ns	ns

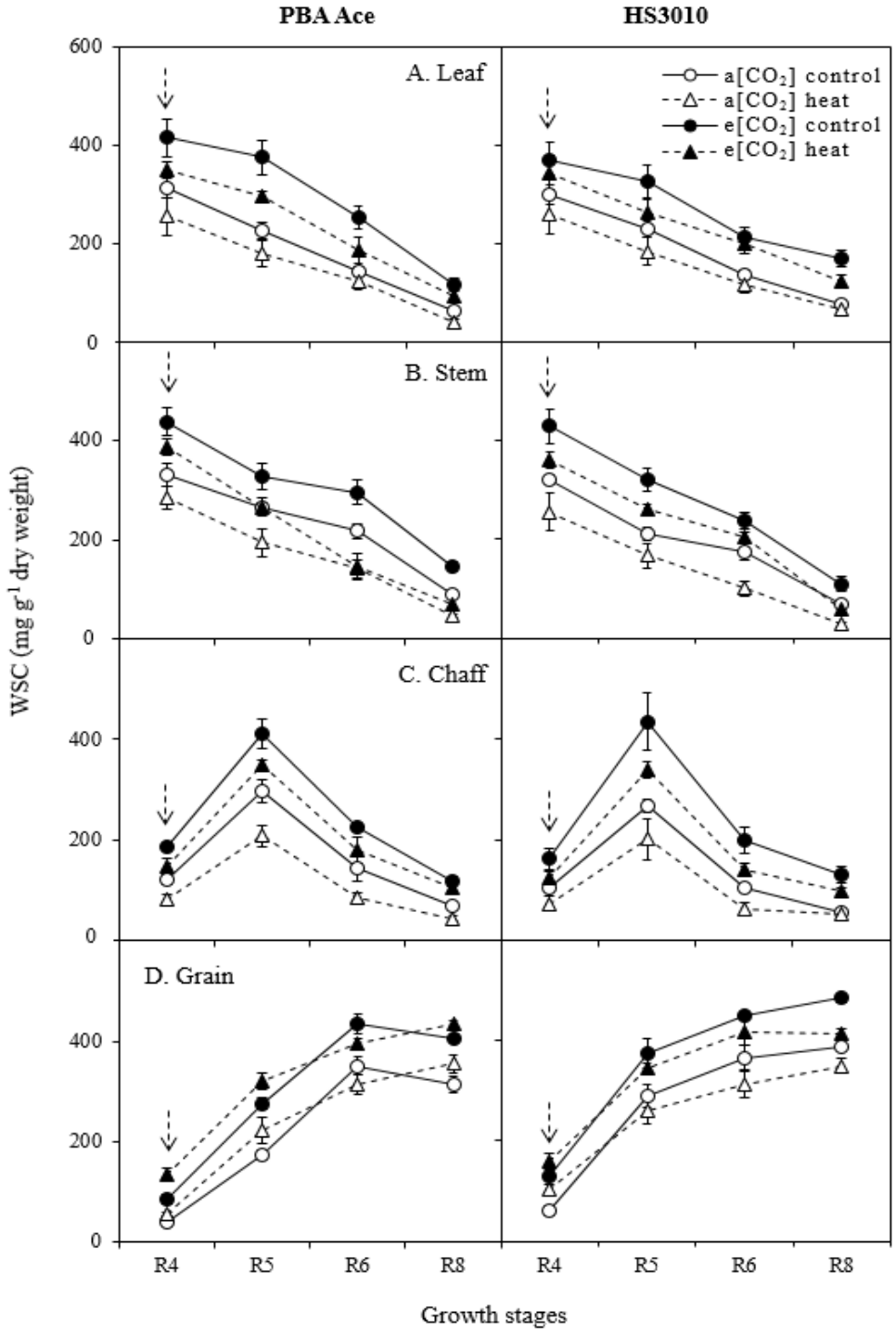


Fig. 6. 2 Water-soluble carbohydrates concentration (WSC) in leaf (A), stem (B), chaff (C) and grain (D) of two lentil genotypes measured at flat pod (R4), early seed (R5), full seed (R6) and physiological maturity (R8) stages grown under ambient (~ 400 ppm) or elevated (~ 550 ppm) CO_2 concentrations in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Arrow represents R4 stage when heat shocked was imposed. Data points represent mean values and standard errors of 4 replicates. Open symbols (○, Δ) for a[CO_2], filled symbols (●, ▲) for e[CO_2]; circles (○, ●) for control, triangles (Δ, ▲) for heat. Statistics are reported in Table 6.2.

Table 6. 2 ANOVA results for water-soluble carbohydrates (WSC, mg g^{-1} dry weight), total free amino acid concentration (µmol g^{-1} dry weight), N concentration (mg g^{-1} dry weight) in different organs at flat pod (R4), early seed (R5), full seed (R6) and physiological maturity (R8) from control and heat lentil plots grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility, Horsham, Australia. P values indicate the significance of the effect of CO_2, genotypes (CV), heat as well as their interactions, ns represent statistically non-significant ($p \geq 0.1$). Data are presented in Fig. 6.2 to 6.4.

Parameters		CO_2	CV	Heat	CO_2 × CV	CO_2 × Heat	CV × Heat	CO_2 × CV × Heat
WSC concentration (mg g^{-1} dw)								
R4	Leaf	0.009	0.0218	0.006	ns	ns	ns	ns
	Stem	0.007	ns	0.001	ns	ns	ns	ns
	Chaff	0.002	0.079	<0.001	ns	ns	ns	ns
	Grain	0.005	0.045	0.0008	ns	ns	ns	ns
R5	Leaf	0.002	ns	0.007	ns	ns	ns	ns
	Stem	0.015	0.092	0.001	ns	ns	ns	ns
	Chaff	0.001	ns	<0.001	ns	ns	ns	ns
	Grain	<0.001	0.005	ns	ns	ns	0.063	ns
R6	Leaf	0.001	ns	0.068	ns	ns	ns	ns
	Stem	0.009	0.084	0.001	0.078	ns	0.012	0.016
	Chaff	0.001	0.028	0.001	ns	ns	ns	ns
	Grain	0.001	ns	0.012	ns	ns	ns	ns
R8	Leaf	0.005	0.008	0.029	ns	ns	ns	ns
	Stem	0.009	0.007	<0.001	ns	ns	ns	ns
	Chaff	0.006	ns	0.029	ns	ns	ns	ns
	Grain	0.001	0.025	ns	ns	ns	0.021	ns
AA concentration (µmol g^{-1} dw)								
R4	Leaf	0.079	0.083	0.001		ns		ns
	Chaff	0.007	ns	0.001	ns	ns	ns	ns
	Grain	0.071	ns	0.001	ns	ns	ns	ns
R5	Leaf	0.003	ns	0.001	ns	0.003	ns	ns
	Chaff	<0.001	0.028	0.001	0.001	0.012	0.074	0.039
	Grain	<0.001	ns	<0.001	ns	ns	ns	ns
R6	Leaf	0.003	0.012	0.001	ns	ns	ns	ns
	Chaff	0.040	0.050	0.001	ns	ns	ns	ns
	Grain	ns	ns	0.001	ns	<0.001	ns	ns
R8	Grain	ns	ns	0.001	0.020	<0.001	ns	ns
N concentration (mg g^{-1} dw)								
R4	Leaves	ns	ns	0.004	ns	ns	ns	ns
	Stems	ns	ns	0.048	ns	ns	ns	ns
	Chaff s	ns	ns	0.007	ns	ns	ns	ns
	Grains	0.002	ns	<0.001	ns	ns	ns	ns
R5	Leaves	0.087	ns	<0.001	ns	ns	ns	ns
	Stems	ns	ns	0.002	ns	0.047	ns	ns
	Chaff s	0.026	ns	0.002	0.055	ns	ns	ns
	Grains	0.016	0.008	0.001	ns	ns	ns	ns
R8	Leaves	ns	ns	0.001	ns	ns	ns	ns
	Stems	ns	ns	0.001	ns	ns	ns	ns
	Chaff s	0.029	ns	ns	ns	ns	ns	ns
	Grains	0.076	ns	<0.001	ns	<0.001	ns	ns

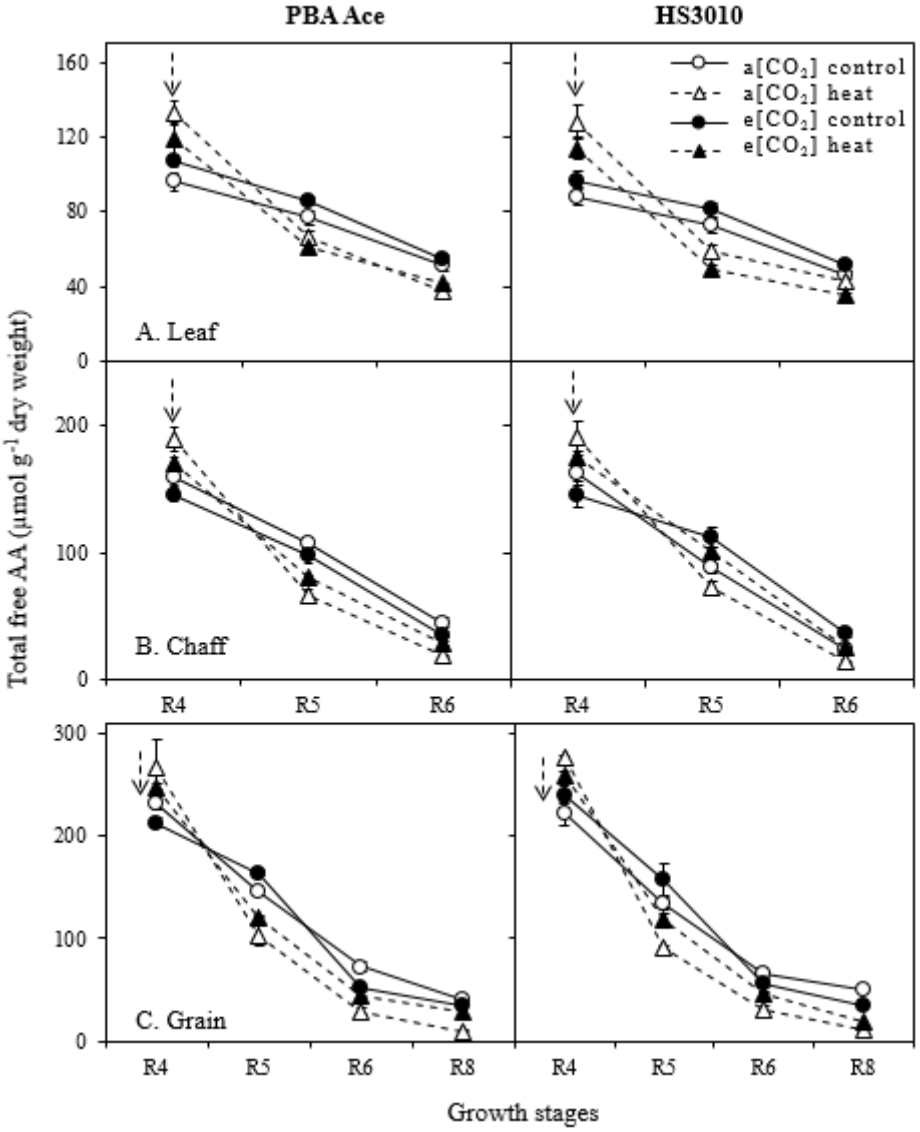


Fig. 6. 3 Total free amino acids (AA) concentration in leaf (A), stem (B) and grain (C) of two lentil genotypes measured at flat pod (R4), early seed (R5), full seed (R6) and physiological maturity (R8) stages grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Arrow represents R4 stage when heat shocked was imposed. Data points represent mean values and standard errors of 4 replicates. Open symbols (○, Δ) for a[CO_2], filled symbols (●, ▲) for e[CO_2]; circles (○, ●) for control, triangles (Δ, ▲) for heat. Statistics are reported in Table 6.2.

6.3.3 Water Soluble Carbohydrates Concentration

Under e[CO_2], water-soluble carbohydrates concentration ([WSC]) increased in leaf, stem, chaff, and grain throughout the developmental stages (Fig. 6.2 A-D and Table 6.2). On average across all developmental stages, cultivars 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]. There was a genotypic difference, but the significance across the growth stages dependant on plant component.

Heat stress increased grain [WSC] only temporarily at flat pod stage (R4) (Fig. 6.2 D and Table 6.2). Plants that undergone heat stress had lower [WSC] in later stages. On average across all stages, the decreases in [WSC] were 17%, 24%, 23% and 16% in leaf, stem, chaff, and grain, respectively.

6.3.4 Total free amino acids concentration

Total free amino acid concentration ([AA]) of leaves slightly increased (5-10%) under e[CO_2] compared to a[CO_2] at R4 stage but decreased at later stages (Fig. 6.3 A and Table 6.2). In chaff and grain, [AA] was 2% and 5% lower under e[CO_2] respectively at the R4 stage but this decrease disappeared at later growth stages (Fig. 6.3 B-C). Significant interactions between CO_2 and cultivar were found for chaff at R5 and grains at the R8 stage. HS3010 had 20% higher [AA] in chaff at R5 than PBA Ace under a[CO_2]. Elevated [CO_2] decreased grains [AA] of HS3010 by 20% at R8.

The interaction between CO_2 and heat was significant for [AA] in leaf and chaff at R5 and grains at R6 and R8 stages. Heat significantly decreased (19%) [AA] in leaf and chaff under both a[CO_2] and e[CO_2], but the magnitude of this increase was greater under a[CO_2] than e[CO_2]. 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 (10-40%) in heat treated plants towards the final harvest. Heat 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 the R5 stage.

6.3.5 Nitrogen concentration and N yield

On average, e[CO_2] decreased [N] in leaf and stem (Fig. 6.4 A-B and Table 6.2). 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 stage and decreased by 12% at R8 (Fig. 6.4 C). Grain [N] was significantly lower (18%) at R4 and greater (9%) at R5 stage under e[CO_2], but this effect disappeared at R8 (Fig. 6.4 D). HS3010 had greater (9.4%) chaff [N] compared to PBA Ace under e[CO_2] at R5 stage.

Leaf, stem, and chaff [N] were significantly decreased by heat in all stages, on average by 20-30%, compared to controls. Heat stress decreased (4-10%) grain [N] under e[CO_2] at R4 and R5 stages 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 6.3).

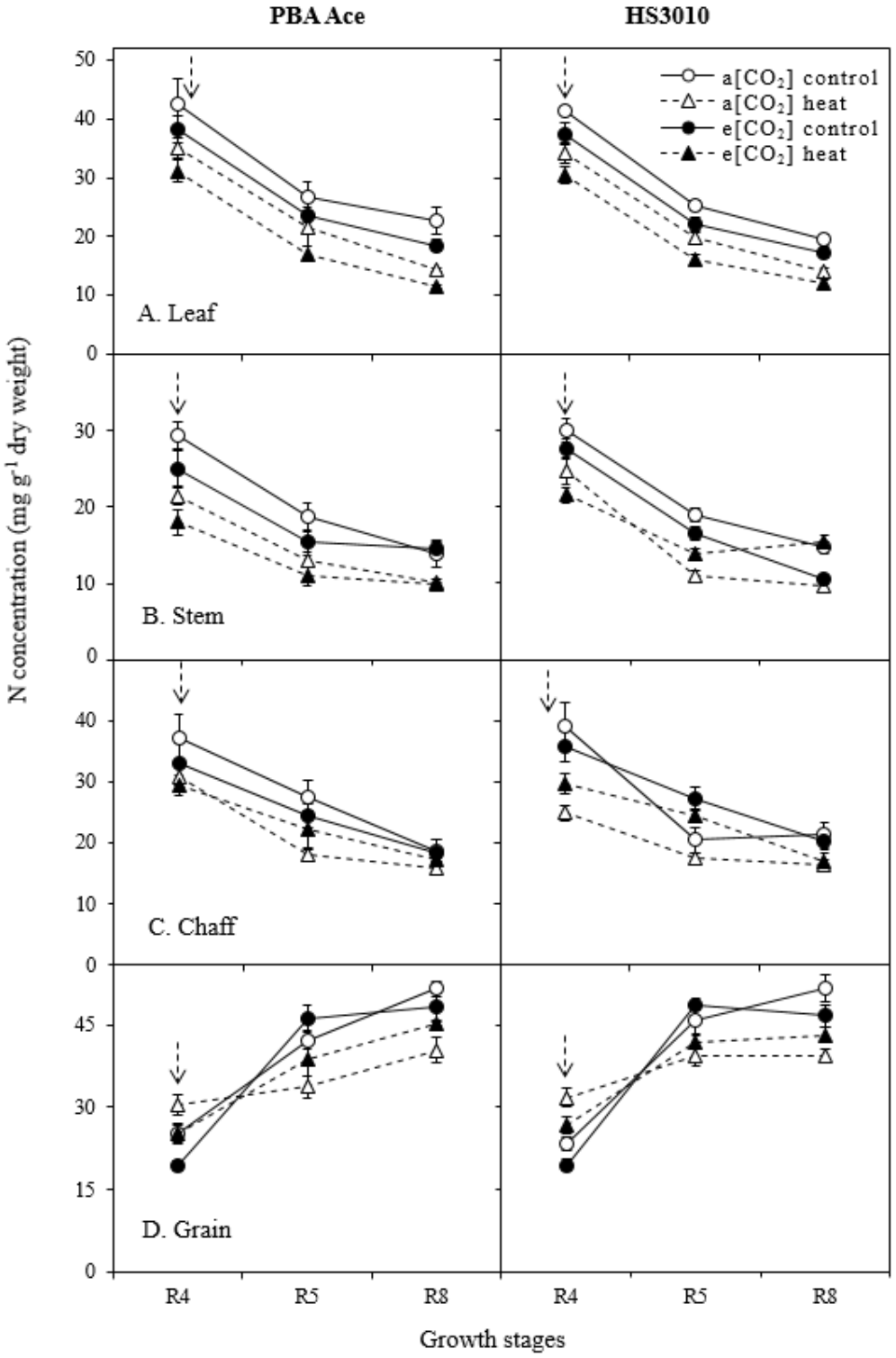


Fig. 6. 4 N concentration in leaf (A), stem (B), chaff (C) and grain (D) of two lentil genotypes measured at flat pod (R4), early seed (R5), full seed (R6) and physiological maturity (R8) stages grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Arrow represents R4 stage when heat shocked was imposed. Data points represent mean values and standard errors of n=4 replicates. Open symbols (○, Δ) for a[CO_2], filled symbols (●, ▲) for e[CO_2]; circles (○, ●) for control, triangles (Δ, ▲) for heat. Statistics are reported in Table 6.2.

Table 6. 3 Effect of e[CO_2] on total N content (kg ha^{-1}), N_2 fixation (Ndfa, kg ha^{-1}) and soil N uptake (Nds, kg ha^{-1}) at physiological maturity (R8) of two lentil genotypes collected from control and heat plots grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility, Horsham, Australia. Data represent mean values, and standard errors of n=4 replicates. P values indicate the significance of the effect of CO_2, genotypes (CV), heat as well as their interactions, ns represent statistically non-significant ($p \geq 0.1$).

