INFLUENCE OF CARBON AVAILABILITY ON CARBON AND NITROGEN DISTRIBUTION IN NODULATED PLANTS

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von MSc. **Gabriela Alejandra Pereyra Ojeda**

geboren am 24.03.1983 in Caracas, Venezuela

Gutachter:

1. Prof. Dr. Beate Michalzik
   Institut für Geographie, Friedrich-Schiller-Universität, Jena, Deutschland
2. Prof. Dr. Susan Trumbore
   Max-Planck-Institut für Biogeochemie, Jena, Deutschland

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List of abbreviations

The following list summarizes the abbreviations mentioned in this thesis:

$[CO_2]$ Atmospheric carbon dioxide concentration

$\delta^{13}C$ Carbon isotopic ratio

$\delta^{15}N$ Nitrogen Isotope ratio

A_{max} Photosynthetic rate at light saturation

AG Aboveground plant tissues

BG Belowground plant tissues

Ci/Ca Intercellular-to-atmospheric CO_2 concentration ratio

CO_2 Carbon dioxide

E Transpiration

g_s Stomatal conductance

g_m Mesophyll conductance

J_{max} Maximum rate of electron transport

LAR Leaf area ratio

NAR Net assimilation rate

NSC Non-structural carbohydrates

PPFD Photosynthetic photon flux density

R_{Dark} Dark respiration

R_{Light} Light-inhibited respiration

RGR Relative growth rate

RubisCO Ribulose-1,5,-bisphosphatecarboxylase enzyme

RuBP Ribulose-1,5,-bisphosphate

SD Standard deviation

SE Standard error

TPU Triophosphate use

Vc_{max} Maximum rate of carboxylation

Vo Photorespiration

Vo/ Vc Oxygenation-to-carboxylation ratio

VPD Vapor pressure deficit

CHAPTER 1

Introduction

1.1. Motivation

During the late Pleistocene (approximately 11,700 years ago) and prior to the modern Industrial Era, the atmospheric CO_2 concentration ($[CO_2]$) was relatively stable and between 180-200 ppm (Tripati *et al.*, 2009). By contrast, since the beginning of industrialization, atmospheric $[CO_2]$ has increased by a rate averaging 0.9 ± 0.1 ppm $year^{-1}$ (NOAA, 2016), and is expected to further increase with fossil fuel burning.

Rising atmospheric $[CO_2]$ has increased global temperatures, and as levels continue to increase, global climate change is predicted to alter patterns of precipitation as well. In some regions, precipitation is expected to increase, and in others to decrease (IPCC, 2014). Changes in climate will alter plant physiology and carbon (C) dynamics (Sevanto & Dickman, 2015). For example, a direct effect is that elevated $[CO_2]$ levels increase photosynthesis and carbon (C) storage in terrestrial ecosystems (IPCC, 2014), but a sustained increase in productivity at elevated $[CO_2]$ also requires an enhanced nutrient supply to match the increase in C storage (Pastore *et al.*, 2016). This is confirmed in short-term experiments, where elevated $[CO_2]$ enhances photosynthesis and biomass growth during initial years, but progressive nitrogen (N) limitation ultimately constrains growth (Luo *et al.*, 2004). However, the impact of increased atmospheric $[CO_2]$, changes in nutrient availability and more frequent droughts may differ depending on plant nutrient acquisition strategies (van de Kooi *et al.*, 2016).

Legumes have the ability to use some of the C they fix to obtain N through symbiosis with N-fixing, nodule-inducing bacteria, collectively known as rhizobia. It has been hypothesized that under elevated $[CO_2]$ legumes will have a competitive advantage over other non N-fixing plant functional types, because they can 'spend' the excess C to obtain a potentially limiting nutrient (Rogers *et al.*, 2009). Nevertheless inefficient symbiotic associations may still be constrained by C and N availability within the symbiotic system, or by the ability to use available resources, at least under detrimental environmental conditions, and/or reflecting developmental constraints (Libault, 2014, Tharall *et al.*, 2000, Voisin *et al.*, 2010). From a plant's physiological perspective, future scenarios of

environmental change raise a fundamental question: how will changes in the environmental conditions affect the balance between C allocation, symbiotic N exchanges, and plant growth?

Plant C allocation to rhizobia and N supply to plants by rhizobia are often studied independently. In this thesis, I address the interactive effects of C allocation and symbiotic N exchanges in nodulated plants with rhizobia symbiosis. First, I explore the effect of rhizobium strains on plant development and drought response for a common tropical legume plant. Second, I investigate changes in plant growth and development as a function of [CO_2], for a species (*Medicago sativa* L.) that grows more readily in greenhouse conditions in Germany.

1.2. Plant C allocation

Plants assimilate C during photosynthesis and redistribute the resulting photosynthates (e.g., nonstructural carbohydrates) across plant organs and functional C sinks including respiration, growth, defense, and storage (Hartmann & Trumbore, 2016). Within the plant, C allocation is determined by changes in source-sink relationships in response to C availability, which in turn, is regulated differently depending on plant functional type, plant development, and also environmental factors, such as atmospheric [CO_2], water availability, irradiance, temperature, etc. (Carbone & Trumbore, 2007, Dickson, 1991, Lemoine *et al.*, 2013).

Measuring the extent of regulation of environmental factors on plant C-availability and its impact on C-allocation is very challenging (Sevanto & Dickman, 2015). Litton *et al.* (2007) describes three different perspectives of how C allocation can be addressed in forests: biomass, flux, and partitioning. These perspectives can also be used at the individual scale: biomass is the amount of material present at a given time; flux is the C inflow-outflow dynamic per unit of time; and partitioning measures how photosynthates (net C inflow) are allocated among different specific C sinks (Sevanto & Dickman, 2015). At the individual plant scale, these sinks include respiration, growth of roots, shoots, or leaves, transfer of fixed-C to microbial symbionts or the rhizosphere, and production of defense or storage compounds. Storage can act as a buffer for times when C supply and demand are out of balance (Hoch, 2015). Under stress that limits the inflow of C, such as drought or low [CO_2], it is also important to distinguish the degree to which plants meet metabolic and sink demands from recent photosynthates versus redistribution of C stores. Such changes in allocation patterns have been studied using C stable isotopes ($^{13}CO_2$) in pulse or long labelling

experiments (Blessing *et al.*, 2015, Epron *et al.*, 2012) through tracking the fixed isotope label into respiration fluxes, tissue growthn and storage compounds.

Plant C allocation can be even more complex when plants are not only distributing C across tissues or internal functional sinks but also exchanging with symbionts (Hartmann & Trumbore, 2016). Understanding the patterns of C allocation in legumes is interesting because changes in allocation may influence N-fixation in root nodules, which represent an additional sink that might change its strength in response to changes in C fixation or growth (Aranjuelo *et al.*, 2014, Fischinger & Schulze, 2010, Minchin & Witty, 2005). Observed legume responses to changes in C availability linked to rising atmospheric [CO_2] or drought include changes in C assimilation and C uptake, and altered biomass or nonstructural carbohydrate partitioning between above- and-belowground tissues (Ainsworth & Long, 2005, Larrainzar *et al.*, 2014). Yet, little is known about plant response to very low [CO_2], as in past glacial periods (Gerhart & Ward, 2010), and how plant responses in low [CO_2] conditions might inform how stressed plants respond to C limitation. This is particularly relevant in learning the evolutionary adaptations of legumes in terms of growth and development to C availability, and in this way, to understand and predict the responses of modern legumes to rising atmospheric [CO_2] (Liu *et al.*, 2016).

1.3. Biological nitrogen fixation

Legumes are a broad and common classification for plants of the *Fabaceae* family. Legume species, including beans, lentils, peas, or peanuts, are among the most important agricultural crops worldwide (Aranjuelo *et al.*, 2014). They also play an important role in ecosystem productivity by increasing overall N availability. Legumes are often considered 'pioneer' plants that can colonize disturbed areas and are often used in restoration plantations to mitigate land degradation (Miles *et al.*, 2006). Roughly 20,000 legume species are able to establish symbiotic associations with nodule-inducing *Rhizobiaceae* bacteria (Rogers *et al.*, 2009).

In legumes, N-fixation takes place mostly in root-nodules. The formation of a root nodule is preceded by an elaborate molecular courtship and the controlled infection of the host plant cell by rhizobia (Rogers *et al.*, 2009). Two main types of nodules have been described in legumes, determinate and indeterminate. Determinate nodules lose meristematic activity shortly after initiation, thus growth is due to cell expansion resulting in mature nodules that are spherical in

shape. Indeterminate nodules maintain an active apical meristem that grows proportionally to C supply. Generally, this results in nodules with cylindrical shape, which may be extensively branched (Monahan-Giovanelli *et al.*, 2006) (Figure 1.1a).

Within the nodules, the bacteria are isolated from the infected host by the symbiosome membrane that regulates the resource exchange between both symbionts (i.e., bacteroids and plant) by forming a diffusive barrier that also limits the entry of O_2 (Figure 1.1b) (Day *et al.*, 2001). Sucrose (Suc) transported into the root system by the plant via the phloem is cleaved in the infected plant cells via the glycolytic pathway to produce phosphoenolpyruvate (PEP). From PEP, malate is produced and the C supply to the bacteroid achieved via oxaloacetate (OAA) (Figure 1.1b). Within the bacteroids, malate is oxidized by the tricarboxylic acid cycle (TCA) to provide reducing equivalents for the nitrogenase complex and for the respiratory chain (RC) that fuels the nitrogenase complex with ATP. The nitrogenase enzyme complex at the bacteroid converts atmospheric N_2 to NH_3 (N_2 + 8 H^+ + 8e^- + 16 ATP→ 2 NH_3 + H_2 + 16 ADP +16 P_i), a reaction that is quite sensitive to the presence of O_2. The resulting ammonium (NH_4^+) is then assimilated via the xylem, converted to asparagine via the asparagine synthase (AS) pathway, and exported into the root xylem. The presence of high concentrations of leghemoglobin in the infected plant cell, which gives active nodules their characteristic pink color, increases the flux of O_2 through the cytoplasm and controls the concentration of free O_2 available to the respiratory chain in the bacteroid (Figure 1.1b).

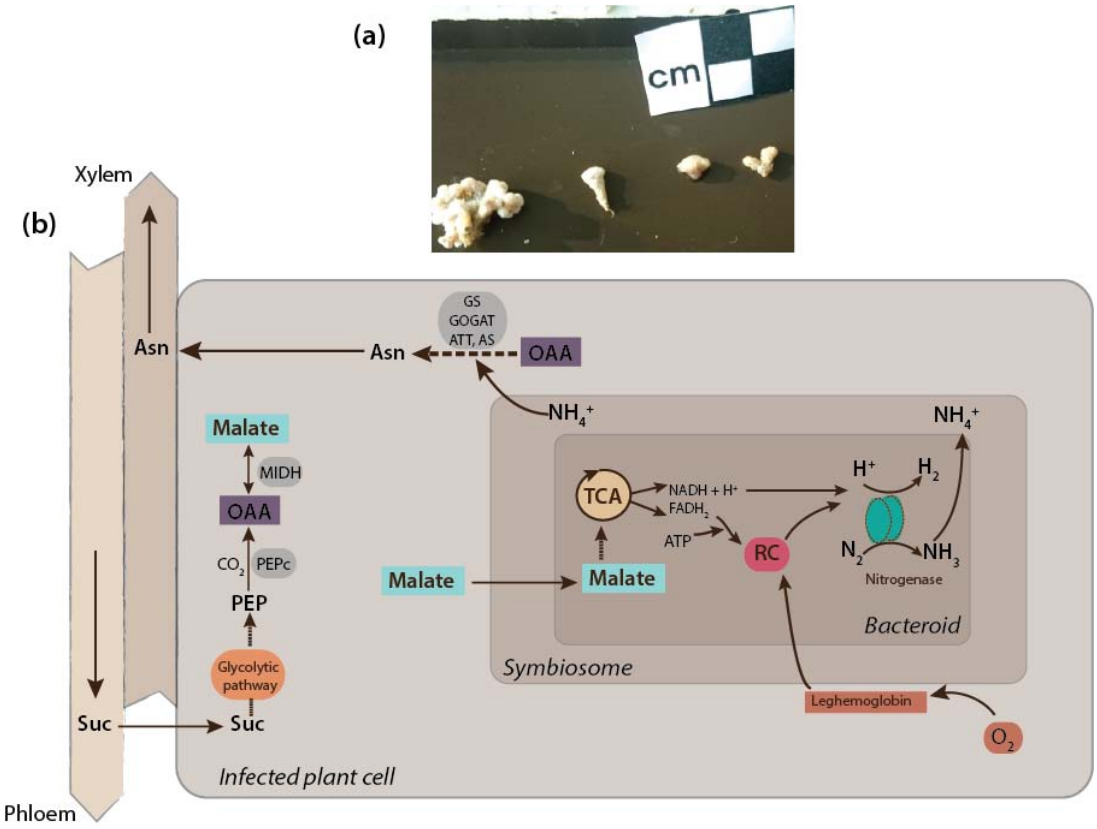

Figure 1.1. (a) Indeterminate nodules harvested from *Medicago sativa* plants inoculated with *Ensifer meliloti*, and (b) Schematic representation of an indeterminate root nodule showing the symbiotic exchange of C and N between the plant and rhizobia. The involved enzymes are mentioned in the text, except: MDH, Malate dehydrogenase GS, glutamine synthetase; GOGAT, glutamate synthase; AAT, aspartate. Modified after Fischinger (2009) and Rogers *et al.* (2009).

1.4. Processes conditioning N-fixation in legumes under changing environmental conditions

Given the large energy requirement of the N-fixation reactions this process relies on the supply of photosynthates by the plant (Hardy & Havelka, 1976). Moreover, studies have shown that, under different environmental conditions, such as elevated CO_2 and low water availability, altered photosynthetic rates change the C supply to the bacteroid, and in turn, might alter nitrogenase activity (Ariz *et al.*, 2015, Larrainzar *et al.*, 2014).

Drought and changes in atmospheric [CO_2] can potentially affect allocation to rhizobia through mechanisms associated with C supply. Drought has been shown to temporarily increase NSC in above-ground tissues through a decline in sink activity. In studies with *M. sativa* and *M. truncatula* plants, sinks related to N fixation were maintained despite reduced photosynthetic activity

(Aranjuelo *et al.*, 2011, Larrainzar *et al.*, 2009). Additionally, under elevated [CO_2] total fixed N did not increase in *M. sativa* despite a potentially higher C supply to the nodules (Ariz *et al.*, 2015). These results suggest that plants under changing environmental conditions adjusted their C supply to sustain nodule catabolism according to plant N demand (Aranjuelo *et al.*, 2013).

Plant responses to elevated [CO_2] have been extensively studied, especially in crops and forage legumes, such as *Glycine max* (soy bean) and *M. sativa* (alfalfa) (Ainsworth *et al.*, 2002, Aranjuelo *et al.*, 2014). Responses observed at elevated [CO_2] in N-fixing plants may affect C-assimilation, NSC content in aboveground and belowground tissues and nodule biomass (Aranjuelo *et al.*, 2014, Ariz *et al.*, 2015, Rogers *et al.*, 2009). On the other hand, the effect of elevated [CO_2] on specific N-fixation rates and/or the total amount of N fixed per plant is variable (Aranjuelo *et al.*, 2014). The regulation of specific N-fixation rates has been attributed to up or-down regulation of organic acid supply involved in respiration mechanism by the bacteroids (Figure 1.1b) and consequently, nitrogenase activity. The different responses of legume N-fixation rates to increased CO_2 observed in these studies may therefore be due to different environmental conditions, plant species, and bacteria strains (Aranjuelo *et al.*, 2008, Fischinger & Schulze, 2010).

1.5. Thesis outline and research aims

Including the introduction (Chapter 1), this thesis comprises six chapters, in which I examine the influence of C availability on plant and rhizobia C and N pools in different rhizobia-legume associations.

A unique feature of this study is that I forced legume plants to gain N solely from symbiotic exchanges with rhizobia, i.e., plants were forced to spend NSC to rhizobia in order to obtain N. The N-fixing bacteria used in this thesis produced indeterminate nodules, therefore, growth and development of both plants and rhizobia were regulated by the C and N trade-off between them.

This manipulative experimental design, used in two separate studies made it possible to explore the importance of environmental factors such as drought and atmospheric [CO_2] on C allocation and overall N-gain in different rhizobium-legume associations.

In **Chapter 2**, I evaluated the influence of the identity of the rhizobium strain on overall biomass and total C and N content in plant tissues and determined how these parameters change during a short but intense drought.

We hypothesized that during seedling establishment and drought, the identity of the rhizobium strain would affect the overall amount of N fixed by the plants. We compared two different legume-rhizobia associations to test if *Mesorhizobium loti* or *Rhizobium tropici*, would affect the overall amount of N fixed by *Leucaena leucocephala* plants during seedling establishment.

We also hypothesized that drought would limit plant growth and nodule energy supply to varying degrees in the different *Leucaena*-rhizobia associations, i.e., the degree of down-regulation of N-fixation due to reduced C availability.

Additionally, **Chapters 3, 4, and 5** present the results of a series of experiments where the symbiotic association studied was *Medicago sativa* and *Ensifer meliloti* (*Sinorhizobium meliloti*) (Figure 1.2). In these experiments, I manipulated C supply by growing the plants under controlled atmospheric [CO_2] (Figure 1.2). The main aims in these series of experiments were to:

1. Understand changes in plant C allocation to growth and overall N-gain in response to different atmospheric [CO_2].

   We hypothesized that plant growth rates would increase with atmospheric [CO_2], but overall growth would be related to tissue N content, and thus, N-fixation.
2. Identify the influence of atmospheric [CO_2] on leaf-level physiological processes, like photosynthesis and respiration, as well as light inhibition of respiration and photorespiration, but also total N and amino acids concentrations and non-structural carbohydrates in plant leaf and root tissues.

   We hypothesized that, at low [CO_2], the maximum carboxylation rates will decrease due to increments in the oxygenation-to-carboxylation ratio of RubisCO. Moreover, the relation between respiration rates measured in the light and respiration rates measured in the dark would be greatest in plants with higher oxygenation-to-carboxylation ratios of RubisCO, likely low [CO_2] plants.

   However, at low [CO_2] levels, more of the fixed C would be allocated belowground to get N, resulting in higher root non-structural carbohydrate (NSC) concentrations and specific root respiration rates (respiration per unit biomass). Thus, NSC concentration in low [CO_2] leaves may reflect, in part, greater C allocation and transport to root tissues.
3. Investigate, using a whole-plant approach, how different atmospheric [CO_2] influences

allocation to other sinks, like respiration, as well as storage, and if potential differences in C allocation patterns could be explained by the C cost of symbiotic N-fixation.

We hypothesized that plants grown at low [CO_2] would assimilate overall less C, but would allocate proportionally more C to respiration, particularly to belowground tissues in order to maintain N-fixation.

All results and achievements of this thesis are finally summarized and discussed in Chapter 6, together with an outlook for future research perspectives.

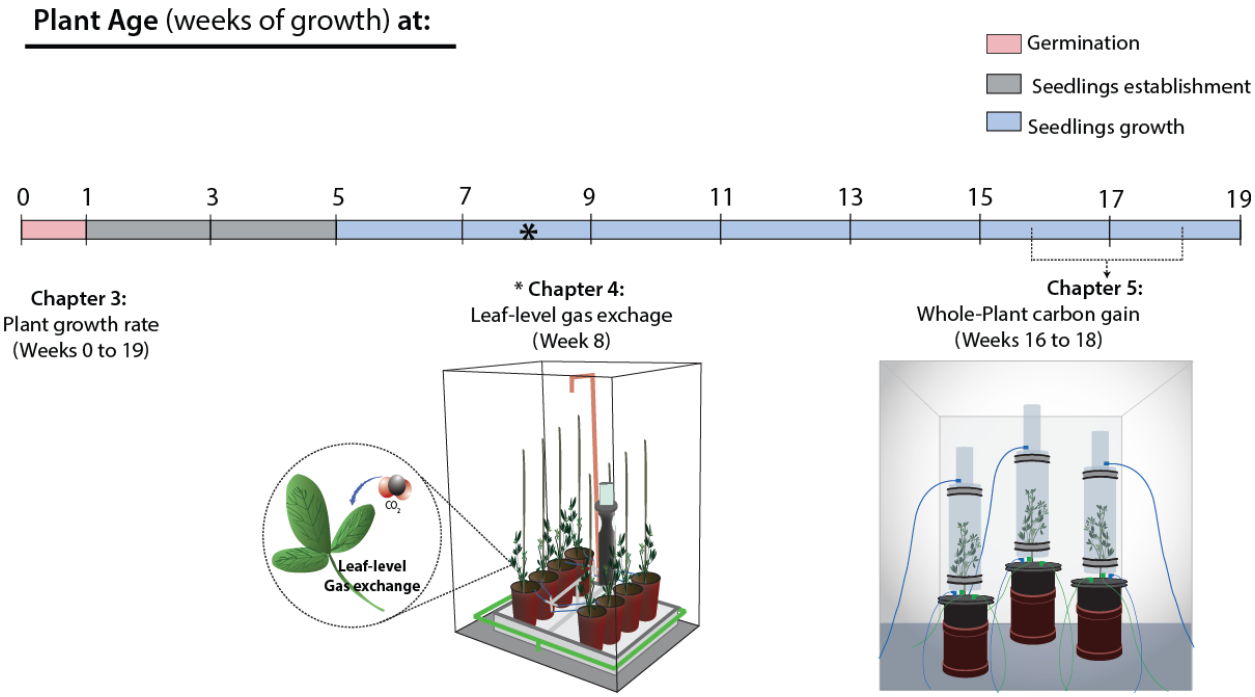

Figure 1.2. Schematic representation of research topics and approaches of chapters 3, 4 and 5 related to a single large experimental manipulation of [CO_2] for growing plant/rhizobium consortia line. The experiment overall lasted 19 weeks. Results reported in chapter 3 are based on harvests starting at week one and then for biomass and tissue composition conducted every other week until the end of the experiment. Chapter 4 reports leaf-level gas exchange measurements performed during week 8, after plants had achieved interaction with rhizobia and were in their vegetative growth phase. In Chapter 5, the whole-plant carbon gain is analyzed. To do so, we placed three plants from each [CO_2] treatment in whole-plant chambers separated into above- and below-ground compartments during 72 hours.

CHAPTER 2

Influence of rhizobia inoculation on biomass gain and tissue nitrogen content of *Leucaena leucocephala* seedlings under drought

Source: Pereyra et al. Influence of rhizobia inoculation on biomass gain and tissue nitrogen content of *Leucaena leucocephala* seedlings under drought. Forest 6 (10): 3686-3703.

Abstract:

Anticipated increases in the frequency of heat waves and drought spells may have negative effects on the ability of leguminous trees to fix nitrogen (N). In seedlings of *Leucaena leucocephala* inoculated with *Mesorhizobium loti* or *Rhizobium tropici*, we investigated how the developmental stage and a short drought influenced overall biomass and the accumulation of carbon and N in plant tissues.

In early developmental stages, the number of nodules and nodule biomass were correlated with total plant biomass and $\delta^{15}N$, and nodules and roots contributed 33-35% of the seedlings' total N. Seedlings associated with *R. tropici* fixed more N and exhibited higher overall biomass compared to *M. loti* seedlings. Four and a half months after inoculation (140 DAI) a short (15-day) drought inhibited seedling growth and caused a decline in total plant N, with the smallest decline in *R. tropici* seedlings. After 15 days of drought, i.e. 155 DAI, nodules had accumulated proline, but total amino acid concentration did not change.

Our results indicate that N-fixation is independent of seedlings growth. Also, *R. tropici* is a better choice than *M. loti* as a symbiont for *Leucaena* seedlings for forest restoration and agroforestry applications under increasingly drier conditions.

Keywords: Symbiosis; Nodulation; Nitrogen fixation*; Rhizobium tropici*; *Mesorhizobium loti*; Reforestation; ^{15}N abundance; Agroforestry.

2.1. Introduction

Leguminous tree species play an important role in ecosystem productivity and diversity due to their association with nitrogen (N)-fixing diazotropic soil bacteria called rhizobia (Thrall *et al.*, 2011). This symbiosis can increase net plant productivity (Spehn *et al.*, 2002) and mitigate land degradation through the use of fast-growing N-fixing trees or shrub 'pioneers' in restoration plantations (Miles *et al.*, 2006). However, anticipated changes in climate, like more frequent drought and heat waves (IPCC, 2014), may have the potential to negatively impact the usefulness of leguminous species or legume-symbiont partners for restoration purposes (Zahran, 1999).

N fixation takes place mostly in root nodules that provide an environment where symbiotic bacteria convert atmospheric dinitrogen (N_2) into ammonia. Plants provide organic-acids as an energy source and in exchange are supplied with fixed N (Miller *et al.*, 2007). This symbiotic interaction is usually highly specific; each rhizobium species and/or strain interacts with only a specific group of legumes (Wang *et al.*, 2012). Such specificity can be expressed at early stages of the interaction, i.e., during bacterial infection and nodule development, or at later stages when N-fixation takes place (Wang *et al.*, 2012). However, some rhizobia species, like *Rhizobium* sp. strain NGR234 and *Mesorhizobium* sp., are known to be less plant-specific and to interact with several legume species (Giller & Bala, 2000, Perret *et al.*, 2000, Wang *et al.*, 2012).

Variations in plant-rhizobia interactions can influence the successful establishment of legume trees during early developmental stages (i.e., seedlings) (Tharall *et al.*, 2000) and influence the benefit accrued from the symbiosis. Such benefits are usually evaluated based on traits measured in the plant (e.g., total plant biomass, N content) (Tharall *et al.*, 2000), nodules (e.g., number of nodules, nodule biomass) (Larrainzar *et al.*, 2014, Sanginga *et al.*, 1989) or nodule physiological parameters (e.g., apparent nitrogenase activity in relation to respiration) (Larrainzar *et al.*, 2014). Nodule traits like number of nodules (Tricot *et al.*, 1997) and nodule biomass (Voisin *et al.*, 2003) clearly depend on plant carbon (C), and are sensitive to environmental factors (i.e., drought stress) which can influence plant C supply (Zahran, 1999, Zahran, 2001).