N allocation (kg ha^{-1})		PBA Ace				HS3010			
		a [CO_2]		e[CO_2]		a [CO_2]		e[CO_2]	
		Control	Heat	Control	Heat	Control	Heat	Control	Heat
Leaf N	Ndfa	14.6±2.4	14.6±1.5	29.2±2.5	27.2±1.2	12.9±2.4	18.4±0.4	25.1±3.3	23.1±2.2
	Nds	7.3±2.3	12.0±1.1	7.1±2.1	9.7±2.8	6.8±0.6	13.2±1.9	12.1±2.1	11.2±3.4
	Total	21.9±1.2	26.7±1.8	36.4±2.3	37.1±2.4	19.7±1.7	31.6±1.7	37.2±1.4	35.1±3.4
Stem N	Ndfa	14.1±1.0	13.1±2.4	19.4±2.6	20.5±1.5	10.9±1.1	15.8±1.3	21.1±1.8	15.3±4.2
	Nds	5.6±0.8	10.4±2.5	6.0±1.2	11.9±1.9	5.7±1.3	8.2±1.9	7.9±1.9	6.3±1.1
	Total	19.8±1.6	23.5±2.8	25.4±1.5	32.4±3.3	16.7±1.1	24.1±3.2	29.1±2.7	31.6±4.9
Chaff N	Ndfa	4.9±0.2	4.1±1.2	3.4±0.3	5.1±1.2	2.8±1.3	3.3±1.1	3.3±0.4	2.8±0.3
	Nds	1.6±0.2	1.4±0.2	0.8±0.5	1.8±0.6	1.1±0.5	1.1±0.5	0.9±0.2	0.8±0.3
	Total	6.5±0.4	5.5±1.4	4.3±0.6	6.9±1.8	3.8±1.9	4.4±1.3	4.3±0.3	3.7±0.5
Grain N yield	Ndfa	67.1±10.9	25.1±1.5	83.2±15.1	49.6±5.8	34.1±8.2	10.3±0.7	34.5±1	22.3±2.9
	Nds	14.1±3.0	19.4±2.9	8.8±3.3	16.5±4.9	10.4±1.9	12.1±0.8	8.1±2.2	13.7±3.8
	Total	81.1±9.1	44.6±4.3	92.1±13.1	66.1±10.2	44.5±7.4	22.5±0.9	42.5±14.2	36.1±6.2
Total NDFA (%)		66.4±2.0	46.6±3.9	76.1±3.8	60.9±2.5	59.4±1.7	44.8±2.7	64.2±1.4	59.5±2.2
Total fixed N		86.5±8.2	44.6±3.3	121.6±14.7	90.7±4.4	50.4±5.9	36.9±1.5	72.9±7.8	43.7±3.4
Total soil N uptake		43.1±2.0	53.8±4.6	36.6±4.6	51.7±3.8	34.4±4.1	45.8±3.2	40.2±3.1	43.7±4.2
Total N content		129.5±9.2	100.4±1.8	158.2±12.3	142.5±4.8	84.9±9.5	82.7±2.1	113.1±10.3	106.5±6.4

ANOVA		**[CO_2]**	**CV**	**Heat**	**[CO_2] × CV**	**[CO_2]× Heat**	**CV × Heat**	**[CO_2]× CV × Heat**
Leaf N	Ndfa	0.007	ns	ns	ns	ns	ns	ns
	Nds	ns	ns	0.0133	ns	ns	ns	ns
	Total	0.001	ns	0.056	0.007	ns	ns	ns
Stem N	Ndfa	0.003	ns	0.008	ns	ns	0.009	ns
	Nds	ns	0.065	0.074	ns	ns	ns	ns
	Total	0.004	ns	0.005	ns	ns	ns	ns
Chaff N	Ndfa	ns	0.073	ns	ns	ns	ns	ns
	Nds	ns	ns	ns	ns	ns	ns	ns
	Total	ns	0.073	ns	ns	ns	ns	ns
Grain N yield	Ndfa	ns	ns	<0.001	ns	ns	ns	ns
	Nds	ns	0.051	0.039	ns	ns	ns	ns
	Total	ns	0.001	0.001	ns	ns	0.046	ns
Total NDFA (%)		0.001	0.006	0.001	ns	ns	0.063	ns
Total fixed N		0.004	0.002	0.001	ns	ns	0.008	ns
Total soil N uptake		ns	0.003	0.001	0.045	ns	ns	ns
Total N content		0.020	0.001	0.002	ns	ns	0.029	ns

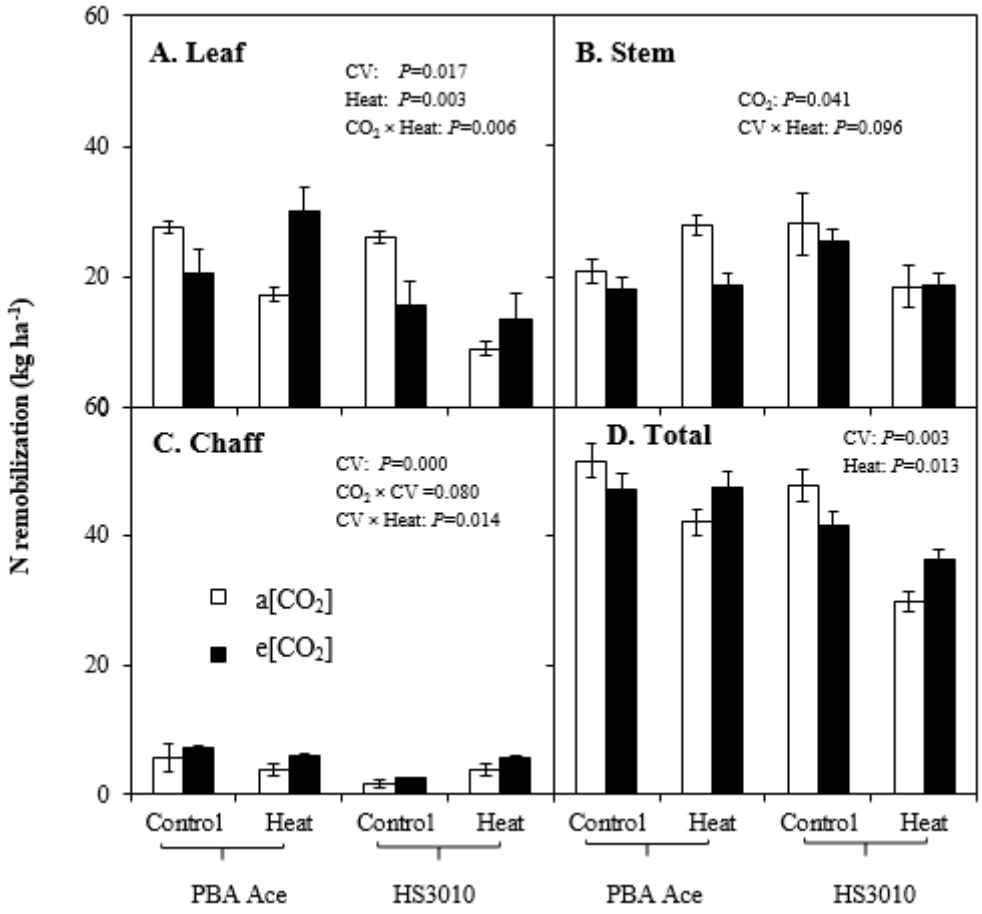


Fig. 6. 5 N remobilization (kg ha^{-1}) in leaf (A), stem (B), chaff (C) and total (D) of two lentil genotypes measured at physiological maturity (R8) stages grown under ambient [CO_2] (a[CO_2], ~400 µmol mol^{-1}) or elevated [CO_2] (e[CO_2], ~550 µmol mol^{-1}) in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Arrow represents R4 stage when heat shocked was imposed. Data points represent mean values and standard errors of n=4 replicates. Open bars for ambient [CO_2], filled bars for elevated [CO_2].

6.3.6 N_2 fixation, allocation, and remobilization

N sources and allocation were evaluated at maturity (R8, Table 6.3). 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 taken up 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 up took greater amount of soil N than HS3010. Heat stress 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 increased the amount of N taken up from the soil. An interaction between cultivar and heat was significant for a total amount of N_2 derived from the atmosphere and total N content in the above-ground biomass. Reduction of the 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 was decreased by heat (Fig. 6.5), but there were differences between organs. Elevated [CO_2] depressed leaf N remobilization without heat stress but stimulated leaf N remobilization by 35% after heat stress (Fig. 6.5A). In chaff, more N was remobilized in HS3010 after heat stress than in PBA Ace (Fig. 6.5C).

6.4 Discussion

The experimental heat wave applied in this study caused reductions in A_{net} and g_s. Preventing excessive water loss through stomatal closure can limit evaporative cooling and also restrict CO_2 input into the leaf. Whilst stomatal closure is a strategy to save water before further damages happen, but it also restricts CO_2 input to the leaf resulting in reduced carbon assimilation (Wang et al., 2016). Both may affect the photosynthetic process (Foyer et al., 2012). Chlorophyll fluorescence measurements (F_v/F_m) provide useful information on photosystem II processes under stress conditions (Johnson and Maxwell, 2000). In this study, the otherwise significant decrease of g_s under e[CO_2] was not evident during the heat wave, suggesting transpirational cooling operated similarly to a[CO_2]. In addition, the F_v/F_m value remained above 0.6 in e[CO_2] grown plants even under the heat wave, indicating that potential damages to photosystem II were less compared to a[CO_2] (F_v/F_m values <0.5) (Bauweraerts et al., 2014; Buchner et al., 2015a).

Lentil crops grown under rainfed conditions in Mediterranean-type-environments commonly experience high temperature combined with drought episodes (Leport et al., 1999) and such conditions are predicted to become even more frequent. Heat stress not only constrains CO_2 assimilation but also significantly affects the carbohydrate translocation to grains and depress grain filling, causing a grain yield loss in lentil (Delahunty et al., 2018; Sita et al., 2017). Previous results reported from the same experiment (Bourgault et al., 2018) showed that grain yield of e[CO_2]-grown lentil exposed to heat wave was equivalent to grain yield of a[CO2]-grown lentil without the heat wave event. In the present study, heat stress significantly reduced the A_{net} and WSC accumulation in lentil, but lentil grown in e[CO_2] consistently maintained greater WSC than a[CO2]-grown plants even after a heat wave, which can explain the maintenance of grain yield at a[CO_2] level. These results are in close agreement with previous reports on wheat at the same site (Macabuhay et al., 2018; Tausz-Posch et al., 2013). These studies found that e[CO_2] partially moderated the adverse effect of heat stress on CO_2 assimilation rate and increased stem WSC translocation to grain. This might be associated with less physiological damage caused by heat waves under e[CO_2]-grown plants as reflected from F_v/F_m measurements in this study.

6.4.1 Effect of e[CO_2] and heat wave on N_2 fixation of lentil

In this present study, e[CO_2] increased N_2 fixation of lentil by 30% compared to a[CO_2] grown ones. The increased N_2 fixation was associated with greater active nodule biomass observed at the R4 and R5 stages. A similar increase in N_2 fixation under e[CO_2] grown soybean from the R5 to R8 stages was also reported as a result of greater nodule biomass (Li et al., 2017). This stimulation of N_2 fixation is associated with the enhanced photosynthesis under e[CO_2] (Rogers et al., 2004) which provides sufficient C sources for maintaining nodule function and N_2 fixation (Cabrerizo et al., 2001).

N_2 fixation was decreased by the heat wave (40%) compared to the non-stressed control. A study on groundnut suggested that decreased N_2 fixation under heat stress is linked to increased nodule mortality and senescence (Prasad et al., 2002). In this study, the greater biomass of dried, dead and senescence nodule in heat stressed plant could explain the decrease of N_2 fixation. As in non-stressed plants, there were more viable nodules which could continue to fix N_2. Even at the R5 stage, the increased amounts of inactive nodules under heat stressed plants further indicated greater nodule mortality following the heat wave event. Hungria and Franco (1993) reported that decreased of active nodule number led to decline of N_2 fixation following heat stress. Similar results were obtained by Sita et al. (2017) confirming that nodule formation was suppressed under heat stress as a result of lower carbohydrate availability. In this study, the reduction of WSC in leaves due to heat stress might have affected carbon supply for nodule formation and activity, a point that needs to be examined further in nodules.

The interactive effects of elevated [CO_2] and heat wave on N_2 fixation can be explained by the changes in the number or biomass of active nodules and the carbohydrate pools available to support nodule activity (Keerio and Wilson, 1998). In response to the first research question, results of this study demonstrated 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 maintaining 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, even under the heat wave lentil grown in e[CO_2] fixed similar amounts of N_2 as lentil grown in a[CO_2] under non-stressed/control conditions. Similar results were also observed for 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; Hartwig et al., 1994). 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 (Cabrerizo et al., 2001). In this study, AA concentration actually declined in leaves at e[CO_2], which according to the above-mentioned model would lead to a relief of any inhibition of N_2 fixation. An increase of free AA could be a protective response or could be an indication of slowing protein synthesis because AA intended to be used for protein synthesis simply accumulate in free pools (Kaplan et al., 2004). Alternatively, a faster rate of protein turnover can also contribute to increasing amino acids pools especially proline (Singh et al., 2016). Accumulation of proline as an osmo-regulant under heat stress has been reported previously (Harsh et al., 2016; Lv et al., 2011). The lack of AA accumulation under e[CO_2] during the heat wave in our study suggests that shoot N demand was maintained, which potentially avoided the N feedback mechanism. The combination of increased nodulation and maintained shoot N demand under e[CO_2] may partially offset the negative impact of a heat wave on N_2 fixation.

6.4.2 Effect of e[CO_2] and heat on N allocation patterns of lentil

In the second research question, this study investigated how e[CO_2] changes N allocation patterns under a heat wave. Elevated [CO_2] increased the proportion of fixed N incorporated into biomass and decreased soil N uptake as investigated in lentil previously (Parvin et al. 2018). This is also consistent with the findings on *Medicago trunculata* L. (Guo et al., 2013) and *Trifolium repens* L. (Zanetti et al., 1996) where N_2 fixing legumes decreased N uptake from soil under e[CO_2]. In contrast, the decrease of N_2 fixation under and after the heat wave was associated with greater soil N uptake. This could possibly be associated with an increase in soil N uptake during grain filling, when the N demand of the developing grain was high and not fully met by the lower fixation rates. Total N content also decreased in response to the heat wave, and this reduction can be explained by a decrease of total biomass (Bourgault et al., 2018). In grain, a decrease in N allocation from both Ndfa or Nds reduced grain N yield in response to a heat wave. Because N allocation to grain is important for grain N yield and grain N concentration, such impairments may affect grain quality (Zhang et al., 2019).

6.4.3 Effect of e[CO_2] and heat wave on grain N concentration ([N]) and N remobilization

In the present study, grain [N] in lentil decreased in response to a heat wave. 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 grain (Sehgal et al., 2017). In agreement with that study, N remobilization significantly declined in lentil in response to heat stress. As a decrease in grain [N] is a strong indication for a similar reduction in grain protein, inhibition of protein synthesis may have played a role, as observed by Sita et al. (2017). In this present study, a peak in grain amino acid concentration immediately after heat stress also indicated the inhibition of protein synthesis, similar to Andean lupin (*Lupinus mutabilis*) (Zu, 2009).

Elevated [CO_2] caused a reduction in grain [N] (Bourgault et al., 2017; Bourgault et al., 2016; Parvin et al., 2018), However, the combination of e[CO_2] and heat wave generally restored grain N concentration to levels above the ones obtained in a[CO_2] and heat wave conditions. Elevated [CO_2] may result in a decreased grain [N] and therefore decreased nutritional quality, but heat stress is likely to offset this adverse effect. Because e[CO_2] accelerated leaf N remobilization under heat stress to some extent. Studies by Pinter et al. (2000) and Long et al. (2006) who reported that increasing canopy temperature of soybean sped up the loss of leaf N under e[CO_2]. These results encompass the possibility that e[CO_2] enhanced faster leaf N translocation into grain when exposed to a heat wave, this could help to partially improve grain N/protein concentration (Macabuhay et al., 2018; Wang et al., 2019).

6.4.4 N_2 fixation and allocation differed between two genotypes in response to heatwave but was not affected by e[CO_2]

Knowledge of intra-specific variability in N_2 fixation in response to e[CO_2] and/or heat stress is a pre-requisite to select for superior genotypes for future climate scenarios. In this study, N_2 fixation varied between two genotypes, as PBA Ace fixed 27% more N from the atmosphere than HS3010. But the lack of genotype × [CO_2] interaction suggests that e[CO_2] stimulated N_2 fixation in both genotypes. A significant interaction between genotype and heat wave was observed for N_2 fixation, where reduction in N_2 fixation was greater for HS3010 (-45%) than for PBA Ace (-30%), similar to reported on *Phaseolus vulgaris* L. exposed to a heat wave (Hungria and Franco, 1993;

Hungria and Vargas, 2000b). Dry weight of active nodules decreased in response to the heat wave, and this was more pronounced in HS3010 genotype than PBA Ace genotype. PBA Ace produced more nodules, which is in accordance with earlier findings (Parvin et al., 2018) and may be attributed to the ability to maintain greater WSC concentrations in leaves under heat stress. Greater nodulation response and WSC production was also observed in heat-tolerant lentil lines (Sita et al., 2017) and was suggested as candidate trait for screening genotypes for heat tolerance.

There is a close positive relation between N_2 fixation and grain N yield in legumes (Anglade et al., 2015; Li et al., 2017). Results of this study demonstrated that heat wave reduced the grain N yield and this response was notably higher in HS3010. This could be explained by genotypic variability in biomass accumulation and N_2 fixation under stress conditions (Bourgault et al., 2018; Parvin et al., 2018). Greater grain N yield in "PBA Ace" was associated with greater biomass and greater N accumulation into biomass (i.e. total N content). In contrast, the decrease in grain N yield for HS3010 under heat wave resulted from a decrease in N_2 fixation in line with lower N accumulation into biomass and decreased N translocation to grain. Despite consistent variation of N_2 fixation and grain N yield, results of the present study suggest that there is no evidence supporting genotypic selection under the combination of [CO_2] × heat wave environments.

6.5 Conclusion

This study of N_2 fixation in lentil exposed to a simulated heat wave suggested that N_2 fixation decreased in response to the heat wave but e[CO_2] maintained N_2 fixation above that of a[CO_2] non-stress control conditions. 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, with 'PBA Ace' performing better than 'HS3010', associated with increased nodulation and WSC accumulation. The presence of genotypic variability in response to a heatwave can be used for large screening of heat-tolerance genotypes for breeding programs, but from this study there was no evidence regarding genotypic selection under a future climatic condition.

6.6 References

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Chapter 6: Supplementary materials

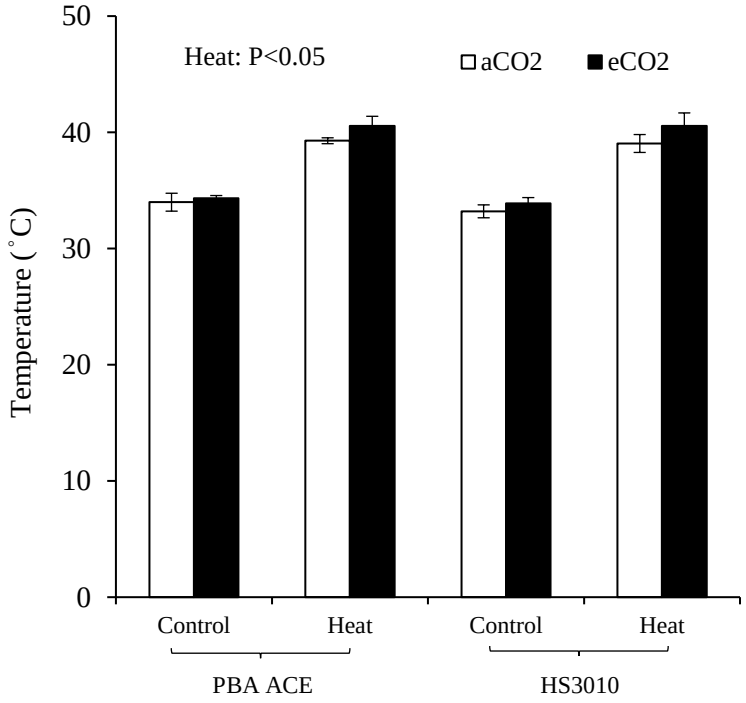


Fig. 6.1S Averaged canopy temperature (°C) during heat treatments recorded using IRT from two lentil genotypes grown under ambient (~ 400 ppm) or elevated (~ 550 ppm) CO_2 concentrations in the Australian Grains Free Air Enrichment (AGFACE) facility, Horsham, Australia. Data points represent mean values and standard errors of 12 replicates. P values indicate the significance of the effect of CO_2, heat, genotype (CV) as well as their interaction, only significant ($p>0.05$) relations were shown.

Chapter 7: Carbon sink strength of nodules and other organs of faba bean (*Vicia faba* L.) grown under elevated [CO_2] and different water supply

(vii) Abstract

Photosynthetic stimulation of legumes grown under elevated [CO_2] is determined by the ability of sink tissues, importantly nodules to utilize these assimilates. Drought is likely to influence not only photosynthesis but also sink activity of nodules. Little is known about carbon sink strength of nodules and other organs being affected by the interaction of e[CO_2] and drought. Therefore, this study wanted to clarify whether elevated [CO_2] maintains carbon sink strength under elevated [CO_2] and drought and if so, whether this sink strength avoids photosynthetic down-regulation. Faba bean plants were grown in pots filled with sand in a controlled glasshouse at ambient (400 ppm) or elevated (700 ppm) atmospheric [CO_2] and two watering supply either well-watered and drought. Plants were exposed to ^{13}C pulse-labeling at their growing CO_2 concentration using an air-tight custom-built growth chamber for 6 hrs and harvested 24-hr chase period. ^{13}C partitioning patterns were examined and photosynthetic responses parameters were estimated. Soluble sugars, total free amino acids, N_2 fixations were analysed at harvest. Results showed that under well-watered conditions, e[CO_2] grown plants showed no photosynthetic acclimation and maintained both maximum photosynthetic carboxylation capacity (V_{cmax}) and maximum capacity for electron transport (J_{max}). A contrasting trend was observed in drought stressed plants, where photosynthetic acclimation under e[CO_2] grown plants was accompanied by lower (-29%) V_{cmax} and lower (-17%) J_{max}. Such acclimatory responses were linked with sink strength of faba bean grown under e[CO_2] and drought. Under well-watered conditions, e[CO_2] increased sink strength (i.e. total ^{13}C incorporation) of all organs through greater sink size but the sink strength of nodules depended on greater sink activity. Drought-decreased nodule sink activity with greater extent under e[CO_2] and associated with less ^{13}C export to nodules. These responses were associated with increased ^{13}C built-up in leaves, decreased Rubisco activity (V_{cmax}) and maximum electron transport rate (J_{cmax}), suggesting plants were photosynthetically acclimated under drought. These results suggest that nodule sink strength plays a vital role in plant acclimation to climatic extremes.

Keywords: Climate change, drought, legume, photosynthetic acclimation, sink strength, root exudates

7.1 Introduction

Atmospheric CO_2 concentration ([CO_2]) is the major driver of global climate change and has increased by more than 40% since pre-industrial times, from 280 ppm to currently more than 400 ppm. According to RCP 8.5 scenario, atmospheric [CO_2] may surpass 700 ppm by the end of this century (IPCC, 2014). Increased [CO_2] stimulates photosynthesis of C_3 crops and increases growth and yield through the so-called "CO_2 fertilisation effect" (Leakey et al., 2009). However, initial stimulation of photosynthesis may diminish during long-time exposure under elevated [CO_2] (e[CO_2]), a phenomenon known as "photosynthetic acclimation" or "downward acclimation" (Long et al., 2004).

Photosynthetic downward acclimation is related to the insufficient size of the 'sink', that is, the organs and tissues consuming photosynthates (Ainsworth et al., 2004). Growth under e[CO_2] commonly creates a situation where

carbohydrate production in photosynthesis is greater than the utilization by the sink tissues (Arp, 1991). High [CO_2] greatly increases the supply of carbohydrates, but if the sink size is sufficient, this may not affect photosynthetic capacity (Lewis et al., 2002; Sanz-Sáez et al., 2010). Insufficient sink size may lead to a feedback on the photosynthetic apparatus, whereby maximum carboxylation rate of Rubisco (V_{cmax}) and maximum rate of electron transport (J_{max}) are downregulated under long-term exposure to e[CO_2] (Ainsworth and Rogers, 2007; Leakey et al., 2009; Long et al., 2004; Nowak et al., 2004). Continuous production or growth of organs, such as sustained leaf growth (Ruiz-Vera et al., 2017) or indeterminate production of generative organs (Ainsworth et al., 2004) has been shown to minimise downward acclimation. Faster export rate of C to the sink organ also modulate sink activity (Ainsworth and Bush, 2011). The large quantity of leaf carbohydrates accumulated during the photoperiod is likely to be exported to sink organs at night, in addition to moderate the carbon sink of leaf maintenance respiration. For example, fast-growing poplar (*Populus* spp.) trees, which exported more than 90% of photosynthate to the actively growing sinks during the night, were able to maintain maximal stimulation of photosynthesis at e[CO_2] (Davey et al., 2006; Leakey et al., 2009).

Alternatively, in conjunction or in addition to a source-sink mediated feedback, photosynthetic down-regulation is associated with a decrease in tissue N concentration, commonly observed in e[CO_2] grown plant (Rogers et al., 1998; Tausz et al., 2013). Among other mechanisms, lower tissue N concentrations may result from an inhibition in nitrate assimilation or from excessive stimulation of biomass growth that is not matched by N uptake; decreased tissue N concentration may also be the consequence rather than the cause of photosynthetic down-regulation, because the photosynthetic apparatus accounts for a large proportion of N in green tissues (Bloom et al., 2014; Taub and Wang, 2008). There is some experimental evidence that high/excessive N supply accelerates rather than mitigates acclimation of C_3-crops (Farage et al., 1998; Jifon and Wolfe, 2002; Sanz-Sáez et al., 2010) which would suggest that N availability is not the sole mechanism governing down-regulation under e[CO_2] (Sims et al., 1998). Keeping the balance between supply and demand for N during the growth and maintaining an equilibrium between production and utilization of carbohydrate to constrain C:N ratio at the tissue and whole plant levels is crucial (Jifon and Wolfe, 2002; Leakey et al., 2009).

Legumes may have a superior capability to maintain C and N balance by allocating additional fixed C/photosynthates to nodule symbionts (*Rhizobia*). This does not only constitute a potentially large and flexible carbohydrate sink, it also stimulates N_2 fixation in tune with carbohydrate supply (Aranjuelo et al., 2008; Rogers et al., 2006). In one experiment, e[CO_2] grown perennial ryegrass displayed photosynthetic acclimation expressed as decreased V_{cmax} and J_{max} values, whereas clover maintained higher V_{cmax} and J_{max}. The legume clover maximized photosynthetic C gain by adjusting the balance between C and N metabolism at e[CO_2] (Rogers et al., 2009). That the additional C sink of nodules can help to avoid photosynthetic acclimation (Ainsworth et al., 2004; Aranjuelo et al., 2008) was demonstrated in an experiment where a non-nodulating soybean cultivar showed downregulation, but a nodulated isogenic line did not (Ainsworth et al., 2004). Experimental evidence suggests that nodule number, mass, and activity increase under e[CO_2], which would increase their sink strength and enable greater C export from source leaves (Voisin et al., 2003b). Sink strength of different organs, whole plant C-movement and partitioning of recently fixed C can be determined directly with $^{13}CO_2$ isotope labeling (Bromand et al., 2001; Orians and Colin, 2006; Voisin et al., 2003a), but to date, there are only a few such direct studies

addressing how e[CO_2] modifies C transport to nodules and how the relative sink strength of different organs of nodulated legumes is affected by e[CO_2] (Rogers et al., 2009).
Direct effects of [CO_2] on plants aside, as part of e[CO_2]-driven climate change precipitation patterns, are predicted to become more variable and extreme in many regions, often causing longer and more severe drought events with the potential to modify C-uptake capacity (Knapp et al., 2015). Particularly legumes grown in Mediterranean dryland environments are already experiencing terminal drought and this will become more significant (Leport et al., 1999; Maalouf et al., 2015; Peoples et al., 2001). Drought affects photosynthesis, that is, the C source, but also organ expansion and growth, the C sink (Pallas et al., 2013).

Source tissues are net exporters of C or N required for plant growth, while sink tissues are net importers of these resources. In legumes, leaves are net sources of C but sinks for N, while root and nodules tissues are net sources of N but sinks for C (White et al., 2016). In addition, C and N are released through the roots as exudates also constitute a further sink, as this phenomenon contributes to resource drawdown. Root exudates modify the rhizosphere to provide a desirable environment for beneficial microorganisms under water stress (Calvo et al., 2017). A synthesis of manipulation experiments has shown that sink and source regulate each other by feedback mechanisms (White et al., 2016) but the number of studies investigating details of drought impact on sink strength of different organs is very limited. In legumes, drought limitation on photosynthesis may decrease carbohydrate supply to nodules, deplete carbohydrate concentrations in the nodules, and therefore constrain nodule activity and N_2 fixation. On the other hand, drought can also directly decrease nodule function even if the supply of photosynthates is still sufficient, a situation marked by an accumulation of sugars in nodules (McDowell, 2011; Serraj et al., 1999). Furthermore, drought limitation to shoot growth may limit N demand and export from nodules, where this can lead to an accumulation of N-compounds. Accumulating N might act as a feedback mechanism to downregulate N_2 fixation, which would then limit the C sink strength of nodules. Despite the critical role of the nodule C-sink to regulate photosynthesis, its magnitude and limitation imposed by drought are unclear and few if any studies have directly quantified the nodule C-sink strength in response to drought.

It has been demonstrated that e[CO_2] can maintain nodule activity under drought by increasing photosynthetic C-export and decreasing the drought-induced accumulation of N-compounds (Aranjuelo et al., 2014; Serraj et al., 1998). Elevated [CO_2] not only stimulates photosynthesis and therefore, assimilate supply to nodules, e[CO2] also decreases stomatal conductance (g_s), and this may reduce canopy water loss, which could avoid or at least delay soil drying, and consequently any reductions in N_2 fixation directly associated with dry soil (Luis et al., 1999; Serraj et al., 1998). Multiyear FACE study by Gray et al. (2016) from a high rainfall agro-ecosystem demonstrated that e[CO_2]-induced soil water savings under drought was variable and the effect was diminished in the longer-term. Regardless, a related study observed increased nodulation (+230 %) under the interactive effect of e[CO_2] and drought (Gray et al., 2013). Whether such nodulation response can maintain activity, consuming more photosynthate and therefore maintain greater C-sink under drought has not been directly quantified.

To quantify sink strength directly under the interactive effect of e[CO_2] and drought, experiments were conducted with faba bean in glasshouse chambers either in ambient [CO_2] (~400 ppm) or elevated [CO_2] (~700 ppm) and exposed them to one of two watering regimes, a high rainfall scenario or the simulation of a dry season in a typical

Mediterranean agro-ecosystem over two consecutive year. Faba bean was chosen, because it has higher nodulation and N_2 fixation potential but also greater drought sensitivity than most other cultivated legumes (Dayoub et al., 2017). Plants were grown in sand culture, which allows proper development of nodules and maximises their sink capacity by avoiding inhibition by soil N. Soil N can arrest nodule growth and activity for diverting photosynthate to NO_3^- assimilation process instead of nodule symbionts (Butterly et al., 2016; Peoples et al., 2012). The $^{13}CO_2$ pulse-labeling technique was employed to estimate sink strength and allocation patterns of assimilated ^{13}C within plant tissues. This experimental set up allowed to address the following research questions:

1. How does e[CO_2] modify the sink-strength of nodules and other organs through greater activity or size?
2. How does drought affect C sink strength of nodules and other organs?
3. Does e[CO_2] maintain nodule sink strength under drought through increasing C supply and conserving soil water surrounding the nodules?
4. Does nodule C sink strength contribute to overcoming photosynthetic acclimation under e[CO_2] and drought?

7.2 Materials and methods

7.2.1 Glasshouse conditions

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

7.2.2 Plant material

Faba bean (*Vicia faba* cv. 'Fiesta') was grown in polyvinyl chloride (PVC) columns (15 cm diameter × 60 cm long) containing white washed sand (10 kg per column) to ensure that the only N source was atmospheric N_2. Seeds were inoculated with commercial Group F peat-based inoculum (WSM1455, *Rhizobium leguminosarum*, NoduleN^{TM}, New Edge Microbials Pty Ltd, Albury, NSM, Australia) before sowing. Inoculated seeds were hand sown at a depth of 2 cm (4 seeds per column). Seedlings were thinned to the two most vigorous plants per column one week after sowing. Plants were watered twice a week with Hoagland N-free nutrient solution (Hoagland and Arnon, 1938) containing the following macro and micro-nutrients: 3 mM KH_2PO_4, 3 mM $CaCl_2$, 3 mM MgSO4, 0.015 mM MnSO4, 0.075 mM H_3BO_3, 60 gL^{-1} FeSO4, 0.0015 mM CuSO4, 0.006 mM ZnSO4, 0.0003 mM H_2MoO_4. To avoid salt accumulation at the bottom of the columns, each plant was irrigated with deionized water once per week.

7.2.3 CO_2 treatments

Based on future climate change scenarios, [CO_2] is predicted to surpass 700 ppm by 2100. To represent plant performance by 2100, [CO_2] in glasshouse experiment was fixed at 700 ppm. Two glasshouse chambers were used to apply two [CO_2] treatments; i.e. ambient CO_2 (a[CO_2]) (~400 ppm) and elevated CO_2 ([CO_2]) (~700 ppm) from June to July in each of two consecutive years (2016 and 2017). To avoid unspecific chamber effects, columns and CO_2 treatments were shifted weekly among chambers. Moreover, columns were arranged randomly in the respective glasshouse chamber.

7.2.4 Water treatments

In each [CO_2] chamber, columns were randomly assigned to one of two groups ten days after sowing (DAS): eight columns to well-watered and eight to drought per CO_2 chamber each year. Water treatments approximated the variability of rainfall in the south-eastern Australian dryland cropping area, using June-July amounts of a high rainfall season (2013) and a low rainfall season (2014) as guidance. Well-watered plants received a mean of 40 mL water each day, corresponding to average precipitation of 2.4 mm day^{-1}, whereas drought treated plants received 15 ml water equivalent to 0.9 mm rain day^{-1}. Before sowing, 100 ml water was applied to each column. Water status of the column was monitored using time domain reflectometer (TDR, Theta Probe, ML3, Delta-T Devices, Cambridge, UK) every three days intervals from sowing to harvest at 20, 40 and 60 cm respectively. Averaged soil water within the column is reported in Fig. 7.1.

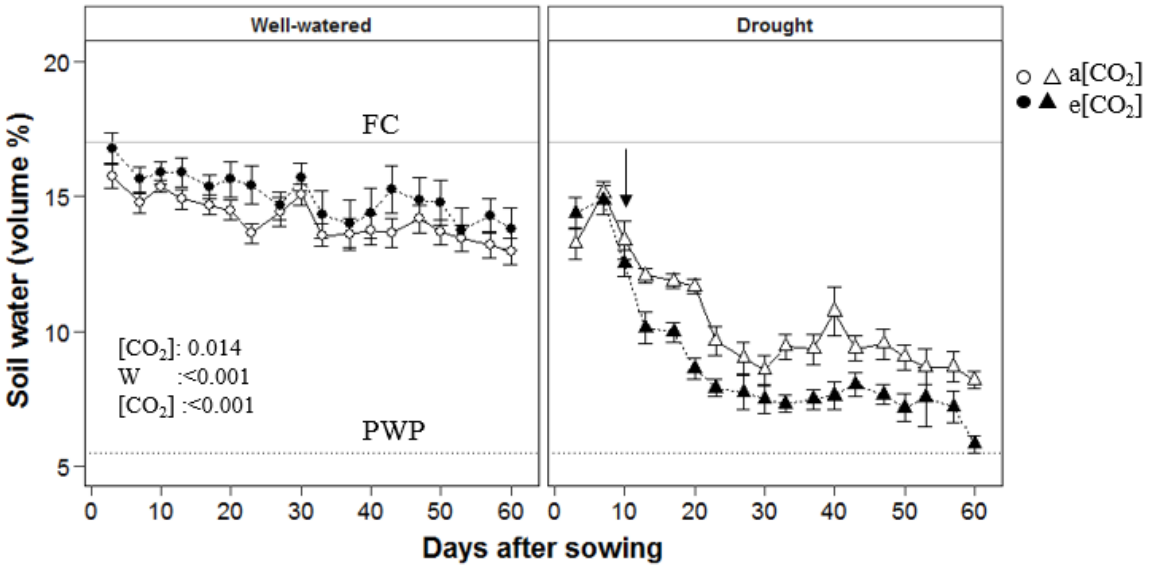


Fig. 7. 1 Volumetric soil water content (%) during the growing period of faba bean under two CO_2 concentrations i.e. ambient [CO_2] (~400 µmol mol^{-1}; open symbols O, Δ) and elevated [CO_2] (~550 µmol mol^{-1}; closed symbols ●, ▲) and two water (W) regimes (well-watered; circles and drought; triangles). Arrow indicates the onset of the drought treatment. Data are means ± 1 SE (n=8). The continuous horizontal line indicates field capacity (FC) and dotted line permanent wilting point (PWP).

7.2.5 $^{13}CO_2$ pulse labeling

On clear sunny days at stem elongation stage (according to BBCH-scale mentioned by Lancashire et al. (1991); about 60 DAS), plants were ^{13}C pulse-labeled at their growing CO_2 concentration. $^{13}CO_2$ pulse-labeling was carried out in an air-tight growth chamber (100 cm long × 70 cm wide × 60 cm height; made of highly transparent plexiglass) fitted onto a stainless-steel frame of 50 cm height (Lucia et al., 2014). A detailed description and illustration of the chamber set up were given by Butterly et al. (2015) and slight modifications were made to better utilise $^{13}CO_2$ by the plant canopy. Briefly, eight columns with *Vicia faba* plants were sealed into fitting holes in the bottom of the plexiglass chambers so that the canopies were inside the chamber. The top of each column was sealed with plastic around the plant stem to prevent direct CO_2 absorption by the below ground organs of the plants. Inside the chamber, the canopies were well-ventilated with vertically mounted electric fans. Two 12-V fans were fitted inside the PVC duct at the diagonally opposite end of the chamber, and an air pump was used to invert and circulate air within the chamber. Temperature and relative humidity (RH) were monitored and logged by a portable sensor (LU-MCH-383SD, ECEFast, Australia**)** mounted just above the canopy. Chambers conditions were 20±2°C temperature, 40±3% RH and 1000-1200 µmol m^{-2} s^{-1} of photosynthetic photon flux density. To maintain desirable RH, chambers were connected with silica gel traps to absorb excess transpiration. A soda lime trap containing 1M NaOH was connected with the chamber. To avoid plant photosynthesis before the chamber CO_2 was replaced by labeled $^{13}CO_2$, the chambers were covered with black plastic before starting the treatment. [CO_2] inside the chamber was monitored and recorded during the labeling period with an infrared gas analyser (IRGA, Li-6400, Li-Cor Inc, Lincoln, NE, USA) (Fig. 7.2). After the canopies were sealed into the chamber, the soda lime trap was started and [CO_2] approximating zero (trapped by soda lime), $^{13}CO_2$ was generated by the addition of H_2SO_4 (1M) through the rubber septum into a vial containing $Na_2{}^{13}CO_3$ (98% atom excess, 99 % CP, Sigma-Aldrich, Miamisburg, USA) (Bromand et al., 2001). Once $^{13}CO_2$ was emitted, the fan ensured homogenisation of [CO_2] and chamber was uncovered to start photosynthesis. The $^{13}CO_2$ concentrations of a[CO_2] and e[CO_2] chamber were constantly maintained to 400±10 or 700±10 ppm, respectively (Palta and Gregory, 1997; Wang et al., 2016; Zong and Shangguan, 2016) by continuously adjusting the emission of $^{13}CO_2$ inside the chambers (Fig. 7.2). At the end of the labeling period, air from the chamber was forced to pass through a soda lime cartridge that trapped the unassimilated $^{13}CO_2$. At each chosen [CO_2], plants were exposed to a $^{13}CO_2$ enriched atmosphere during active photosynthesis period for 6 hours (9.00-15.00) and then transferred to their respective growth conditions in the glasshouse.

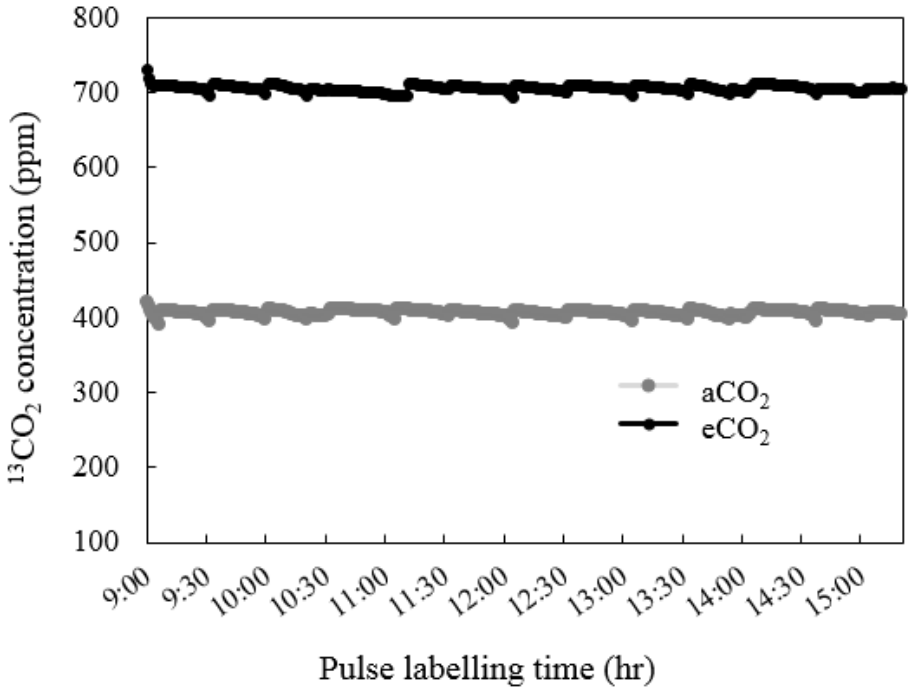


Fig. 7. 2 $^{13}CO_2$ concentration inside the ambient [CO_2] and elevated [CO_2] chambers during $^{13}CO_2$ pulse-labeling period (6hrs) showing the target [CO_2] of ~ 400±10 and ~700±10 ppm, respectively.

7.2.6 Photosynthetic parameters

Light-saturated photosynthetic [CO_2] uptake (A_{sat}, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and internal [CO_2] concentration (Ci, ppm) measurements were performed on the day after $^{13}CO_2$ labeling. Fully expanded apical leaves were measured using an infrared gas analyser (IRGA) system (Li- 6400, Li-Cor, Lincoln, NE, USA). Leaves were placed in the measurement cuvette and allowed to reach steady state at their growth [CO_2] (i.e. 400 ppm or 700 ppm) at a saturating light level of 1500 mol m^{-2} s^{-1} before starting the measurements. The measurement was performed using the Li-Cor Auto program 'A/Ci curve' and IRGAs were matched at each step. Block temperature was held constant at 25 °C, and relative humidity was stabilized at 45–55% during measurements. Once a steady state of photosynthesis (A) was reached, measurements of A, C_i, and g_s were recorded at the growth [CO_2]; [CO_2] was then decreased step-wise to 50 µmol mol^{-1}, increased again to the growth [CO_2], and then increased stepwise to 1800 ppm [CO_2]. A minimum of 10 data points was collected for each plant and the ACi data were fitted to the biochemical model of photosynthesis (Farquhar et al., 1980) to obtain maximum rate of carboxylation (V_{cmax}), maximum electron transport rate (J_{max}) and respiration (R_d) following the methods outlined by Long and Bernacchi (2003). The 'plantecophys' package implemented in R was used to estimate V_{cmax}, J_{max} and R_d with the function "fitaci" (Duursma, 2015). V_{cmax} and J_{max} were expressed per unit leaf area.

The photosynthetic N use efficiency (PNUE) was measured as the ratio between A (µmol CO_2 m^{-2} s^{-1}) and N content (g N m^{-2} leaf area). The leaf efficiency of N utilisation (ENUleaf) was calculated as the relation between total dry mass and leaf N concentration (g leaf dry matter g^{-1} N).

7.2.7 Biomass sampling

Plants were harvested 24 hours after pulse labeling. Twenty-four hours chasing period is well suited to characterise the allocation fate of new photosynthates (Kouchi et al., 1986). Plants were separated into leaf, stem, root, and nodule. Sub-samples of leaf and nodule tissues were immediately frozen in liquid N_2 and stored in -80^{0}C for amino acid analysis. Leaf area was measured using a leaf area meter (LI-3100C, LI-COR, Lincoln, NE, USA). All biomass was dried at 65^{0}C for 72 hours and weighed.

7.2.8 N concentration, N_2 fixation, and ^{13}C isotopic analysis

The finely ground plant tissue samples (leaves, stems, roots, nodules) were analysed for total N (% of tissue dry weight), total C (% of tissue dry weight), ^{15}N (atom %) and ^{13}C (atom %) by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon, Crewe, UK) coupled to an elemental analyzer (Thermo Scientific Flash 2000). N content was calculated per organ from N concentration (%N × 10) multiplied by organ biomass and the sum of all organs expressed as mg N plant$^{-1.}$ As the plants were grown in pure sand, all N accumulated in the biomass was considered as derived from N_2 fixed from the atmosphere.

The $^{13}CO_2$ uptake by leaves and translocation to other organs was determined by multiplying the C content of an organ fraction with the ^{13}C excess (atom %) of this fraction over the ^{13}C (atom %) of an unlabeled reference group as described by Fischinger and Schulze (2010):

^{13}C fixed (mg) =C (mg) ×[^{13}C labelled (mg^{13}C)-^{13}C reference (mg^{13}C)]/100(mg C)

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

Nodule activity was estimated dividing the amount of N_2 fixed by nodule dry weight (Parvin et al., 2018).