Methods for estimating time-integrated rates of symbiotic N fixation in plant tissues rely on differences in the ^{15}N content of biologically fixed N (with $\delta^{15}N$ values close to 0‰) compared to N taken up by plants from the soil (in tropical forests, $\delta^{15}N$ values much greater than 0‰) (Boddey *et*

al., 2000). $\delta^{15}N$ values can vary substantially between the organs and tissues of a single plant (Wanek & Arndt, 2002). For example, N-fixing plants commonly exhibit ^{15}N enrichment of nodules and a depletion of ^{15}N in shoots (Unkovich, 2013), while averaged over the whole-plant, $\delta^{15}N$ values are close to atmospheric N_2 (i.e., close to 0‰) (Unkovich, 2013). Tissue specific $\delta^{15}N$ values have been related to the rate of nitrate reduction in roots and shoots (Unkovich *et al.*, 2000), drought stress (Robinson *et al.*, 2000), and changes in the amount of N-fixed relative to the N mass of nodules (Kohl *et al.*, 1983).

Drought can reduce the amount of C available to rhizobia for fixing N, which can lead to reductions in the total amount of N in plant tissues of legumes (Zahran, 1999). Stomatal closure associated with drought decreases the amount of new photosynthetic products. However, it can also reduce sinks for nonstructural carbohydrates (NSC) and reduce translocation of sugars to roots in drought-affected plants. Together, these can lead to accumulation of NSC in above-ground tissues and depletion in roots, which in turn negatively affects N metabolism in the rhizobial symbiont (Gil-Quintana *et al.*, 2013, Mengel, 1994, Muller *et al.*, 2011). Drought also induces changes in metabolite concentrations (i.e. amino acids) within the nodules, including increased concentrations of glutamine (Neo & Layzell, 1997) and/or asparagine (Sulieman *et al.*, 2010). Such changes act as a signal for down-regulation of N-fixation (Gil-Quintana *et al.*, 2013). In this paper, we report the effect of drought on an important legume shrub species and explore the effect of rhizobium strain on plant development and drought response.

Leucaena leucocephala (L.) de Wit, hereafter *Leucaena*, often referred to as the "wonder tree", is a widely used woody legume species in the tropics and sub-tropics (Orwa *et al.*, 2009) and is an efficient means for sustaining productivity of agroforestry systems (Shelton, 1998). For example, *Leucaena* improves soil fertility through biological N-fixation (up to 500 kg ha^{-1} $year^{-1}$ of N) in tropical areas of southern Mexico (Casanova-Lugo *et al.*, 2014) and subtropical areas of Australia (Shelton & Scott, 2007). *Leucaena* readily produces nodules in association with 8 different rhizobia strains isolated from 62 pan-tropical soils, including *Rhizobium* sp. (Oono & Denison, 2010) and *Mesorhizobium loti* (Jarvis *et al.*, 1997). It is clear that multipurpose legume tree species, such as *Leucaena* are important for ecosystem restoration and agroforestry. However, during critical developmental stages (i.e., seedling establishment), little is known about the effect of the rhizobia strain on the biomass gain and tissue N content and how these are affected by drought stress.

In this study, our aims were to evaluate the influence of the identity of the rhizobium strain on: (i) the overall biomass and the total C and N content in plant tissues and, (ii) how these parameters changed during a short but intense drought. We hypothesized that during seedling establishment and drought the identity of the rhizobium strain would affect the overall amount of N-fixed by the plants. We also hypothesized that drought would limit plant growth and nodule energy supply to varying degrees in the different *Leucaena*-rhizobia association, i.e., the degree of down-regulation of N-fixation due to reduced C availability. To test these hypotheses we inoculated *Leucaena* seedlings with two different rhizobial strains, *M. loti* or *Rhizobium tropici,* and measured plant and nodule biomass, as well as tissue C and N concentrations and ^{15}N isotope enrichment during seedling development. We then compared how these traits were changed by a short drought period, and included measures of NSC and nodule amino acids to test whether limitations in energy supply affected plant growth, in particular, rhizosphere responses.

2.2. Experimental Section

2.2.1. Biological material and general conditioning

Plant growth conditions

Leucaena seeds were purchased from a commercial retailer (Sunshine-Seeds, Ahlen, Germany). We scarified seeds in 95% sulphuric acid for 10 minutes to ensure quick and homogenous germination. After rinsing thoroughly with distilled water, seeds were soaked in distilled water over night. We placed the treated seeds in Petri dishes containing distilled water and covered with parafilm™ (Pechiney Plastic Packing Company, IL, USA) to increase air humidity and kept them at 30 °C for 48 hours. The pre-germinated seeds were individually transplanted into small pots (0.3 L) containing sterilized C- and N-free vermiculite (oven dried at 100°C for 8 hours) and were grown under controlled temperature at 25/20 ± 1 °C (day/night) in the greenhouse at the Max Planck Institute for Biogeochemistry, Germany. Natural sunlight was supplemented during cloudy days with high-pressure sodium vapor lamps (400 W Gro-Lux®, Osram Sylvania Ltd., Danvers, MA) providing a minimum photosynthetic photon flux density (PPFD) of 400-500 μmol $m^{-2}s^{-1}$. All seedlings were irrigated every other day to substrate saturation (i.e., until drainage appeared in trays). Pots were fertilized weekly with a N-free nutrient solution with twice the concentrations of the standard Hansen (1994) nutrient solution, also to substrate saturation (approx. 50 mL per pot), forcing plants to rely exclusively on symbiotic N-fixation as a source of N. The nutrient solution contained:

$MgSO_4 \cdot 7H_2O$ (2.0 mM), KH_2PO_4 (0.5 mM), K_2HPO_4 (0.5 mM), $CaCl_2 \cdot 2H_2O$ (2.0 mM), K_2SO4 (1.0 mM), EDTA $NaFe^{III}O$ (0.2 mM), H_3BO_3 (0.09mM), $MnCl_2$ (0.004 mM), $ZnCl_2$ (0.0008 mM), $CuCl_2 \cdot 2H_2O$ (0.0003 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.0001 mM), $CoCl_2$ (0.00002 mM). Growth conditions were maintained throughout the entire experiment until the interruption of irrigation/fertilization during the drought pulse (see below).

Bacterial material

Strains of *Mesorhizobium loti* (Jarvis *et al.*, 1997) (Strain type: DSM No. 2627, Strain designation: NZP 2037) and *Rhizobium tropici* (Romero-Martinez *et al.*, 1991) (Strain type: DSM No. 11418, ATCC 49672, CIAT 899. Strain designation: HAMBI 1163) were used for the inoculation of *Leucaena* seedlings. Both strains were purchased at the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The bacterial strains were cultivated on a YEM (yeast extract mannitol) medium and the culture was incubated at 30 °C (± 1.0 °C) for four days.

2.2.2. Experimental design

Experiment 1: Growth and early development

The first experiment assessed growth and stoichiometric responses of *Leucaena* to different rhizobia strains (*Leucaena* + *M. loti*, *Leucaena* + *R. tropici)* during early seedling development. We planted 80 pots with germinated *Leucaena* seeds. After twenty days of growth, 35 seedlings were inoculated with the pure liquid culture of *M. loti* and another 35 with *R. tropici.* This delay of 20 days was chosen to ensure homogenous seed germination prior to inoculation, and to avoid low inoculation success observed for *L. leucocephala* when immediate inoculation using seed coating was used (Forestier *et al.*, 2001). Furthermore, we carried out inoculation pre-tests that showed liquid (1 mL) inoculation once seeds were germinated (i.e., seedlings one to two weeks old) yielded the best results in terms of the production of nodules over the subsequent weeks. Both bacteria cultures were applied to the surface of the soil with a liquid solution (1 mL of liquid inoculum, in the late-exponential growth phase for the culture) and randomly divided into subgroups according to their treatment. Ten pots received no bacteria strain inoculum and were used as non-fixing control plants (i.e., uninoculated plants).

Harvest days (i.e., 60 and 140 days after inoculation, DAI) were chosen based on preliminary

greenhouse experiments with inoculated woody legumes. These experiments demonstrated that nodulation using liquid inoculum was achieved by 60 DAI (Mrema *et al.*, 1997). Also, other greenhouse and garden experiments studying the effectivity of rhizobia for nodulation, found early N-fixation and biomass accumulation in harvested *Leucaena* seedlings between 120 and 180 days after inoculation (Forestier *et al.*, 2001, Kadiata *et al.*, 1996, Sanginga *et al.*, 1989, Thies *et al.*, 1991). We harvested at 60 DAI assuming nodulation by this point (i.e., symbiosis was established), and then harvested half of the seedlings at 140 DAI when tissue lignification was visible, assuming that nodules were active and fixing N_2 (Figure 2.1). Control seedlings (i.e., uninoculated) died approximately ten days after the first harvest (i.e., at 70 DAI) and no data were obtained for control plants at 140 DAI. Active N-fixation was identified via visual assessment of the harvested nodules based on their red/pink coloration. We kept 60 pots for the subsequent short-drought experiment (Experiment 2).

Experiment 2: Short drought pulse

For experiment 2 (short drought) we used a subgroup of plants from experiment 1 (60 pots) (Figure 2.1). Those plants were irrigated every other day until drainage appeared in trays and were fertilized weekly until 140 DAI when the short drought pulse started. In droughted plants, both irrigation and fertilization were completely discontinued. Control (i.e., irrigated) plants were irrigated (every other day) and fertilized (weekly) throughout the drought experiment with the nutrient solution, and both groups (control and drought) were harvested at the end of the 15-day drought period (155 DAI). Seedlings were harvested at 0 (140 DAI) and 15 days after drought initiation (155 DAI) at the end of the experiment (n=6 per treatment for each time point).

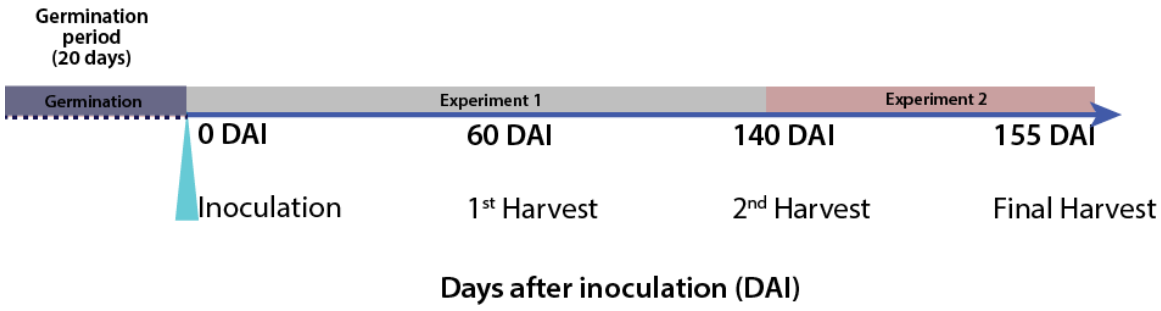

Figure 2.1. Schematic representation of the experimental time line. Experiment 2 is based on a subsample of plants from experiment 1. The drought pulse lasted 15 days and plants were harvested for analysis at the start and end of this period.

2.2.3. Sample processing and analysis

Sample processing

Harvested plants (n=6 for each sampling point) were separated into leaves, branches/stems, roots, and nodules. Tissues form half of the harvested plants (n=3) were oven-dried to constant weight at 70 °C, their dry weight recorded, and the material was stored for subsequent elemental and isotope analysis. Tissues of the other half (n=3) of the plants harvested in the drought experiment, were immediately frozen in liquid nitrogen and stored at -80 °C for NSC analyses.

Nitrogen concentration and isotopic analysis

Subsamples (c. 5-20 mg DW) of oven-dried material were ground in a ball mill to fine powder (Retsch MM200, Haan, Germany), then measured using an elemental analyser (VarioMax Elementar Analysensysteme GmgH, Hanau).

The total amount of N derived from biological fixation was calculated using the N balance method (Unkovich *et al.*, 2008), where plants were grown in N-free media (i.e. vermiculite substrate) and the only source of N for growth, apart from seed- and inoculum-N, was N_2 fixation. This was calculated as:

$$\text{Total Plant } N_{fixed}\,(g) = N_{(Leaves+Branches+Roots)}\,(g) - N_{(in\ sown\ seed\ +\ in\ inoculant)}\,(g) \qquad \text{Eqn. 2.1}$$

for a given plant tissue the amount of N was calculated from the amount of dry biomass and its measured N concentration:

$$\text{Tissue N}(g) = (N_{tissue}\ \text{concentration}\ (\text{mg g}^{-1}\ \text{DW}) * \text{sample DW}\ (g))/1000 \qquad \text{Eqn. 2.2}$$

The isotopic analysis of ^{15}N abundance was made in a subsample of each tissue (*c.*1 mg DW), and analyzed for $\delta^{15}N$ (expressed relative to atmospheric N_2) at the Stable Isotope Laboratory of the Max Planck Institute for Biogeochemistry, using a Delta+ XL Isotope Ratio Mass Spectrometry (IRMS, ThermoFinnigan, Bremen, Germany).

Water status

In the drought experiment, substrate water content (SWC) was defined as the percentage of the

maximum mass of water retained by the substrate (i.e., field water capacity). SWC was estimated by weighing the pots between 07.00 and 08.00 h on days 0 and 15 following the day irrigation was stopped. SWC was estimated from the difference in fresh weight (FW) and dry weight (DW) of the substrate contained in pots measured after each harvest (n=6 per treatment):

$$\text{SWC}\ (\%) \ = \ 100 * (\text{FW} - \text{DW}/\text{FW}_{\text{max}}) \qquad \text{Eqn 2.3}$$

NSC and amino acid concentrations

Total NSC concentrations were estimated from the sum of measurements of three major sugars within each tissue: glucose, fructose, and sucrose. Shock-frozen samples (n=3 for each tissue type) of leaves, roots and nodules were vacuum freeze-dried for 96 h and milled (Retsch MM200, Haan, Germany) to fine powder. After grinding, 5 to 30 mg of the sample was added to 1 mL of distilled water. The mixture was vortexed and incubated for 10 min at 65°C in a thermomixer and then centrifuged for 6 min at 12000 g. The supernatant was removed with a pipette, stored on ice and the water extraction procedure was repeated twice and the three extracts combined. Sugars in the extract were measured using high/pressure liquid chromatography with pulse amperometric detection (HPLC-PAD) on a Dionex ICS 3000 ion chromatography system equipped with an autosampler (Raessler *et al.*, 2010). Due to a freezer failure NSC in leaf and root samples of drought-control (irrigated) *M. loti* seedlings could not be measured.

An aliquot of the nodule sugar extracts was pipetted into tin cups and assayed with a Finnigan MAT DeltaPlus XL EA-IRMS (ThermoFinnigan GmbH, Bremen, Germany), coupled to an autosampler. Amino acid concentrations were quantified on the same extracts prepared for nodule NSC analyses, and were diluted at a ratio of 1:10 (v:v) in water. Amino acid measurements on the diluted extracts were performed at the Max Planck Institute for Chemical Ecology in Jena using liquid chromatography (LC-MS/MS) as described in Docimo *et al.* (2012), coupled to an API 5000 tandem mass spectrometer (Applied Biosystem, Darmstadt, Germany). All samples were spiked with ^{13}C and ^{15}N labelled amino acids (algal amino acids ^{13}C, ^{15}N, Isotec, Miamisburg, OH, USA) at a concentration of 10µg of the mix per mL solution. The concentration of individual labelled amino acids in the mix was determined by HPLC-fluorescence detection analysis after pre-column derivatization with ortho-phthaldialdehyde-mercaptoethanol using external standard curves made from standard mixtures (amino acid standard mix, Fluka plus Glutamine, Asparagine and Tryptohan,

also Fluka). Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N, labelled amino acid internal standard, except for tryptophan and asparagin: tryptophan was quantified using ^{13}C, ^{15}N-Phenylalanine applying a response factor of 0.42. Asparagin was quantified using ^{13}C, ^{15}N-Asparagine applying a response factor of 1.0.

2.2.4. Statistical analysis

Mean (± SE) values of the measured parameters were tested for differences between strains and across harvest days by a two-way ANOVA (strain and harvest days as factors for experiment 1 and experiment 2) using Type I sum of squares using R (v.2.15.0, R Foundation for Statistical Computing, 2012) and the package Agricolae, version 1.1-8 (de Mendiburu, 2014). When ANOVA indicated a significant overall treatment effect, a multiple comparison test was carried out (*post hoc* Least Significant Difference test) to compare parameter means across harvest days within rhizobia strain. To determine whether whole plant ^{15}N discrimination was different from atmospheric N_2 (0‰), a *t*-test was performed across harvest days within strains.

2.3. Results

2.3.1. Rhizobium strain effects on seedling carbon and nitrogen content

At 60 DAI, the symbiosis of *Leucaena* with both inoculated rhizobia strains was clearly established, and had substantial effects. Uninoculated plants were shorter and had on average less biomass than inoculated plants. Leaf tissues in uninoculated plants had half the N concentration found in leaves of inoculated seedlings (Table 2.1). Approximately ten days after the harvest (i.e., at 70 DAI), uninoculated seedling died, and no data were obtained for control plants at 140 DAI.

At this early stage of development, differences observed between plants infected with different rhizobia were mostly belowground. *R. tropici* plants produced fewer nodules, but these had similar biomass compared to nodules on plants with *M. loti*. The average root length of *R. tropici* seedlings was almost 2-fold longer than *M. loti* seedlings (Table 2.1). The N concentration in root tissues differed very little across treatments, but the ^{15}N isotopes showed the source of root N differed. Roots of inoculated seedlings had depleted bulk $\delta^{15}N$ (*c.* -1.4‰) when compared to atmospheric ^{15}N (0 ‰) and roots of control seedlings (*c.* +3.4‰) (Table 2.1). However, the whole-plant $\delta^{15}N$ (calculated from the weighted total N contibutions of shoots, roots and nodules to the whole plant)

was not significantly different from 0 ‰ (Table 2.1).

At 140 DAI, stems started to lignify and all inoculated seedlings looked similar aboveground. The number of leaves, average plant height, and tissue C and N concentrations were not significantly different between plants associated with the two rhizobia strains. However, total plant biomass was significantly greater in *R. tropici* seedlings (4.9 ± 0.8 g) compared to *M. loti* seedlings (3.2 ± 0.5 g) (Table 2.1). Thus, overall, *R. tropici* plants had fixed more N and C at this stage.

Table 2.1. Leaf, root and nodule traits of pot-cultured *Leucaena leucocephala* inoculated with different rhizobium strains (*Mesorrhizobium loti* or *Rhizobium tropici*) and their respective controls (uninoculated plants). Numbers represent mean ± SE (n=5 at 60 DAI and n=3 at 140 DAI). Different letters indicate significant differences across harvest days within rhizobia strain ($P<0.05$, Least significant differences (LSD) test, following ANOVA). Asterisks indicated the differences between atmospheric N_2 (0‰) and total-plant $\delta^{15}N$ were analyzed by Student's t-test.

	Harvests				
	60 DAI			**140DAI**	
Trait	*M. loti*	*R. tropici*	Control	*M. loti*	*R. tropici*
Plant height (cm)	5.8 ± 0.4^{b}	5.4 ± 0.4^{b}	4.2 ± 0.4^{b}	12.2 ± 1.4^{a}	14.8 ± 0.6^{a}
Plant biomass (g)	0.5 ± 0.1^{c}	0.5 ± 0.1^{c}	0.3 ± 0.1^{c}	3.2 ± 0.5^{b}	4.9 ± 0.8^{a}
Total plant $\delta^{15}N$(‰)	-0.9 ± 0.3	-0.4 ± 0.2	1.8 ± 0.4*	-0.4 ± 0.3	-0.6 ± 0.2
Root-to-shoot ratio	0.4 ± 0.0^{b}	0.3 ± 0.0^{b}	0.6 ± 0.1^{a}	0.6 ± 0.0^{a}	0.7 ± 0.0^{a}
No. of leaves (plant^{-1})	7.0 ± 1.4^{bc}	8.4 ± 1.2^{b}	2.7 ± 0.9^{c}	24.7 ± 1.8^{a}	22.0 ± 3.8^{a}
Leaf C (%)	42.1 ± 0.3^{bc}	42.9 ± 1.7^{abc}	40.6 ± 0.3^{c}	43.6 ± 0.2^{ab}	45.6 ± 0.2^{a}
Leaf N (%)	2.4 ± 0.4^{b}	2.1 ± 0.3^{b}	1.2 ± 0.0^{c}	3.4 ± 0.2^{a}	3.8 ± 0.2^{a}
Leaf $\delta^{15}N$(‰)	-1.4 ± 0.1	-1.0 ± 0.4	-1.6 ± 0.4	-0.8 ± 0.1	-0.8 ± 0.3
Root length (cm)	12.7 ± 4.0^{b}	21.0 ± 2.0^{ab}	22.9 ± 2.1^{a}	13.8 ± 0.6^{ab}	23.5 ± 5.2^{a}

Root C (%)	40.0 ± 1.8^{b}	41.1 ± 0.0^{ab}	42.4 ± 0.1^{ab}	41.8 ± 0.3^{ab}	43.6 ± 1.3^{a}
Root N (%)	1.7 ± 0.2	1.6 ± 0.0	1.7 ± 0.0	1.5 ± 0.1	1.6 ± 0.2
Root δ^{15}N(‰)	-1.4 ± 0.5^{b}	-1.4 ± 0.4^{b}	3.4 ± 0.1^{a}	-1.8 ± 0.4^{b}	-1.8 ± 0.2^{b}
Nodules (plant^{-1})	12.4 ± 2.2^{a}	7.0 ± 0.5^{b}	0	13.7 ± 0.5^{a}	13.3 ± 0.6^{a}
Nodule biomass (mg)	11.4 ± 0.9^{b}	14.4 ± 1.5^{b}	0	78.3 ± 12.4^{a}	70.1 ± 8.8^{a}
Nodule C (%)	41.8 ± 0.1^{b}	41.4 ± 0.2^{b}	0	44.7 ± 1.3^{a}	43.1 ± 0.8^{ab}
Nodule N (%)	4.3 ± 0.0^{b}	4.5 ± 0^{b}	0	4.4 ± 0.1^{b}	5.6 ± 0.2^{a}
Nodules δ^{15}N(‰)	4.9 ± 0.2^{b}	5.2 ± 0.2^{ab}	0	7.4 ± 0.8^{ab}	7.9 ± 1.2^{a}

Nodule biomass was a much better predictor of total plant N-fixed (Figure 2.2b) than plant nodule count (Figure 2.2a). While the number of nodules per plant almost doubled in *R. tropici* seedlings at 140 DAI compared with 60 DAI, nodule count did not increase in *M. loti* seedlings. Total nodule biomass significantly increased between 60 and 140 DAI and was very similar between strains (Table 2.1). Values of δ^{15}N remained constant for leaf and root tissues, but increased an additional 2.5 ‰ to 2.7 ‰ δ^{15}N in nodules between 60 DAI and 140 DAI, respectively for *M. loti* and *R. tropici*, with final δ^{15}N values in nodules of *c.* 8 to 9‰ greater than leaf or root ^{15}N values (Table 2.1).

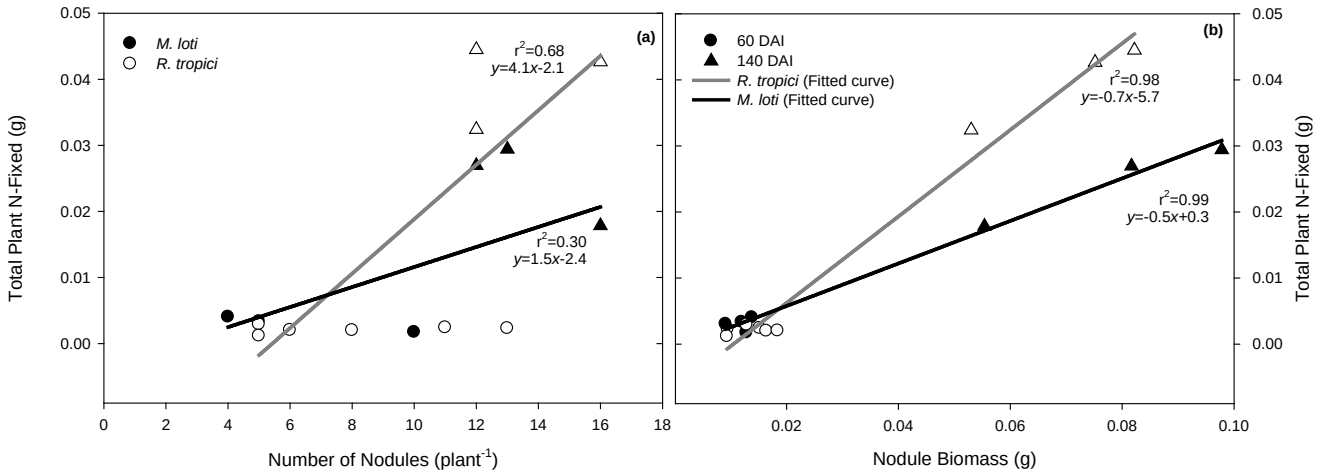

Figure 2.2. Total content of fixed nitrogen per plant as a function of plant nodule count (a) and nodule biomass (b) in *Leucaena leucocephala* seedlings inoculated with *Mesorhizobium loti* (filled bars) and *Rhizobium tropici* (open bars). Different symbols indicate single measurements at 60 DAI (circles) and 140 DAI (triangles). Fitted curves show the linear regression between parameters. Equations and determination coefficient are included in each figure.