7.2.9 Estimation of sink strength

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

Sink strength (nmol C s^{-1}) = sink size (g) × sink activity (nmol C g^{-1} s^{-1})

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

Biomass-specific ^{13}C accumulation in organ fractions was estimated as described by Orians and Colin (2006). Because plants differed in biomass and the amount of ^{13}C fixed, relative biomass (organ fraction biomass/whole-plant biomass) and relative ^{13}C accumulation (organ fraction ^{13}C/whole-plant ^{13}C) were estimated. From these values, the biomass specific ^{13}C accumulation (relative ^{13}C accumulation/relative biomass) was calculated. Tissues with higher biomass specific ^{13}C accumulation were considered the strongest sink for ^{13}C.

7.2.10 Measurement of root exudates

After harvesting the plants, intact root systems including the rhizosphere sand adhering to the roots were removed from the columns and immersed in 100 mL deionized water for 30 minutes. Eluates were filtered through

membrane filter (0.45 µm, EMD Millipore TM, Thermo Fisher Scientific, NSW, Australia) to remove sand particles and root debris, and aliquots were analysed for total C, ^{13}C excess (atom%), total N, ^{15}N (atom%) by isotope ratio mass spectrometry (IRMS) (Hydra 20–20, SerCon, Crewe, UK) coupled to an elemental analyser. To estimate the amount of ^{13}C released by root exudation, the above equation was followed (Fischinger and Schulze, 2010) and ^{13}C in exudates was expressed per g root dry weight basis (Phillips et al., 2009). The allocation rate to belowground pools is greatest within the first day of pulse labeling and strongly correlated with photosynthetic C gains (Pausch and Kuzyakov, 2018). Unable to detect ^{13}C excess (atom %) in bulk sand, it was assumed that all ^{13}C exudated by the roots was present in the rhizosphere zone.

7.2.11 Biochemical analysis

Leaves and nodules tissues were analysed for soluble sugar and starch. Briefly, total soluble sugar was determined from oven dried and finely ground leaves and nodule tissues with the anthrone method (Yemm and Willis, 1954) modified for use in a plate reader (Tecan Sunrise, Tecan, Austria (Tausz-Posch et al., 2015). Measurements were done at 600 nm using D-fructose as standard and total sugar concentration expressed as mg fructose-equivalent g^{-1} dry weight. The retained pellets (Sheng et al., 1993) were used for analysis of starch at 505 nm as described by (Edwards et al., 2011) using glucose as standard.

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

Xylem sap was collected by the centrifugation method from the freshly harvested stem sections (Dannel et al., 1995). For xylem sap collection, the stem was cut with a sharp blade and to avoid contamination, close the phloem and remove cell bleeding; the cut surface was rinsed with 1 M $CaCl_2$ solution for 1 minute (Fischinger and Schulze, 2010). Immediately after collection, the concentration of AA in the sap was measured with the acid ninhydrin method and was expressed as µM.

7.2.12 Statistical analysis

The experiment consisted of 2 [CO_2] × 2 levels of water supply (well-watered and drought) × 4 replications (columns with two plants each in each year). A similar set of reference plants was grown under similar [CO_2] and watering supply level with four replications and therefore, total experiment consists of 32 columns (16 used for pulse labeling and 16 for reference) in each of two years. As in a first analysis, there were no statistical differences between years, data from both years were analysed jointly. Values presented in this experiment correspond to mean values of data collected during the two consecutive years (n=8).

Linear mixed-effect models were fit by REML using the R package "nlme" (Pinheiro et al., 2017) considering [CO_2] and water regimes as fixed effect and replications as random effect. The results were accepted as significant at $P < 0.05$. P values between 0.05-0.1 are presented for discussion purposes.

7.3 Results

7.3.1 Soil water

The well-watered plant had soil water close to field capacity (Fig. 7.1). Drought decreased soil water as the season progressed and reached a permanent wilting point at harvest. Elevated [CO_2] had higher soil water content in the well-watered plant. In contrast, drought decreased soil water with greater extent under e[CO_2] than a[CO_2].

7.3.2 Plant growth and N status

Elevated [CO_2] significantly increased total biomass of faba bean and this increase was greater under well-watered (+49%) than drought conditions (+39%) (Table 7.1). Leaf and stem biomass increased under e[CO_2] but decreased both under drought. Root biomass was affected by an interaction of [CO_2] and watering regimes such that e[CO_2] grown plant invested more biomass into roots, leading to an increased root: shoot ratio and this effect was greater extent under well-watered than drought conditions (Table 7.1). Regardless of the watering regime, e[CO_2] increased nodule biomass (~40%), whereas drought decreased it by 25% irrespective of [CO_2]. Nodule number was stimulated by e[CO_2], and this response was greater under well-watered (50%) than drought (43%) conditions. Also, drought decreased nodulation by 1.5 folds in both a[CO_2] and e[CO_2] (Table 7.1). Elevated [CO_2] increased nodule activity (+28%) in well-watered plants and decreased it by 14% in drought treated plants (significant interaction, Table 7.1).

Drought depressed leaf [N] to a greater extent under e[CO_2] (29%) than a[CO_2] (5%). Drought also depressed root [N] and nodule [N] but did not affect stem [N] (Table 7.1). No statistical difference was observed for area- based N_{leaf}. Drought decreased specific leaf area (SLA) and ENU_{leaf} by 6% compared to well-watered plants.

7.3.4 Leaf gas exchange

Greater carbon assimilation (A) was observed under e[CO_2], when photosynthesis was measured at growth [CO_2] (Table 7.1). The stimulation of A by e[CO_2] was greater under well-watered (50%) than drought conditions (20%). Stomatal conductance (g_s) was lower under e[CO_2] which was further accentuated by drought. The increase of A and decrease of g_s by e[CO_2] was accompanied by increased intrinsic water use efficiency (iWUE), as a tendency to increase to a greater under drought than well-watered conditions (Table 7.1).

Under well-watered conditions, e[CO_2] grown plants had no depression of maximum photosynthetic carboxylation capacity (V_{cmax}) and appeared to have even greater maximum capacity for electron transport (J_{max}) compared to a[CO_2] grown plants (Fig. 7.3 A, B). A contrasting trend was observed in drought stressed plants, where an acclimatory response to e[CO_2] was accompanied by lower (-29%) V_{cmax} and lower (-17%) J_{max} compared to a[CO_2] grown plants. The ratio of J_{max} to V_{cmax} was always greater under e[CO_2] and this pattern was maintained under drought (Fig. 7.3 C). Respiration rate as derived from A/Ci curves (Rd) was stimulated by e[CO_2] (+82%) when plants were well-watered but decreased by 55% under drought (Fig. 7.3 D).

Table 7. 1 Gas exchange parameters, biomass, nitrogen concentration ([N]), sugars and amino acids concentrations of faba bean grown under two CO_2 concentrations (a[CO_2], ~400 µmol mol^{-1} and e[CO_2], ~700 µmol mol^{-1}) and two water (W) regimes (Well-watered and drought) and harvested at 60 DAS. Gas exchange parameters were measured at growth [CO_2]. Means ±SE (n=8) and two-way ANOVA results are shown. dwt: dry weight, N_{leaf}: leaf [N] m^{-2} leaf area, SLA: specific leaf area, NUE: nitrogen use efficiency, ENU_{leaf}: N utilization efficiency by leaf, $PNUE_{leaf}$: photosynthetic N use efficiency. Unit for A: µmol CO_2 m^{-2} s^{-1}, g_s: mol m^{-2} s^{-1}, iWUE: µmol mol^{-1}.

Growth	Well-watered		Drought		P-value		
parameters	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	[CO_2]	W	[CO_2] × W
Biomass							
Total biomass (g $plant^{-1)}$	4.77±0.22	7.12±0.21	3.55±0.13	4.95±0.19	<0.001	<0.001	0.013
Leaf biomass (g $plant^{-1}$)	1.23±0.13	1.81±0.11	1.08±0.08	1.63±0.09	<0.001	0.065	ns
Stem biomass (g $plant^{-1}$)	2.27±0.14	2.58±0.08	1.74±0.04	2.12±0.15	0.004	<0.001	ns
Root biomass (g $plant^{-1}$)	1.04±0.13	2.44±0.13	0.58±0.11	0.97±0.09	<0.001	<0.001	<0.001
Root: shoot (R/S) ratio	0.374±0.045	0.628±0.039	0.265±0.040	0.334±0.043	0.001	<0.001	0.034
Leaf area (cm^2 $plant^{-1}$)	377.10±10.33	550.32±20.63	273.66±20.06	411.01±19.78	<0.001	<0.001	ns
Nodule biomass (g $plant^{-1}$)	0.231±0.02	0.290±0.02	0.159±0.01	0.246±0.01	<0.001	<0.001	ns
Nodule number ($plant^{-1}$)	150.76±8.89	227.13±8.24	101.85±5.13	145.67±6.04	<0.001	<0.001	0.033
Nodule activity (mg N_2 fixed g^{-1} nodule biomass)	765.15±56.36	942.25±91.89	663.89±51.94	627.28±34.94	0.465	<0.023	0.025
N_2 fixation (mg $plant^{-1}$)	164.88±7.15	250.28±8.66	123.08±7.17	139.29±4.79	<0.001	<0.001	<0.001
N concentration							
Leaf [N] (% dwt)	5.19±0.28	5.31±0.30	4.92±0.19	3.75±0.16	ns	<0.001	0.008
Stem [N] (% dwt)	2.12±0.18	2.43±0.17	2.19±0.12	1.98±0.11	ns	ns	0.090
Root [N] (% dwt)	2.94±0.05	3.14±0.15	2.66±0.15	2.52±0.11	ns	0.001	ns
Nodule [N] (% dwt)	6.85±0.27	6.83±0.17	6.33±0.27	5.78±0.15	0.071	<0.001	0.086
N_{leaf} (g N m^{-2})	1.70±0.13	1.65±0.11	1.96±0.14	1.64±0.16	ns	ns	ns
ENU_{leaf} (g DM g^{-1} N)	0.72±0.05	1.12±0.07	0.56±0.05	1.00±0.06	<0.001	0.021	ns
SLA (cm^2 g^{-1} leaf dwt)	399.10 ±33.40	317.95±25.03	234.66±23.40	233.76±18.97	ns	<0.001	ns
Gas exchange parameters							
Photosynthesis (A)	20.33±0.48	30.64±0.62	15.04±0.52	18.29±1.50	<0.001	<0.001	<0.001
Stomatal conductance (g_s)	0.29±0.01	0.21±0.03	0.16±0.03	0.09±0.01	<0.001	0.001	<0.073
iWUE	70.14±2.89	159.08±18.89	140.58±38.69	210.57±15.75	<0.001	0.002	0.079
$PNUE_{leaf}$ (µmol CO_2 s^{-1} g^{-1} N)	12.36±0.92	19.25±1.32	7.95±0.62	10.67±0.81	0.001	<0.001	0.047

Abbreviations: ns, not significant (P>0.1)

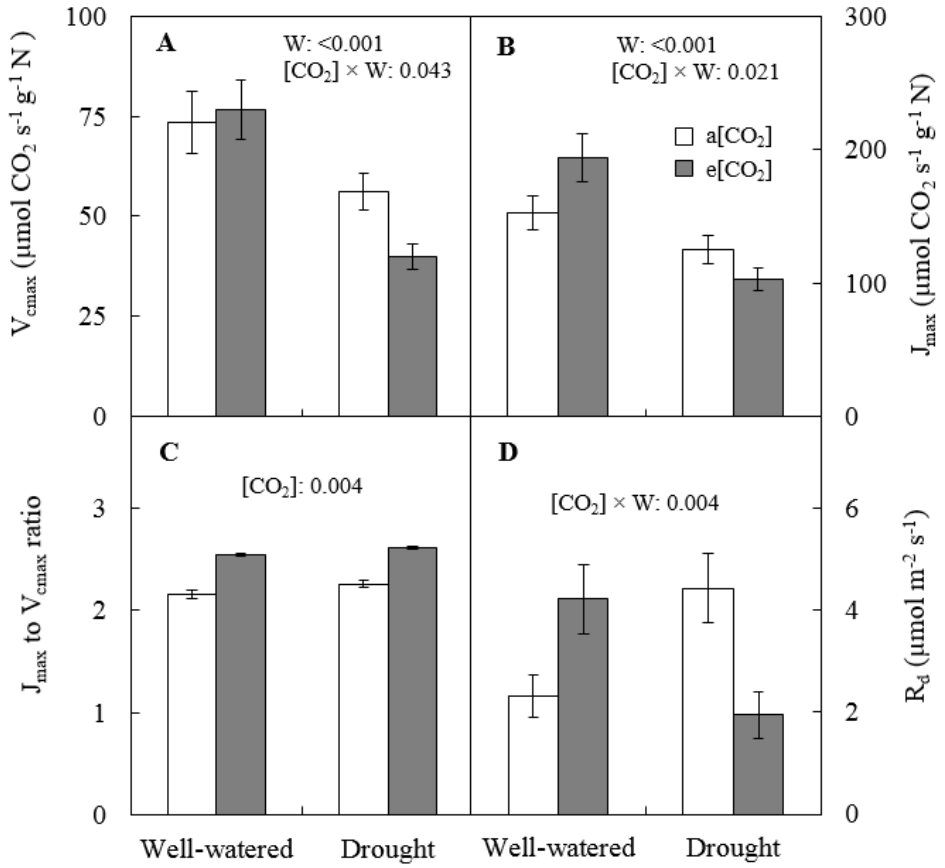


Fig. 7. 3 A. Maximum velocity of RuBP carboxylation by Rubisco (V_{cmax}), B. Maximum rate of photosynthetic electron transport (J_{max}), C. Ratio of J_{amx} to V_{cmax} and D. Respiration in leaves of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, white bars) or e[CO_2] (~700 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) and measured at 60 DAS. Means and ±SE (n=8) in each bar. Only significant relations ($P<0.05$) are shown.

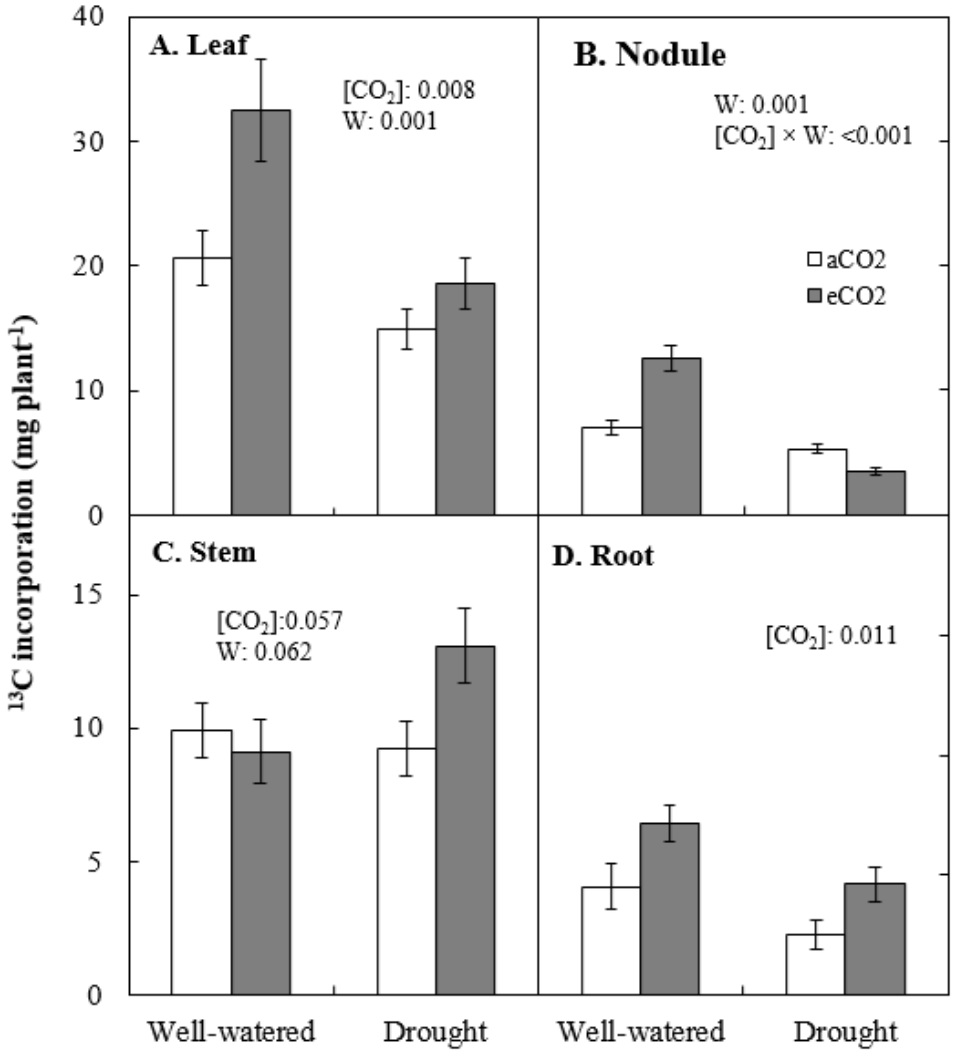


Fig. 7. 4 Total ^{13}C incorporation in leaf (A), nodule (B), stem (C) and root (D) of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, white bars) or e[CO_2] (~700 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) and harvested at 24hrs chase period. Means and ±SE (n=8) in each bar. Only significant relations (P<0.10) are shown.

7.3.5 ^{13}C incorporation and C sink strength

Measured after a 24h chase period following a six (6) h labeling period, the total amount of ^{13}C incorporated into biomass was greater in e[CO_2] grown plants, associated with greater photosynthesis and greater leaf biomass and area (Fig. 7.4; Table 7.1). In leaves, e[CO_2] grown and well-watered plants had more ^{13}C incorporated, and there was no interaction (Fig. 7.4 A). Under well-watered conditions e[CO_2]-grown plants incorporated more ^{13}C into nodules than under a[CO_2], but this was reversed under drought (Fig. 7.4 B). Incorporation to stems was not significantly affected by either water or CO_2 treatments (Fig. 7.4 C), and incorporation in roots was greater under e[CO_2] (Fig. 7.4 D).

Elevated [CO_2] increased nodule sink activity in well-watered conditions, but decreased it under drought, with a similar trend for leaves. Sink activity of roots and stems were unaffected by drought or e[CO_2] (Fig. 7.5). Elevated [CO_2] increased ^{13}C released by roots, and this increase was greater magnitude under drought than well-watered conditions (significant interactions, [CO_2] × W) (Fig. 7.6).

7.3.6 N_2 fixation

Elevated [CO_2] increased total N_2 fixation to a greater extent under well-watered (+60%) than drought conditions (+21%) (Table 7.1). Nodule specific ^{13}C fixation showed the similar patterns to specific N_2 fixation under e[CO_2] grown plants, as they were increased in well-watered plants (+25%, +40%) and decreased (-15%, -115%) in drought treated ones (Fig. 7.7).

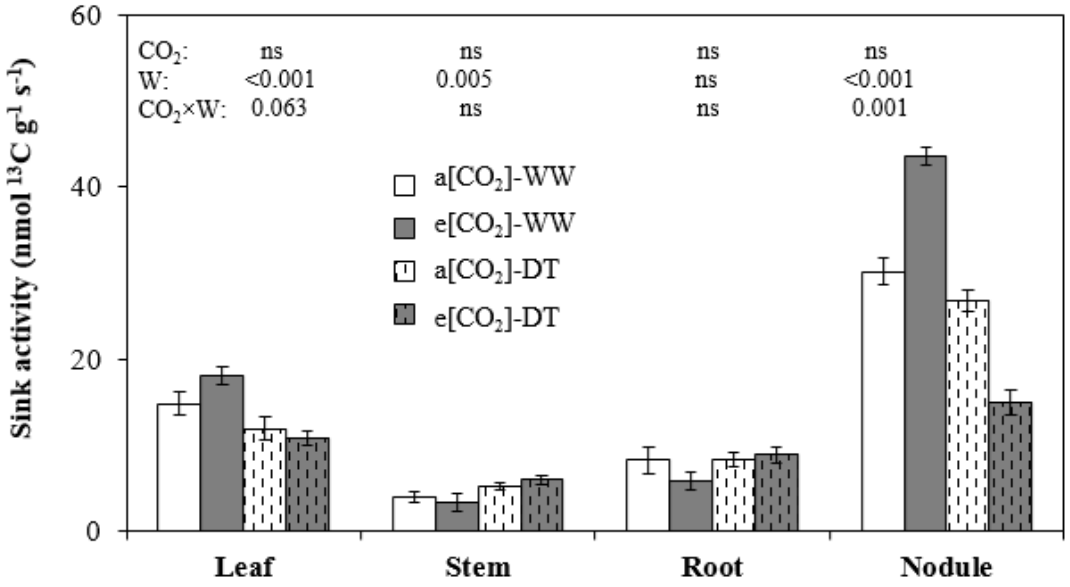


Fig. 7. 5 Sink activity of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, white bars) or e[CO_2] (~700 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) and harvested at 60 DAS after 24 hrs chase period. Means and ±SE (n=8) in each bar. Only significant relations ($P<0.05$) are shown. DW: dry weight, WW: well-watered, DT: drought.

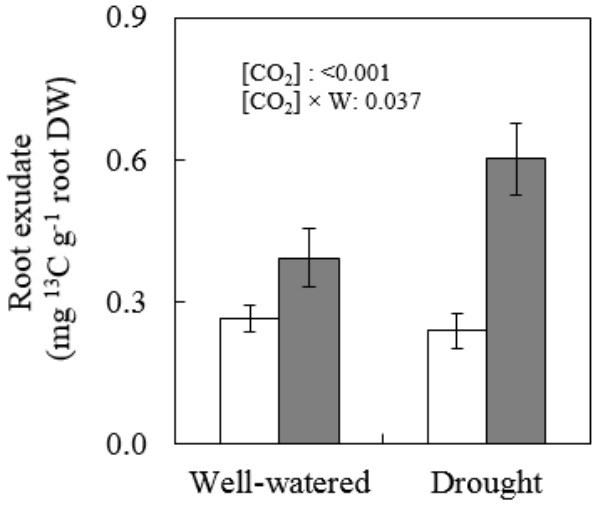


Fig. 7. 6 Amount of ^{13}C released by root exudation after 24-hr chasing period of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, white bars) or e[CO_2] (~700 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) and harvested at 60 DAS. Means and ±SE (n=8) in each bar. Only significant relations (P<0.05) are shown.

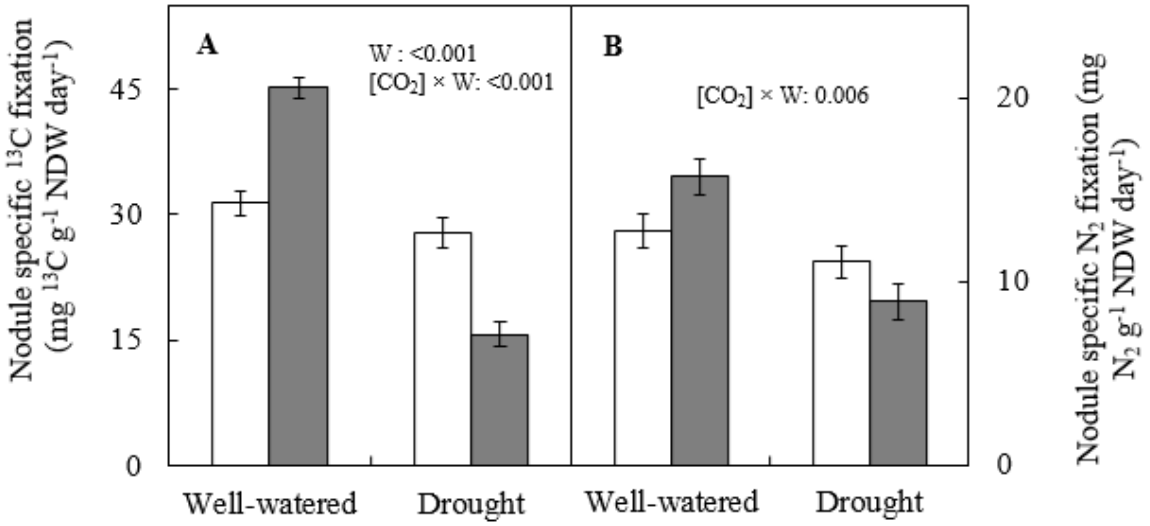


Fig. 7. 7 Nodule specific ^{13}C fixation (A) and specific N_2 fixation (B) of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, white bars) or e[CO_2] (~700 µmol mol^{-1}, grey bars) and two water (W) regimes (well-watered and drought) and harvested at 60 DAS. Means and ±SE (n=8) in each bar. Only significant relations (P<0.05) are shown.

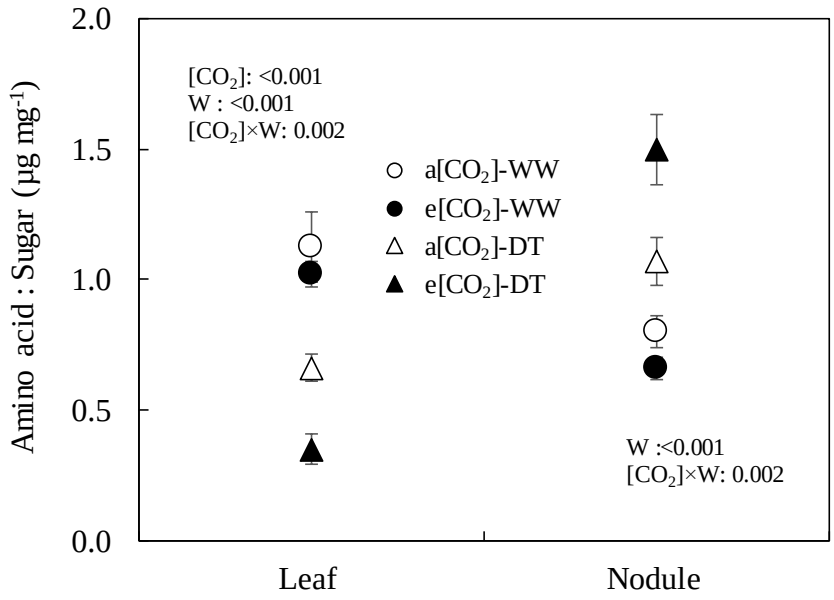


Fig. 7. 8 Ratio of free amino acid to sugar in leaf and nodule of faba bean grown under a[CO_2] (~400 µmol mol^{-1}, open symbols) or e[CO_2] (~700 µmol mol^{-1}, closed symbols) and two water (W) regimes (well-watered and drought). Means and ±SE (n=8) at each point. Only significant relations (P<0.05) are shown. WW: well-watered (circles), DT: drought (triangles). Data expressed in µmol amino acids per mg sugar per g dry weight basis.

Table 7. 2 Carbohydrates and amino acids concentrations of faba bean grown under two CO_2 concentrations (a[CO_2], ~400 µmol mol^{-1} and e[CO_2], ~700 µmol mol^{-1}) and two water (W) regimes (Well watered and drought) and harvested at 60 DAS. Means ±SE (n=8) and two-way ANOVA results are shown. dwt: dry weight.

Growth	Well-watered		Drought		P-value		
parameters	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	[CO_2]	W	[CO_2] × W
Leaf							
Sugars (mg g^{-1} dwt)	120.69±10.09	129.39±6.00	158.22±10.04	250.68±13.40	0.001	<0.001	<0.001
Starch (mg g^{-1} dwt)	159.23±9.30	160.90±5.83	191.56±9.97	283.80±13.24	0.001	<0.001	<0.001
Free AA (µmol g^{-1} dwt)	129.20±5.881	133.59±7.06	102.31±6.49	83.21±8.60	ns	0.005	ns
Nodule							
Sugars (mg g^{-1} dwt)	304.03±18.87	358.95±22.94	275.26±17.84	231.42±15.85	ns	<0.001	0.015
Starch (mg g^{-1} dwt)	185.74±20.59	250.92±20.54	159.95±18.73	182.62±14.50	0.036	0.025	ns
Free AA (µmol g^{-1} dwt)	239.44±19.27	229.37±5.17	285.78±15.20	335.97±18.16	ns	<0.001	0.042
Xylem sap							
Free AA (µM)	10.97±0.349	9.08±0.202	13.70±0.651	15.36±0.553	ns	<0.001	<0.001

Abbreviations: ns, not significant (P>0.1)

7.3.7 Carbohydrate and amino acid concentrations

Drought increased concentrations of sugars and starch in leaves to a greater extent under e[CO_2] than a[CO_2]. In contrast, nodules contained higher concentrations of sugars under well-watered conditions than under drought (Table 7.2). Starch concentrations were on average slightly greater in both organs under e[CO_2], but drought stimulated starch accumulation in leaves and depletion in nodules. Drought decreased total free amino acid (AA) concentrations in leaves but increased them in nodules (Table 7.2) and this increase was greater e[CO_2] (+46%) than a[CO_2] (+19%). Concentrations of AA were also greater in the xylem sap of drought treated plants and whilst e[CO_2] slightly depressed xylem sap concentrations of amino acids under well-watered conditions, it slightly increased them under drought (resulting in significant [CO_2] × W interactions, Table 7.2). In leaves, the ratio of amino acids to sugars was lower under drought and e[CO_2], and the combination depressed the ratio most (significant interaction [CO_2] × W, Fig. 7.8). In nodules, the ratio of amino acids to sugars increased under drought and this increase was greater under e[CO_2] (significant interaction, [CO_2] × W) (Fig. 7.8).

7.4 Discussion

In this study, drought decreased g_s by 50% than well-watered plants and the averaged value at or above 0.10 mol m^{-2} s^{-1} revealed that plants were mild to moderate drought stress. Decreased photosynthesis through stomatal closure is commonly reported under moderate stress but was unlikely to occur any metabolic or physiological damage (Flexas et al., 2006a; Flexas et al., 2006b). Also, soil water content was gradually declined as the plant growth advanced but was nearly or above the PWP at harvest, indicating plants were moderate drought stress.

It has been a long-held paradigm that "CO_2 fertilization effect" will be greater under dry conditions because of reduced g_s (Kimball, 2016). This lower g_s would be expected to soil water saving, however the indirect effects of e[CO_2] on plant water use determinants i.e. greater plant growth, leaf biomass or more precisely transpiring leaf area and elevated canopy temperature offset lower g_s, leading to increase soil water use under drought (Manea and Leishman, 2014). Consistent with this study, reduced g_s under e[CO_2] was overcompensated by increased leaf area matched with greater soil water depletion under drought conditions. As a result, stimulation of total plant biomass by e[CO_2] was greater under well-watered than drought condition in this study. This might be associated lack of soil water saving under e[CO_2], consistent with earlier findings from AGFACE site (Parvin et al., 2018) and overseas (Gray et al., 2016). Studies reported that CO_2-induced changes in total biomass growth might induce the modification of the sink strength through regulating sink size and or sink activity (Ainsworth et al., 2004; Aranjuelo et al., 2011; Fatichi et al., 2014).

7.4.1 Elevated [CO_2] had greater sink strength of all organs due to increased sink size but greater sink activity was only detected in nodules

This study investigated the sink strength (sink size × activity) of different organs directly using the $^{13}CO_2$ pulse labeling approach (Voisin et al., 2003c). Sink size, i. e. the total C content of the sink organ reflects the fact that larger organs are likely to accumulate more C than smaller ones, while sink activity (uptake rate per organ mass) reflects the fact that metabolically more active tissues import C per unit of time (White et al. 2016). Measured after the 24h chase period following a 6h labeling period, leaves had the greatest C sink strength, about double the strength of nodules, stems, or roots. This ranking was dominated by sink size, whereas nodules ranked first in

terms of sink activity, i.e. they imported most C per g organ weight per unit of time. Greater accumulation rate of $^{14}C/^{13}C$ have demonstrated in previous experiments showed strong sink activity of nodules for recent photo-assimilates (Lawrie and Wheeler, 1973; Voisin et al., 2003c), but these studies were conducted under a[CO_2].

Result of this study demonstrated that e[CO_2] increased sink strength (i.e. total ^{13}C incorporation) of leaves, roots and nodules by 40-50% than a[CO_2] in a well-watered plant. Increased sink strength might be correlated with greater sink size, sink activity or combination of both. Evidence suggested that sink strength and sink size are often intercorrelated, but sink strength is probably not usually related to sink size (Marcelis, 1996). The present study showed that e[CO_2] regulated the sink strength through increased sink size of leaves, roots and nodules, yet sink activity increased exclusively in nodules. Because, increased biomass under e[CO_2] including the development of new sinks such as new leaves, more roots and greater nodulation (nodule number and biomass) increased sink size of most organs (Leakey et al., 2009; Long et al., 2006). Linear relationship between sink size/biomass and ^{13}C incorporation has been demonstrated in pea (Voisin et al., 2003b). Interestingly, increased sink activity of nodules under e[CO_2] suggested that nodules were a far more competitive sink for recently fixed C due to increased respiratory activity (C costs of N_2 fixation are mainly due to respiration), as reflected by ^{13}C fixation per unit of nodule mass, and is consistent with other reports (Aranjuelo et al., 2008; Voisin et al., 2003b; Voisin et al., 2003c). Greater ^{13}C fixation under e[CO_2] was also linked with greater N_2 fixation by the nodules. While sink strength of e[CO_2] grown plant increased because of greater sink size, the results of our findings suggest that nodule sink strength was modulated through greater sink activity rather than sink size.

7.4.2 Drought-induced growth limitation affected sink strength of all organs through decreasing sink size but decreasing sink activity in nodules

In this study, the biomass growth of all organs was decreased under drought compared to well-watered plant. As a result, the sink strength also decreased in line with biomass growth due to decreasing sink size or organ expansion, leading to proportionally lower ^{13}C assimilation under drought stressed plants. The decrease of photosynthetic C gain and sink capacity has been suggested under drought (Muller et al., 2011; Pallas et al., 2013). Studies reported that assimilate distributions also decrease by the drought due to inhibition of vascular transport and reduction of sink demand (Galvez et al., 2005). Corroborating of this findings, ^{13}C partitioning data showed that under mild drought stress, sink activity of nodules was significantly reduced, which gives clear proof of a limitation in recently fixed ^{13}C transport to bacteroids that is likely to cause a decline in nodule activity. Because, sink activity of nodules is determined by the recently fixed C. However, the sink activity of leaf slightly reduced under drought, but stem and roots were unchanged. Taken together, this evidence shows that sink strength of other organs except nodules is mediated by a direct constraint on growth rather than acting via direct C supply. In contrast, the drought-induced impairment of nodule sink strength in faba bean appears to be caused by C limitation and nodule sink activity is finely tuned with C availability.

7.4.3 Elevated [CO_2] decreased nodule activity and sink strength even further under drought

Previous studies suggested that e[CO_2] can ameliorate the effects of drought on nodule activity either by maintaining greater water content in the soil surrounding root nodules or by maintaining greater supply of C to the nodules and greater N demand in growing plant organs (Gray et al., 2013; Rogers et al., 2009). The drought

treatment applied in this study showed greater depletion of soil water under e[CO_2]. Soil conditions may have been even drier under e[CO_2] and this coincided with decreased nodule N_2 fixation activity (Table 7.1). The results are in line with multiyear FACE study in soybean (Gray et al. 2016) because greater leaf area offset e[CO_2]-induced soil water saving via lower gs, leading to lower soil moisture under drought with little to no stimulation of N_2 fixation (Gray et al., 2016).

Decreased fixation activity of drought-stressed faba bean was reported in previous studies conducted (Abdelhamid et al., 2011; Streeter, 1993). Consistent with these studies, N_2 fixation activity of nodules decreased under drought. ^{13}C labeling in our study showed that nodule C sink strength decreased markedly under drought, especially at e[CO_2], and whilst sink activity was nearly unchanged by drought alone, it was strongly depressed by the combination of drought and e[CO_2]. This suggests that C transport may be more limiting under drought in e[CO_2] grown plants, which is an indicator of sink limitation of C in nodules. Altered sensitivity of stomata to drought signals and impairing nodule functionality also were thought to contribute to this response. Because e[CO_2]-grown plants showed a stronger response to stomatal closure under soil drying. Drought-induced decrease of ^{13}C enrichment of nodule tissue with greater extent under e[CO_2] was also a sign of greater diminishment of nodule sink strength.

7.4.4 Drought-induced inhibition of nodule sink activity led to photosynthetic acclimation under e[CO_2]

In well-watered plants, the concentration of soluble sugars was not increased by e[CO_2] in leaves, but sugar concentrations increased in nodules, indicating rapid export of additional assimilate to the nodules, which showed increased C sink strength under e[CO_2]. A positive relationship between assimilate consumption/export and photosynthetic stimulation has been reported previously (Ainsworth and Bush, 2011; Ribeiro et al., 2012). Increases in C availability for nodules, when grown at e[CO_2], may enhance nodular activity and in turn supply more N for plant growth (Guo et al., 2013; Irigoyen et al., 2014). However, decreased sink capacity/activity may lead to increased leaf carbohydrate level under e[CO_2] growth conditions (Ainsworth et al., 2004; Rogers et al., 2004), which then can act as a signal to down-regulate the capacity of the photosynthetic apparatus. In the present study, decreased nodule sink activity as reflected from ^{13}C diminishment by drought caused increased water-soluble carbohydrate (i.e. soluble sugar) in leaves with greater extent under e[CO_2] grown plant, associated with photosynthetic down-regulation. Studies reported that a reduction in sink capacity resulted in an imbalance between photosynthate production and export (Ainsworth and Bush, 2011). This response is consistent with (Myers et al., 1999), who reported that experimentally lowered sink strength resulted in rapid feedback inhibition of leaf-level photosynthetic capacity in loblolly pine. Furthermore, the consequent C source/sink imbalance under drought might have induced the downregulation of photosynthesis (Aranjuelo et al., 2009).

Analyses of ACi curve parameters revealed that e[CO_2] diminished leaf photosynthetic rates of faba bean only under drought when nodule C sink strength was sharply reduced. Because, down-regulation of nodule activity caused inhibition of N_2 fixation under drought even under e[CO_2], leading to decrease leaf [N] concentration. As a result, the RuBP carboxylation rate of Rubisco (V_{cmax}) decreased in association with maximum electron transport rate (J_{cmax}), suggesting a decrease in the allocation of N to Rubisco. Drought- induced decreases in Rubisco activity has been observed in C_3 crops (Flexas et al., 2006a; Flexas et al., 2006b; Perdomo et al., 2017). It has also been

frequently observed that down-regulation of photosynthesis at e[CO_2] is greater when N is limiting (Irigoyen et al., 2014; White et al., 2016 and references therein), although the modulation of nodule C sink strength under different water supply and its relationship to photosynthetic down-regulation have not been previously studied.

In summary, faba bean plants were maintained photosynthetic stimulation under e[CO_2] due to a greater C sink strength of nodules rather than other organs, as confirmed by ^{13}C enrichment/greater sink activity of nodules tissues in well-watered plants. Because, assimilated C was transported rapidly to the nodules due to the greater sink activity and avoided sugar accumulation in source leaves. These findings confirmed that nodules sink activity of e[CO_2] grown plants was depleted sharply under drought, resulted in a decrease of C-transport to nodules and subsequent C accumulation in leaves provoked photosynthetic down-regulation. These results suggest that nodule C sink strength is a key factor in the responsiveness of legumes grown under future atmospheric [CO_2] enriched environments.

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Chapter 8: Grain minerals quality of dryland legumes as affected by elevated CO_2 and drought: A FACE study on lentil (*Lens culinaris*) and faba bean (*Vicia faba*)

(viii) Abstract

Stimulation of grain yield under elevated [CO_2] grown plants is often associated with the deterioration of grain quality. This effect may be further complicated by the frequent occurrence of drought, as predicted in most of the climate change scenarios. Lentil and faba bean were grown in the Australian Grains Free Air CO_2 Enrichment facility either under ambient CO_2 concentration (a[CO_2], ~400 ppm) or elevated [CO_2] (e[CO_2], ~550 ppm), and with two contrasting watering regimes (faba bean) or over two consecutive seasons contrasting in rainfall (lentil) to investigate the interactive effect of e[CO_2] and drought on selected grain minerals (Fe, Zn, Ca, Mg, P, K, S, Cu, Mn, Na) concentrations. Dry growing environments increased grain minerals concentration (Fe, Zn, Ca, K, S, Cu) but decreased grain mineral yield (g mineral per plot surface area) and vice-versa in a wet growing environment. Elevated [CO_2] decreased Fe, Zn, P and S concentrations in both crops. However, the relative decrease of Fe, Zn, P and S concentrations under e[CO_2] was greater under dry (20-25%) compared to wet (4-10%) growing conditions. Principal component analysis showed that greater yield stimulation under e[CO_2] was associated with the reduction of Fe and Zn concentration indicating a yield dilution effect, but this was not consistently observed for other minerals. Even if energy intake is kept constant to adjust for lower yields, decreased legume micronutrients densities under e[CO_2] may have negative consequences for human nutrition, especially under drier conditions and in areas with less access to food.

Keywords: Climate change, nutritional quality, grain legumes, dry environments, Fe and Zn concentration

8.1 Introduction

Grain concentration of nitrogen (N) and thus protein as well as grain concentration of other micronutrients including iron (Fe) and zinc (Zn) commonly decrease under elevated atmospheric CO_2 concentrations (e[CO_2]) (Fernando et al., 2012; Fernando et al., 2014; Goicoechea et al., 2016; Högy et al., 2013). More than 2 billion people already suffer from Fe and Zn deficiency worldwide (Kumssa et al., 2015) and it has been estimated that e[CO_2] may increase the number of people at risk of Zn deficiency by 138 million by 2050 (Myers et al., 2015).

In legumes, the decrease of grain [N]/protein under e[CO_2] is small or largely absent (Gray et al., 2016; Gray et al., 2013), owing to their capacity to fix atmospheric N_2 (Rogers et al., 2006). Apart from N, grain mineral concentrations are also reduced by e[CO_2] in legumes as in non-legume crops (Loladze, 2002; Loladze, 2014). A meta-analysis by Myers et al. (2014) revealed that the concentration of Fe and Zn ([Fe], [Zn]) in field pea and soybean grains decreased under e[CO_2] in line with rice and wheat.

The rise in global atmospheric [CO_2] is driving an increase in global mean temperature and therefore climate change, with predicted follow-on effects on precipitation patterns, such as increases in severity and frequency of drought events in many cropping areas (IPCC, 2014). Particularly in Mediterranean-type climates, heat wave

events (short periods of high temperatures) are likely to become more frequent and more severe: a 1-in-10-years event is expected to become a 1-in-3-years event by the middle of the 21st Century (IPCC, 2014). Legumes, especially those grown in water-limited Mediterranean environments, already experience intermittent drought at some stage in their vegetative growth period, and terminal drought throughout their reproductive period when temperatures are higher, and rainfall is diminishing. More extreme conditions may lead to lower grain yields and poor grain quality (Farooq et al., 2017; Sehgal et al., 2017).

Among cultivated legumes, faba bean (*Vicia faba* L.) and lentil (*Lens culinaris* MEDIK.) rank third and fourth by global production volume, followed by soybean and field pea (FAO, 2016). Lentil, normally grown in dryland conditions, has high protein content and therefore plays a major role in the food and nutritional security of millions, particularly among low-income Asian families (Erskine et al., 2011). Faba bean also provides food and feed, contributes positively to the N economy of dryland agriculture (Denton et al., 2017) and is rich in protein (Multari et al., 2015). Nitrogen-fixing beneficial rhizobia bacteria living in the root nodules of legumes can also help plants to cope up with drought stress (Vurukonda et al., 2016). Exopolysaccharides produced by rhizobia provides a microenvironment that holds water and dries up more slowly than the surrounding environment thus protecting the bacteria and plant roots against desiccation (Hepper, 1975), and thereby possibly also creating conditions conducive to other symbioses, such as mycorrhizas, which are considered beneficial for mineral uptake (Goicoechea et al., 2016). Therefore, legumes inoculated with rhizobia might also establish mycorrhizal symbiosis in the field, and this type of association may modify the legumes response under climate change conditions (Baslam et al., 2014; Gavito et al., 2001).