2.3.2. Drought-induced changes in water availability and number of nodules per plant

During a 15-day drought at 140 DAI (four and a half months after inoculation) the substrate water content (SWC) declined gradually from approximately 86.7 and 90.8% (Day 0) to 41.1 and 48.7% (Day 15) for *M. loti* and *R. tropici* seedlings, respectively (Table 2.2). All drought-affected seedlings showed signs of stress in that their leaves folded inward. However, in droughted seedlings, traits like the number of leaves, C and N concentration of leaves and root-to-shoot-ratio did not change (Table 2.2). Irrigated plants also did not change, except for a significant increase over the 15-day period, i.e., 155 DAI, in root biomass and root-to-shoot ratio for *R. tropici* seedlings (Table 2.2). Thus, growth of plants inoculated with M. loti was not affected by drought during this period.

R. tropici seedlings had more but lighter nodules when exposed to drought compared to both irrigated seedlings and *M. loti* seedlings under drought (Table 2.2). NSC increased in roots and nodules of seedlings of both strains under drought or irrigation, with the largest increases in nodules of *R. tropici* plants. In contrast, NSC also increased in leaves of *M. loti* plants affected by drought (Table 2.2).

Roots were more depleted in ^{15}N than leaves, compared to the atmospheric ^{15}N concentration (i.e.,

$\delta^{15}N$ <0‰) (Table 2.2). Nodule $\delta^{15}N$ values increased (>0.5 to 0.8 ‰) and their N concentration decreased for both rhizobia strains after 15 days of drought (Table 2.2).

Table 2.2. Effect of a short drought on leaf, root and nodule traits of pot-cultured *Leucaena leucocephala* inoculated with different rhizobium strains (*Mesorrhizobium loti* or *Rhizobium tropici*) and their respective controls (irrigated plants). Numbers represent mean ± SE (n=3). Harvests were made at the beginning of the short drought (day 0, 140 DAI) and after 15 days at the end of the drought (day 15, 155 DAI). Different letters indicate significant differences across harvest days within rhizobia strain (*P*<0.05, Least significant differences (LSD) test, following ANOVA).

	Days after the short-drought pulse					
	0 (140 DAI)		15 (155 DAI)		Irrigated plants	
	M. loti	*R. tropici*	*M. loti*	*R. tropici*	*M. loti*	*R. tropici*
Soil water content (%)	87.7±2.0^{a}	90.8±2.9^{a}	41.1±4.2^{b}	48.7±2.4^{b}	89.0±2.0^{a}	90.5±2.9^{a}
Root-to-shoot ratio	0.7±0.1^{b}	0.8±0.0^{b}	0.7±0.0^{b}	0.7±0.0^{b}	0.8±0.0^{b}	0.9±0.1^{a}
Number of leaves (plant^{-1})	24.7±2.3	22.0±3.8	23.3±0.7	25.3±3.3	22.0±1.2	24.0±0.0
Leaf biomass (g)	0.4±0.1^{ab}	0.6±0.1^{a}	0.4±0.0^{b}	0.4±0.0^{b}	0.3±0.1^{b}	0.6±0.1^{a}
Leaf C (%)	43.6±0.1	45.6±0.7	45.3±2.3	44.5±0.1	44.9±1.2	44.1±0.1
Leaf N (%)	3.4±0.2^{ab}	3.8±0.2^{a}	3.3±0.2^{ab}	3.6±0.2^{ab}	3.1±0.4^{b}	3.5±0.0^{ab}
Leaf $\delta^{15}N$(‰)	-0.8±0.1	-0.8±0.3	-0.9±0.2	-1.1±0.4	-1.4±0.2	-0.9±0.3
Leaf NSC (%)	4.9±0.3^{b}	4.4±0.3^{b}	8.6±0.7^{a}	5.2±0.3^{b}	--	4.1±0.3^{b}
Root length (cm)	13.8±0.6^{bc}	23.5±5.2^{ab}	13.3±2.5^{c}	23.6±5.1^{a}	12.9±0.9^{c}	14.7±1.2^{abc}
Root biomass (g)	0.5±0.1^{b}	0.7±0.1^{ab}	0.5±0.0^{b}	0.6±0.1^{ab}	0.4±0.1^{b}	0.9±0.1^{a}
Root C (%)	41.8±0.3^{ab}	43.6±1.3^{a}	38.2±1.1^{b}	41.5±0.8^{ab}	39.7±1.2^{b}	38.8±0.9^{ab}

Root N (%)	1.5±0.1	1.6±0.2	1.7±0.1	1.7±0.1	1.5±0.1	1.4±0.1
Root $\delta^{15}N$(‰)	-1.8±0.4	-1.8±0.2	-2.2±0.2	-1.7±0.4	-2.7±0.8	-2.2±0.4
Root NSC (%)	4.7±1.9^{b}	6.4±0.2^{b}	9.0±0.8^{ab}	10.0±1.0^{ab}	--	14.3±2.9^{a}
No. of nodules (plant^{-1})	13.6±0.5^{b}	13.3±0.6^{b}	6.7±0.3^{b}	16.3±2.9^{ab}	35.0±6.6^{a}	11.3±4.4^{b}
Nodule biomass (mg)	78.3±12.4^{ab}	70.1±8.8^{ab}	60.1±17.1^{ab}	56.2±16.2^{ab}	41.3±3.0^{b}	86.3±14.2^{a}
Biomass per nodule (mg nodule^{-1})	4.8±0.7^{ab}	5.3±1.0^{ab}	9.3±3.2^{a}	4.0±0.7^{ab}	1.2±0.2^{b}	11.4±5.0^{a}
Nodule C (%)	44.7±1.3^{a}	43.1±0.7^{ab}	40.7±0.4^{b}	42.6±0.7^{ab}	42.7±0.3^{ab}	42.4± 0.3^{ab}
Nodule N (%)	4.4±0.1^{c}	5.5±0.2^{a}	4.2±0.1^{c}	4.3±0.2^{c}	5.0±0.1^{b}	4.4±0.0^{c}
Nodule $\delta^{15}N$(‰)	7.4±0.8	7.9±1.2	7.9±0.5	8.7±0.6	8.0±0.9	8.7±0.6
Nodule NSC (%)	7.4±0.6^{ab}	2.4±0.1^{c}	10.1±1.8^{ab}	8.8±1.4^{ab}	9.3±0.7^{ab}	13.9± 1.2^{a}

Amino acid concentrations in nodules decreased slightly during the drought treatment and compared to irrigated seedlings (Figure 2.3a), especially specially in *M. loti*. Glutamic acid (Figure 2.3b) and asparagine (Figure 2.3c) represented ~63% and ~66% of the total amino acid (by mass) analyzed in nodules of *R. tropici* and *M. loti*, respectively. Drought affected mostly asparagine in nodules of *M. loti* and *R. tropici,* causing reductions of 44% (Figure 2.3c). In contrast, proline significantly increased after 15 days of drought (Figure 2.3d) at similar rates from 10.3 ± 0.8 to 58.6 ± 3.7 nmol g^{-1} in nodules of *M. loti* seedlings, and from 2.8 ± 0.7 to 48.6 ± 5.8 nmol g^{-1} in nodules of *R. tropici* seedlings.

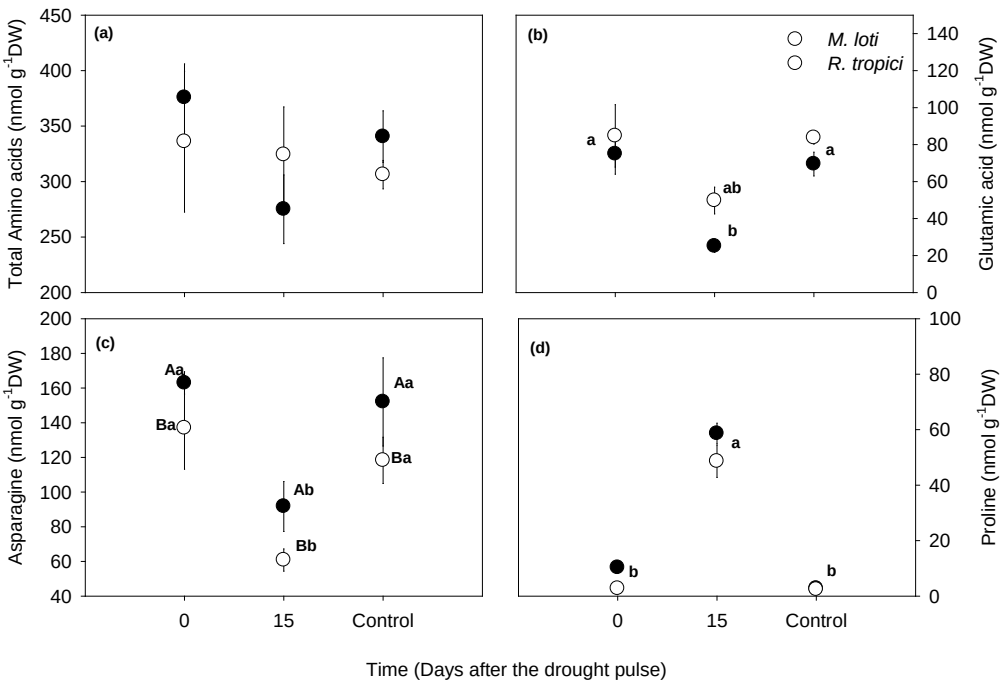

Figure 2.3. Averages of total amino acids (a), glutamic acid (b), asparagine (c) and proline (d) in nodules of *Leucaena leucocephala* inoculated with *Mesorrhizobium loti* (filled symbols) and *Rhizobium tropici* (open symbols) under a short drought or irrigated. Values represent means ± SE (n=3). Different upper-case letters denote significant differences between strains, lower-case letters across harvest days (*P*<0.05, Least significant differences (LSD) test, following ANOVA).

2.4. Discussion

The choice of N-fixing trees for forest restoration and agroforestry in drought-prone systems is usually based on plant growth traits (e.g., survival rate, fast vs. slow growth, C and N tissue concentrations) (Casanova-Lugo *et al.*, 2014, Ceccon *et al.*, 2015). The use of such traits has led to variable growth responses to heterogeneous environmental conditions, differences in propagation success in tree nurseries, and the initial performance of trees (Craven *et al.*, 2011, Hall *et al.*, 2011). However, the contribution of the rhizobia strain associated with the various plants has received less attention, especially in the plants' response to drought.

Our study demonstrates that different rhizobium strains in the *Leucaena*-rhizobium symbiosis had substantial impacts on growth and development, both during early seedling stages and during drought.

2.4.1. Effects of rhizobium on nodulation, seedling growth, and total plant N

A common understanding in legume-rhizobia symbiosis is that larger plants can support more nodules and can establish themselves earlier (Giller & Bala, 2000), which in turn translates into faster growth, higher biomass, and more N in plant tissues (Kiers *et al.*, 2013). However, this was not the case in our experiment; the number of nodules did not increase over time in *M. loti* seedlings, a phenomenon that has also been observed in other studies on *L. leucocephala* inoculated with *M. loti* (Strains: MAFF 303099 and R7A) (Hubber *et al.*, 2004, Hubber *et al.*, 2007). Such a regulation of nodule formation was attributed to the presence of secretion systems, which are large protein complexes that are exported across bacteria membranes and cell walls (Nelson & Sadowsky, 2015). The regulation of the total number of nodules in *M. loti* seedlings was associated with negative effects on biomass and N accumulation in *Leucaena*.

In our study, *Leucaena* was an obligate N-fixer at early growth stages, and seedlings regulated N content via changes in their whole plant biomass (Menge *et al.*, 2009) as indicated by the fact that N was fixed at the same rate per unit of biomass independent of growth phase (Figure 2.2). Seedlings inoculated with *R. tropici* had higher biomass accumulation and N-fixation. The hypothesis of our study was confirmed, as N-fixation and biomass accumulation differed between inoculated strains, a result also observed for *Acacia* sp. inoculated with different rhizobia strains (i.e., phylotypes) (Bever *et al.*, 2013). Furthermore, the amount of N-fixed during a given period was strongly related to nodule biomass, but not to nodule count (Figure 2.2b). Similar results have been observed in *M. truncatula,* where higher N-fixation rates (measured as apparent nitrogenase activity) were observed in association with the strain that yielded higher nodule biomass (Larrainzar *et al.*, 2014).

Belowground tissues contributed to 33-35% of the seedlings' total N, three-fold higher than reported for pasture legumes (Kohl *et al.*, 1989). Belowground tissues therefore contain a substantial proportion of total N fixed by plants, as has been observed in field experiments, albeit to a lesser degree (7-10%) (Ohyama & Kumazawa, 1980). The observed differences are probably related to differences in plant type (pasture legume vs. woody legume) and/or to the type of manipulation (field experiment vs. greenhouse experiment). However, when N content and tissue $\delta^{15}N$ are integrated over the whole plant, applying a mass balance approach, we did not observe isotope discrimination against ^{15}N compared to atmospheric N_2 (i.e., the $\delta^{15}N$ for the whole plant was ~0‰) (Unkovich, 2013). We found that $\delta^{15}N$ values of nodules increased with time and were enriched compared to atmospheric N_2, while other plant tissues were depleted in $\delta^{15}N$ by comparison. This

has also been observed in other studies of legume shrubs and trees inoculated with *Rhizobium* sp. (Boddey *et al.*, 2000). The reason for such large enrichment of ^{15}N in nodules is still debated (Unkovich, 2013). One hypothesis is that deamination reactions produce ^{15}N-depleted ammonia (NH_3), which is exported out of the nodules, leaving residual N that is enriched in ^{15}N (Kohl *et al.*, 1989) and causing nodule $\delta^{15}N$ increases. The subsequent assimilation of the exported NH_3 into growing plant tissues would thus explain their depletion in ^{15}N (Ohyama & Kumazawa, 1980). We observed concurrent changes in N concentration in aging leaves associated with an ^{15}N enrichment of *c.* 0.6‰, so that discrimination processes other than N-fixation must have been operating (Unkovich, 2013). Methodological approaches using ^{15}N to partition total N into symbiotically fixed versus soil-uptake N in leaf tissues need to consider this post-fixation fractionation in their calculations.

2.4.2. Leucaena symbiosis during drought: root length and N-fixation

Leucaena is known as a drought tolerant species with a deep rooting system (Nasr Esfahani *et al.*, 2014). Under short-term drought stress, *Leucaena* seedlings associated with *R. tropici* had greater root length than *M. loti* seedlings. This is interpreted as a survival strategy during soil water depletion (Zahran, 1999) by allowing plants to explore a greater soil volume.

Drought caused plant growth to cease and resulted in slight reductions of plant N in *R. tropici* seedlings, which might indicate greater N use without resupply from N-fixation. On the other hand, *M. loti* seedlings stopped growing before the short drought started. This might be related to the regulation of the number of nodules discussed earlier, rather than regulation of N-fixation due to drought. Regulation of the symbiotic N-fixation during drought is complex and not yet fully understood (Larrainzar *et al.*, 2009). Other pot experiments with short drought spells (7 days) have shown a reduction of N-fixation via impairment of bacteroids C metabolism (Nasr Esfahani *et al.*, 2014) and/or accumulation of amino acids (i.e., N-feedback) (Gil-Quintana *et al.*, 2013). Apart from a strong accumulation of proline, a common drought stress response, the total amino acid concentration in nodules did not increase after 15 days and hence cannot be explained by N-feedback mechanisms in our experiment. However, the accumulation of NSC in nodules might indicate that levels of sucrose synthase, a key enzyme in the hydrolysis of sucrose to hexoses in nodules, was reduced by drought (González *et al.*, 1998), leading to a limitation of respiratory substrate for bacteriods (Ladrera *et al.*, 2007). The respiratory substrate for bacteriods is malate,

derived from the catabolism of hexoses to oxaloacetate and ultimately, reduced to malate, due to the enzyme malate dehydrogenase (Vance & Gantt, 1992). Such a mechanism has been observed in pea and soybean (*Glycine max*), but not in *M. truncatula* (Larrainzar *et al.*, 2009) and may lend support to our hypothesis that drought down-regulated N-fixation due to reduced C availability to fuel bacteriods' respiration in *R. tropici* seedlings.

In a restoration effort of degraded agricultural land in Mexico, establishment rate and drought survival were greater for legume seedlings that grew for two months under controlled conditions before planting (Martínez-Garza *et al.*, 2013). Applying our results to field conditions, *Leucaena* seedlings grown with *R. tropici* until lignification occurs are likely to gain more biomass and increase N tissue content compared to those grown with *M. loti*. The *R. tropici* symbiosis also allowed increases in root length, which might enable the plant to better respond to drought conditions by exploring distant soil water pools. However, a co-limitation of phosphorus (P) should always be considered in tropical ecosystems, where low availability of P may prevent N fixers from having a significant advantage (Davinson *et al.*, 2004). While there has been a clear emphasis on how drought affects leguminous trees and their performance in reforestation efforts or agroforestry, there are major knowledge gaps in how other abiotic stressors affect the rhizobia-host symbiosis.

2.5. Concluding remarks and implications

Under the controlled conditions of our manipulation, *Leucaena* was an obligate N-fixer and N-fixation occurred during the whole seedling growth phase. However, when *Leucaena was* inoculated with *R. tropici* it fixed more N and gained more biomass than seedlings associated with *M. loti*. Though both associations ceased fixing N during drought, *R. tropici* seedlings had on average longer roots, and their nodules accumulated more proline, suggesting that seedlings inoculated with *R. tropici* are likely better able to survive drought. For better results in forest restoration and agroforestry applications, we recommend the growth of *Leucaena* trees in association with *R. tropici* under controlled conditions until lignification occurs, before transplanting the seedlings to the field.

CHAPTER 3

Influence of atmospheric [CO_2] on growth and biomass partitioning of *Medicago sativa* L.

Abstract

Plant carbon (C) allocation to growth can be affected by changes in C availability, for example, from changes in atmospheric [CO_2]. In nodulated plants, C availability may also influence nitrogen (N) fixation by heterotrophic bacteroids depending on exchange rates between plants and bacteroids. The aim of this study was to compare growth rates, biomass allocation and nitrogen content in *Medicago sativa* grown from seeds and inoculated with *Ensifer meliloti* under different atmospheric [CO_2]: Pleistocene, 170 ppm; ambient, 400 ppm and projected future, 700 ppm.

M. sativa plants grown at 400 and 700 ppm were much larger. After 11 weeks of growth these plants had partitioned more biomass to roots than plants grown at 170 ppm and flowering onset started after week 15, but flowers were aborted approximately 3 to 5 days after they appeared. After 19 weeks, 170 ppm plants were still in the vegetative growth stage, had fewer nodules, lower relative growth rates, but allocated less of their total C to the production of leaves, compared to plants grown at higher [CO_2] levels. Despite differences in biomass partitioning and relative growth rate, C and N concentrations in plant tissues remained similar over time and across treatments.

Our results suggest that C limitation slowed plant development in nodulated *Medicago sativa* plants and caused plants to allocate more C to roots and nodules, thereby maximizing the potential to fix N. Moreover, based on our results, *M. sativa* as a N-fixer might not have a competitive advantage over non-fixing plant in terms of growth under higher [CO_2] compared with plants grown at atmospheric [CO_2].

Keywords: relative growth rate, N-fixation, carbon cost, alfalfa, ^{15}N, elevated [CO_2].

3.1. Introduction

Rising atmospheric CO_2 concentrations increase plant C availability (Gerhart & Ward, 2010, Nicotra *et al.*, 2010). Plants can acclimate to these conditions through phenotypic plasticity, defined as the range of phenotypes i.e., different observable characteristics of a single genotype can express in response to its environmental cues (Nicotra *et al.*, 2010). For example, by adjusting in phenology, leaf area per unit of leaf mass (SLA), plant height or flowering onset (Poorter *et al.*, 2009, Sanz-Sáez *et al.*, 2015, Westoby, 1998). However, because plants have evolved over the past million years with mostly low [CO_2], acclimation responses to increasing CO_2 may be constrained by evolutionary adaptations (Rico *et al.*, 2013, Sage & Coleman, 2001, Tissue & Lewis, 2012).

In general, elevated [CO_2] can stimulate growth, while low [CO_2] (i.e., Pleistocene epoch [CO_2], 170 ppm) usually reduces growth compared to current [CO_2] (Lewis *et al.*, 2010, van der Sleen *et al.*, 2015, van Kleunen & Fischer, 2005). Additionally, changes in [CO_2] may alter C allocation to growth versus respiration, storage, and defense, but also allocation across plant tissues (Ainsworth & Long, 2005, Poorter *et al.*, 2012).

In theory, plants adjust their relative growth rates (RGR, defined as the rate of biomass increase in per unit of biomass in a given time interval) of their tissues to avoid resource limitation (Dippery *et al.*, 1995, Pons & Poorter, 2014). Thus, C will be allocated to those tissues (leaves or roots) to maximize the acquisition of the most limiting resource (Shipley & Meziane, 2002). At low [CO_2], plants should hence favor C allocation to biomass in above-ground structures (i.e., increase leaf area and therefore C assimilation) over below-ground structures (e.g., roots and therefore, nutrient acquisition) (Dippery *et al.*, 1995, Gerhart & Ward, 2010, Poorter *et al.*, 2012).

Nutrient availability also regulates plant responses to elevated [CO_2] (Campbell & Sage, 2006). For example, nitrogen (N) is a limiting nutrient in many temperate and boreal ecosystems, and interactions of N limitations under elevated [CO_2] have been studied more often than interactions with other macronutrients, such as phosphorus and potassium (Taub & Wang, 2008). Plants grown at elevated [CO_2] often show a decrease in tissue N concentrations (Poorter *et al.*, 1997), especially when compared to plants grown at low [CO_2] (i.e., 200 ppm) (Campbell & Sage, 2006). Such decreases in N concentration are related to dilution by increased non-structural carbohydrates (NSC) content, alteration of plant phenology, or changes in root architecture in ways that reduce specific N uptake capability (Bernacchi *et al.*, 2007, Coleman *et al.*, 1993, Gifford *et al.*, 2000). However, the

effect of increased [CO_2] on tissue N concentration is smaller in legumes than in other plants (Jablonski *et al.*, 2002, Taub & Wang, 2008).

The symbiotic association of legume plants with N-fixing bacteria takes place in root nodules and can stimulate photosynthetic C assimilation by increasing tissue N content (Kaschuk *et al.*, 2009). Under elevated [CO_2], the interaction of C and N metabolism in legumes has been hypothesized as a stimulator of the plant's growth (Libault, 2014). Higher [CO_2] increases C supply to nodule-associated bacteria and allows the bacteria to fix more N. This, in turn, allows plants to maintain or even increase tissue N concentrations allowing overall greater total plant biomass (Sulieman *et al.*, 2015). However, N-fixation is an energy-demanding process and nodule-associated bacteria can be a large sink for C (Minchin & Witty, 2005). Therefore, changes in growth under elevated [CO_2] likely result from the balance between higher photosynthetic rates (likely, via increased leaf N content) and greater C cost from fueling nodules with carbohydrates.

At the moment, it is unclear to what extent growth rates and plant tissue stoichiometry in N-fixing plants are affected by changes in [CO_2]. In non-fixing plants grown at low [CO_2] (i.e., 150 ppm) RGR was lower, while biomass proportions in leaves were greater in plants grown at elevated [CO_2] (i.e., 700 ppm), which had a greater proportion of their total biomass in roots (Dippery *et al.*, 1995, Sage & Coleman, 2001). In N-fixing plants under elevated [CO_2], greater growth and photosynthetic rate would imply a larger supply of C supply for nodules, and hence higher N-fixation, N availability and ultimately, higher competitive advantage over non-fixing plants (Aranjuelo *et al.*, 2014, Bertrand *et al.*, 2007). However, it is uncertain whether elevated [CO_2] provides N_2-fixing plants a competitive advantage over non-fixing plants in terms of growth and development despite greater C costs for N acquisition.

In this study, our aim was to evaluate differences in plant C allocation to growth and total N-gain for an N-fixing legume grown under different [CO_2]. Beginning just after germination, plants were grown at three levels of [CO_2]: 170 ppm (as occurred during late Pleistocene epoch), 400 (ambient) and 700 ppm (projected future). We hypothesized that at higher [CO_2], growth rates would be greater due to enhanced C assimilation and N-fixation, thereby providing a competitive advantage over plants grown at low and atmospheric [CO_2]. To test this hypothesis we inoculated *Medicago sativa* L. (Alfalfa, also called Lucerne) seedlings with *Ensifer meliloti (Synorhizobium meliloti)*, an N-fixing bacterium, and grew the plants in a N-free substrate that made them entirely dependent on

symbiotic nitrogen fixation In this way, we forced *M. sativa* plants to obtain all of their N by C allocation to *E. meliloti*. Hence, plants had to spend C in the obligate symbiosis for N acquisition. We then harvested replicates cohorts of plants in different stages of the experiment, measured total C and N concentrations in different tissues, and then derived allocation patterns from these measurements.

3.2. Materials and methods

3.2.1. Bacterial material

A strain of *Ensifer meliloti* (Strain type: DSM1981, TAL 380) was used for the inoculation of *M. sativa* and were purchased at the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). The bacterial strains were cultivated on a YEM (yeast extract mannitol) medium and the culture was incubated at 30°C (± 1° C) for three days.

3.2.2. Plant growth conditions

M. sativa seeds, purchased at a commercial retailer (Bioland Hof Jeebel, Salzwedel, Germany), were individually weighed, placed in microplate wells containing distilled water, and kept at room temperature for 48 hours. Once germination had occurred, healthy seeds (visual assessment) were transplanted individually into small pots (0.3 L) filled with a sterilized 1:2 vermiculite:sand mixture (oven dried at 100°C during 8 hours), and inoculated with a pure liquid culture of *E. meliloti* (1 mL) in the late-exponential phase culture.