The mechanisms responsible for the decrease in mineral concentrations are not clear (Feng et al., 2015; Loladze, 2014). Drought alone or in combination with e[CO_2] can change grain mineral composition by altering the mechanism of nutrients/water uptake or changing the nutrient deposition to grains (Etienne et al., 2018; Houshmandfar et al., 2018; McGrath and Lobell, 2013). Decrease nutrients uptake per unit of root mass is one of the suggested mechanisms related with the decline in mineral concentration under e[CO2] grown plants (Taub and Wang, 2008). Such decrease in root uptake capacity could result from changes in root systems, such as less efficient root system architecture or decreased uptake capacity per unit root length (Bahrami et al., 2017), or from reduced transpiration-driven mass flow of nutrients due to decreased *g*s under e[CO2] (Houshmandfar et al., 2018; McGrath and Lobell, 2013). Dry conditions may elicit e[CO_2]-induced decreases in grain [N] of legumes (Bourgault et al., 2017; Bourgault et al., 2016) and data from cereals suggest that drought intensifies e[CO_2]-induced relative decreases in concentrations of other grain minerals too (Fernando et al., 2014). As decreases in the nutritional value of legumes under increasing atmospheric [CO_2] are already considered significant, it is important to assess the combined effects of e[CO_2] and drought on legume mineral concentrations.

The effect of e[CO_2] and drought on grain [N]/protein has been examined under Free-Air CO_2 Enrichment Facility (FACE) for some legumes (for example, field pea, soybean, lentil) (Bourgault et al., 2016; Gray et al., 2016; Parvin et al., 2018), but observations of effects on other minerals elements have received less attention. The decrease in concentrations of some essential minerals (Fe and Zn) has been documented for legumes in a global analysis (Myers et al., 2014), but it is still uncertain how and to what extent these and other elements are affected by e[CO_2]

× drought interactions. For the crops analysed in this study, it was shown that soil water was depleted faster under e[CO_2] under dry conditions, which would cause an escalation of drought effect (Parvin et al. 2018 for lentil; Parvin et al. 2019 for faba bean). As these results also pointed to limited N acquisition into grains, it seems likely that the composition of other important grain minerals is affected by the e[CO_2] × drought interaction, too. To address this question, this study reports on two independent but related experiments, one on lentil and one on faba bean, conducted within the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility in a semi-arid temperate dryland cropping region, representative of widely-distributed water limited agro-ecosystems worldwide. In AGFACE, crops were grown under a CO_2 concentration projected for 2050 (e[CO_2], ~550 ppm) or ambient [CO_2] (a[CO_2], ~400 ppm) over two contrasting growing seasons sharply differed in seasonal rainfall for lentil or experimentally imposed drought during a growing season for faba bean. Particularly, the objectives of this study were (i) to investigate the changes in grain mineral concentrations of lentil and faba bean under e[CO_2] and drought alone or in combination, (ii) to investigate whether e[CO_2] would further decrease the mineral yield and nutritional values of these legumes under dry conditions. Furthermore, we tested the hypothesis that e[CO_2]-stimulation of grain yield even under drought dilutes grain mineral concentration through carbohydrate dilution mechanism.

8.2 Materials and methods

8.2.1 Experimental design and growing conditions for lentil

The experimental design and site description are reported previously in Chapter 2. Briefly, this study used the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility located near Horsham, Victoria, Australia ($36^0$45′S, $142^0$06′E, 127m above sea level) to grow lentil in 2015 and 2016 at either ambient [CO_2] or elevated [CO_2] under fully open-air conditions. The meteorological data are reported in Table 8.1. The soil type in the study area is a Murtoa clay consisting of ~35% clay at the surface and 60% in 1.4 m depth, classified as a Vertosol according to the Australian Soil Classification (Isbell, 2002). In both seasons, soil samples were collected prior to sowing and analysed for physicochemical characteristics. The initial soil characteristics of the experimental site are given in Table 8.2. A detailed description of the AGFACE site and the performance of the CO_2-delivery system is given by (Mollah et al., 2009; Mollah et al., 2011). The 2015 growing season ('**dry season**') was extremely dry according to local conditions with growing season rainfall (128 mm) well below the long-term average (274 mm). In contrast, the 2016 season ('**wet season**') was well above (334 mm) the long-term average (Table 8.1). A PR2 Profile Probe (Delta-T Devices, Cambridge, UK) was used in pre-installed access tubes (ATS1/ATL1, Delta-T Devices Ltd., Burwell, Cambridge, UK) each year to monitor the soil water throughout the growing season every week. Seasonal averaged soil water content (v/v %) up to 1 m depth profile was ~18% in 2015 and ~30% in 2016.

In each year, four octagonal areas ('rings') with elevated CO_2 concentration (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 this experiment. Rings were 12m in diameter in 2015 and 16m in 2016. The horizontal tubes injecting CO_2 were raised as the crop grew to maintain them about 15 cm above canopy height. For 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, approximately 12 h but varied according to day length. CO_2 injection commenced near crop emergence and continued until physiological

maturity. CO_2 concentration was monitored using sensors installed centrally in each ring (IRGA, SBA-4, PP Systems, Amesbury, MA, USA) and meteorological data were recorded from the onsite-weather station (Table 8.1). More details are provided in Mollah et al. (2009).

Table 8. 1 Minimum (T_{min}) and maximum (T_{max}) temperatures, rainfall (mm) and global solar radiation (GSR) logged on-site in the 2015 and 2016 growing seasons in the Australian Grains Free Air CO_2 Enrichment (AGFACE) facility, Horsham, Victoria, Australia. Values are reported on a monthly average basis.

Parameters	Month's								Total seasonal rainfall (mm)
	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec	
2015									
T_{min} (average, °C)	5.35	3.46	1.52	2.49	3.29	8.01	9.16		
T_{max} (average, °C)	17.40	13.59	12.95	14.29	17.93	27.91	26.33		
Rainfall (mm)	3.21	39.25	28.62	24.12	27.8	1.80	3.82		128
GSR (MJ m^{-2})	9.94	6.25	8.22	11.56	17.31	24.14	26.61		
2016									
T_{min} (average, °C)	7.93	3.84	4.74	3.56	5.02	5.68	7.45	8.73	
T_{max} (average, °C)	18.86	13.69	13.22	15.24	15.36	18.35	25.24	27.68	
Rainfall (mm)	23.01	41.4	54.24	46.01	107.8	84.52	18.54	8.45	384
GSR (MJ m^{-2})	8.86	7.41	7.23	12.05	14.47	22.43	28.39	28.78	

Table 8. 2 Soil physiochemical properties of lentil (2015 and 2016) and faba bean (2016) grown in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia. Values are average of 50 soil samples collected from 0 to 120 cm depth from all plots in the AGFACE site on initial soil samples. For faba bean, soil samples were collected from adjacent to lentil plots in 2016 and showed similar physiochemical properties.

Soil properties	**2015**	**2016**
pH ($CaCl_2$)	7.77	7.73
Electrical conductivity (dS m^{-1})	0.57	0.55
Organic C (%)	0.41	0.42
Total N (%)	0.11	0.08
Ammonium-N (mg kg^{-1})	2.25	1.39
Nitrate-N (mg kg^{-1})	2.48	0.09
P (Colwell) (mg kg^{-1})	5.35	6.54
K (Colwell) (mg kg^{-1})	354.58	355.26
S (KCl) (mg kg^{-1})	278.79	132.45
Fe (DTPA) (mg kg^{-1})	16.72	15.42
Cu (DTPA) (mg kg^{-1})	1.22	1.21
Mn (DTPA) (mg kg^{-1})	2.72	2.65
Zn (DTPA) (mg kg^{-1})	0.44	0.40
Ca^{2+} (meq/100 g)	20.61	21.37
Mg^{2+} (meq/100 g)	9.12	8.72
K^{+} (meq/100 g)	0.93	0.90
Na^{+} (meq/100 g)	8.27	8.28

Two lentil genotypes (PBA Ace and 05H010L-07HS3010) were grown in each season at a sowing density of 150 plants m^{-2} and row spacing of 24.4 cm in subplots (6 rows or 1.5 m by 4 m length) in each ring. No nitrogen fertilizer was added but inoculated seeds (Group F®, WSM1455, *Rhizobium leguminosarum*, peat-based inoculant, NoduleNTM, NewEdge Microbials Pty Ltd. Albury, NSW, Australia) were hand sown on 22 May 2015 and 01 June 2016. Before sowing, plots were fertilized with superphosphate at the rate of 9 kg P and 11 S ha^{-1}. To control weeds, pre-emergence herbicides (simazine, dimethenamid-P, trifluralin) were also applied. Plants were harvested at physiological maturity from the central 4 rows by 0.30 m length (corresponding to 0.29 m^2) on 9 November and 12 December in 2015 and 2016, respectively. Total plant biomass and other agronomic parameters at harvest is reported in Chapter 2.

8.2.2 Experimental design and growing conditions for faba bean

The experimental design and site description are reported previously in Chapter 5. The faba bean experiment was conducted in a sub-facility of the AGFACE facility known as SoilFACE (Soil Free Air CO_2 Enrichment) at the same site in 2016 and the meteorological data are reported in Table 8.1. SoilFACE consisted of eight round bunkers sunk into the ground (3.7 m diameter; 1.2 m depth). Bunkers were at least 28 m apart and each bunker

was treated with either ambient [CO_2] (~400 ppm) or elevated e[CO_2] (~550 ppm), resulting in four replicates arranged in a completely randomized design (Butterly et al., 2015). The FACE system used to achieve the e[CO_2] level was similar in design and used the same reticulated CO_2 supply as the facility previously described by Mollah et al. (2009), except that smaller rings (4.0 m diameter) were used. The CO_2 exposure regimes were similar to the one described for the lentil experiment.

Plants were grown in polyvinyl chloride (PVC) columns (15 cm in diameter, 60 cm long) each filled with 14 kg of soil and placed into the bunkers on steel brackets (60 cm above from the bunker soil surface) so that the surface was level with the surrounding paddock. Additional soil columns (parts of other experiments) filled the bunker volume achieving paddock-like conditions. The experimental soil was a Vertosol (Isbell, 2002) collected adjacent to the site and the physiochemical properties of soil collected in 2016 are presented in Table 8.2. There were two columns per bunker with one column per water treatment (see below).

Faba bean (*Vicia faba* L. cv. Fiesta) seeds were inoculated with commercial Group F peat-based inoculum (*Rhizobium leguminosarum*, NoduleN, New Edge Microbials Pty Ltd, Albury, NSW, Australia) before sowing. Inoculated seeds were hand sown on 24 May 2016 at a depth of 2 cm with uniform germinated seeds in PVC columns (4 seeds per column). Seedlings were thinned to the two most vigorous plants at 15 days after sowing (DAS). Volumetric soil water content (v/v %) was measured weekly at three depths (20, 40, 60 cm) in the soil columns by time domain reflectometer (Theta Probe, ML3, Delta-T Devices, Cambridge, UK) throughout the growing period. At 60 days after sowing, columns were assigned into two groups i.e. **wet** (80% ± 5% field capacity, equivalent to ~30% v/v%) and **dry** (30% ± 5% field capacity, equivalent to ~18% v/v%) treatment. Rainfall was withheld for dry columns. The soil water content of wet and dry columns was maintained by watering to weight every week (Rab *et al.* 2011). Plants were harvested at physiological maturity on 21 November 2016. Total plant biomass and other agronomic attributes at harvest is reported in Chapter 5.

8.2.3 Grain sample preparation

After harvesting the plants, pods were separated, oven dried (at 40°C for 72 h), threshed and aspirated (Vacuum separator, Kim seed, Australia) to remove the pod walls and any dust. Subsequently, grains were weighed to estimate yield. Dried grains were ground into fine powder using a ball mill grinder (TissueLyser II, Qiagen, Chadstone, Victoria, Australia) and stored in air-tight containers.

8.2.4 Chemical analyses

Grain mineral concentrations were analysed by inductively-coupled plasma atomic emission spectrometry (ICP-AMS) (Applied Research Laboratories, 3580B, Switzerland) as described by Zarcinas et al. (1987). Briefly, 100 mg of ground grains were digested overnight with ultrapure concentrated nitric acid (HNO_3) in glass tubes. For the second stage of this digestion procedure, HCl was added and the final volume was adjusted to 25 mL. The digestion was carried out at a temperature of ~ 140°C for a period of 6 hours. After digestions, aliquots were analysed by ICP-AMS using appropriate standard for each mineral (Fe, Zn, Ca, Mg, P, K, S, Cu, Mn, Na) (Sigma Aldrich, Castle Hill, NSW, Australia). Mineral concentrations were expressed as mg kg^{-1} of grain dry weight.

Grain mineral yield was the products of grain yield and grain mineral concentration and expressed as g mineral per plot surface area.

8.2.5 Statistical analysis

The lentil experiment was designed as a split-plot design (growing season as main plot and [CO_2] as sub-plots). Analysis of variance (ANOVA) was performed after a linear mixed-effect model fit using the REML procedure of R package "nlme" (Pinheiro et al., 2017) considering the growing season and [CO_2] as fixed effect and ring numbers as random effect. No genotype or genotype by [CO_2] and growing season interaction effect were found to be statistically significant. Therefore, we only report the growing season and CO_2-driven effects.

The faba bean experiment was a split-plot design with ([CO_2] as main plots and water regimes as sub-plots. ANOVA was performed as for lentil (Pinheiro et al., 2017), considering [CO_2] and water regimes as fixed effects and ring numbers as random effects.

For both experiments, Levene tests were conducted to check the homogeneity of variance across groups (function LeveneTest from R package "DescTool") and data were natural logarithm transformed where necessary. Principal component analysis (PCA) was used to determine the associations among the grain yield and mineral elements. PCA was based on a correlation matrix and is presented as biplot ordinations of treatment combinations (PC scores). Statistical effects were considered significant at $P<0.05$.

Table 8. 3 Grain minerals concentrations and grain yield of lentil and faba bean 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 wet (2016) season for lentil and under dry and wet treatments for faba bean. Grains were analysed at maturity. Means and SE of n=8 (lentil) or n=4 (faba bean) replicates. W denotes water regimes (dry vs wet season for lentil and dry vs wet treatment for faba bean). Non-significant effects are reported as ns (P>0.01).

Mineral	**DRY**		**WET**		**P-value**		
concentrations	a[CO_2]	e[CO_2]	a[CO_2]	e[CO_2]	[CO_2]	W	[CO_2] × W
Lentil							
[Fe] (mg kg^{-1})	89.94±5.21	71.74±4.08	62.13±2.80	59.39±3.28	<0.001	0.008	0.048
[Zn] (mg kg^{-1})	68.03±4.25	49.46±0.82	32.63±0.92	30.61±0.67	<0.001	<0.001	0.020
[Ca] (g kg^{-1})	0.89±0.006	0.85±0.06	0.73±0.06	0.74±0.04	ns	0.023	0.032
[Mg] (g kg^{-1})	0.96±0.01	0.95±0.01	0.97±0.01	0.96±0.01	ns	ns	ns
[K] (g kg^{-1})	10.49±0.08	10.05±0.09	9.11±0.10	9.14±0.04	ns	0.004	ns
[P] (g kg^{-1})	4.62±0.04	4.11±0.05	3.80±0.09	3.76±0.08	0.044	ns	0.045
[S] (g kg^{-1})	2.22±0.04	1.89±0.01	1.93±0.02	1.86±0.02	0.011	ns	0.037
[Na] (mg kg^{-1})	17.95±0.51	12.76±0.37	14.32±0.45	13.08±0.43	ns	0.035	ns
[Mn] (mg kg^{-1})	13.28±0.25	11.82±0.14	11.82±0.15	11.32±0.21	ns	ns	ns
[Cu] (mg kg^{-1})	8.98±0.17	8.65±0.13	10.27±0.17	10.03±0.09	ns	0.006	ns
[C] (mg g^{-1})	431.41±6.28	443.9±4.25	380.07±4.28	391.85±3.21	0.040	<0.001	ns
Grain yield (g m^{-2})	126.42±17.54	148.74±23.45	315.68±22.60	503.61±22.65	<0.001	<0.001	0.002
Faba bean							
[Fe] (mg kg^{-1})	129.65±4.41	100.82±4.21	104.96±1.82	96.28±1.85	0.004	<0.001	0.044
[Zn] (mg kg^{-1})	68.28±6.30	51.82±2.45	33.72±2.44	31.07±1.32	0.001	<0.001	0.037
[Ca] (g kg^{-1})	1.38±0.15	1.11±0.08	1.16±0.06	0.99±0.05	0.056	ns	ns
[Mg] (g kg^{-1})	1.35±0.04	1.21±0.05	1.20±0.03	1.16±0.01	0.027	0.011	ns
[K] (g kg^{-1})	12.16±0.80	10.85±0.22	11.74±0.29	10.75±0.56	0.099	ns	ns
[P] (g kg^{-1})	4.95±0.36	4.19±0.26	4.11±0.27	4.09±0.06	0.085	ns	0.039
[S] (g kg^{-1})	1.65±0.08	1.50±0.04	1.50±0.02	1.45±0.02	0.044	0.032	0.048
[Na] (mg kg^{-1})	103.01±7.15	83.88±6.52	67.85±7.42	62.37±7.97	ns	0.008	ns
[Mn] (mg kg^{-1})	13.45±0.44	11.39±0.23	10.46±0.52	10.81±0.57	ns	ns	ns
[Cu] (mg kg^{-1})	3.29±0.54	2.68±0.19	2.46±0.21	2.40±0.23	ns	ns	ns
[C] (mg g^{-1})	435.26±3.24	447.08±4.25	420.21±3.24	430.25±1.27	0.021	<0.001	ns
Grain yield (g $column^{-1}$)	27.59±0.93	34.11±0.67	36.72±1.78	58.38±2.25	<0.001	<0.001	0.001

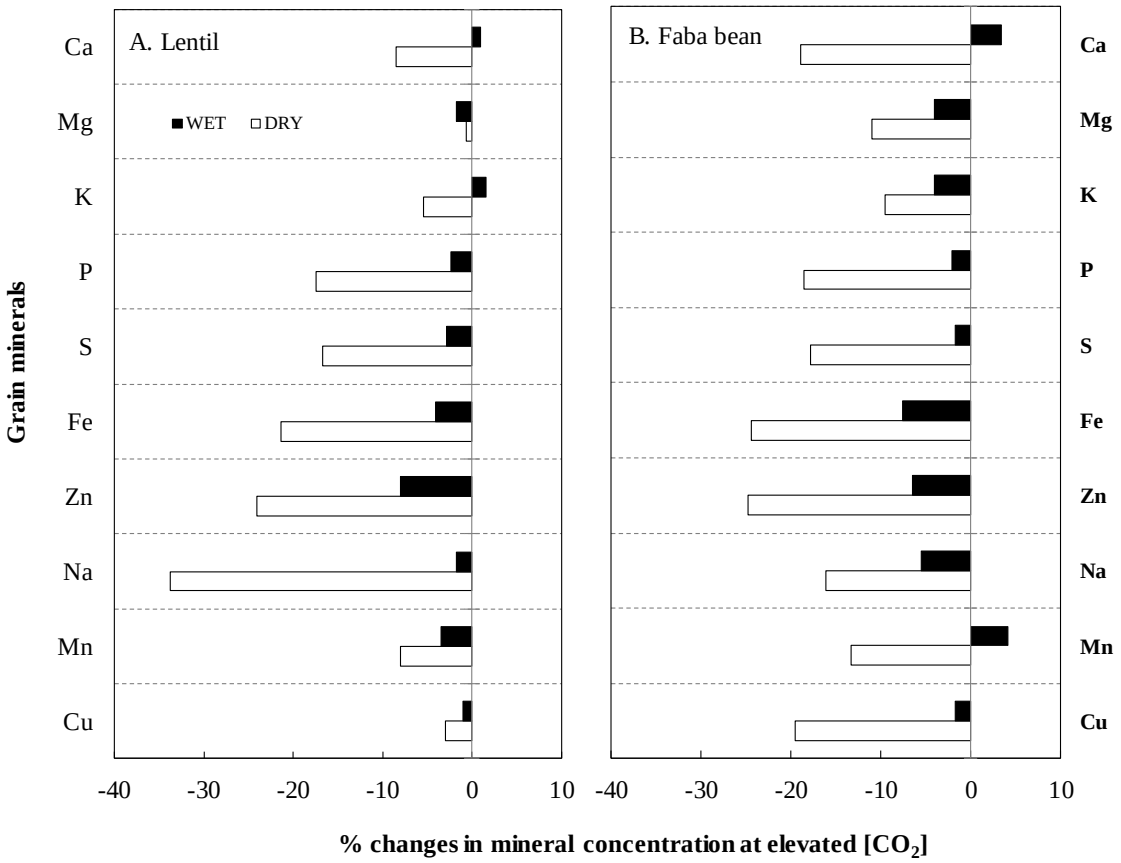


Fig. 8. 1 Percentage change of grain mineral concentration under elevated [CO_2] relative to ambient [CO_2] of lentil (A) and faba bean (B) during a dry (2015, white bars) and a wet (2016, black bars) season for lentil and under dry and wet treatment for faba bean grown under ambient [CO_2] (~400 ppm) or elevated [CO_2] (~550 ppm) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia. Each bar represents a means of n=8 (lentil) or n=4 (faba bean) replicates.

8.3 Results

8.3.1 Lentil

Elevated [CO_2] significantly decreased Fe and Zn concentrations ([Fe], [Zn]) of lentil grains. This decrease was relatively greater in the dry season (20-24%) than in the wet season (4-8%) (Table 8.3 & Fig. 8.1A). Concentrations of P and S also decreased under e[CO_2] to a greater extent under the dry than the wet growing season (Table 8.3). For K and Na, absolute concentrations were greater in the dry (2015) than the wet season (2016), whereas [Cu] was slightly greater in the wet than the dry season. Elevated [CO_2] decreased [Ca] in the dry but not in the wet season and did not significantly affect concentrations of K, Na or Cu (Table 8.3). There was no significant effect of any treatment on [Mg] and [Mn]. Even where [CO_2] × season interactions were not significant, the [CO_2]-induced relative decrease in concentration was proportionally greater in the dry season (Fig. 8.1A). The PCA showed strong associations between grain yield and mineral elements (Fig. 8.2A). This association was supported by negative correlations among these variables except [Ca] and [Cu].

Mineral yield of all tested elements decreased in the dry season and among them Fe and Zn showed greater decrease under e[CO_2] (Fig. 8.3A, Table 8.4). Grain mineral yield of Fe, Zn, S, P, Na increased in the wet season with greater extent under e[CO_2] than a[CO_2]. The ratio of mineral elements (Fe, Zn, P, S, Na) and C showed greater depletion under e[CO_2] in the dry than the wet season (Table 8.5).

8.3.2 Faba bean

Grain [Fe] and [Zn] decreased under e[CO_2] to a greater extent in the dry (~22-25%, Fe and Zn, respectively) than wet treatment (~9%, for Fe and Zn, respectively; Table 8.3 & Fig. 8.1B). Grain [Ca], [Mg], [P] and [S] decreased under e[CO_2] and only [P] and [S] showed greater reduction under dry treatment (Table 8.3 & Fig. 8.1B). Grain [Na] was 18% greater in the dry than wet treatment. There was no significant change of [Cu], [Mn] and [K]. Grain yield was negatively associated with the concentrations of all measured mineral elements (Fig. 8.2B).

Grain mineral yield of Fe, Zn, Mn, Mg, K, P, and S significantly decreased under e[CO_2] in the dry treatment but increased in the wet treatment (Fig. 8.3B, Table 8.4). Elevated [CO_2] decreased the stoichiometric ratio between minerals and C, and this decrease was greater under dry than wet conditions (Table 8.5).

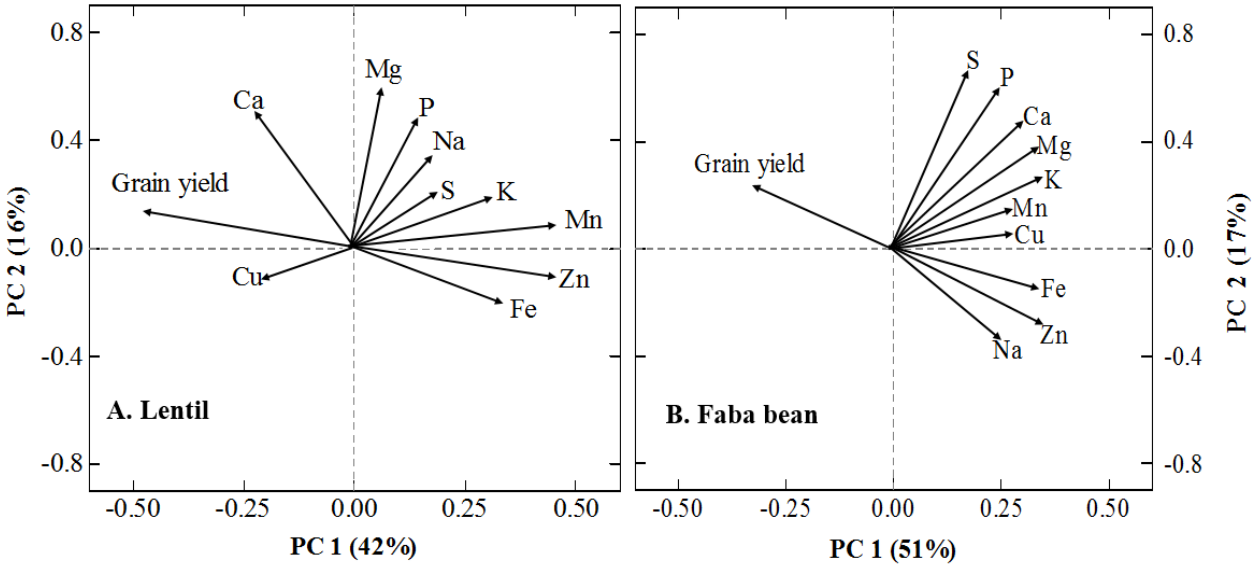


Fig. 8. 2 Biplot of principal component (PC) analysis (based on correlation matrix) of grain yield and grain mineral concentration for lentil (A) and faba bean (B) grown under ambient [CO_2] (~400 ppm) and elevated [CO_2] (~ 550 ppm) in two environments (wet and dry) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia. Values shown in parentheses are the percentage of the total variance explained by the first two PCs.

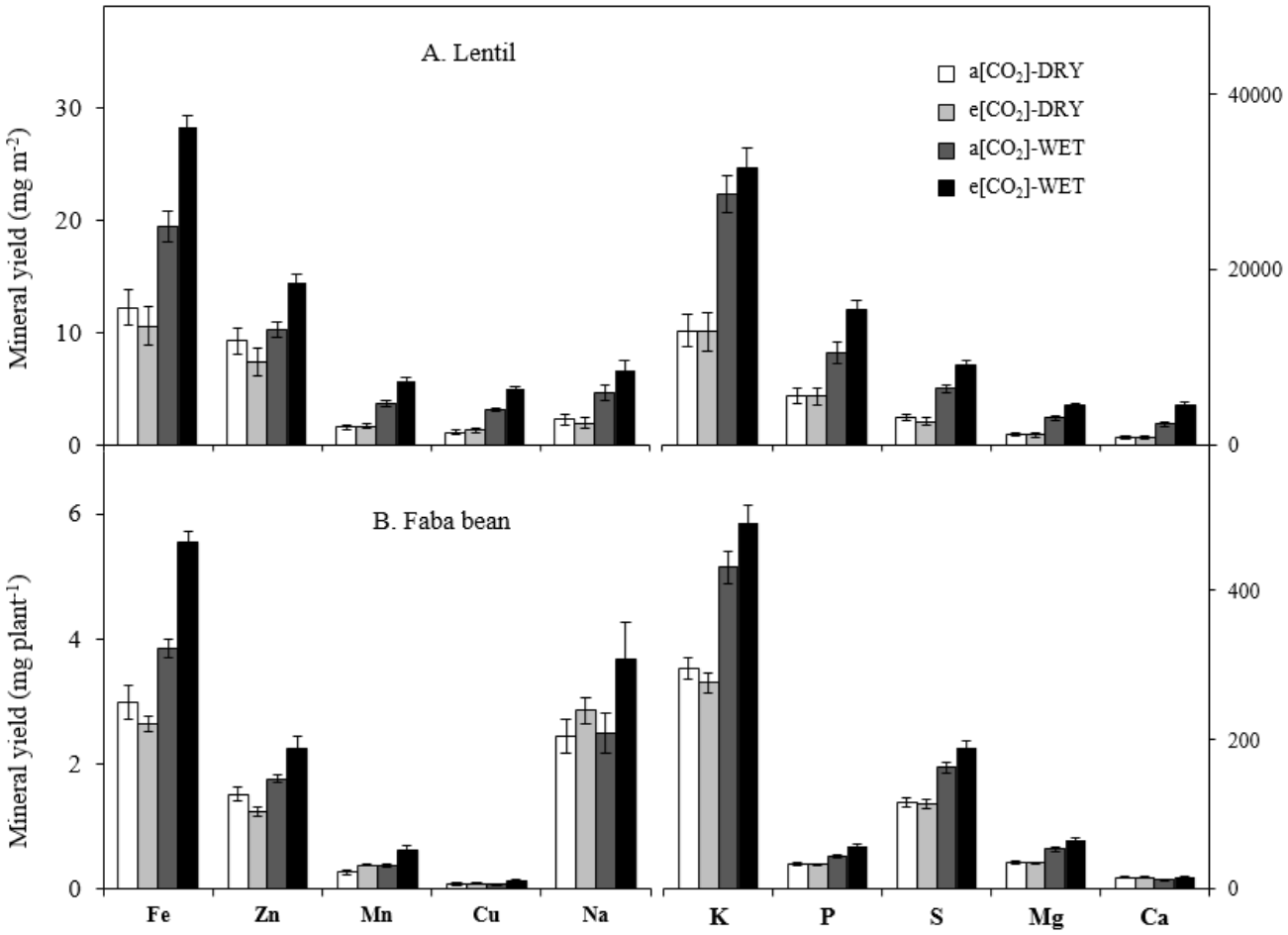


Fig. 8. 3 Grain mineral yield of lentil (A) and faba bean (B) at maturity grown under ambient [CO_2] (a[CO_2],~400 ppm, white bars) or elevated [CO_2] (e[CO_2], ~550 ppm, black bars) in the Australian Grains Free Air CO_2 Enrichment facility, Horsham, Australia during a dry (2015) and a wet (2016) season for lentil and under dry and wet treatment for faba bean. Grains were analysed at maturity. Each bar represents the means and SE of n=8 (lentil) or n=4 (faba bean) replicates. Significant effects are reported in Table 8.4.

T**able 8. 4** Statistical significance (P-values) of grain mineral yield of lentil and faba bean presented in **Figure 8.3.** W denotes water regimes (dry vs wet season for lentil and dry vs wet treatment for faba bean). Non-significant effects are reported as ns (P>0.01).

	P-value		
Mineral yield	$[CO_2]$	W	$[CO_2] \times W$
Lentil			
Fe	0.011	<0.001	0.096
Zn	0.002	<0.002	0.089
Na	<0.001	<0.001	0.096
Mn	0.079	<0.001	ns
Cu	0.021	<0.001	ns
Ca	0.002	<0.001	ns
Mg	0.056	<0.001	ns
K	0.011	<0.001	ns
P	<0.02	<0.001	ns
S	0.012	<0.001	0.068
Faba bean			
Fe	0.001	<0.001	0.016
Zn	<0.001	0.045	0.025
Na	0.097	ns	ns
Mn	0.002	0.002	0.09
Cu	0.068	0.080	ns
Ca	<0.037	0.003	ns
Mg	<0.001	0.003	0.032
K	0.011	0.001	0.029
P	0.018	0.001	0.015
S	0.005	<0.001	0.043

Table 8. 5 Stoichiometric ratio of carbon and mineral elements in grains of lentil and faba bean 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 wet (2016) season for lentil and under dry and wet treatments for faba bean. Grains were analysed at maturity. Means and SE of n=8 (lentil) or n=4 (faba bean) replicates. Unit for [C]: mg g^{-1} dry weight and for other minerals: mg kg^{-1} dry weight.

Stoichiometric ratio	DRY			Wet		
	a[CO_2]	e[CO_2]	% Change at e[CO_2]	a[CO_2]	e[CO_2]	% Change at e[CO_2]
Lentil						
[Fe]: [C]	0.21	0.15	-34.63	0.16	0.15	-7.86
[Zn]: [C]	0.16	0.11	-37.49	0.08	0.07	-6.60
[Ca]: [C]	2.06	1.97	-4.71	1.69	1.72	+1.35
[Mg]: [C]	2.23	2.20	-1.05	2.25	2.18	-3.19
[K]: [C]	24.32	23.30	-4.38	21.12	21.19	+0.33
[P]: [C]	10.71	8.92	-20.00	8.81	8.72	-1.06
[S]: [C]	5.15	4.38	-17.46	4.47	4.31	-3.76
[Na]: [C]	0.04	0.03	-40.67	0.03	0.03	-9.48
[Mn]: [C]	0.03	0.03	-8.67	0.03	0.03	-4.42
[Cu]: [C]	0.02	0.02	-3.82	0.02	0.02	-2.39
Faba bean						
[Fe]: [C]	0.30	0.23	-32.70	0.25	0.22	-11.62
[Zn]: [C]	0.16	0.12	-35.97	0.08	0.07	-11.12
[Ca]: [C]	3.19	2.48	-28.29	2.28	2.30	+0.71
[Mg]: [C]	3.12	2.71	-15.13	2.86	2.70	-5.92
[K]: [C]	28.07	24.27	-15.65	27.70	25.47	-8.74
[P]: [C]	11.43	9.37	-21.91	9.78	9.34	-4.68
[S]: [C]	4.04	3.38	-19.59	3.57	3.37	-5.92
[Na]: [C]	0.24	0.19	-26.72	0.16	0.15	-9.28
[Mn]: [C]	0.03	0.03	-21.85	0.02	0.03	+0.93
[Cu]: [C]	0.01	0.01	-26.68	0.01	0.01	-4.95

8.4 Discussion

In this study, the use of the AGFACE facility enabled us to investigate the changes in grain mineral composition under future climate change scenario. Experiments were conducted under two contrasting water regimes (faba bean) or two contrasting rainfall season (lentil). Consistently, dry growing environments shortened the grain filling period and thereby lowered the grain yield. In contrast, wet growing environments extended the grain filling duration and increased the grain yield 2-3 folds compared to dry growing environments. These typical differences in grain yield between dry and wet growing environments may lead to changes in grain mineral concentration as observed in wheat (Fernando et al., 2014).

In lentil and faba bean, drought increased most of the grain minerals concentrations but decreased mineral yield. The micronutrients "concentration effect" under drought stress has been reported in several studies (Guzmán et al., 2016) A negative relationship between grain yield and concentration is reported previously in wheat (Murphy et al., 2008). These results are in agreement with reports of increased concentration of some grain minerals following drought, whereby reduced photosynthesis has probably led to reduced carbohydrate accumulation in grains (Farooq et al., 2017) as reported in lentil (Parvin et al., 2018).

In Mediterranean-type climates, terminal drought during grain filling period is typically accompanied by higher temperature, which would exacerbate the drought effect on grain yield and quality (Mahrookashani et al., 2017). Decreased stomatal conductance under e[CO_2] may limit transpirational cooling and reduce the potential to mitigate high-temperature effects (Ruiz-Vera et al., 2013). The interactive effect of high temperature and e[CO_2] has been reported to decrease growth and yield in soybean (Heinemann et al., 2006) or leave them unchanged in lentil (Bourgault et al., 2018; Delahunty et al., 2018). In the lentil experiment, heat wave effects were detected in the dry season, especially during the onset of grain filling period (in October 2015), when extremely dry soil (Parvin et al., 2018) was coupled with high temperature (Table 8.1). Compared to the wet season, this would have reduced mineral uptake and deposition into grains. If e[CO_2] exacerbated the heat effect, the extent of grain mineral yield decrease would be greater under e[CO_2]. Results of this study are in agreement with Fernando et al. (2012) who investigated the combined effect of e[CO_2] and high temperature on grain mineral composition of wheat and reported that the reduction of most nutrients was highest at [eCO_2] in their late sowing treatments (which pushed grain filling further into the hotter season).

In addition to the growing environment, e[CO_2] consistently decreased the concentration of grain mineral elements (Fe, Zn, P, S, K, Mg) and the relative decrease of grain [Fe], [Zn], [P] and [S] under e[CO_2] was greater when grown in the drier conditions (either in the dry season or in the dry treatment). Similar observations have been noted previously in field pea and soybean in a meta-analytical study (Myers et al., 2014), although direct experimental investigation of the interactive effect of e[CO_2] and drought has not been previously conducted. Decreased concentrations of other grain minerals under e[CO_2] were also studied in FACE systems, particularly in wheat under irrigated or high rainfall conditions (Högy et al., 2009), but these studies did not assess the potential interaction effect of e[CO_2] and drought. The magnitude of reduction of these grain minerals was in line with reductions in grain protein observed in wheat, soybean, and lentil (Fernando et al., 2012; Gray et al., 2013; Parvin et al., 2018). Similar results were also found for [Zn] in maize kernels and wheat under drought stress (Erbs et al., 2015; Goicoechea et al., 2016).

Several mechanisms may explain the decline of grain mineral concentration under e[CO_2]. For example, decreased stomatal conductance (g_s), as widely observed under e[CO_2] may decrease the uptake of nutrients that are dependent on transpiration driven mass flow (Houshmandfar et al., 2018; McGrath and Lobell, 2013). Greater relative decrease in g_s in dry environmental conditions under e[CO_2] was reported for lentil (Parvin et al., 2018) and also observed for faba bean (Parvin et al., 2019). This mechanism applies directly to drought conditions, where the diffusion rate of nutrients in the soil, uptake by the roots and nutrient transport to the shoots are limited by decreases in g_s and consequently, transpiration rate (Hu and Schmidhalter, 2005). However, reduced mineral uptake under drought is commonly overcompensated by limited C assimilation in grains due to shortening the grain filling duration together with accelerated leaf senescence that led to faster remobilization of minerals to the grains (Etienne et al., 2018), so that mineral concentration in grains increase. However, despite this increase in the concentration of most of the grain mineral elements, e[CO_2] decreased concentrations, so that total mineral yields were significantly lower.

Yield stimulation under e[CO_2] and a concurrent 'dilution effect' has also been described as a potential mechanism decreasing mineral concentration (Poorter et al., 1997). In this study, the decline in mineral concentration occurred in association with yield stimulation under e[CO_2] for both crops (Fig. 8.2). However, the magnitude of this decline was not the same for all elements, for example [Ca] and [Cu] were even positively related to yield in lentil. The greatest decline was observed for [Zn] than [Fe] in both legumes (based on PCA score). Loladze (2002) used the effect size of the CO_2 stimulation of biomass/yield (δ) to predict element dilution [% dilution = $-\delta/(1+\delta)$]. For example, a biomass stimulation of 25% leads to an expected 20% dilution (concentration decrease) for a given element. In our study, yield stimulation by e[CO_2] was greater in the wet (~60%) than the dry (17-24%) growing environments, but in contrast, the relative decrease of grain mineral concentration under e[CO_2] was greater in the dry than the wet environments. Therefore, the hypothesis of this study was partially supported in line with the previous notion that e[CO_2] depression of mineral concentration cannot be completely explained by yield stimulation (Loladze, 2014; McGrath and Lobell, 2013). Mineral composition is closely related to the nutritional status of the plant and affected by mineral deficiency and interaction between minerals (Maillard et al., 2016). For example, S and K deficiencies may increase Mo and Na uptake due to up-regulation of K-transporters that also transport Mo and Na. A better knowledge of how the mineral composition of grains is modified by e[CO_2] and drought requires future experiments in which both drought conditions and mineral nutrient supplies are finely tuned in a growing environment. Moreover, microbial association with legumes particularly mycorrhizal symbiosis under drought conditions may help to alleviate drought stress and improve mineral acquisition in grains, which needed a better understanding under changing environmental conditions i.e. rising [CO_2] and water availability (Baslam et al., 2014; Goicoechea et al., 2016).

Results on the stoichiometric ratio between minerals and C are in agreement with (Loladze, 2002) who reported that increased carbohydrate accumulation and lower nutrient acquisition due to reduced transpiration/mass-flow under e[CO_2] can alter plant stoichiometry, decreasing the ratio between the nutritional and the caloric value of crops and increasing the micronutrient malnutrition problem in human diets. Results of this study further show that this e[CO_2] reduction of (nutritional value):(caloric value) of legume grains might be further aggravated under drought conditions.

In summary, this study confirms that e[CO_2]-induced decrease of grain mineral concentration strongly depended on water availability and that the relative decrease was greater under dry growing environments for Zn, Fe, P, S. In wet environments, the relatively lower decrease in grain minerals concentrations was balanced by a greater grain yield under e[CO_2], resulting in increased mineral yield. In contrast, lower grain yield under dry environments offset the increase in mineral concentration, resulting in decreased mineral yields, this was exacerbated by e[CO_2], especially for Fe and Zn. This is concerning because many legumes are grown in areas of low rainfall or where regular droughts occur (Stefaniak and McPhee, 2015). Large populations in developing countries rely on legumes not only for daily protein but also for mineral intake, especially Fe and Zn. A recent global synthesis study reported that e[CO_2] could cause an additional 175 million people to be Zn deficient and 1.4 billion women and child would lose >4% of dietary Fe, which could increase the prevalence of anaemia by 20% (Smith and Myers, 2018). These results suggest that increasing atmospheric [CO_2] will decrease mineral concentration, most under drought conditions and even if similar dietary energy intake can be achieved, the mineral density of legume grains down under dry conditions will decrease most in a future e[CO_2] atmosphere. Because drought event is expected to increase with high temperature under projected rising [CO_2] environments, which would further intensify the decrease of grain quality.