Pots were placed randomly in 12 glass chambers (96 pots per chamber) and [CO_2] treatments randomly assigned to chambers, (1) Pleistocene: 170 ppm, (2) ambient: 400 ppm and (3) projected future: 700 ppm. Custom-made growth chambers (80 cm high x 75 cm long x 45 cm wide, c. 250 L volume) (Figure 3.1), were mounted in the greenhouse at the Max Planck Institute for Biogeochemistry (Hartmann *et al.*, 2013). Plants were grown at 32/18 °C (day/night, ± 2 °C) and natural sunlight was supplemented with high-pressure sodium vapor lamps (400 W Gro-Lux®, Osram Sylvania Ltd., Danvers, MA) providing a minimum total photosynthetic photon flux (PPF) of 400-500 μmol $m^{-2}s^{-1}$ at daytime. After four weeks of growth/inoculation, surviving seedlings were transplanted to bigger pots (1.0 L) within growth chambers and these pots were also filled with a sterilized 1:2 vermiculite:sand mixture and inoculated with a pure liquid culture of *E. meliloti* (1 mL)

to ensure infection during the second week following transplantation.

All pots were irrigated/fertilized using an automated irrigation system that allowed maintaining humid substrate field capacity (i.e., irrigation until drainage water appeared in trays) without opening the chambers (Figure 3.1). Pots were fertilized weekly, with a N-free double-concentrated (as the standard Hansen (1994)) nutrient solution (NS) making plants growth rely exclusively on symbiotic nitrogen fixation. The solution contained $MgSO_4 \cdot 7H_2O$ (2.0 mM), KH_2PO_4 (0.5 mM), K_2HPO_4 (0.5 mM), $CaCl_2 \cdot 2H_2O$ (2.0 mM), K_2SO_4 (1.0 mM), $EDTA \cdot NaFe_{III}O$ (0.2 mM), H_3BO_3 (0.09mM), $MnCl_2$ (0.004 mM), $ZnCl_2$ (0.0008 mM), $CuCl_2 \cdot 2H_2O$ (0.0003 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.0001 mM), $CoCl_2$ (0.00002 mM). Despite consecutive harvests (see below), that reduced the number of plants per chamber, irrigation and application of NS had to be increased over time due to growing biomassFrom week 0 to week 4, 0.35 L of water and 0.35 L of NS were given to all treatments each week. From week 4 to week 19, 700 ppm and 400 ppm chambers were given 0.7 L $week^{-1}$ of water and 0.7 L $week^{-1}$ of NS, and to 170 ppm chambers were given 0.35 L $week^{-1}$ of water and 0.35 L $week^{-1}$ of NS.

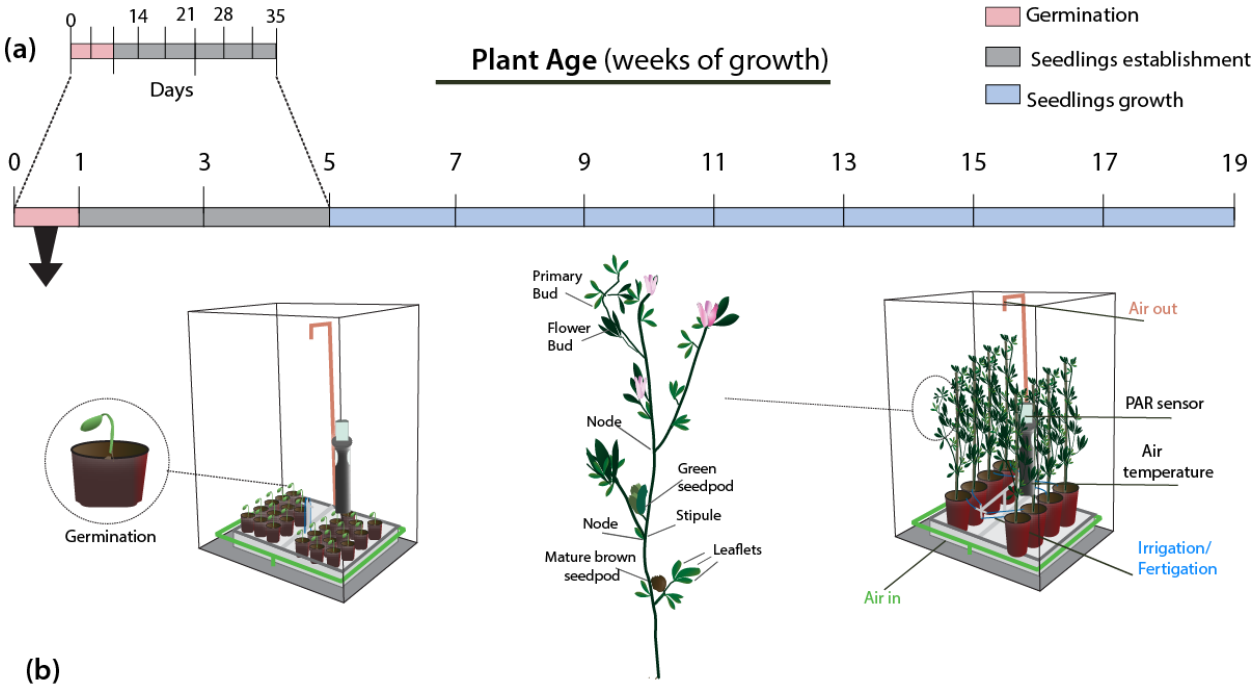

Figure 3.1. (a) Schematic representation of the experimental time line. The experiment lasted 19 weeks and plants were harvested for analysis every other week until the end of the experiment, except for the first five weeks of growth (grey) during which harvests were carried out every three days (tick mark indicating week of harvest). (b) Schematic representation of the germination period (week 1), leaf gas exchange measurements (week 8) and plant growth stage in the glass chambers.

3.2.3. Harvest and samples analysis

Harvest

Plants from each CO_2 treatment (n =3-5 per sampling period) were harvested every two weeks from day 0 of growth until week 19 of growth. Except for the first five weeks of growth, in which seedlings of each [CO_2] treatment were harvested every three days (n =5) (Figure 3.1a). At the harvest, total leaf area was determined using a leaf area meter (LI-3100 leaf area meter, Li-Cor Inc. Lincoln Nebraska, USA) and the number of nodules per plant was counted. At the completion of harvest, plants were separated into leaves, stems, roots and nodules. These were oven-dried to constant weight at 70°C, their dry weight recorded, and material was stored for subsequent elemental analysis.

As *M. sativa* plants developed, phenological changes in the plants were observed on individual stems. Over the experimental period, plants pass through vegetative, bud and flower stages (Table 3.1b). However, numerous stems at various stages of development were found within a single plant growing in a given [CO_2] treatment. We used the mean stage of development method defined by Kalu and Fick (1981) to quantify the stage of development in each harvest. The mean stage of development by count (MSC) was determined by examining individual stems and classifying them according to the staging categories shown in Table 3.1. MSC in a given harvest *(j)* was calculated as the average of the individual stage categories present in a plant, weighted for the number of stems in each stage:

$$\mathrm{MSC} = \frac{\sum_j (N° \, Stems \; x \; Stage)}{Total \; N° \; Stems} \qquad \text{Eqn. 3.1}$$

Table 3.1. Descriptions of *M. sativa* developmental stages. Modified after Mueller and Teuber (2007)

Developmental Stages		Stem length	Buds	Flowers	Seed Pods
Vegetative	Stage 0	≤ 15.0 cm	-	-	-
	Stage 1 (Mid Vegetative)	16-30 cm	-	-	-
	Stage 2 (Late vegetative)	≥31 cm	-	-	-

Bud	Stage 3 (Early bud)	1-2 nodes	-	-
	Stage 4 (Late Bud)	≥3 nodes		
Flowering	Stage 5 (Early Flower)	1 node	1 open flower	-
	Stage 6 (Late Flower)	≥2 nodes	Open flowers	-
Seed Pod	Stage 7 (Early seed pod)	1-3 nodes		Green seed pods
	Stage 8 (Late seed pod)	≥ 4 nodes		Green seed pods
	Stage 9 (Ripe seed pod)			Mostly brown, mature seed pods

Nitrogen concentration and isotopic analysis

Samples were ground with a ball mill (Restch MM200, Haan, Germany) and subsamples (c.1-5 mg DW) were analyzed at the Stable Isotope Laboratory of the Max Planck Institute for Biogeochemistry, using a Delta+ XL Isotope Ratio Mass Spectrometry (IRMS, ThermoFinnigan, Bremen, Germany). ^{15}N was expressed relative to atmospheric N_2 ($\delta^{15}N$).

For a given plant tissue, the amount of N content was calculated by multiplying from the amount of dry tissue biomass and with its measured N concentration:

$$\text{Tissue N(g)} = (\text{N}_{\text{tissue}}\ \text{concentration (mg g}^{-1}\ \text{DW}) * \text{sample DW (g)})/1000 \qquad \text{Eqn. 3.2}$$

The total amount of N derived from biological fixation was calculated using the N balance method (Unkovich *et al.*, 2008), where plants were grown in N-free media (i.e., sand:vermiculite substrate) and the only source of N for growth, apart from seed- and inoculum-N, was N-fixation. This was calculated as:

$$\text{Total Plant } N_{fixed}\,(g) = N_{(Leaves+Branches+Roots)}\,(g) - N_{(in\ sown\ seed\ +\ in\ inoculant)}\,(g) \quad \text{Eqn. 3.3}$$

3.2.4. Growth measurements and C cost of fixed N

We calculated for each harvested plant the, average RGR (unit dry mass increase per unit of dry mass) and net assimilation rate (NAR, unit dry mass increase per unit leaf area) and their standard errors were calculated using the analysis tool developed by Hunt *et al.* (2002) in Microsoft Excel®. Average LAR (leaf area ratio) was calculated as total leaf area/total plant biomass. These parameters are defined as:

$$RGR = LAR \times NAR \quad \text{Eqn. 3.4}$$

To estimate the amount of C used by the plant to fix the N used to grow tissues, we computed the whole-plant C released per unit of fixed N (C_{cost}). As all of the N in the plant (except that inherited from seeds) came from N-fixation, we used N content of both above ground and belowground C tissue. The C cost at the time *j,* at a given plant tissue, above (AG) or belowground (BG), was calculated as:

$$C_{cost(j)} = \text{Total DW (g)} \times \frac{AG\ or\ BG\ DW\ (g)}{Total\ \ DW\ (g)} \times \text{Tissue } [N]\,(\text{gN } 100\text{g}^{-1}\text{DW}) \times 7.8\ \text{gC g}^{-1}\text{N} \quad \text{Eqn. 3.4}$$

The respiratory/C cost of symbiotic N-fixation, 7.8 gC g^{-1}N (9.1 mol CO_2 mol^{-1}N) was based on the value reported in Fischinger and Schulze (2010) for vegetative growth of *Pisum sativum* L.

3.2.5. Statistical analysis

Mean values of the measured parameters were tested for differences for each time step (i.e., harvest) across CO_2 treatments by an ANOVA using R (v.3.1.0, R Foundation for Statistical Computing, 2012), after checking the assumption of heteroscedasticity across groups with a Levene test (Table 3.S2). When ANOVA indicated a significant overall treatment effect, a multiple comparison test was carried out (Tukey's honest significance test (Tukey's HSD, $\alpha<0.05$) to compare parameter means between treatments.

3.3. Results

3.3.1. Development and biomass production

By the end of week 1, the symbiosis of *M. sativa* with *E. meliloti* was established across [CO_2] treatments and indeterminate nodules started to develop. At this early stage of establishment, the ^{15}N discrimination of the seedlings' tissues was used to determine the contribution of N from fixation vs. N from seeds.

After 2 to 3 weeks of growth (15 to 21 days) ^{15}N isotope ratios had started to decrease (<0‰) in leaves and roots compared with seeds ^{15}N (~2.34‰) and atmospheric ^{15}N (0‰) (Figure 3.2a,b). During week 5, when all the plants were still at stage 0 of development, the leaf and root bulk tissues were ^{15}N depleted (<0‰) across [CO_2] treatments and we therefore considered that from week five on all N available in the plant was acquired through biological N-fixation (Figure 3.2a-c).

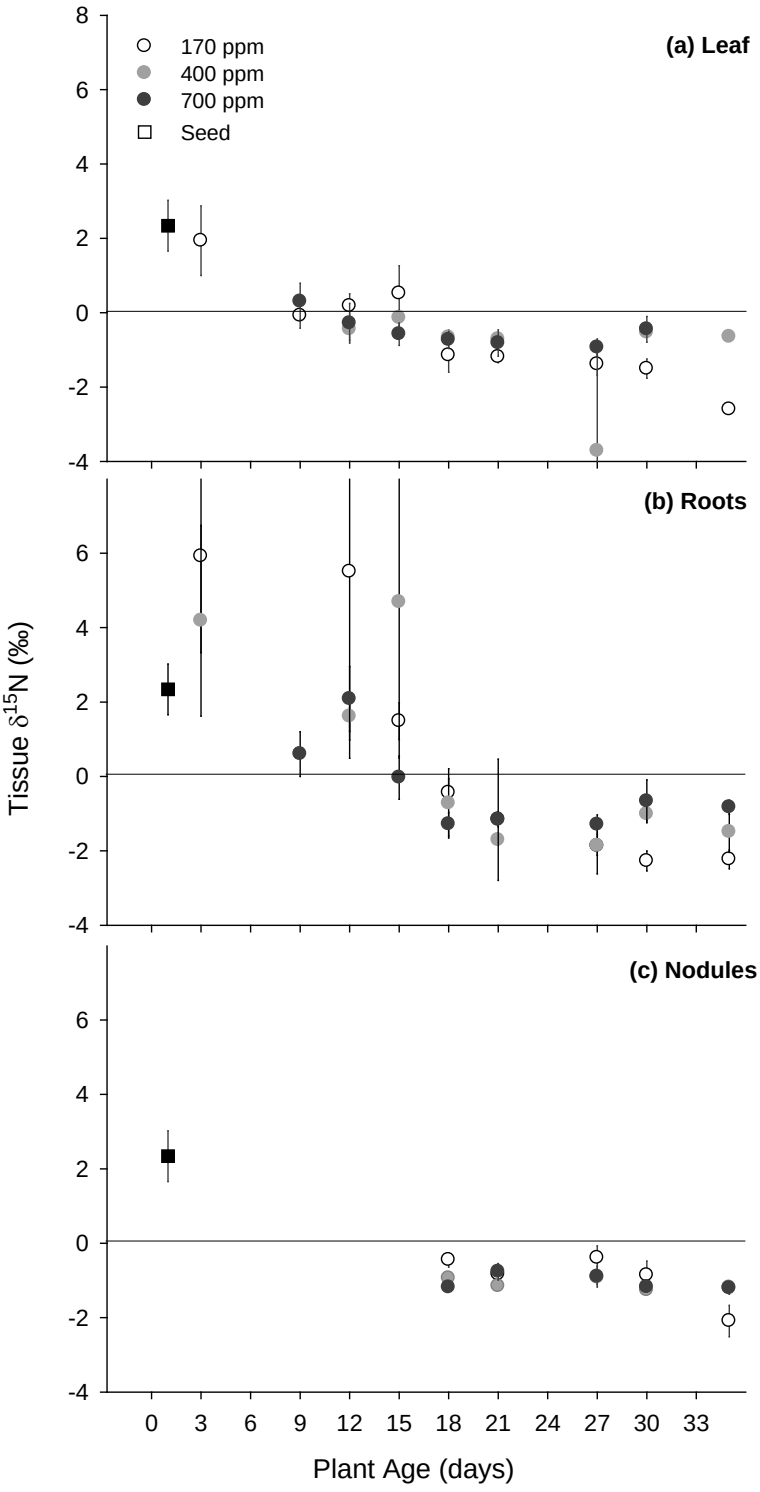

Figure 3.2. ^{15}N abundance during early plant development in aboveground (a), roots (b) and nodules (c) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170, 400 and 700 ppm (circles), and seeds (square). Values represent means ± 1 SE (n = 4).

Plants entered the vegetative phase (SMC= 0, see eqn.3.1) already at the second week of growth; however, the length of this phase varied between [CO_2] treatments. Plants grown at low [CO_2] were still in the vegetative phase after 19 weeks (SMC = 1.9), while plants grown at ambient and elevated [CO_2] showed the first visible signs of bud and flower production at week 15 (SMC=5.0), but the flowers were aborted approximately 3 to 5 days after they had appeared (Figure 3.3).

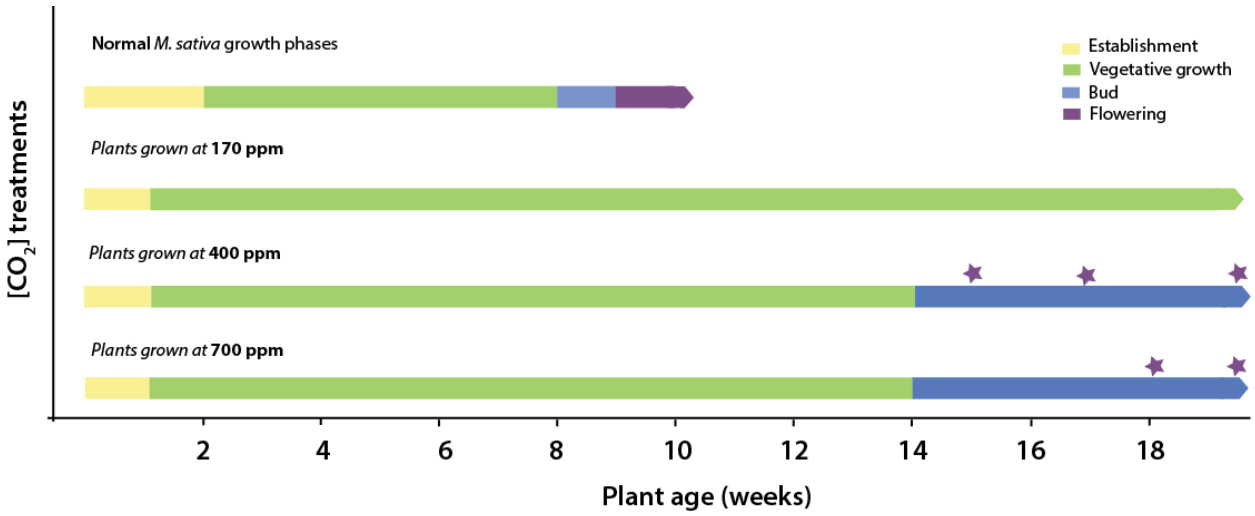

Figure 3.3. (a) *Medicago sativa* developmental stages in plants inoculated with *Ensifer meliloti* and grown at three atmospheric [CO_2]: 170, 400 and 700 ppm. The stars indicate the onset of flowering in the higher [CO_2] level from week 15.

In terms of whole plant biomass, the lowest growth was observed in plants grown under 170 ppm [CO_2]. Plants grown at 400 and 700 ppm were much larger than those grown at 170 ppm, but there was no difference between plants grown at 400 and 700 ppm (Figure 3.4). At the end of the experiment, plants grown at 170 ppm had approximately 7-fold lower biomass (Figure 3.4) and were also shorter, had fewer leaves per plant, smaller leaf area; and fewer nodules per plant compared to plants grown at higher [CO_2] levels (Table 3.S1).

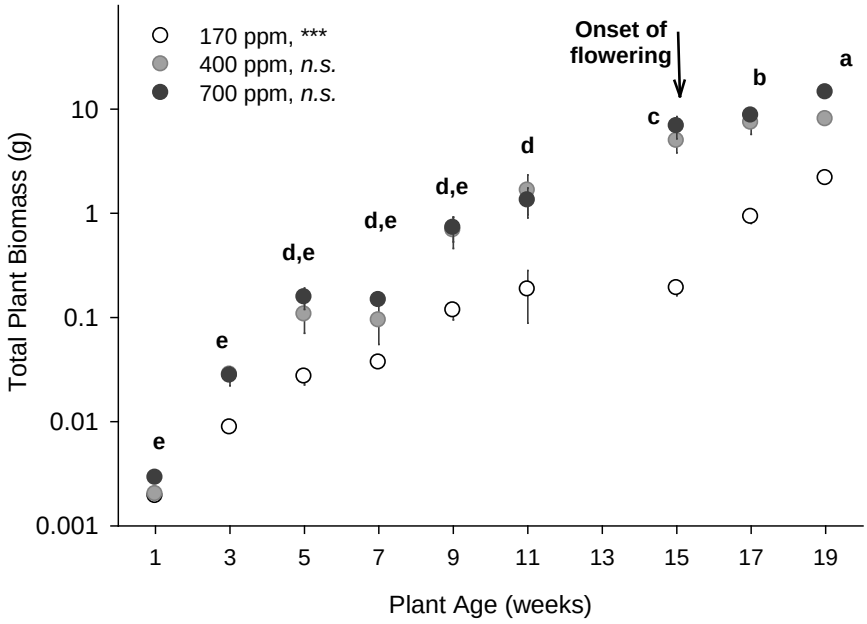

Figure 3.4. Total plant dry mass of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170, 400 and 700 ppm. Values represent means ±1 SE (n=5 until week 15, n=3 week 17 to week 19). Statistically significant effects of CO_2 levels are shown in legend and different letters indicate significant differences across plant age (weeks) (P<0.05) (Tukey's honest significant test (HSD following significant ANOVA). The arrow at week 15 shows the onset of flowering in the higher [CO_2] levels. Note log scale used on y-axis.

3.3.2. Biomass partitioning and growth rates

The proportion of biomass allocated to above- and belowground tissues differed between plants grown at higher [CO_2] compared to plants grown at 170 ppm (Figure 3.5a-c). However, in all treatments, aboveground tissues (leaves and stems) had a higher biomass proportion compared to roots and nodules. The proportion of biomass allocated to nodules was only between 1 and 3 % of the total plant biomass and this proportion was constant over time (Figure 3.5a-c).

After nine weeks of growth, the proportion of biomass allocated to aboveground tissues decreased in plants grown at 400 and 700 ppm (Figure 3.5b,c), while the proportion of biomass allocated to roots in relation-to-total plant mass increased. At low [CO_2] this trend was not the same, and a substantial shift to higher root proportions occurred only at week 19 (Figure 3.5a). At the last harvest, week 19, plants grown at elevated [CO_2] had similar proportions of biomass in aboveground tissues and roots (Figure 3.5c).

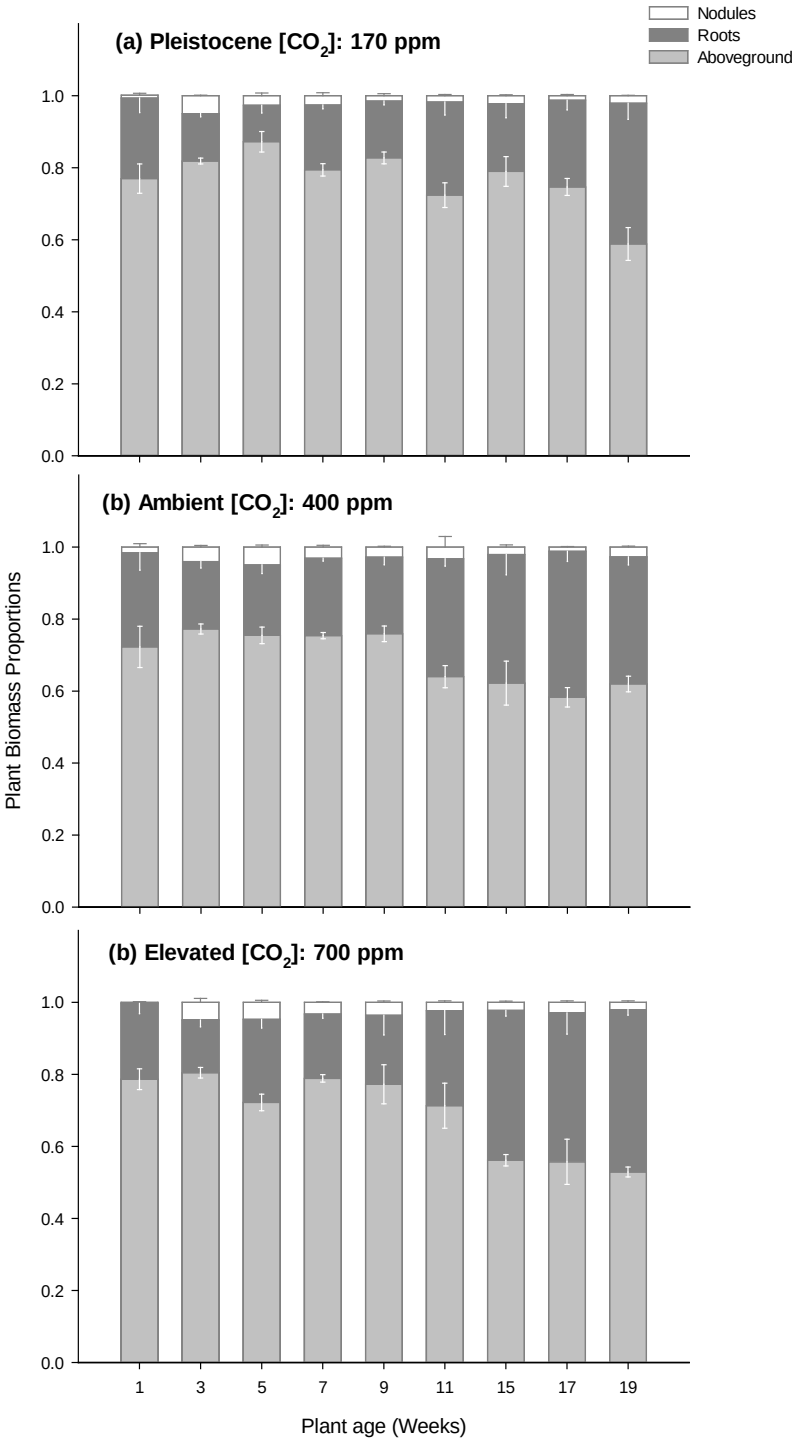

Figure 3.5. Partitioning of biomass between aboveground (leaves and stems), roots and nodules as a function of plant age for *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2] levels: (a) 170, (b) 400 and (c)700 ppm. Values represent means ± 1 SE (*n*=5 until week 15, *n*=3 week 17 to week 19).

High [CO_2] increased relative growth rates (RGR) (Figure 3.6a). Leaf area ratio (LAR, total leaf area/total plant biomass) did not change within [CO_2] treatments over time, and 170 ppm plants showed higher LAR compared to plants grown at 400 and 700 ppm (Figure 3.6b).

Trends in net assimilation rates (NAR) were similar to RGR (Figure 3.6c). As plants aged, RGR decreased at 400 ppm and 700 ppm (Figure 3.6a), while in plants grown at 170 ppm, RGR and NAR were low and did not vary over time (Figure 3.6a,c).

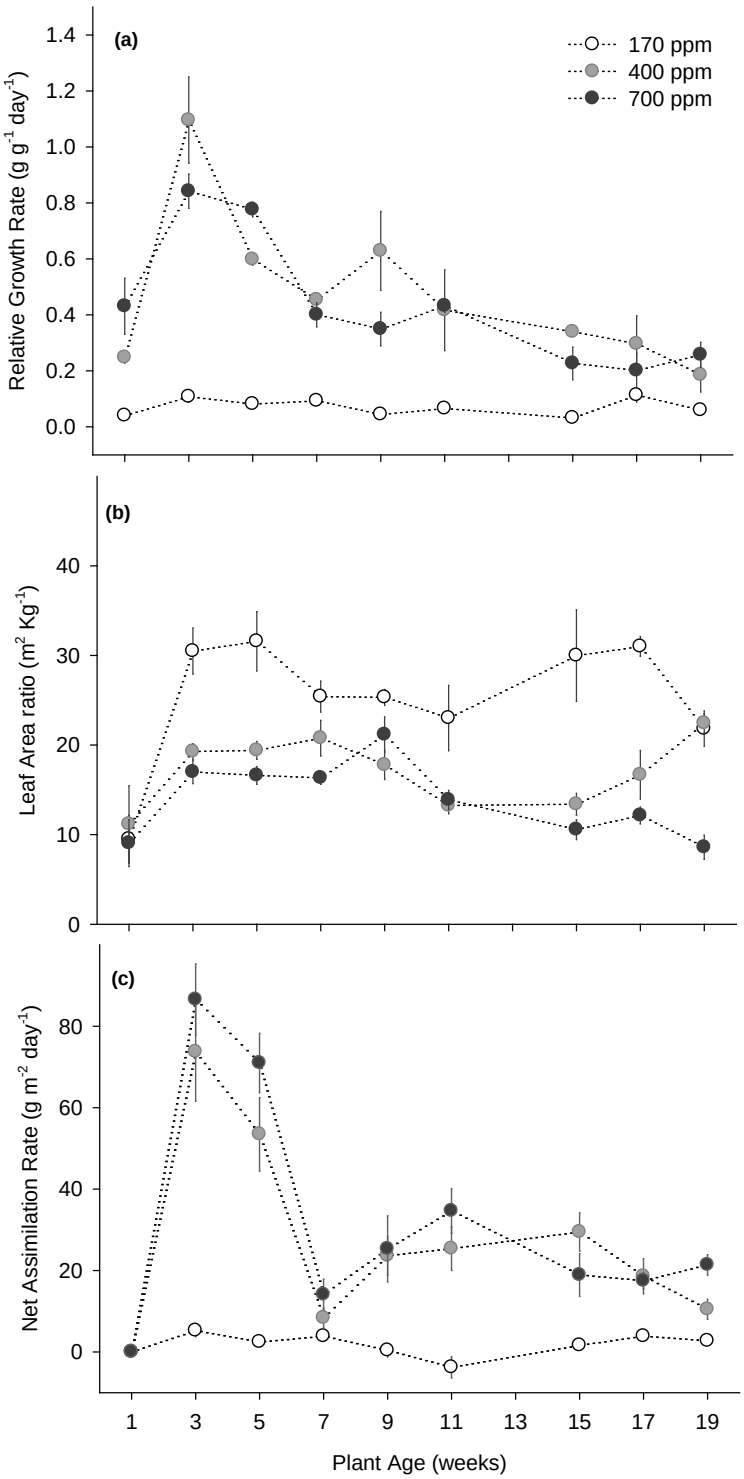

Figure 3.6. Relative growth rate (a), leaf area ratio (b) and, net assimilation rate (c) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2] levels: 170, 400 and 700 ppm. Values represent means ± 1 SE (n=5 until week 15, n=3 week 17 to week 19), both calculated after Hunt *et al.* (2002).

3.3.3. N distribution and C cost of N-fixation

Aboveground tissues (i.e., leaves and stems) generally had greater N concentrations (Figure 3.7) oscillated more, and were more variable between harvests than N concentrations in roots. However, we did not see significant differences in N concentrations between treatments in either tissue. The lowest N concentration in aboveground tissues was observed at week 15 in plants grown at 400 ppm (20.8 ± 5.0 mg g^{-1}), while the highest was observed at week 17 in plants grown at 170 ppm (43.0 ± 2.1 mg g^{-1}) (Figure 3.7a). Similarly, no significant differences were observed across treatments in the N concentrations of roots (Figure 3.7b). The average N concentration in roots was 18.8 ± 2.9 mg g^{-1} in 170 ppm plants, 20.6 ± 1.6 mg g^{-1} in 400 ppm, and 18.6 ± 1.7 mg g^{-1} in 700 ppm plants.

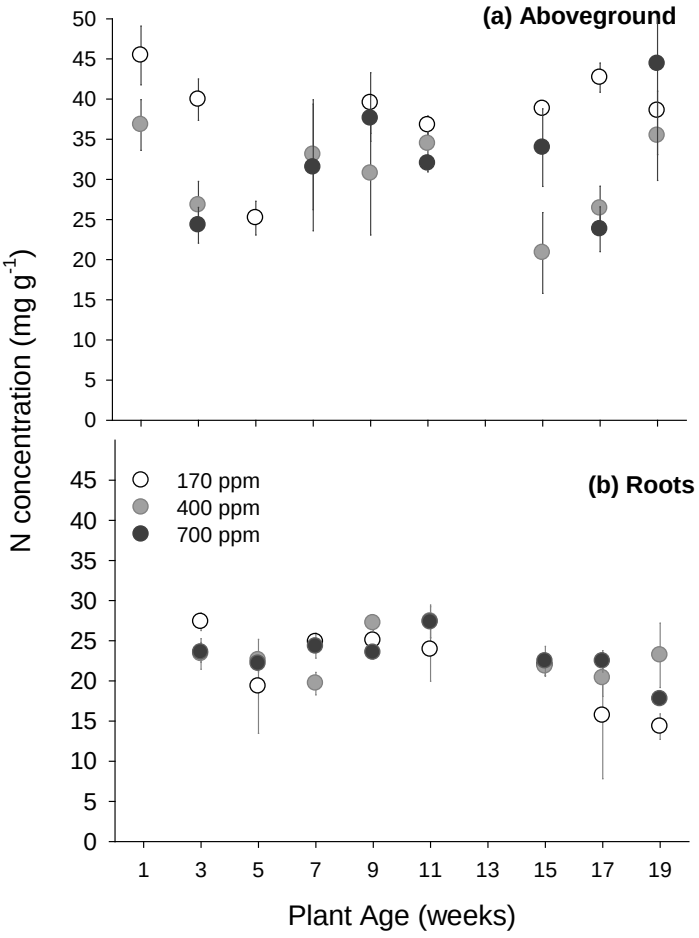

Figure 3.7. Nitrogen concentration in aboveground (a) and roots (b) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170, 400 and 700 ppm. Values represent means ± 1 SE (*n* =5 until week 15, *n* =3 week 17 to week 19).

Total N content, did increase over time across CO_2 treatments, especially between vegetative (week 11) and flowering (week 19) in higher CO_2 treatments (Figure 3.8a). The specific amount of C respired to fix N (g C g^{-1} DW, equation 3.4) was higher in plants grown at 170 ppm compared to plants grown at 400 and 700 ppm (Figure 3.8b). At establishment (week 3), 170 ppm plants were respiring 31% and 38% more C than 400 and 700 ppm plants per unit biomass, respectively. At week 19, while still in the late vegetative stage, plants grown at 170 ppm reached their minimum C cost, 2.0 ± 0.1 gC g^{-1}DW. In plants grown at 400 and 700 ppm, the C cost was maintained from establishment (week 3) until the end of the experiment (week 19), except for 400 ppm plants during early reproductive phase (week 15,) where the C cost dropped by ~46% (Figure 3.8b).

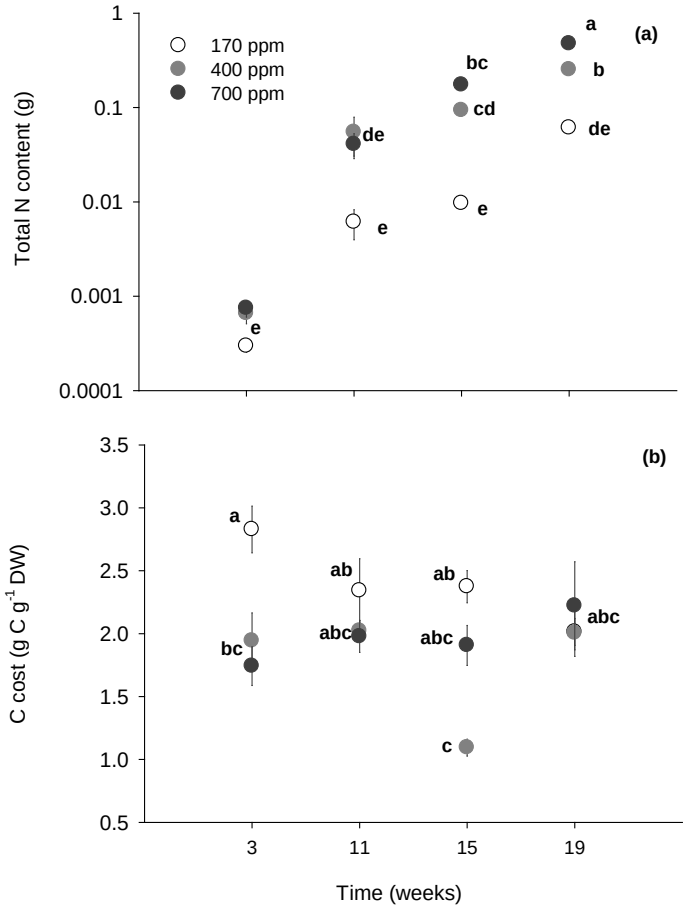

Figure 3.8. Total N content (a), and the specific amount of C respired to fix N per unit biomass (C cost) (b) in *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170, 400 and 700 ppm. Values represent means ± 1 SE (n =5 from week 3 to week 11, n =3 week 15 to week 19). Different letters indicate significant differences across plant age (weeks) (P<0.05) (Tukey's honest significant test (HSD following significant ANOVA). Note log scale used on Figure 3.8a's y-axis.

3.4. Discussion

N-fixation in nodulated *M. sativa* was positively correlated with atmospheric [CO_2], although the rate of increase of N-fixation slowed beyond current ambient atmospheric condition. Differences in plant response to the transition from Pleistocene to ambient [CO_2] were greater than those from ambient to elevated [CO_2]. The overall variation in the rate of C allocation to growth was strongly related to the net assimilation rate and the amount of C respired to fix N, yet N concentration in tissues was maintained across [CO_2] treatments and plant developmental stages.

M. sativa plants grew faster under ambient and elevated [CO_2] than under low [CO_2]. Overall, atmospheric [CO_2] affected plant phenology and flowering onset. Under natural conditions, flowering onset in *M. sativa* is initiated by changes in temperatures and photoperiod after approximately ten weeks of growth (Major *et al.*, 1991). In our experiment, where temperature was constant and natural sunlight was supplemented by greenhouse lighting, flowering onset started after 15 weeks of growth. Changes in [CO_2] affected time of flowering has been observed in *Arabidopsis thaliana* (Ward & Strain, 1997). Flowering at low [CO_2] was delayed and at higher [CO_2] we did not observe differences. In contrast, recent results indicate that elevated [CO_2] reduced the number of days to flowering, due to the positive effect on growth, specifically the increased number of leaves (Song *et al.*, 2009).

In our experiment, differences in RGR were related to changes in the whole-plant net assimilation rate. Net assimilation rate was proposed as the best indicator of RGR in response to light for herbaceous species (Shipley, 2002). Similar to our findings, differences in RGR due to changes in [CO_2] in *Abutilon theophrasti,* an annual weed, and in *Eucalyptus* trees, were associated with the net assimilation rate and not with the C invested in leaf area relative to plant size (LAR) (Dippery *et al.*, 1995, Ghannoum *et al.*, 2010). Furthermore, we did not observe differences in relative growth rates, plant size, number of nodules, ontogenetic changes or tissue N concentration between elevated and ambient [CO_2]. In contrast, biomass increments have been observed at elevated [CO_2] in *Medicago truncatula* grown at elevated [CO_2] over 7 weeks. In *M. truncatula* (cv. Jemalong), the larger biomass observed at elevated [CO2] was associated with a greater number of nodules and up-regulation of the N-fixation genes, which enhanced the overall percentage of N derived from the symbiotic fixation, and thus foliar N concentration (Guo *et al.*, 2013).

Biomass production and plant growth in *M. sativa* plants at different developmental stages were

affected by rising atmospheric [CO_2] and not by N availability. C allocation to growth and N fixation shifted among above- and belowground organs at different developmental stages to maintain sufficient N concentration for plant growth. For example, low [CO_2] plants had similar foliar N concentration compared to higher [CO_2] treatments, but allocated relatively more C to aboveground tissues (higher LAR). Low [CO_2] plants, however, grew less due to the increased C cost per unit of fixed N. Therefore, our original hypothesis was rejected, as N-fixation was not limited by belowground C availability and changes in growth were related to differences in C allocation and not to N availability. Increasing C cost of N-fixation in leaves with plant age has been observed in *Pisum sativum* (Voisin *et al.*, 2003), and has also been shown in *M. sativa* inoculated with *E. meliloti* (strain 102F78), where higher N demand in leaves was associated with enhanced C assimilation rates at elevated [CO_2] (Ariz *et al.*, 2015).

Considering that *M. sativa* is a common forage legume, both increased growth rates and hastened development from high [CO_2] may increase forage production (Dumont *et al.*, 2015). Moreover, it has been hypothesized that elevated [CO_2] reduces soil N availability by increasing N immobilization and denitrification in soil, at least in temperate grasslands (Simonin *et al.*, 2015). The fact that N concentration was maintained in aboveground tissues suggest that litter and fodder quality of *M. sativa* plants will be enhanced compared to other forage legumes, like white clover (*Trifolium rapens*), where N concentration decreased at elevated [CO_2] (Frehner *et al.*, 1997).

3.5. Conclusion

Current knowledge of the evolutionary responses of plants to low [CO_2] is limited (Becklin *et al.*, 2014), not to mention our understanding of N-fixers and how low atmospheric [CO_2] has impacted their growth since the last glacial maximum. However, based on our results, N-fixers did not have a competitive advantage during the Pleistocene epoch, when C was potentially more limiting than N (McLauchlan *et al.*, 2013). On the other hand, in a future world of elevated [CO_2], N-fixation may be advantageous if soil N availability is reduced. However, biological N-fixation at elevated [CO_2] may be limited by other soil nutrients, like molybdenum and phosphorus (van Groenigen *et al.*, 2006). Additionally, the C cost to fix N per unit of plant mass produced will increase. Hence, a deeper understanding of how elevated [CO_2] influences not only plant growth, but also C sink-and-source dynamics and physiological processes like photosynthesis and respiration, is necessary to predict the competitive advantage of this forage legume in the future.

3.6. Supplementary data

Table 3.S1. Plant traits of pot-cultured *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent mean ±1 SE (n= 5 until week 15, n= 3 week 17 and 19). Different letters indicate statistically significant differences within a parameter across [CO_2] levels over time at $P<0.05$.

	Plant height (cm)			Nitrogen content (g m^{-2})			Number of leaves ($plant^{-1}$)			Leaf area (cm^2)		
Plant Age (weeks)/[CO_2] (ppm)	**170**	**400**	**700**	**170**	**400**	**700**	**170**	**400**	**700**	**170**	**400**	**700**
1	1.82±0.25 g	1.20±0.15 g	1.44±0.17 g	4.21±0.17 a	2.96±0.17 ab	2.51±0.1 ab	0.80±0.20 d	0.80±0.20 d	0.80±0.20 d	0.18±0.06 e	0.25±0.11 e	0.28±0.09 e
3	4.26± ±0.49 fg	9.68± ±1.43 fg	8.76± ±1.74 fg	1.30± ±0.12 b	1.22±0.14 b	1.42±0.04 b	3.2±0.37 d	4.6±0.24 d	4.2±0.37 d	2.65±0.31 e	5.54±0.94 e	4.74±1.01 e
5	6.30±1.09 fg	17.50±2.55 efg	20.26±2.55 efg	0.78±0.08 b	1.62±0.04 b	1.59±0.04 ab	6.0±0.63 d	10.0±3.64 d	13.8±3.64 d	9.01±1.94 e	18.52±6.16 e	26.04±6.16 e
7	9.17±1.46 fg	11.00±3.22 fg	21.12±0.51 efg	1.05±0.01 b	1.51±0.27 b	2.26±0.43 ab	9.3±1.33 d	8.8±1.25 d	13.4±1.60 d	9.35±0.55 e	17.37±5.57 e	24.34±3.58 e
9	22.40±4.86 defg	27.86±5.26 cde	38.42±2.72 cde	1.45±0.15 ab	1.45±0.40 b	1.74±0.16 ab	21.7±2.85 cd	40.2±14.82 cd	58.6±13.82 cd	29.35±5.21 e	109±30 e	146±31 e
11	15.23±4.47 efg	41.90±1.97 cde	48.48±11.81 bcd	1.53±0.27 ab	1.53±0.20 ab	2.21±0.15 ab	23.3±9.33 cd	61.0±18.61 cd	65.8±22.47 cd	36.82±14.1 e	201±71 de	184±58 de
15	17.13±3.14 efg	88.94±18.80 a	89.80±11.13 a	1.29±0.23 b	1.24±0.37 b	1.59±0.28 ab	36.3±0.33 cd	212±42 bcd	231±51 b	57.42±12.4 e	682±194 c	693±124 c
17	21.03±2.29 efg	76.47±13.72 ab	89.13±8.70 a	1.16±0.11 b	1.22±0.23 b	1.43±0.49 b	96.3±16.37 cd	185±47 bcd	341±61 b	288±46 de	1155±100 b	1053±129 b
19	34.50±2.54 cdef	93.17±13.69 a	68.50±2.53 abc	1.35±0.32 ab	1.36±0.24 ab	1.46±0.22 ab	143±11 bcd	677±26 a	619±26 a	460±12 cd	1788±34 a	1866±185 a

Table 3.S1 (continuation)

Plant Age (weeks)/[CO_2] (ppm)	Aboveground mass (g)			Root mass (g)			Nodule mass (mg)			Number of nodules (plant^{-1})		
	170	**400**	**700**	**170**	**400**	**700**	**170**	**400**	**700**	**170**	**400**	**700**
1	0.00±0.00 d	0.00±0.00 d	0.00±0.00 d	0.00±0.00 e	0.00±0.00 e	0.00±0.00 e	0.02±0.01 f	0.03±0.03 f	0.01±0.01 f	1.40±0.87 e	1.00±0.77 e	0.60±0.40 e
3	0.01±0.00 d	0.02±0.00 d	0.02±0.00 d	0.00±0.00 e	0.01±0.00 e	0.00±0.00 e	0.44±0.06 f	1.19±0.26 f	1.42±0.41 f	2.40±0.24 e	3.60±0.93 e	3.60±1.12 e
5	0.02±0.00 d	0.08±0.03 d	0.12±0.03 d	0.00±0.00 e	0.02±0.00 e	0.04±0.00 e	0.71±0.02 f	5.52±2.44 f	7.68±2.44 ef	4.50±0.77 e	21.80±1.60 e	12.60±1.60 e
7	0.03±0.01 d	0.07±0.03 d	0.12±0.01 d	0.01±0.00 e	0.02±0.00 e	0.03±0.00 e	0.94±0.30 f	2.99±1.03 f	4.91±0.54 f	4.67±1.33 e	11.80±5.30 e	19.80±8.49 e
9	0.09±0.02 d	0.53±0.15 d	0.58±0.14 d	0.02±0.00 e	0.15±0.07 e	0.14±0.08 e	1.72±0.69 f	19.67±7.38 ef	26.99±6.86 def	10.33±1.45 e	51.80±10.02 e	49.20±9.81 e
11	0.13±0.07 d	1.09±0.44 d	0.96±0.32 d	0.05±0.03 e	0.56±0.24 e	0.36±0.11 e	3.20±1.05 f	43.38±13.98 def	31.10±9.60 def	20.33±1.76 e	72.60±17.35 de	58.20±23.41 e
15	0.15±0.02 d	3.14±0.91 c	3.91±1.0 bc	0.04±0.01 e	1.80±0.33 cd	2.91±0.63 bc	4.23±0.77 f	105±5 cd	156±19 bc	9.33±3.67 e	111±45 cde	103±29 cde
17	0.69±0.07 d	4.36±0.75 bc	4.95±0.26 b	0.22±0.05 e	3.04±0.91 bc	3.68±0.84 b	11.23±1.65 ef	86.21±8.37 cde	260±60 a	57.00±12.50 e	201±66 bc	297±64 b
19	1.28±0.03 d	5.10±0.36 b	7.82±0.75 a	0.85±0.20 de	2.90±0.16 bc	6.66±0.57 a	45.19±7.23 def	224±16 ab	301±85 a	127±33 cde	472±120 a	201±41 bcd

Table 3.S2. Analysis of variance for growth analysis traits including those *P* values that represent the significance levels of the Levene test for the homogeneity of variance. Also, shown are the *P* values for the statistically significant effects of CO_2 levels and plant age (weeks) (*P*<0.05 Tukey's honest significant test (HSD) following significant ANOVA).

		P values		
	Levene Test	Treatment	Week	Treatment x Week
Total Plant DW (g)	0.212*	$3.6e^{-16}$***	$2.2e^{-16}$***	$2.2e^{-16}$***
Plant Height (cm)	0.002**	$5.56e^{-14}$***	$2.2e^{-16}$***	$1.24e^{-10}$***
N content (mg cm^{-2})	0.05*	0.38	$1.58e^{-5}$***	0.51
Number of leaves ($plant^{-1}$)	0.125*	$7.59e^{-5}$***	$2.2e^{-16}$***	$6.38e^{-8}$***
Leaf area (cm^2)	0.001**	$2.2e^{-16}$***	$2.2e^{-16}$***	$2.2e^{-16}$***
Above ground mass (g)	0.002**	$6.97e^{-15}$***	$2.2e^{-16}$***	$2.2e^{-16}$***
Root mass (g)	0.025*	1.75^{-13}***	$2.2e^{-16}$***	$2.2e^{-16}$***
Nodule mass (mg)	$8.32e^{-6}$***	$4.71e^{-11}$***	$2.2e^{-16}$***	$5.42e^{-15}$***
Nodules	0.090*	$1.22e^{-5}$***	$2.2e^{-16}$***	$5.95e^{-9}$***

Significance codes: *** *P*<0.001, ** *P*< 0.01, * P<0.05

CHAPTER 4

Influence of atmospheric [CO_2] concentration on photosynthesis and respiration in nodulated *Medicago sativa* L.

Abstract

Plant carbon (C) allocation and plant metabolic processes can be affected by changes in C availability, such as changing atmospheric [CO_2].

We investigated how long-term exposure to different atmospheric [CO_2] (170 ppm, 400 ppm and 700 ppm) influences plant acclimation (plastic responses) by modification of morphological, physiological, and chemical traits in N-fixing *Medicago sativa* growing under obligated symbiosis.

In leaves of 8 week old plants, mesophyll and biochemical limitations influenced both C-assimilation and respiration. Additionally, light inhibited respiration, but the degree of inhibition decreased as atmospheric [CO_2] increased. Differences in respiration rates between leaves and roots reflected the demands for sucrose loading, not differences in metabolic costs associated with tissue N concentration, which was constant across [CO_2] treatments.

Our results suggest that acclimation responses of plant C-balance to long-term exposure to sub-atmospheric and elevated [CO_2] are driven by changes in C allocation. Low [CO_2] plants (i.e., 170 ppm) reduced metabolic cost despite a greater investment cost for N-fixation in order to maintain a positive C-balance and continue growth, whereas elevated [CO_2] plants increased their respiratory energy cost.

Keywords: Alfalfa, Nitrogen fixation, Photorespiration, Respiration, Kok effect, A/*Ci* curves

4.1. Introduction

Rising atmospheric CO_2 concentration ($[CO_2]$) has considerable implications for plants. Their history of adaptation to lower $[CO_2]$ conditions, however, may constrain their physiological, chemical, and/or morphological responses to currently rising $[CO_2]$ (Sage & Coleman, 2001, Tissue & Lewis, 2012). Changes in $[CO_2]$ influence both processes of carbon (C) assimilation and respiration (González-Meler *et al.*, 2009, Sage & Coleman, 2001), while allocation to growth, reproduction or symbiosis may have the greatest implications for land-atmosphere C balance.