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Chapter 9: General discussion and conclusion

This thesis investigates N_2 fixation of dryland legumes under predicted future elevated atmospheric [CO_2], including interactions with drought, heat waves, and genotypes. The effect of e[CO_2] on N_2 fixation of legumes was investigated under different growing conditions, and experimental designs using both the Free Air CO_2 Enrichment (FACE) and controlled glasshouse facilities. Key findings are summarized in Fig. 9.1 and discussed below:

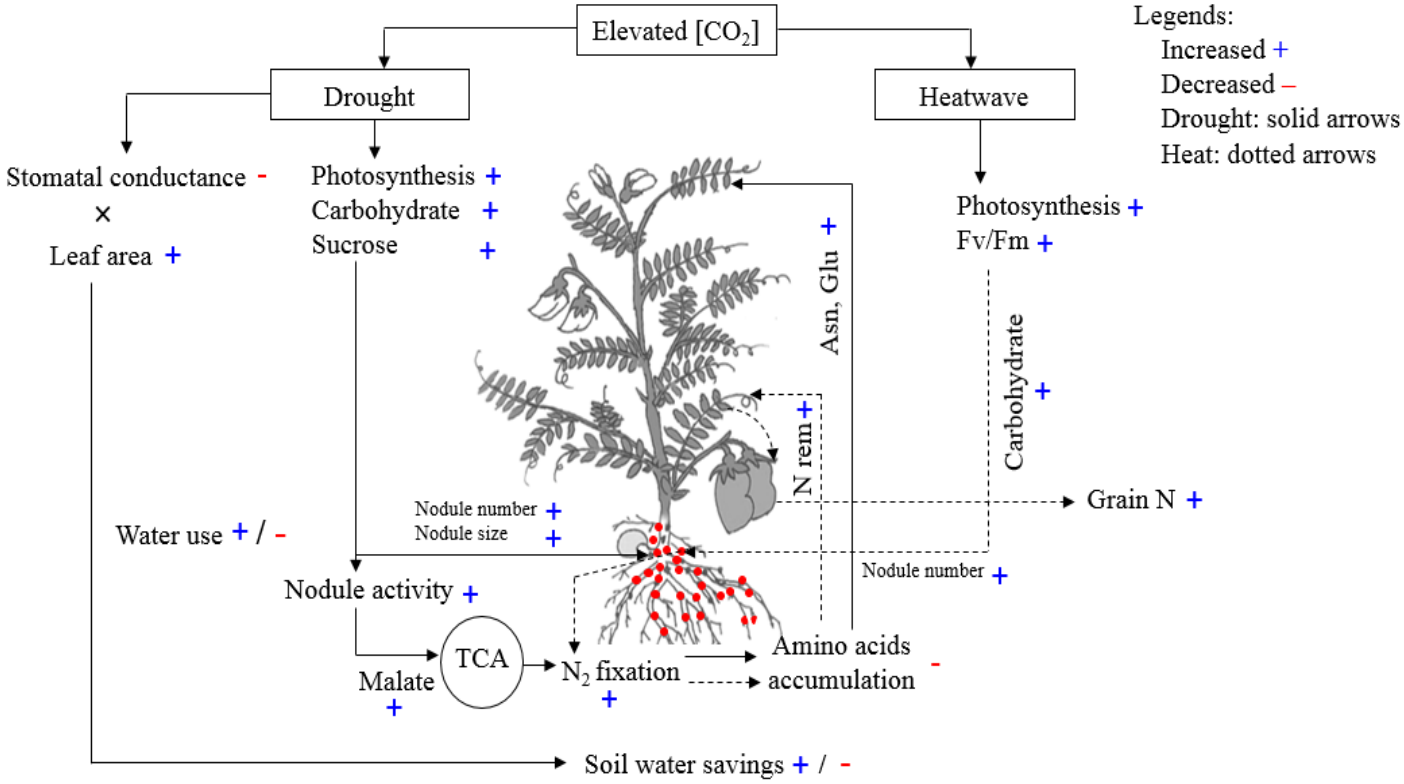


Fig. 9. 1 A summary of the interactive effect of elevated [CO_2] and drought/heat wave on photosynthesis, carbohydrate supply, N_2 fixation and grain N concentration. Greater photosynthesis under elevated [CO_2] increased carbohydrate (in the form of sucrose) supply to nodules, which increased nodule number, nodule size and nodule activity and thus, sustained N_2 fixation mechanism under stress condition. Under drought, elevated [CO_2]-induced reduction of stomatal conductance may conserve soil water by reducing crop water use. However, these effects might be counteracted by leaf area stimulation. Sucrose in nodules converted to malate in the TCA cycle, which provided ATP and energy to fix atmospheric N_2 and synthesized amino acids. Increased N demand under elevated [CO_2] grown plant avoided amino acids in nodules and exported it to the shoot as asparagine (Asn) and glutamine (Glu). Overall mechanisms help to continue N_2 fixation and maintain shoot N demand, leading to avoidance of N_2-feedback inhibition. Under heat, greater photosynthesis and chlorophyll florescence (fv/Fm) provided increased carbohydrate supply to maintain greater nodulation and N_2 fixation process. In addition, faster leaf N remobilization helped to maintain grain N concentration under e[CO_2]. Red negative sign (-) refer to the decrease in relative response under elevated [CO_2] compared to ambient [CO_2], while blue positive sign (+) show an increase in relative response. Solid arrows represent drought effect, while dotted arrows show heat effect.

9.1 Elevated [CO_2] stimulated N_2 fixation of dryland legumes but this effect was transient under stress conditions, and genotypic variability in this response pattern was also detected

Previous findings suggested that e[CO_2] increased nodule size and number, specific nitrogenase activity and consequently increased total N_2 fixation in legumes such as *Trifolium repens*, *Lupinus albus*, *Pisum sativum*, and *Glycine max* (Butterly et al., 2016; Lee et al., 2003; Rogers et al., 2009; Zanetti et al., 1996). Little attention has been given to how e[CO_2] interacts with drought or heat waves to maintain such stimulation.

In accordance with the first objective **(Chapter 1),** this thesis investigated the response of N_2 fixation of three important legumes (lentil, field pea and faba bean) under a wide range of environmental growing conditions **(Chapter 2, 4, 5)**. Consistently, e[CO_2] increased nodule number, nodule biomass, and activity of these legumes which revealed that there was a combination of mechanisms responsible for increasing N_2 fixation under e[CO_2]. Among these three phenomena, nodule activity is most important but highly sensitive to water stress. It has been suggested that decreased stomatal conductance under e[CO_2] delays the onset of drought by conserving soil water (Serraj et al., 1998). Findings of this thesis revealed that conservation of soil water under e[CO_2] was dependant on the intensity and the duration of drought **(Chapter 2, 4, 5)**. As a consequence, e[CO_2] maintained a greater soil water content during relatively short-dry spells through reduced gs (**Chapter 4, 5**) but this effect diminished in the longer term when greater soil water depletion under long-term seasonal drought was even greater under e[CO_2] **(Chapter 2)**. Drought more generally affects carbohydrate supply to nodules, causing accumulation of N-compounds in nodules and reduced nodule permeability for O_2 diffusion (Minchin et al., 1985; Naya et al., 2007). This thesis indicated that increased photosynthesis under e[CO_2] provided greater carbohydrate supply to nodules under short-term drought, which partly maintained N_2 fixation **(Chapter 2, 4)**. Accumulation of N-compounds (i.e., amino acids) was not apparent in e[CO_2] grown legumes, indicating that the greater carbon assimilation under e[CO_2] may have helped to overcome N_2-feedback inhibition (Fig. 9.1).

Another explanation for the avoidance of N-feedbacks under e[CO_2] would be that increased biomass growth under e[CO_2] demands more N, and this increased demand stimulates N_2 fixation when soil N availability is insufficient. Greater biomass and yield response under e[CO_2] was observed when legumes grown in optimum water supply, but drought limited these effects in line with biomass growth; therefore total N assimilation, including N_2 fixation was reduced **(Chapter 2, 4, 5)**. Finally, this thesis work provides further evidence that e[CO_2] maintained greater N_2 fixation even under drought, but the stimulatory effect was aligned with biomass stimulation, and this was strongest under sufficient soil water availability.

In addition to drought, heat waves are likely to be encountered by dryland crops under projected future climate. Combined e[CO_2] and heat wave treatments showed that e[CO_2] mitigated the deleterious effect on N_2 fixation by the heat wave. Because of greater CO_2 assimilation, e[CO_2] maintained greater nodulation response following a heatwave. As a result, N_2 fixation was greater in e[CO_2]-grown lentil than a[CO_2] grown ones when both were exposed to a heatwave (**Chapter 6**).

The capacity for total N_2 fixation in response to e[CO_2] and water availability varied among lentil genotype; one genotype, PBA Ace had the most significant increase in N_2 fixation under e[CO_2] in a dry season (**Chapter 2**). In contrast, a comparison genotype, HS3010 did not exhibit any increase in N_2 fixation under e[CO_2] during the dry season. In the wet season, both genotypes showed similar stimulation of N_2 fixation under e[CO_2]. The observed genotypic variability in this thesis revealed that the pattern of N accumulation for lentil was proportional to dry matter accumulation, which suggests a tight coupling between net photosynthesis and N accumulation or N_2 fixation. Furthermore, e[CO_2]-derived greater biomass accumulation and nodulation capacity of one genotype even in the dry season further confirmed that this genotype outperformed the comparison one in e[CO_2] environments. This study reveals that genotypic variability in response to N_2 fixation under e[CO_2] and drought is existed. Since this thesis investigated only one or two genotypes, large scale screening of genotypes to identify the traits which can maintain N_2 fixation longer by exploiting potential water savings mechanism and confer superior benefit under future climatic conditions warrants further research.

9.2 Elevated [CO_2] maintained grain N concentration by extending N_2 fixation through grain filling period under optimum soil water availability, or by accelerating N remobilization under a heat wave

Under e[CO_2] grain quality changes; most importantly grain nitrogen concentration ([N]) and therefore protein concentrations decrease, particularly in cereal, raising concerns about a decrease in nutritional quality. However, legumes have the potentiality to overcome this deterioration of grain [N], through stimulation of N_2 fixation under e[CO_2] (Gray et al., 2013).

In order to address the second objective of this thesis (**Chapter 1**), changes in grain [N] of lentil and faba bean was investigated under the interaction of e[CO_2] and drought. Elevated [CO_2]-induced stimulation of N_2 fixation maintained grain [N] of both lentil and faba bean **(Chapter 2, 5**) only when provided with well-watered conditions. However, under drought conditions, the effect of e[CO_2] was in line with some earlier studies in a semi-arid environment, which showed small but significant decreases in grain [N] (Bourgault et al., 2017; Bourgault et al., 2016). N_2 fixation and allocation patterns **(Chapter 2, 5)** showed that N_2 fixation contributed to the highest proportion of seed N, which is in line with reports on soybean and clover under e[CO_2] (Li et al., 2017; Zanetti et al., 1996). However, there was no effect of [CO_2] on soil N uptake **(Chapter 2, 4, 5)**, consistent with previous findings (Guo et al., 2013; Luscher et al., 2000).

When drought decreases N_2 fixation and soil N uptake grain N demand usually meet by translocation of N assimilated before reproductive phases (Farooq et al., 2017). Consistently, in both lentil and faba bean, remobilization provided about 70-80% of grain N (with a major portion derived from fixation), similar to wheat N remobilization at post-anthesis under limited water-limited environments (Palta et al., 1994). Remobilization was unaffected by e[CO_2], while it was accelerated by drought. However, such increased remobilization of N was not sufficient to maintain grain [N] under e[CO_2] resulting in decreased in grain [N]. In contrast, e[CO_2] maintained grain [N] under well-watered conditions or in a wet growing season when N_2 fixation was extended throughout the grain filling period (**Chapter 2, 5**).

In addition to drought, a heat wave decreased grain [N] of lentil (**Chapter 6**), which may be associated with a reduction in N assimilation/translocation into grains (Sita et al., 2017). Heat wave reduces N_2 fixation and shortens the grain filling period and these both processes negatively affect grain [N] (Sehgal et al., 2017). A decrease was also found in N remobilization in lentil exposed to heat stress. Interestingly, e[CO_2] interacted with the heat wave to restore grain [N] to higher levels than observed under a[CO_2]. This might be related to e[CO_2]-induced changes in not only N_2 fixation but also N remobilization patterns in lentil subjected to heat waves. This thesis work showed that e[CO_2] accelerated leaf N remobilization under heat wave **(Fig. 9.1)**, which offset the decrease of grain [N] in lentil (**Chapter 6**). This is in agreement with a previous report on wheat from the AGFACE site, where e[CO_2] interacted with heatwave to maintain grain N/protein concentration (Macabuhay et al., 2018). The increase of grain [N] under heat and e[CO_2] might be associated with reductions in the starch deposition to grain, which influences grain [N] by allowing more N per unit of starch as observed in soybean (Thomas et al., 2003).

9.3 Elevated [CO_2] increased C-supply to nodules in well-watered plants and modified C- and N- metabolisms to mitigate the effect of drought on N_2 fixation

Tight coupling between assimilate consumption/export and stimulation of photosynthesis/N_2 fixation has been reported previously (Ainsworth and Bush, 2011; Fischinger et al., 2010; Ribeiro et al., 2012). C partitioning towards nodules would contribute to overcoming leaf carbohydrate build-up, with the consequent avoidance of photosynthetic acclimation (Aranjuelo et al., 2014). Increased C availability in nodules under e[CO_2] may enhance the nodule activity and in turn supply more N for plant growth (Guo et al., 2013; Irigoyen et al., 2014).

To address the third objective of this thesis (**Chapter 1**), the relationship between C-supply and stimulation of photosynthesis/N_2 fixation were evaluated by exposing plants to $^{13}CO_2$ pulse-labeling (**Chapter 7**). Results of this experiment showed that well-watered plants under e[CO_2] accumulated a greater amount of ^{13}C and transported to nodules. In association with greater ^{13}C export to nodules, both photosynthesis and N_2 fixation were stimulated. Under drought conditions, e[CO_2] although increased the accumulation of ^{13}C in leaves but failed to effectively export it to the nodules, which indicates poor sink strength of nodules. The poor sink capacity/activity may lead to increased leaf carbohydrate concentration under e[CO_2] growth conditions (Ainsworth et al., 2004; Rogers et al., 2004), which then can act as a signal to down-regulate the capacity of the photosynthetic apparatus. Consistent with these findings, in this study photosynthetic down-regulation was observed under e[CO_2] grown faba bean only when exposed to drought conditions.

Exchange of C and N metabolites between bacterial symbionts and host plants is considered as the crucial factor of controlling symbiotic N_2 fixation (Voisin et al., 2003). In this study, alterations in the C-and N-related metabolism and their linkage with stimulation of photosynthesis/N_2 fixation under the interactive effect of e[CO_2] and seasonal drought was investigated in lentil (**Chapter 3**). Results of this chapter demonstrated that e[CO_2]-derived stimulation of photosynthesis/N_2 fixation was highly dependant on the availability of soil water and can be explained with the abundance/ depletion of carbohydrate, organic acids and amino acids related metabolites in leaves and nodules. For example, dry season increased the abundance of metabolites related to carbohydrate metabolism (sucrose, trehalose, mannose, galactose) but reduced the abundance of organic acids (malate, succinate, aspartate) and amino acid (asparagine, glutamine, glycine) in leaves. This response is also opposite to

what was observed in nodules, where the amounts of sugars decreased but increased the abundance of a few amino acids (asparagine, glutamine, GABA, proline). In the present study, the nature of the metabolites accumulated in leaves and nodules were similar to what has been frequently reported under drought conditions (Silvente et al., 2012). Studies reported that e[CO_2] moderated the effects of drought on sugar and amino acid metabolism (Zinta et al., 2018), because e[CO_2] led to better conservation of soil water, and thus maintained the activities of the Calvin cycle, for longer during drought (De Souza et al., 2015). This thesis reported that e[CO_2] increased the concentration of sucrose and trehalose in leaves, suggesting that e[CO_2] grown plant increased their osmotic adjustment to the lower water potentials. On the other hand, increased sucrose in leaves under e[CO_2] may be related to photosynthetic acclimation of e[CO_2] grown lentil as observed in the dry season.

In addition, N_2 fixation is regulated by the availability of sucrose to the bacteroids, which determines nodule activity (Larrainzar et al., 2009). Elevated [CO_2] increased the supply of sucrose to nodules with greater extent in the wet season than in the dry season (**Chapter 3**). This may explain greater malate concentration into the TCA cycle, that fuelled nodule rhizobia and greater stimulation of N_2 fixation in the wet season. The reduction of N_2 fixation under drought is also associated with accumulation of several amino acids, for example, N-compounds such as asparagine, glutamine or ureides have been reported to induce N_2 feedback process (Aranjuelo et al., 2013; Serraj et al., 1999). In nodules, an increased abundance of asparagine and glutamine in the dry season indicated feedback inhibition of N_2 fixation, but the accumulation of asparagine was lower under e[CO_2] grown lentil **(Fig. 9.1)**. Increased level of glutamine together with proline under e[CO_2] during this season suggested that the shift in carbon partitioning to glutamic acid metabolism. Because glutamine and proline can be synthesized from the same glutamic acid substrate, playing an important role in protecting plants during different environmental stresses (Gil-Quintana et al., 2013). Besides, there was an increased abundance of osmoprotectant (i.e., trehalose, mannitol, inositol) under e[CO_2] in the dry season, suggesting that e[CO_2] induced changes in C-and N-metabolites increases legume tolerance to drought.

9.4 The decrease of grain mineral concentration under elevated [CO_2] was lower when soil water was sufficient

In order to support the fourth objective of this thesis, grain mineral quality under the interactive effect of e[CO_2] and drought was investigated (**Chapter 8**). Consistent with the findings on wheat grain from the AGFACE site, e[CO_2] consistently decreased the concentration of grain mineral elements (Fe, Zn, P, S, K, M) in lentil and faba bean. Similar observations have been noted previously in field pea and soybean in a meta-analytical study (Myers et al., 2014), although direct experimental investigation of the interactive effect of e[CO_2] and drought has not previously conducted. In this thesis, there was significant interaction between e[CO_2] and drought for several grain mineral elements which confirmed that the relative decrease of grain [Fe], [Zn], [P] and [S] under e[CO_2] was more prominent when grown in the drier conditions (either in the dry season or the dry treatment). Similar results were also found for [Zn] in maize kernels and wheat under drought stress (Erbs et al., 2015; Goicoechea et al., 2016).

Yield stimulation under e[CO_2] and a concurrent 'dilution effect' has also been described as a potential mechanism decreasing mineral concentration (Poorter et al., 1997), but the results of this study did not fully match this overall mechanism. Because, yield stimulation by e[CO_2] was higher in wet (~60%) than dry (17-24%) season, but in

contrast, the relative decrease of grain mineral concentration under e[CO_2] was more prominent in the dry than the wet environments. Also, [Cu] and [Ca] increased under e[CO_2] in line with increasing grain yield, suggesting that the process of mineral deposition into developing grain is limiting or highly variable with the type of minerals. This result partially supports the previous notion that e[CO_2] depression of mineral concentration cannot be entirely explained by yield stimulation (Loladze, 2002) and complement with a recent report on wheat from AGFACE site (Houshmandfar et al., 2018). Minerals accumulation in seed depends on many factors such as their availability into soil water then uptake by root through mass flow or diffusion into plants and finally translocated to the growing seeds (Etienne et al., 2018). Both e[CO_2] and drought alone or in combination may affect these uptake or translocation pathways (McGrath and Lobell, 2013). Lower depletion of grain mineral concentration under e[CO_2] during the wet season/well-watered conditions might be associated with increased mineral uptake or increased amount of mineral deposition into grains. Exactly which mechanism can regulate grain mineral quality under the rising [CO_2] environments will be an important focus for future research.

9.5 Potential limitation of the study and future recommendation

The results in thesis are limited to one or two genotypes for all investigated legume species and environmental growing conditions is also limited to one location (Horsham). Genotypic variability in response to the 'CO_2 fertilisation effect' has been reported for legumes (Bishop et al., 2015; Ziska et al., 2016), which can assist to select germplasm with more responsive of nodule attributes and consequently N_2 fixation under e[CO_2]. All the experiments in this thesis deployed either only one or two genotypes, which made it impossible to draw explicit conclusions about genotypic variability in CO_2-responsiveness. Therefore, pre-screening amongst wide range of germplasm to detect e[CO_2]-responsive genotypes is suggested for future studies (Shimono et al., 2019).

In this thesis, root growth and root biomass were investigated under glasshouse and FACE facilities in response to e[CO_2]. Extraction of roots under field condition is extremely labour intensive and very destructive (Kimball et al., 2002). In addition, due to smaller plot size used within the FACE rings, root growth might be affected (Poorter et al., 2012). The precision of root attributes (biomass and length) reported in this thesis is relatively low (Cai et al., 2018). It is therefore, highly recommended to examine root attributes under natural field condition utilizing both non-destructive and destructive techniques to not only obtain data with greater precision but to better understand the whole-plant responses to e[CO_2].

Most climate change scenarios predicted reduction of rainfall and rising temperature in the future (IPCC, 2014). While experimentation on e[CO_2] generally conducted in interaction with either drought or heat wave, it is difficult to predict the real situation, i.e., "triple whammy" (elevated [CO_2], drought and higher temperature) challenges as anticipated to occur together in most of the climate change scenarios. For example, the interactive effect of e[CO_2] (with target [CO_2] both for 2050 and 2100) and drought on N_2 fixation and soil water dynamics have been extensively studied in this thesis. Increasing temperature accelerates leaf gas exchange (Bagley et al., 2015), shortens crop phenological growth (Ruiz-Vera et al., 2018) and affects N_2 fixation mechanisms (Aranjuelo et al., 2007). Variation of these physiological and biochemical responses may significantly affect N acquisition patterns, which is not well-thought-out in this thesis. Therefore, investigating the interactions of these triple challenges is crucial to understanding N_2 fixation mechanisms under a changing climate environment.

9.6 Conclusion

In summary, e[CO_2] stimulated N_2 fixation of legumes through increased nodule number, nodule biomass, and nodule activity. However, this stimulation was highly dependent on the availability of soil water, leading to greater effect under well-watered than drought conditions. Decreased stomatal conductance under e[CO_2] did not conserve soil water because of accelerated biomass/leaf growth, which intensified the effect of drought. Metabolite profiling showed that decreased N_2 fixation under drought was associated with increased level of asparagine, and glutamine in the nodules but the accumulation of asparagine was slightly lower under e[CO_2] grown plants, which helped to avoid N_2-feeback inhibition. In addition, enhanced accumulation of trehalose, inositol, mannitol, proline under e[CO_2] could have offered protection from drought. The depletion of grain quality especially grain [N] and other mineral elements was not apparent in legumes under e[CO_2] when sufficient soil water provided. Differential responses for the two genotypes to the interactive effect of e[CO_2] and water regimes suggests a potential breeding opportunity to maximise the benefit from 'CO_2 fertilisation effect'. Maximising the benefits of legume N_2 fixation from the 'CO_2 fertilisation effect' is essential to increase food security and improving the soil fertility under future climate scenarios.

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