At leaf level, C-assimilation rates are controlled by stomatal, mesophyll, and biochemical limitations (Flexas *et al.*, 2012). The gas exchange responses to changes in $[CO_2]$ are studied by tracking how C assimilation changes as a function of internal $[CO_2]$ (C_i) (Figure 4.1) (Farquhar *et al.*, 1980, Sharkey, 2015). At current atmospheric $[CO_2]$ levels, which have exceeded 400 ppm (NOAA, 2016), C-assimilation in most plants is still directly controlled by the efficiency of the catalytic reaction between $[CO_2]$ and the ribulose-1,5,-bisphosphate carboxylase enzyme (RuBisCO), represented as the steep slope in Figure 4.1. In this range, the efficiency of RuBisCO can be enhanced by increments of the $[CO_2]/[O_2]$ ratio, decreasing the RuBisCO affinity for O_2 and thus photorespiration rates (Sage, 2013).

Since the RuBisCO capacity at current $[CO_2]$ levels is not limiting C assimilation, under elevated $[CO_2]$, C-assimilation might further increase to the point where it is limited by RuBP regeneration (Kromdijk & Long, 2016). Alternatively, there is evidence that C-assimilation rates were lower at low $[CO_2]$. This has been attributed to reduction of the maximum carboxylation rates (Vc_{max}), a measure of RuBisCO capacity (Campbell & Sage, 2006, Sage & Coleman, 2001), and related to the expression of specific genes using signals of accumulated sucrose in the mesophyll cells (Long *et al.*, 2004). However, at the $[CO_2]$ levels found during the more glaciated periods of the late Pleistocene Epoch (e.g., 170-190 ppm) the implications for processes associated with C-assimilation are less studied (Gerhart & Ward, 2010). If C assimilation is limited by the RuBisCO activity at low $[CO_2]$, Vc_{max} decreased due to increments in the oxygenation-to-carboxylation ratio of RuBisCO (Vo/Vc) (Sage, 2013). Thus, the balance between these two opposite processes, carboxylation and photorespiration, has a major influence on the C-source strength under limited C-availability, and in turn influences C-sink processes, like growth and respiration (Ayub *et al.*, 2014).

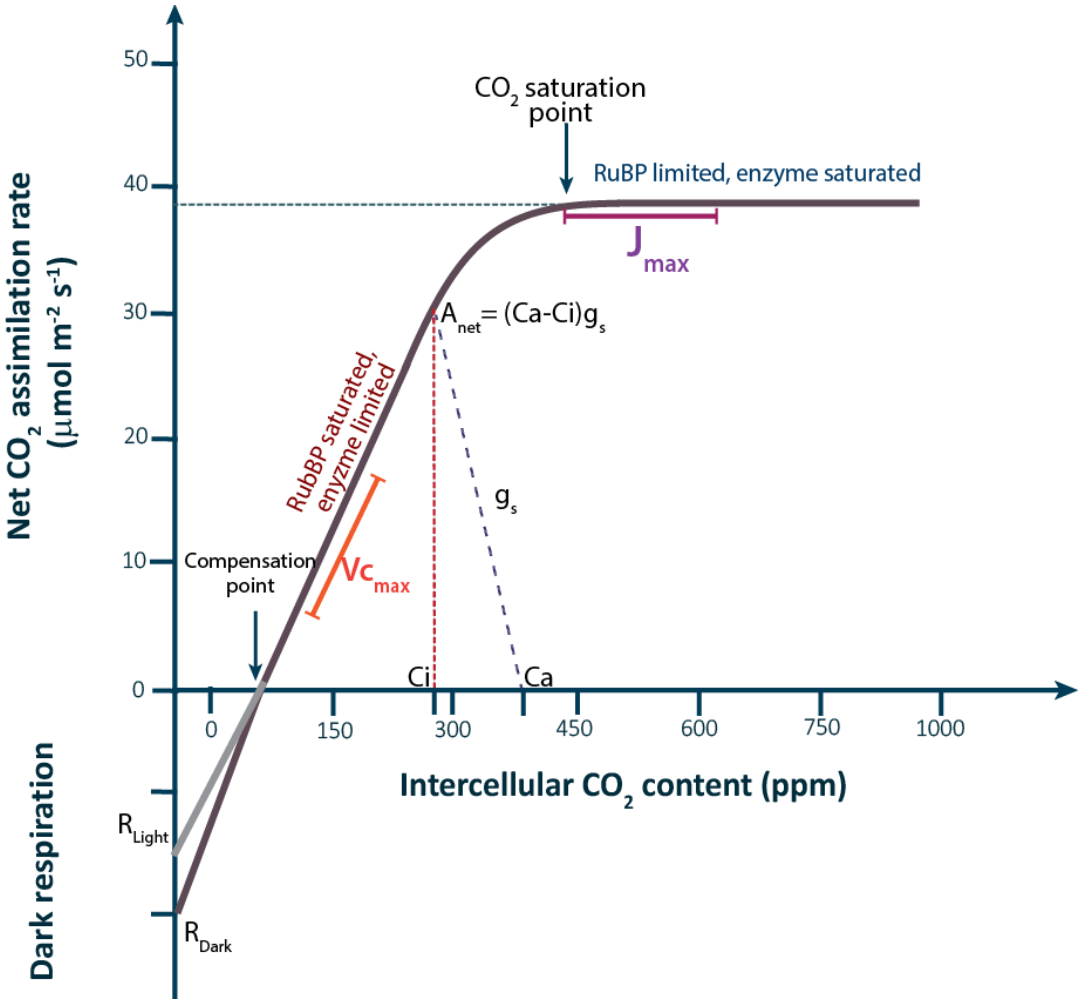

Figure 4.1. Theoretical responses of net CO_2 assimilation rate to intercellular CO_2 concentration for a typical C3 plant. Three important phases are observed: first, in the range above the compensation point the increase of net CO_2 assimilation reflects increased efficiency of RuBP-carboxylase. Here C-assimilation is limited by both the activation of RuBisCO and the stomatal aperture (as A_{net}= (*Ci*-Ca)·gs, where A_{net} is the net assimilation rate, *Ci* is the intercellular CO_2 and Ca the atmospheric CO_2, and gs is the stomatal conductance). The second range is the CO_2 saturation point. Here, RuBP-carb-oxylase is fully activated and the capacity of light harvesting is at its maximum. At this range C-assimilation is limited by the RuBP regeneration capacity. RuBP regeneration capacity, in turn, might be limited by the regeneration of inorganic phosphate (Pi). At this point, the photosynthesis electron transport (blue dashed line) provides ATP and reducing equivalents for anabolic processes and hence fuels starch and sucrose synthesis. The last range occurs at *Ci* below the compensation point where oxygenase activity of RubisCO strongly increases. Depending on ATP consumption in the cytosol, mitochondrial dark respiration is activated (R_{Dark}). In the vicinity of the light compensation point light-inhibited respiration (R_{Light}) is estimated by continuing the response observed above the compensation point, compared to the observed response of R_{Dark} with a greater slope below the break point. The regions of the curve from where the maximum carboxylation rate (*Vc*$_{max}$, orange) and maximum electron transport rate (J*max*, purple) are calculated are shown, as well as CO_2 compensation and saturation point (arrows). Modified after Schulze et al. (2002).

Leaf respiration also varies with [CO_2] in complex ways that include the supply and use of nonstructural carbohydrates (NSC) such as sucrose, and adjustments of metabolic costs (i.e., the demand for ATP by tissues under development) (González-Meler *et al.*, 2009, Leakey *et al.*, 2009b). Leaf respiration is inhibited by light, such that respiration rates measured in the light (R_{Light}) are normally less than those measured in the dark (R_{Dark}) (Griffin & Turnbull, 2013; Figure 4.1). Inhibition of respiration in the light is a consequence of disruption in the linear response of CO_2 assimilation under low levels of radiation (Sharp *et al.*, 1984), and is related to photorespiration and nitrogen availability (Tcherkez *et al.*, 2008, Zaragoza-Castells *et al.*, 2007). Increments of atmospheric [CO_2] have been shown to reduce the inhibition of respiration in the light, from 17-24% for plants grown at elevated [CO_2] to 29-35% for those grown in ambient [CO_2] (Shapiro *et al.*, 2004), and up to 80% for plants growing at pre-industrial Holocene [CO_2] levels (i.e., 290 ppm) (Ayub *et al.*, 2014). Yet, so far, few studies have investigated light inhibition of R_{Light} at [CO_2] at or below pre-industrial Holocene levels (see Ayub *et al.*, 2014). To our knowledge, to date no study has quantified how leaf respiration is inhibited by light at interglacial [CO_2] levels, and the study of Ayub *et al.* (2014) which investigated pre-industrial [CO_2] effects, was done on soy plants not limited by nutrients (including N).

In this study, we investigated the responses of C-assimilation, R_{Light} and R_{Dark} for obligate N-fixing *M. sativa* under interglacial, 170 ppm; ambient, 400 ppm; and projected future, 700 ppm [CO_2]. A unique feature of our study is that we forced *M. sativa* plants to obtain all N by allocating NSC to belowground tissues in order to feed the symbiotic N-fixing bacteria living in root nodules. We also investigated the amounts of NSC and respiration rates in nodulated roots as well as leaf tissues. Our main questions were: (i) how does [CO_2] influence leaf level physiological processes including A and R?; (ii) how does [CO_2] influence light inhibition of respiration (difference between R_{Light} and R_{Dark}), and how that is related to photorespiration rates? Finally, for a plant that must spend C to get N, (iii) how does the availability of [CO_2] affect NSC content in leaf and root tissues, and in turn root respiration rates? We hypothesized that, at low [CO_2], Vc_{max} will decrease due to increments in the oxygenation-to-carboxylation ratio of RuBisCO. Moreover, the relation R_{Light}/R_{Dark} would be greatest in plants with higher oxygenation-to-carboxylation ratios, likely low [CO_2] plants. However, at low [CO_2] levels, more of the fixed C would be allocated below ground to get N, resulting in higher root NSC concentrations and specific root respiration rates (respiration per unit biomass). Thus NSC concentration in low [CO_2] leaves may reflect in part greater C allocation and transport to

root tissues.

4.2. Material and methods

Individual plants used in the experiment were 55 days old (Figure 4.2). The conditions in which the plants were grown are detailed in Chapter 3. Briefly, plants were all grown from sprouted seeds at one of three levels of CO_2 (170, 400, or 700 ppm) and were supplied with water and all nutrients needed for growth except for N, which they could only obtain through N-fixing symbionts. As discussed in Chapter 3, the plants grown at higher CO_2 levels had begun to increase biomass, however all plants were at their mid vegetative stage.

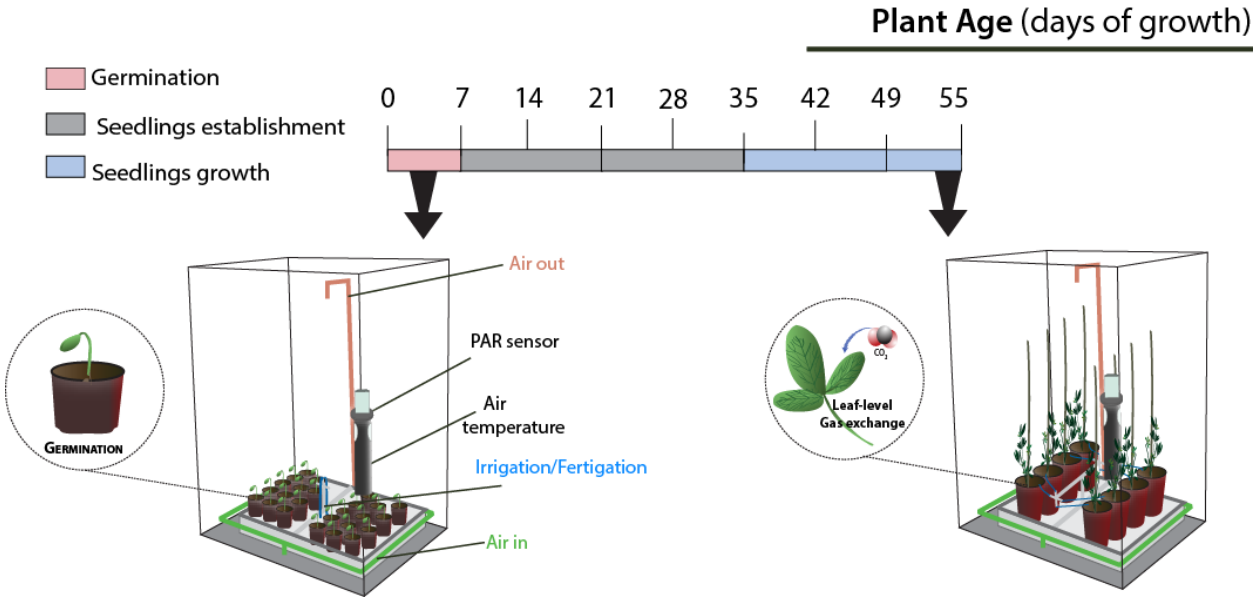

Figure 4.2. Schematic representation of the experimental time line. The gas exchange measurements were performed after 55 days (8 weeks) of growth.

4.2.1. Gas exchange measurements

Leaf-level gas exchange

Gas exchange measurements were conducted within growth chambers on 55-day-old *M. sativa* plants, using one fully expanded trifoliate leaf from the plants' upper canopy. Following the measurement, the leaf and its nearest neighbor were harvested, their leaf area determined using a leaf area meter (Li-Cor 3100, Li-Cor Inc. Licoln Nebraska, USA), after which they were immediately frozen in liquid nitrogen and vacuum freeze-dried for 96 h.

Gas exchange measurements were conducted using a portable photosynthesis measurement system (Li-Cor 6400; Li-Cor Inc. NE, USA). Flow rate through the cuvette was constant at 500 μmol $s^{-1.}$.

Relative humidity was kept between 40 and 50% using a desiccant, and leaf temperature was tracked during each measurement with the leaf temperature sensor in the Li-Cor 6400 chamber, with overall means of 31.2 °C (± 1.0°C), 33.2 °C (± 2.0°C) and 32.6 (± 1.4°C) in 170 ppm, 400 ppm and 700 ppm treatments, respectively. CO_2 concentration inside the leaf cuvette was adjusted to equal the relevant treatment level. Readings (*Amax*) were taken after rates of CO_2 exchange had stabilized, at saturated photosynthetic photon flux density (PPFD) of 2000 μmol photons m^{-2} s^{-1}. Thereafter, the light response of net CO_2 exchange was determined, using 1000, 700, 300, 150, 125, 100, 90, 80, 70, 60, 50, 40, 30, 20, and 0 μmol PPFD. Rates of respiration in darkness (R_{Dark}) were assessed after 30 min of zero irradiance. CO_2 response (A/*Ci*) curves were performed immediately after light response measurements at light saturation (2000 μmol PPFD).

A/*Ci* curves were fit with the PCE model version 2.0 (Buckey & Díaz-Espejo, 2015, Sharkey, 2015) developed by Sharkey *et al.* (2007). Four parameters were estimated: Vc_{max}, TPU, J*max* and g_m (respectively, the maximum carboxylation rate of RuBisCO, the maximum rate of triose phosphate use, the maximum potential electron transport rate and mesophyll conductance). Using the same model, both parameters the compensation point in the absence of dark respiration (Γ*) and the concentration at the site of carboxylation (Cc) at A*max* were obtained to calculate photorespiration as a fraction of carboxylation (*Vo*/*Vc*) as:

$$\frac{vo}{vc} = \frac{2\Gamma *}{Cc} \qquad \text{Eqn. 4.1}$$

Given the inhibition of respiration by light, respiration rates during the day (R_{Light}) were assessed from the light response curves using the Kok method (Kok, 1948). This method is based on the fact that photosynthetic responses to light are generally linear at low irradiances but, in the vicinity of the light compensation point, there is a break in the linear relationship with a greater slope below than above the break point (Heskel *et al.*, 2013). The slope at low irradiance (below the break) extends to R_{Dark}, at 0 PPFD, and the slope at higher irradiance (above the break) extrapolates to R_{Light} (Figure 4.3). The approach can be applied to any CO_2 level (Shapiro *et al.*, 2004, Yin *et al.*, 2011). R_{Light} was computed from the y-axis intercept of the linear fit to each light response curve (Table 4.S1).

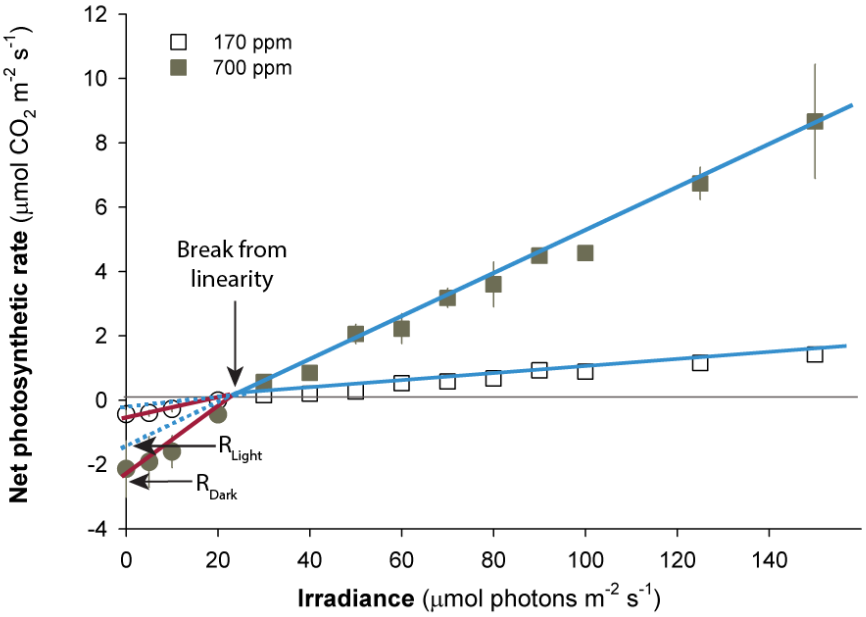

Figure 4.3. Example of light response curves for *M. sativa* at two atmospheric [CO_2]: 170 ppm (white) and 700 ppm (grey). Squares represent the average (±1 SE, n=5) of the higher- light response (slope from above the break) extrapolated to 0 PPFD to estimate R_{Light} (blue line). R_{Dark} is estimated by extrapolation of the slope below the break (red line, circles) to 0 PPFD.

Gas exchange parameters reported per unit of nitrogen were calculated using their maximum rate per unit area (e.g. *Amax* or R_{Dark}) divided by N concentration expressed per unit area.

Root respiration

Belowground respiration was measured on whole root systems, i.e., roots and their attached nodules, detached from the stem after gas exchange measurements. Rates were measured using a portable infrared gas analyzer (Li-Cor 6400; Li-Cor Inc. NE, USA) with a soil CO_2 flux chamber attached (6400-09). Roots were carefully washed to remove traces of the sand-vermiculite substrate. Respiration rate was determined as the linear regression slope from the CO_2 concentration in the chamber (i.e., 'CO2S' out of the Licor 6400) and time length of each measurement (Table 4.S2) and calculated as:

$$Root\ respiration = \frac{(m * v)}{(V_{\text{molar}} * U)} \qquad \text{Eqn. 5.2}$$

where m=linear regression slope (Table 4.S3), v= volume of the respiration chamber (0.991L), V_{molar}=22.4 L mol^{-1} and U represents the unit to which respiration is referred, for example weight (g),

nitrogen (g N) or pot area (0.073 m^2).

4.2.2. Harvest

After leaf gas exchange and root respiration measurements, we measured the total leaf area and number of nodules on the harvested plants. These were separated into two components: aboveground (AG) (i.e., leaves and stem) and belowground (i.e., roots and nodules), oven-dried to constant weight at 70°C, weighed, and then stored for subsequent elemental and isotope analysis.

Carbon and nitrogen concentration and stable isotope analysis

Samples were ground with a ball mill (Restch MM200, Haan, Germany) and subsamples (c.1-5 mg DW) were analyzed at the Stable Isotope Laboratory of the Max Planck Institute for Biogeochemistry, using a Delta+ XL Isotope Ratio Mass Spectrometry (IRMS, ThermoFinnigan, Bremen, Germany). ^{13}C was expressed in reference to the Vienna Pee Dee Belemmite ($\delta^{13}C$) and ^{15}N was expressed relative to atmospheric N_2 ($\delta^{15}N$).

4.2.3. Nonstructural carbohydrates (NSC) and amino acids measurements

Total soluble nonstructural carbohydrate (NSC) concentrations were estimated from the sum of measurements of three major sugars (glucose, fructose, and sucrose) representing mobile compounds and starch as the main non-mobile compound, within each tissue (leaves and roots). Deep-frozen samples (n=5 for each tissue type) of leaves and roots were vacuum freeze-dried for 96 h and milled (Retsch MM200, Haan, Germany) to fine powder. After grinding, approximately 10 mg of the samples were added to 1 mL of distilled water. The mixture was vortexed and incubated for 10 min at 65 °C in a thermomixer and then centrifuged for 6 min at 12000 g. The supernatant was removed with a pipette, stored on ice and the water extraction procedure was repeated twice and the three extracts combined.

The same amount of ground sample (approximately 10 mg) was added to 0.35 mL distilled water, vortexed for 1 min and treated for 10 min in a thermomixer at 65°C. For starch hydrolysis we then added 0.5 mL of 33% perchloric acid and let it incubate in an orbital shaker for 30 min. After centrifugating for 12000 g for 6 min, the supernatant was removed with a pipette and the procedure was repeated on the remaining pellet.

Sample concentrations were diluted (1:5 sugars and 1:55 starch) with bi-distilled water. The sample

extracts were measured using high/pressure liquid chromatography with pulse amperometric detection (HPLC-PAD) on a Dionex ICS 3000 ion chromatography system equipped with an autosampler (Raessler *et al.*, 2010). Peak areas (heights) were converted to concentrations using a calibration curve made with known concentration mixtures of sugars.

An aliquot of the nodule sugar extracts was pipetted into tin cups and assayed with a Finnigan MAT DeltaPlus XL EA-IRMS (ThermoFinnigan GmbH, Bremen, Germany), coupled to an autosampler. Amino acid concentrations were quantified on the same extracts prepared for nodule NSC analyses, and were diluted at a ratio of 1:10 (v:v) in water. Amino acid measurements of the diluted extracts were performed at the Max Planck Institute for Chemical Ecology in Jena using liquid chromatography (LC-MS/MS) as described in Docimo *et al.* (2012) coupled to an API 5000 tandem mass spectrometer (Applied Biosystem, Darmstadt, Germany). All samples were spiked with ^{13}C and ^{15}N labelled amino acids (algal amino acids ^{13}C, ^{15}N, Isotec, Miamisburg, OH, USA) at a concentration of 10µg of the mix per mL solution. The concentration of individual labelled amino acids in the mix was determined by HPLC-fluorescence detection analysis after pre-column derivatization with ortho-phthaldialdehyde-mercaptoethanol using external standard curves made from standard mixtures (amino acid standard mix, Fluka plus Glutamine, Asparagine and Tryptohan, also Fluka). Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N labelled amino acid internal standard. The exceptions were tryptophan and asparagin: tryptophan was quantified using ^{13}C, ^{15}N-Phenylalanine applying a response factor of 0.42. Asparagin was quantified using ^{13}C, ^{15}N-Asparagine applying a response factor of 1.0.

4.2.4. Statistical analysis

Treatment means of measured parameters were tested for differences by a one-way ANOVA ($[CO_2]$ treatment as source factor) or two-way ANOVA ($[CO_2]$ treatment and Respiration, both R_{Light} and R_{Dark}, as source factor with Type I sum of squares using R (v.3.1.0, R Foundation for Statistical Computing, 2012) and the package Agricolae, version 1.1-8 (de Mendiburu, 2014). When differences were significant, a multiple *post hoc* comparison test (Unequal N Tukey's Honestly Significant Difference test) was carried out.

4.3. Results

4.3.1. Photosynthetic parameters and gas exchange characteristics

Plants grown at both ambient and elevated [CO_2] had the highest leaf-level CO_2 assimilation rates, as well as higher (modelled) Vc_{max}, TPU, J*max*, and lower *Vo*/*Vc* ratio compared to plants grown at 170 ppm [CO_2]. Stomatal conductance, however, was maintained across [CO_2] levels, and the *Ci*/*Ca* ratio only differed between plants grown at 170 ppm and those grown at 400 ppm (Table 4.1).

Table 4.1. Leaf level photosynthesis and biochemical estimated parameters of *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent means (n=5 ± SE) for each growth CO_2 level. Different letters represent significant differences among growth levels (*P*<0.05, honest significant differences (HSD) test, following ANOVA). *P* represents the significance level of the ANOVA.

	Growth [CO_2] (ppm)			Significance
Traits	**170**	**400**	**700**	***P***
Amax				
per area (μmol m^{-2} s^{-1})	4.36 ± 1.02^{b}	29.30 ± 4.40^{a}	66.88 ± 12.70^{a}	*P*<0.001
per dry weight (μmol g^{-1} s^{-1})	0.17 ± 0.04^{b}	1.05 ± 0.35^{ab}	2.40 ± 0.50^{a}	*P*<0.01
per nitrogen (μmol gN^{-1} s^{-1})	3.47 ± 0.50^{c}	21.69 ± 5.11^{b}	49.78 ± 9.87^{a}	*P*<0.01
E (mmol H_2O m^{-2} s^{-1})	5.88 ± 0.76^{c}	9.26 ± 0.74^{b}	12.35 ± 0.91^{a}	*P*<0.001
g_s (mol H_2O m^{-2} s^{-1})	0.36 ± 0.05	0.36 ± 0.04	0.55 ± 0.11	NS
Ci/*Ca*	0.78 ± 0.01^{a}	0.65 ± 0.01^{b}	0.71 ± 0.03^{ab}	*P*<0.01
g_m (mol CO_2 m^{-2} s^{-1})	1.86 ± 0.72	0.84 ± 0.55	1.50 ± 0.42	NS
Jmax (μmol m^{-2} s^{-1})	106 ± 23^{b}	196 ± 39^{ab}	341 ± 64^{a}	*P*<0.01
Vc_{max} (μmol m^{-2} s^{-1})	82.6 ± 28.03^{b}	567 ± 83^{a}	471 ± 105^{a}	*P*<0.01
TPU (μmol m^{-2} s^{-1})	6.58 ± 0.78^{b}	19.32 ± 2.99^{a}	26.60 ± 4.51^{a}	*P*<0.01
Vo/*Vc*	0.81 ± 0.02^{a}	0.44 ± 0.2^{b}	0.24 ± 0.01^{c}	*P*<0.001

Amax= Photosynthetic rate at saturation; E=Transpiration; g_s= Stomatal conductance; *Ci*/*Ca*= Intercellular-to-atmospheric CO_2 concentration ratio; g_m= mesophyll conductance; J*max*= electron transport; Vc_{max}= Maximum rate of carboxylation allowed by RubisCO; TPU= Triose phosphate Use; *Vo*/*Vc*= Oxygenation-to-carboxylation ratio.

When A*max* rates were compared to rates representative of the average PPFD (~500 μmol photons m^{-2} s^{-1}) experienced by the plants growing in our greenhouse (A_{500}), it was clear that CO_2 assimilation

was light inhibited in plants grown in the high [CO_2] treatments (Figure 4.4a). On the other hand, plants grown at low [CO_2] were C-limited, as was shown in their constant A*max* responses to intracellular CO_2 content increments (C-supply) (Figure 4.4b).

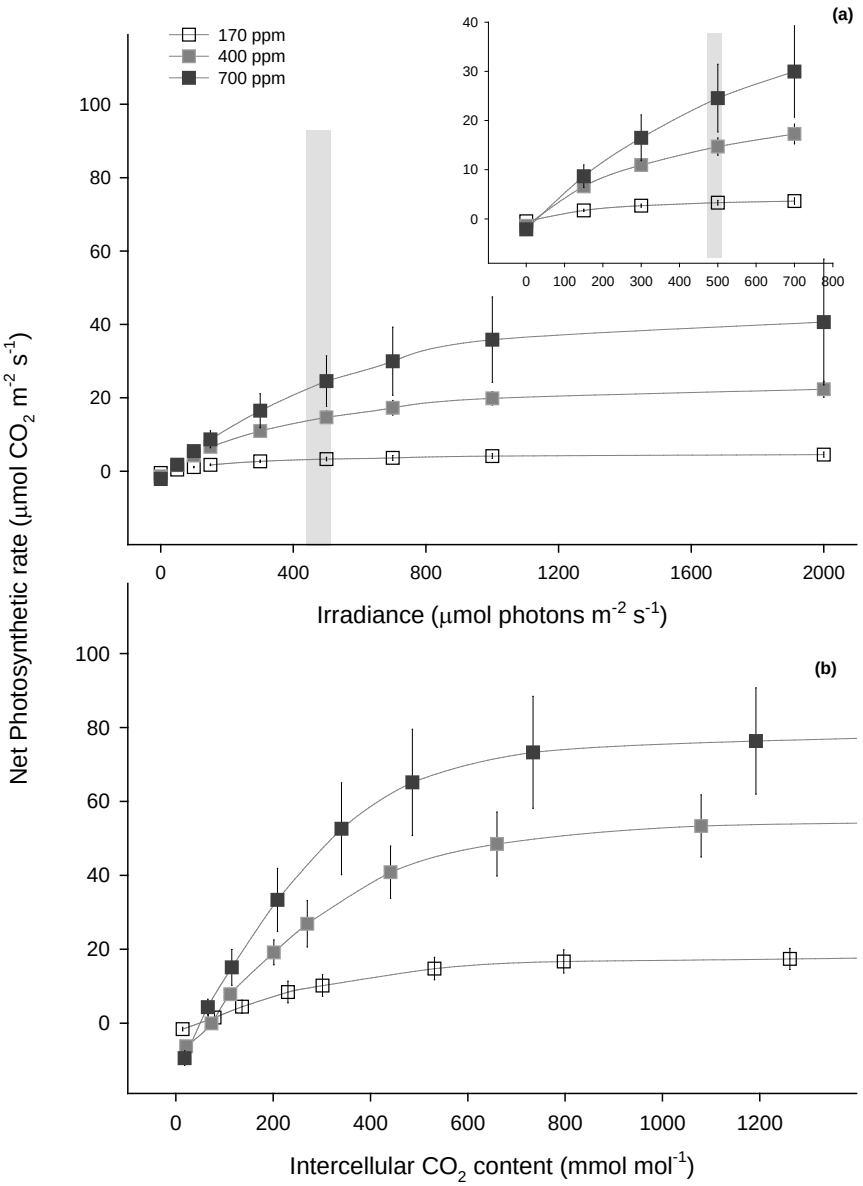

Figure 4.4. Net assimilation curve in response to (a) light and to intercellular CO_2 concentration (b) in *M. sativa* plants measured at contrasting atmospheric [CO_2] growth treatments: 170 ppm (white), 400 ppm (light grey) and 700 ppm (dark grey). In panel (a) the grey vertical line shows the maximum light availability in our greenhouse (~500 PPFD). For the inset plot, axis shows the same parameters as the primary plot. Values represent means ± 1 SE (n=5).

Leaf *R* was 25% and 29% inhibited by light at low and ambient [CO_2], respectively (Table 4.2). Leaf

respiration rates were highest for plants grown at elevated [CO_2] and, were not inhibited by light ($R_{Light} = R_{Dark}$; Table 4.2; Figure 4.2). Leaves grown at elevated [CO_2] had the lowest respiration-to-C assimilation ratio.

While specific respiration rates (expressed either per unit dry weight or N) in leaves increased with [CO_2], specific respiration rates in nodulated roots were two-fold higher in low [CO_2] compared to higher [CO_2] levels (Table 4.2). When combined with larger root biomass in higher [CO_2] levels and expressed per unit pot surface area, this meant that root respiration rates were lower overall at low [CO_2] (Table 4.2). In all cases, no significant differences were observed between the 400 and 700 ppm [CO_2] treatments.

Table 4.2. Average tissue respiration rates. Leaf averages include respiration in the dark and in the light of *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent means (n=5 ± SE, except for root respiration at 170 ppm (n=3) and 400 ppm (n=4) for each growth CO_2 level. Different letters represent significant differences among growth levels (*P*<0.05, honest significant differences (HSD) test, following ANOVA). *P* represents the significance level of the ANOVA.

	Growth [CO_2] (ppm)			Significance
Traits	**170**	**400**	**700**	***P***
Leaf:				
R_{Dark}				
per area (μmol m^{-2} s^{-1})	0.44 ± 0.13^{b}	1.41 ± 0.36^{ab}	2.14 ± 0.68^{a}	*P*<0.1
per dry weight (μmol g^{-1} s^{-1})	0.02 ± 0.00^{b}	0.04 ± 0.01^{ab}	0.08 ± 0.03^{a}	*P*<0.1
per unit nitrogen (μmol gN^{-1} s^{-1})	0.36 ± 0.11^{b}	0.97 ± 0.20^{ab}	1.59 ± 0.51^{a}	*P*<0.1
R_{Light}				
per area (μmol m^{-2} s^{-1})	0.32 ± 0.12^{b}	0.74 ± 0.20^{ab}	1.96 ± 0.94^{a}	*P*<0.01
per dry weight (μmol g^{-1} s^{-1})	0.01 ± 0.00^{b}	0.02 ± 0.01^{ab}	0.07 ± 0.03^{a}	*P*<0.05
per unit nitrogen (μmol gN^{-1} s^{-1})	0.25 ± 0.09^{b}	0.49 ± 0.12^{b}	1.47 ± 0.69^{a}	*P*<0.01
R_{Light}/R_{Dark}	0.75 ± 0.12	0.71 ± 0.13	1.09 ± 0.09	*P*<0.1

$R_{Light}/Amax$	0.08 ± 0.02[a]	0.03 ± 0.01[b]	0.03 ± 0.01[b]	$P<0.01$
$R_{Dark}/Amax$	0.12 ± 0.04[a]	0.05 ± 0.01[ab]	0.03 ± 0.01[b]	$P<0.05$
Root:				
R				
per pot area (μmol m^{-2} s^{-1})	0.15 ± 0.04[b]	0.62 ± 0.13[a]	0.76 ± 0.15[a]	$P<0.01$
per dry weight (μmol g^{-1} s^{-1})	0.07 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	P<0.1
per unit nitrogen (μmol gN^{-1} s^{-1})	2.06 ± 1.03	1.30 ± 0.20	1.22 ± 0.18	NS

$R_{Dark=}$ Dark respiration; $R_{Light=}$ Light-inhibited respiration; *Amax*= Photosynthetic rate at saturation; R= Respiration

4.3.2. Structural and biochemical traits

Leaves grown under different [CO_2] did not differ significantly in N or C concentrations, and had the same SLA (Table 4.3). However, plants grown at ambient and elevated [CO_2] were taller and had more leaves, so that their total leaf area was almost three times greater than plants grown at low [CO_2] (Table 4.S3).

Amino acids made up a small component of total leaf N (Table 4.3). Highest total amino acid concentrations were observed in plants grown at elevated [CO_2], but of all the major amino acids (e.g., glutamic acid, glutamine, serine), only asparagine concentrations were significantly different across [CO_2] levels (Table 4.3).

Table 4.3. Average (n=5± SE) of structural and biochemical traits of the leaves used for gas exchange measurement of *Medicago sativa* plants growing under variable atmospheric CO_2 concentration (170, 400 and 700 ppm). Different letters represent significant differences (P<0.05, honest significant differences (HSD) test, following ANOVA) among growth levels.

Leaf structural and biochemical traits	Growth [CO_2] (ppm)			Significance
	170	400	700	*P*
Structural traits:				
Specific leaf area ($m^2 kg^{-1}$)	40.25± 2.28	33.59 ± 5.77	34.60 ± 2.59	NS
Leaf Carbon (%)	42.47 ± 3.48	36.98 ± 5.51	32.79 ± 5.65	NS
Leaf Nitrogen (%)	5.02 ± 0.25	4.60 ± 0.51	4.70 ± 0.25	NS
Biochemical traits:				
Total Amino acids (nmol g^{-1})	151 ± 14^{b}	217 ± 40^{ab}	303 ± 41^{a}	P<0.05
Glutamic acid (nmol g^{-1})	30.28 ± 7.13	55.90 ± 14.62	75.58 ± 22.18	NS
Glutamine (nmol g^{-1})	11.28 ± 4.09	42.74 ±12.11	54.71 ± 27.42	NS
Alanine (nmol g^{-1})	27.20 ± 5.81	29.88 ±5.65	50.21 ± 9.55	NS
Serine (nmol g^{-1})	22.01 ± 2.23	21.91 ± 4.55	20.29 ± 3.24	NS
Asparagine (nmol g^{-1})	17.61 ± 3.16^{b}	26.59 ± 7.19^{ab}	40.30 ± 6.76^{a}	P<0.05

The $\delta^{15}N$ values in plant tissues were all < 0‰. For plants grown at low [CO_2], $\delta^{15}N$ increased from roots (~-1.8‰) to nodules (~-1.2‰) to leaves (~-0.2 ‰) and are consistent with plants that acquired their N through N-fixation in the nodules (Figure 4.5). Furthermore, in the low [CO_2] plants, $\delta^{15}N$ values in leaves and particularly in roots, were lower, indicating that the amount of fixed N in these tissues was small enough that some influence of the N inherited from the seed was still detected in 5-week old plants.

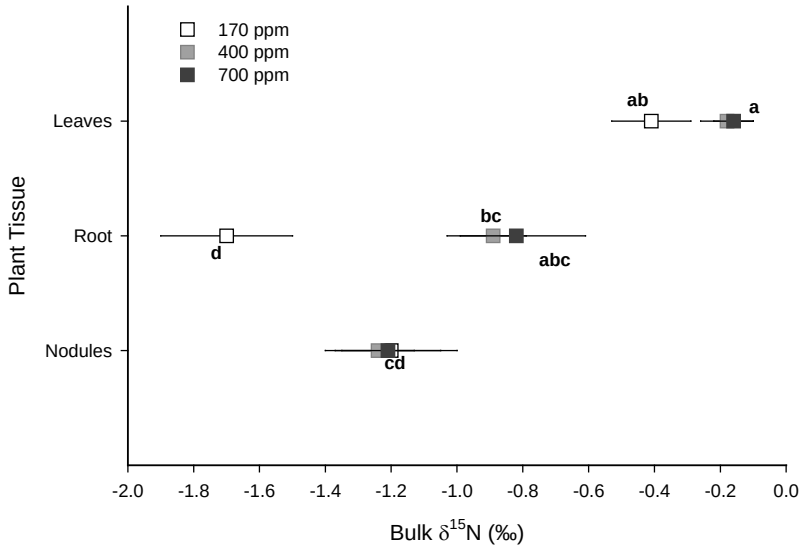

Figure 4.5. Bulk $\delta^{15}N$ in different tissues of *M. sativa* measured at contrasting atmospheric [CO_2] growth levels: 170 ppm (white), 400 ppm (light grey) and 700 ppm (dark grey). Values represent means ± SE (n=5 leaves). Different letters indicate significant differences between plant tissues across growth levels (P<0.05, Honest significant differences (HSD) test).

Overall, roots had higher concentrations of sucrose, glucose, fructose and starch than leaves (Figure 4.6). However, the patterns of variation with [CO_2] differed between the tissues. Roots of low [CO_2] plants had higher concentrations of sucrose, glucose and fructose (Figure 4.6e-g) than plants grown at ambient or elevated levels. Additionally, leaves of low [CO_2] plants had higher concentrations of starch (Figure 4.6d).

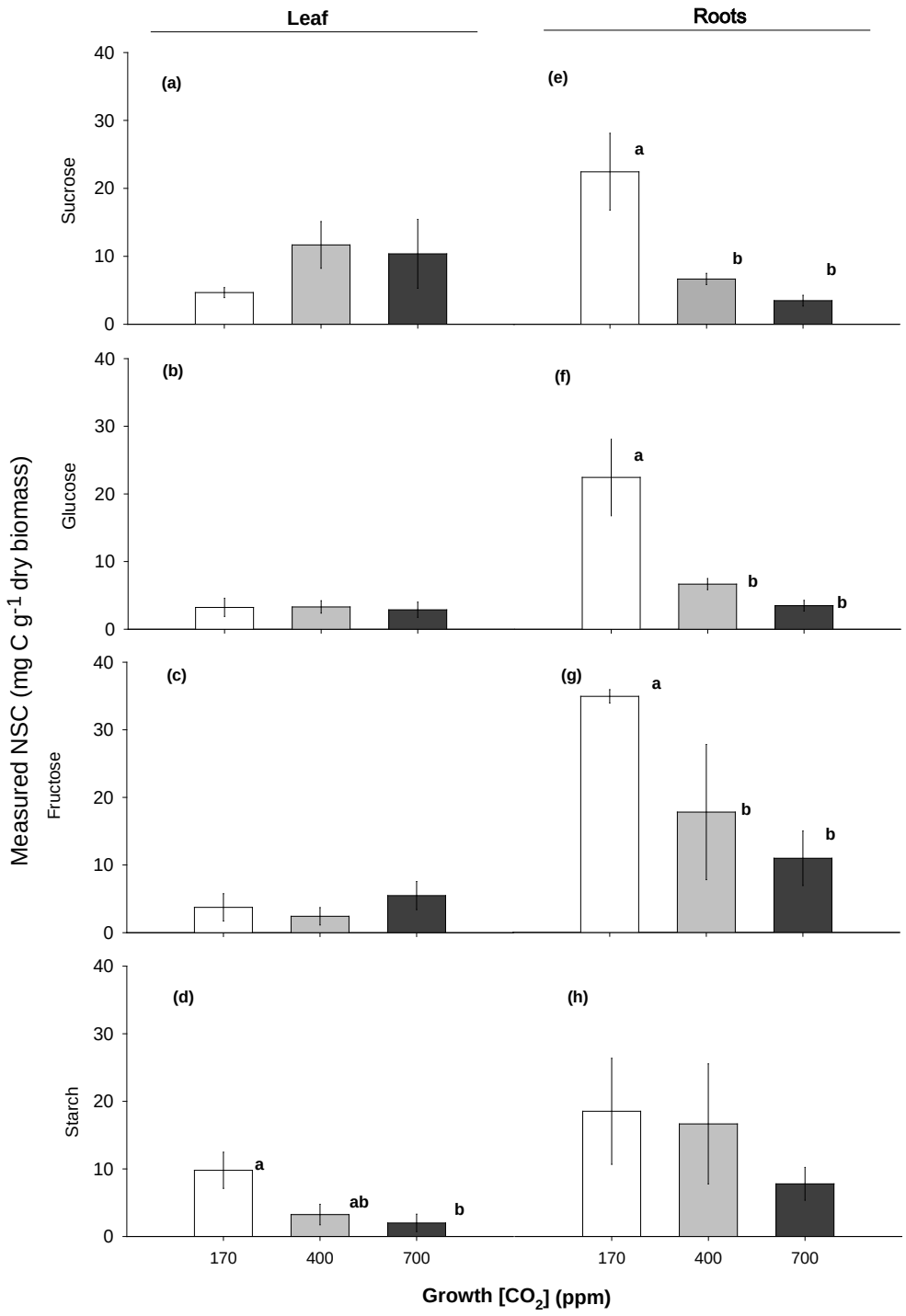

Figure 4.6. Concentration of sucrose, glucose, fructose and starch in leaves (a-d) and roots (e-h) of *M. sativa* in the different [CO_2] growth levels: 170 ppm (white), 400 ppm (light grey) and 700 ppm (dark grey). Values represent means ± SE (n=5 leaves; n=4 roots, except in 170 ppm level where roots n=3). Different letters indicate significant differences (P<0.05, Honest significant differences (HSD) test) across growth levels.

4.4. Discussion

4.4.1. C-assimilation limited by RubisCO carboxylation capacity

In our study, a large increase in net C assimilation rates from low to ambient [CO_2] levels combined with constant *Ci/Ca* ratios across treatments, suggests that C assimilation is responding to biochemical, rather than stomatal limitations. Although our light curves indicated that plants grown at elevated [CO_2] have the capacity to assimilate more C, at the lower light levels in our greenhouse the C-assimilation was clearly light-limited in plants grown at higher [CO_2] levels (Figure 4.4a). Similarly, it has been observed that low light intensity reduces C assimilation in *M. sativa* plants growing under greenhouse conditions (Schmitt *et al.*, 2013). Consequently, light limitation probably reduced not only C-assimilation capacity but also growth in plants grown at elevated [CO_2].

At saturating light and high *Ci* values, assimilation rates will ultimately be limited by either maximum rate of linear electron transport, or by the rate of rate of triose-P usage (TPU) (Sharkey, 2015). In our experiment, C-assimilation increased and was limited by RuBP as indicated by the maintenance of the maximal rate of linear electron transport (J*max*), calculated using the Sharkey (2015) model (Figure 4.4b). TPU rates increased with [CO_2], but C-assimilation did not decline at high *Ci*. Also serine and glycine concentrations were maintained across CO_2 levels. The use of glycine and serine, together with changes in photorespiration are considered indicators of TPU limitation when C-assimilation decreases at high [CO_2] (Harley & Sharkey, 1991, Sharkey, 2015). Therefore, in nodulated *M. sativa* grown at elevated [CO_2], C-assimilation was likely limited by RuBP regeneration and not by TPU.

Low [CO_2] plants were limited by the carboxylation capacity of RuBisCO, and not by RuBP regeneration, as seen when leaves were exposed to higher [CO_2] levels during measurement of photosynthesis parameters (Figure 4.4b). Limitation of carboxylation by RuBisCO at low [CO_2] has been attributed to enhanced photorespiration (Gerhart & Ward, 2010, Sage & Coleman, 2001). In our experiment, the proportion of O_2 relative to CO_2 was higher at low [CO_2]. This suggests that under low [CO_2], given the specificity of RuBisCO, in *M. sativa* plants the chances of biding O_2 instead of CO_2 (i.e., carboxylation capacity) increases.

In plants grown under long-term exposure to low CO_2, there is no consistency in the reported response of C-assimilation to changes in carboxylation capacity (Sage & Coleman, 2001). For

example, *Solanum dimidiatum* (a C3 perennial forb) showed higher carboxylation capacity at low [CO_2] compared to plants grown at higher [CO_2] (200 vs. 500 ppm) (Anderson *et al.*, 2001). On the contrary, Ayub *et al.* (2014) in soybean, observed increments in photorespiration at low [CO_2] (290 ppm). Such increments were explained by the higher demand for tricarboxylic acid cycle (TCA) intermediates maintained by higher N concentration of leaves grown under low [CO_2]. In, C3 leaves the plant nitrogen metabolism is impacted by protorespiratory imbalances (Abadie *et al.*, 2016). Probably, a potential explanation for the inconsistency observed in C-assimilation rates in response to the carboxylation capacity is the leaves' N availability. C-assimilation reductions due to limited carboxylation capacity of RuBisCO might inhibit NO_3^- assimilation (Bloom *et al.*, 2010). Clearly, more long-term studies at low [CO_2] are required to fully understand the photosynthetic responses of a plant's full life cycles and its influence to whole-plant C uptake.

4.4.2. Respiration in light was not inhibited by higher *Vo/Vc* ratio at low [CO_2]

Similar to the study of Ayub *et al.* (2014), we found that light inhibited respiration at low and ambient [CO_2] ($R_{Light} < R_{Dark}$) (Table 4.2). However, Ayub *et al.* (2014) observed the greatest photoinhibition at the highest [CO_2], while we saw no light inhibition of respiration in *M. sativa* leaves grown at elevated [CO_2]. In *Xantrium strumartion* it was observed that the light inhibition at elevated CO_2 was less than it was for ambient [CO_2] plants, presumably due to higher demand for energy and non-structural carbohydrates compared to ambient [CO_2] plants (Wang *et al.*, 2001).

It seems unlikely that we underestimated R_{Light} at 700 ppm derived from the Kok effect, as the estimated mesophyll conductance was greater than the threshold value that could lead to erroneous estimates of R_{Light} (g_m <0.1 mol m^{-2} s^{-1}) (Ayub *et al.*, 2011). The differences could be explained if the extent of light inhibition of respiration in obligate N-fixers is related to energy status or sucrose loading (e.g. increased demand for mitochondrial ATP to support sucrose synthesis) (Kroemer, 1995). Furthermore, we found a positive relationship between R_{Light} and the *Vo/Vc* ratio. Tcherkez *et al.* (2008) suggested that under low CO_2 this positive relationship might reflect, as previously discussed, an increased demand TCA cycle intermediates like 2-oxaglutarate, to adjust glutamic acid production to fuel photorespiration. It is probable that under low [CO_2], in nodulated *M. sativa* plants, greater *Vo/Vc* ratios were fueled by glutamic acid via stimulation of TCA, as a result of enhanced N-availability.

4.4.3. N-fixation was fueled by NSC adjustment under C-limitation

Differences in respiration rates between tissues reflected the demands for sucrose loading (Cannell & Thornley, 2000), and not differences in metabolic costs associated with tissue N concentration. Tissue N concentrations were constant across [CO_2] levels, though roots always had lower N concentration than leaves. Differences among treatments were manifested through changes in above- versus below- ground growth, with low [CO_2] plants having higher leaf area ratios, i.e., allocating more C to the assimilating tissues (see Chapter 3). In contrast, leaves of soy plants in the study of Ayub *et al.* (2014) had similar respiration rates despite variations in N content.

The changes in specific respiration rates for leaf and roots tissues grown under different [CO_2] levels suggests that nodulated *M. sativa* regulated the tissue maintenance cost (i.e., respiration per unit of N), and C allocation both to respiration substrates and growth (González-Meler *et al.*, 2009). Compared with roots, leaves of plants grown at elevated [CO_2] had higher maintenance respiration rates (respiration per unit of N) and higher sucrose concentrations. We did not measure N-fixation rates directly. However, in low [CO_2] plants root respiration per unit biomass was two times higher than in plants grown at higher [CO_2] levels, presumably due to higher rhizobia metabolism and at the expense of nonstructural carbohydrates (NSCs). Plants grown at low [CO_2] had higher NSC and specific respiration rates in roots, but lower NSC in leaves, and lower overall growth rates, compared to plants grown at ambient and elevated [CO_2]. This contrasted with our hypothesis that low [CO_2] plants would have higher sinks compared to supply of NSC and therefore lower NSC in the plant overall, including roots.

Our results suggest that acclimation (plastic) responses of plant C balance to long-term exposure to sub-atmospheric and elevated [CO_2] are driven by changes in C allocation. High specific rates of respiration in low [CO_2] roots are associated with high NSC, indicating plants are allocating a greater proportion of the C they fix to fuel belowground respiration, which suggest that more C needs to be expended to maintain plant N levels. However, our knowledge of the evolutionary responses of plants to low [CO_2] is limited (Becklin *et al.*, 2014), not to mention our understanding of obligated N-fixers and how low atmospheric [CO_2] has impacted their physiology since the last glacial maximum. We suggest further studies at whole plant level in plants grown at late Pleistocene epoch [CO_2] are needed to understand the metabolic cost to maintain and produce leaf area and whole plant biomass (as well as reproductive organs) at low atmospheric [CO_2].

4.5. Conclusions

- C assimilation in plants growing under low [CO_2] was limited by C availability, while C assimilation in plants at higher [CO_2] levels was light-limited. This means that changes in maximum rates of C assimilation may not necessarily translate into higher plant growth under field conditions, if light becomes limiting.

- Photo inhibition of leaf respiration declined as [CO_2] increased. These changes were related to higher oxygenation-to-carboxylation ratio of RuBisCO and not to N availability.

- Leaf N concentration was maintained at low [CO_2] through allocation of a greater amount of the photosynthates to NSC and respiration in roots.

4.6. Supplementary data

Table 4.S1. Linear regression of photosynthesis rate against 0 to 150 PPDF. Coefficients of determination (r^2), y-axis intercepts equivalents to R_{Light} values and standardized major axis slopes are given.

Growth [CO_2] (ppm)	Replicate	r^2	y-axis intercept (R_{Light})	Slope
170 ppm	n_1	0.9790	-0.7677	0.0217
	n_2	0.9901	-0.1755	0.011
	n_3	0.9989	-0.1181	0.0099
	n_4	0.9923	-0.4411	0.0118
	n_5	0.9992	-0.1259	0.0036
400 ppm	n_1	0.9963	-0.5168	0.0644
	n_2	0.9933	-0.0641	0.0523
	n_3	0.995	-0.8152	0.0465
	n_4	0.9921	-1.0869	0.0469
	n_5	0.9959	-1.1938	0.038
700 ppm	n_1	0.991	-0.3844	0.058
	n_2	0.9915	-5.5316	0.1334
	n_3	0.9969	-1.6145	0.076
	n_4	0.9965	-1.8101	0.0601
	n_5	0.9969	-0.4816	0.0274

Table 4.S2. Linear regression calculated from root respiration against time. Coefficients of determination (r^2), slope equivalents to respiration rate and y-axis intercept standardized major axis are given.

Growth [CO_2]	Replicate	r^2	Slope ($\mu mol\ mol^{-1}\ s^{-1}$)	y-axis intercept [CO_2] $\mu mol\ mol^{-1}$
170 ppm	n_1	0.9953	0.0367	445.16
	n_2	0.9834	0.0143	446.79
	n_3	0.9908	0.0241	445.47
400 ppm	n_1	0.9974	0.054	445.84
	n_2	0.9984	0.1388	446.41
	n_3	0.9979	0.1382	446.17
	n_4	0.9871	0.0786	446.27
700 ppm	n_1	0.9959	0.1381	441.19
	n_2	0.9961	0.0615	442.01
	n_3	0.9959	0.0551	441.70
	n_4	0.9995	0.1853	442.05

Table 4.S3. Whole plant, aboveground and belowground traits of *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent means (n=5 ± SE) at each growth CO_2 treatment. Different letters represent significant differences among growth treatments (P<0.05, honest significant differences (HSD) test, following ANOVA). P represents the significance level of the ANOVA.

	Growth [CO_2] (ppm)			Significance
Traits	**170**	**400**	**700**	***P***
Whole-plant traits				
Total Biomass (g)	0.11 ± 0.03^{b}	0.58 ± 0.08^{a}	0.61 ± 0.11^{a}	*P*<0.01
Plant height (cm)	20.00 ± 0.58^{b}	45.98 ± 1.98^{a}	42.5 ± 3.6^{a}	*P*<0.001
Root-to-shoot ratio	0.24 ± 0.02	0.30 ± 0.13	0.30 ± 0.10	NS
Leaf area:Total Biomass (m^2g^{-1})	25.76 ± 2.30^{a}	14.52 ± 2.78^{b}	15.00 ± 1.40^{b}	*P*<0.01
Aboveground traits(AG):				
Leaf area (cm^2)	25.80 ± 5.24^{b}	84.26 ± 15.86^{ab}	86.71 ± 14.93^{a}	*P*<0.05
AG Biomass (g)	0.08 ± 0.00^{b}	0.42 ± 0.05^{a}	0.45 ± 0.08^{a}	*P*<0.01
AG nitrogen (%)	3.78 ± 0.02^{ab}	3.31 ± 0.00^{b}	4.33 ± 0.04^{a}	*P*<0.05
Belowground traits:				
Root Biomass (g)	0.02 ± 0.0	0.13 ± 0.03	0.14 ± 0.03	*P*<0.1
Root length (cm)	29.3 ± 9.17^{ab}	23.38 ± 2.46^{b}	46.88 ± 6.52^{a}	*P*<0.01
Root nitrogen (%)	2.40 ± 0.07	2.03 ± 0.08	2.07 ± 0.18	NS
Nodules ($plant^{-1}$)	20.67 ± 4.18	52.00 ± 10.14^{b}	81.00 ± 26.99^{a}	NS
Nodule biomass (mg)	3.01 ± 0.41^{b}	15.72 ± 2.84^{a}	21.17 ± 4.10^{a}	*P*<0.05
Nodule nitrogen (%)	6.33 ± 0.27	6.12 ± 0.38	7.61 ± 0.41	NS

CHAPTER 5

Influence of atmospheric [CO_2] on carbon allocation of *Medicago sativa* L.

Abstract

Increasing atmospheric CO_2 concentration ([CO_2]) can have positive effects on photosynthetic rates, yet there is no clear consensus as to the effect of changing [CO_2] on the whole-plant C balance and, in plant-rhizobium symbiotic systems, on nitrogen (N) fixation. We investigated how whole-plant C balance and C allocation patterns varied in 16-17 week old nodulated *Medicago sativa* L. plants that had grown since sprouting from seed under [CO_2] equivalent to Late Pleistocene (170 ppm), ambient (400 ppm) and projected future (700 ppm) conditions. We used a stable isotope label to trace the fate of newly fixed-C over a 72 h period, using a chamber design that allowed partition aboveground and belowground fluxes.

Overall, plants grown at higher [CO_2] had much higher total net C fixation and total respiration rates and much greater biomass than those grown at 170 ppm. However, when expressed per gram of dry biomass, specific rates of net C fixation and belowground respiration were highest at low [CO_2]. N concentration in plant tissues was similar across different atmospheric [CO_2]. Concentrations of non-structural carbohydrates (NSC) were highest in leaves of plants grown at 700 ppm, but constant in nodules across [CO_2] treatments. Labeling of $^{13}CO_2$ indicated a fast depletion of new photosynthetic products to support belowground respiration in low [CO_2] plants, while plants grown at higher [CO_2] respired newly-fixed C over a period of several days.

These results suggest that organs, like roots and nodules, represented a major respiratory sink of newly assimilated C, reducing C allocation to growth. Moreover, plant metabolic processes like photosynthesis and respiration were affected by changes in [CO_2], while nitrogen acquisition in such a symbiotic system was not.

Key words: C balance, Alfalfa, growth, N-fixation, nodules, Non-structural carbohydrates.

5.1. Introduction

The availability of CO_2 affects the rate of C assimilation in plants, and hence, their C balance. Whole-plant C balance represents the difference between net assimilation and numerous C sinks, including respiration, growth, defense, and storage. Storage can act as a buffer at times when C supply and demand are out of balance (Hartmann & Trumbore, 2016).

Changes in atmospheric [CO_2] have a direct impact on a plant's C balance (Franks *et al.*, 2012, González-Meler *et al.*, 2004). For example, under elevated [CO_2], enhanced C assimilation has also led to increased growth and non-structural carbohydrates (NSC) reserves (Leakey *et al.*, 2009a, Rogers *et al.*, 2009). On the other hand, C assimilation under low [CO_2] is limited by reduced rates of carboxylation of RuBisCO (Gerhart & Ward, 2010), and thus, C source-sink imbalances are likely to occur, potentially leading to near-zero or even occasionally negative C balance. Under such conditions, it has been suggested that growth is reduced, because ultimately survival depends more on C demands for metabolism than for growth (Sala *et al.*, 2012). In plants grown at low [CO_2] compared to plants grown at ambient [CO_2] this hypothesis may explain the reduction of growth rates, biomass (Temme *et al.*, 2016), NSC content, (Schädel *et al.*, 2010) and leaf mass-based respiration rates (González-Meler *et al.*, 2009). Under low CO_2, lower respiration rates were associated with reduced activity of the alternative (non-cytochrome) pathway, which led to positive plant C balance (Gerhart & Ward, 2010, González-Meler *et al.*, 2009).

Nodulated plants represent a special case in terms of C allocation. Root nodules that host symbiotic rhizobia bacteria in leguminous plants constitute an extra sink for plant C, as the plant supplies C to the bacteria in order to obtain N in return. Changes in C availability should therefore directly affect N-fixation (Aranjuelo *et al.*, 2014). Understanding how C allocation of nodulated legumes is influenced by C availability is of critical importance for predicting responses of legumes to rising atmospheric [CO_2]. Changes in the net C gain associated with altered C availability in legumes may affect biomass, C assimilation, NSC, number of nodules and N-fixation rates per plant (measured as specific nitrogenase activity) (Aranjuelo *et al.*, 2014, Butterly *et al.*, 2016, Rogers *et al.*, 2009). Yet, it is still unclear how and to what extent nodulated plants modify allocation of C to N-fixation against other C sink demands, such as growth, under changing C availability.

Chapter 3 demonstrated that tissue N concentrations of nodulated plants were maintained in leaves and roots grown at the three different CO_2 levels, but biomass production and C partitioning

between above- and belowground were not. At low [CO_2], plants optimized C allocation aboveground by creating higher leaf area ratios, although a greater proportion of fixed C was allocated belowground to support N fixation (greater C cost). In Chapter 4, it was shown that a greater C cost for N fixation and greater specific root respiration rates (respiration per unit biomass) in nodulated plants during vegetative growth were accompanied by lower NSC concentration in leaves, but higher concentration in roots. However, it is still unclear if N-fixation is secured by NSC adjustment at the whole-plant level under low CO_2, similar to what has been observed at elevated [CO_2] nodulated *Pisum sativum* (Butterly *et al.*, 2016).

In this study we determined how daily whole-plant C fluxes varied in nodulated plants under different atmospheric [CO_2], and in particular, how C allocation to nodules was affected. We hypothesized that plants grown at low [CO_2] would overall assimilate less C, but would allocate proportionally more C to respiration, particularly to belowground tissues, in order to maintain N-fixation. In order to address our hypothesis, we quantified the whole-plant C balance using flow-through chambers and $^{13}CO_2$ pulse labeling to trace the fate of new photoassimilates. In addition, we quantified starch and sugars to assess their role as a buffer in an obligate symbiotic association between *Medicago sativa* and *Ensifer meliloti (Sinorrhizobium meliloti).* Growing plants from seeds under different atmospheric [CO_2] created a gradient of C availability: Late Pleistocene, 170 ppm; ambient, 400 ppm and projected future, 700 ppm.

5.2. Materials and methods

Individual plants used in the experiment were: 112, 124 and 120 days old for 170, 400 and 700 ppm treatments, respectively. The conditions for the plants' growth before this experiment are described in detail in Chapter 3. Briefly, all plants were grown from sprouted seeds at three levels of [CO_2] (170, 400, or 700 ppm; $^{13}CO_2$= 7.86‰ ± 1.24‰) and were supplied with water and all nutrients needed for growth except for N, which they could only obtain through N fixing symbionts. At the developmental stage described here, plants had either produced (and aborted) flowers (400 and 700 ppm [CO_2]) or had not produced flowers (low [CO_2]) yet. As discussed in Chapter 3, plants grown at higher [CO_2] levels had begun to increase stores of sugars and starches in their roots (*M. sativa* is a perennial plant).

5.2.1. Experimental design

Three randomly selected plants of each [CO_2] treatment were transferred to whole-plant chambers which were then placed into rooms that supplied controlled climate conditions (growth chambers) (Figure 5.1) (York Industriekälte, Mannheim, Germany). Growth chambers were programmed for a day/night cycle of 13/11 h, using halogen lamps with a constant photosynthetically active radiation of 700 µmol m^{-2} s^{-1}. Air temperature and relative air humidity in the growth chambers were kept constant during the experiment at 25.0°C ± 1.0 °C and 30.0 ± 0.10 %, respectively.

Plants were installed in whole-plant chambers to separate above-and below-ground tissues (Figure 5.1). To do so, we placed aboveground tissues in transparent airtight Plexiglass chambers (~2.4 L), while darkened acrylic lids sealed the upper part of plant pots (1.0 L). Pots were placed in air-tight belowground chambers (in Figure 5.1, dark red section of the belowground chamber). We observed an increase of 3-4 °C in aboveground parts of the whole-plant chambers due to heating from lighting. At the beginning of the experiment the growth substrate was watered to field capacity (volumetric water content *c.* 50%). Because of limited space in the growth chamber, the three [CO_2] treatments were conducted in three different trials, each trial lasting 96 h.

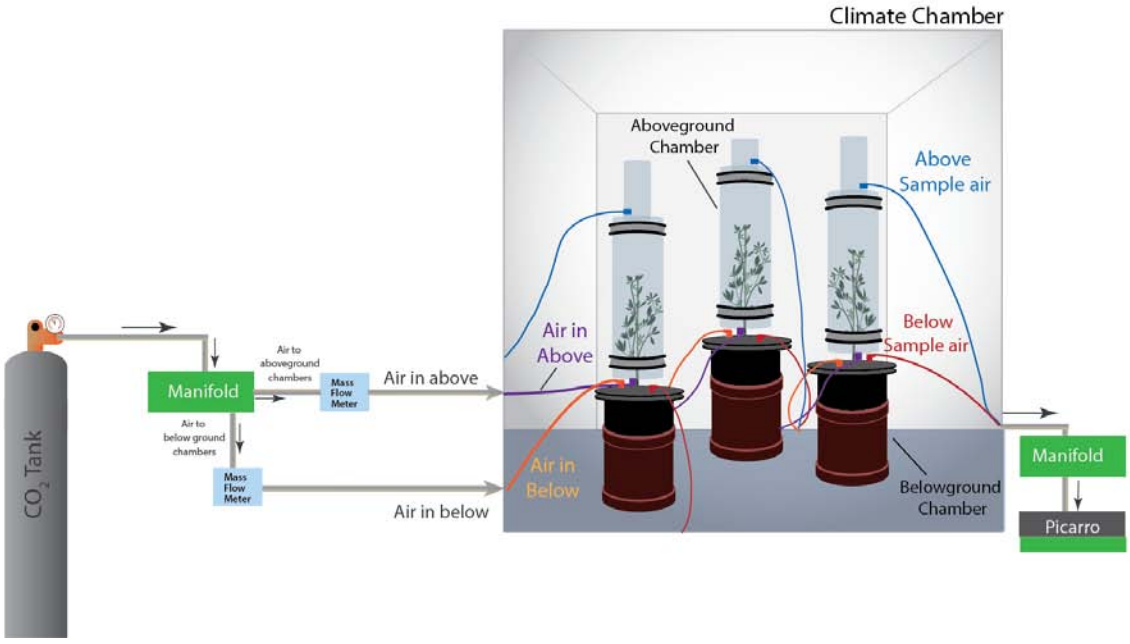

Figure 5.1. Schematic representation of the technical setup for controlling [CO_2] in the whole-plant chambers. The air was added at a given concentration, mass flow controllers (MFC) were used to adjust the flow of the CO_2. The air stream (indicated by arrows) was distributed via an automated magnetic valve system (manifold) in above and below compartments. Isotopic composition as well as gas concentrations of the chambers (above and below) were monitored with a Picarro cavity ring-down spectrometer.

Gas exchange and respiration

Air with constant moisture and vapor pressure deficit (VPD) (2.94 ± 0.47 KPa) was continuously pumped through above- and below-ground compartments at a constant rate, controlled by needle valves outside the climate chamber (aboveground, 3.0 l min^{-1}; belowground 0.6 l min^{-1}). The inflowing air had the following [CO_2]: 159.65 ± 12.92 ppm ($^{13}CO_2$=-11.39 ± 1.55 ‰), 406 ± 15.65 ppm ($^{13}CO_2$= -9.69± 0.25 ‰) and 682.50 ± 28.06 ($^{13}CO_2$= -9.24± 0.41 ‰) ppm, for the 170 ppm, 400 ppm and 700 ppm treatments, respectively.

[CO_2] and [H_2O] were measured from the air exiting the chambers with a Picarro 2131-*i* (Picarro Inc., Santa Clara, CA, USA). Within an hour period, all chambers in the system (6) were measured by switching between above- and belowground chambers in 7.5 min intervals using a custom built valve switching device controlled by electromagnetic valves. During each 7.5 min interval, a second logger-controlled manifold switched between incoming (in) air to outgoing (sample air) air. A core period of 225 s in each 450 s interval was used to compute the difference between inlet and outlet air, which was the basis for our flux measurement (Figure 5.1).

In above- and belowground chambers instantaneous gas exchange and transpiration were defined as:

$$[CO_2 \text{ or } H_2O]_{plants} = [CO_2 \text{ or } H_2O]_{out} - [CO_2 \text{ or } H_2O]_{in} \qquad \text{Eq. 5.1}$$

These were considered constant for the whole one hour cycle and were converted to hourly carbon and water flux (g h^{-1}) at time *i* using the following equation:

$$C_i \text{ or } H_2O_i = \frac{[S]_{out} - [S]_{in}}{22.4} * 10^{-6} * FR * 60 * MW \qquad \text{Eqn. 5.2}$$

where $[S]_{out}$ and $[S]_{in}$ were the CO_2 (or H_2O) concentrations (ppm) of the air leaving and entering the chambers. FR was the air flow rate (3.0 l min^{-1} aboveground and 0.6 l min^{-1} belowground) through the chambers, 22.4 is the molar volume at standard temperature and pressure (l mol^{-1}) and MW was the weighted molecular weight of carbon mol^{-1} CO_2 (12 g C mol^{-1}/44 g CO_2 mol^{-1}) or the molecular weight of water (18.02 g mol^{-1}). The hourly fluxes were then summed to obtain a daily value. The daytime (13 h) CO_2 flux was defined as net C-assimilation and the night time (11 h) CO_2 flux was defined as respiration. The sum of root below- and above-ground respiration on a daily basis is referred to as total respiration. The H_2O flux was defined as transpiration, and water-use efficiency

was calculated as net C-assimilation divided by daytime transpiration (E_d) on a daily basis. Stomatal conductance (G_s, mmol g^{-1} DW d^{-1}) was estimated based on Zhao *et al.* (2013) and McDowell *et al.* (2008) as:

$$G_s = \frac{E_d}{VPD} \qquad \text{Eqn. 5.3}$$

5.2.2. Labelling procedure

After 24 hours of acclimation in the climate chambers, an isotope (^{13}C) label was applied to aboveground chambers over a period of 3 h, starting at 10:00 h. The labeled air with an $\delta^{13}C$ of 350 ‰ ± 0.68 ‰ (158.73 ppm ± 9.05 ppm), 370.68 ‰ ± 0.01 ‰ (397.15 ppm ± 0.07 ppm) and 370.02 ‰± 0.55 ‰ (720.47 ppm ± 0.04 ppm) was flushed at a rate of 3.0 l min^{-1}, in the 170, 400 and 700 ppm treatment, respectively.

5.2.3. Tissue incubation (respiration rates) and harvest

At the end of the experiment, plants of each CO_2 treatment were first incubated (discrete samples) and then harvested. Discrete tissue incubations were performed after leaf area measurements, or in the case of roots, after they were washed, to remove traces of the sand-vermiculite substrate (Figure 5.2).

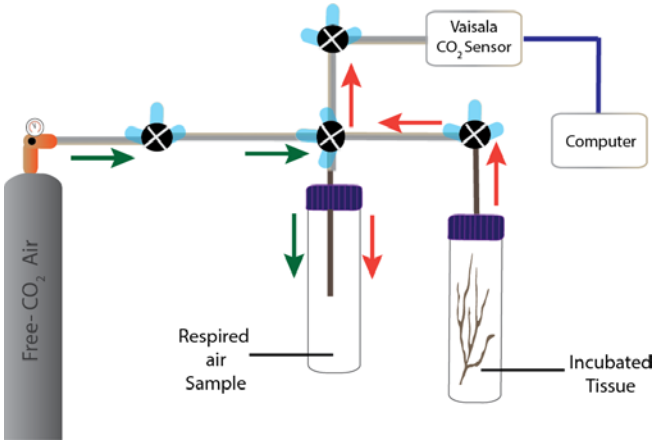

Figure 5.2. Schematic representation of discrete tissue samples incubation system. The air stream (indicated by arrows) was controlled manually and by three way valves (blue). Prior to incubation the system was flushed. Green arrows indicate the flushing airstream, and mass flow controllers (MFC) at the free-CO_2 air tank were used to adjust the flow of the air. Once the system was flushed, the tissue was placed inside a vial and connected to the system through hypodermic needles. Gas concentration increments of the respired air sample were monitored with a Vaisala® sensor and

registered on a computer.

Prior to incubations, the system was flushed with synthetic air to avoid CO_2 contamination from other sources (Figure 5.2), the incubations of discrete tissue samples (e.g., portion of the roots with attached nodules or 2-3 leaflets) were then measured using a closed-circuit system. During the leaflets incubation the vials were darkened to avoid CO_2 assimilation. The [CO_2] was monitored using a Vaisala CARBOCAP® GMM111 and logged. Also, incubation times were measured and recorded.

Specific respiration rates were determined as the linear regression slope of CO_2 concentrations in incubation tubes over time and calculated as mass-specific rates:

$$Respiration\ rate = \frac{(m * v)}{(V_{molar} * DW)} \qquad \text{Eqn.5.4}$$

where m=slope of [CO_2]~incubation time , v= volume of the tube (0.012 L), v_{molar}=22.4 L mol^{-1} and DW represents the dry weight of the incubated tissue.

At harvest, total leaf area was determined using a leaf area meter (LI-3100 leaf area meter, Li-Cor Inc. Lincoln, Nebraska, USA) and the number of nodules per plant was counted. At the end of harvest, plants were separated into leaves, stems, roots and nodules. These were oven-dried to constant weight at 70 °C, their dry weight recorded, and material was stored for subsequent elemental analysis.

5.2.4. Non- structural carbohydrates (NSC)

Total soluble non-structural carbohydrate (NSC) concentrations were estimated from the sum of measurements of three major sugars (glucose, fructose, and sucrose) representing mobile compounds and starch as the main non-mobile compound, within each tissue (leaves, roots and nodules). Only at the moment of harvest (end of experiment), deep-frozen samples (n=3 for each tissue type) of leaves, roots, and nodules were taken and then vacuum freeze-dried for 96 h and milled (Retsch MM200, Haan, Germany) to fine powder. After grinding, approximately 10 mg of the samples were added to 1 mL of distilled water. The mixture was vortexed and incubated for 10 min at 65°C in a thermomixer and then centrifuged for 6 min at 12000 g. The supernatant was removed with a pipette, stored on ice and the water extraction procedure was repeated twice and the

three extracts combined.

The same amount of ground sample (approximately 10 mg) was added to 0.35 mL distilled water, vortexed for 1 min and treated for 10 min in a thermomixer at 65°C. For starch hydrolysis we then added 0.5 mL of 33% perchloric acid and let it incubate in an orbital shaker for 30 min. After centrifuging at 12000 g for 6 min, the supernatant was removed with a pipette and the procedure was repeated on the remaining pellet.

Extracts were diluted (1:5 sugars and 1:55 starch) with bi-distilled water prior to analysis using high/pressure liquid chromatography with pulse amperometric detection (HPLC-PAD) on a Dionex ICS 3000 ion chromatography system equipped with an autosampler (Raessler *et al.*, 2010). Peak areas (heights) were converted to concentrations using a calibration curve made with known concentration mixtures of sugars.

5.2.5. Growth measurements

Carbon and Nitrogen concentration and isotopic analysis

Tissue samples were ground with a ball mill (Restch MM200, Haan, Germany) and subsamples (c. 1 to 5 mg DW) were analyzed at the Stable Isotope Laboratory of the Max Planck Institute for Biogeochemistry, using a Delta+ XL Isotope Ratio Mass Spectrometry (IRMS, ThermoFinnigan, Bremen, Germany). ^{15}N was expressed relative to atmospheric N_2 ($\delta^{15}N$) and ^{13}C composition in reference to Pee Dee Belemnite ($\delta^{13}C$). The label was traced with the Picarro 2131-i by using the $^{13}CO_2$ of the air entering and leaving the chambers in both above- and belowground chambers. $\delta^{13}C_m$ indicates here the ^{13}C composition of the CO_2 assimilated by plants during the labeling period:

$$\delta^{13}C_{\mathrm{m}} = \frac{[CO_2]_{\mathrm{out}} \cdot \delta_{\mathrm{out}} - [CO_2]_{\mathrm{in}} \cdot \delta_{\mathrm{in}}}{[CO_2]_{\mathrm{out}} - [CO_2]_{\mathrm{in}}} \qquad \text{Eqn. 5.5}$$

where δ_{out} and δ_{in} represent ^{13}C composition of the CO_2 in the air leaving and entering the chamber, respectively.

Relative growth rate and leaf area ratio

For each harvested plant, average RGR (unit dry mass increase per unit of dry mass) and their standard errors were calculated using the analysis tool developed by Hunt *et al.* (2002) in Microsoft

Excel ®. Average LAR (leaf area ratio) was calculated as total leaf area/total plant biomass.

5.2.6. Statistical analysis

Mean values of the measured parameters were tested for differences across CO_2 treatment levels with an ANOVA using R (v.3.1.0, R Foundation for Statistical Computing, 2012). When ANOVA indicated a significant overall treatment effect, a multiple comparison test was carried out (Tukey's honest significance test (Tukey's HSD, $\alpha<0.05$) to compare means between individual treatment levels.

5.3. Results

5.3.1. Plant development and biomass

At the end of the experiment, low [CO_2] plants were significantly smaller than plants grown at higher [CO_2] levels (Figure 5.3). The most visible differences were in the root architecture and nodules morphology (Figure 5.3). Although similar in biomass, 700 ppm plants had coarser roots than 400 ppm plants, and in both treatments, a thickening of the tap root was visible. On the other hand, the root system of plants grown at 170 ppm had fewer fine roots, and the few nodules were bigger than those observed in plants grown at higher [CO_2].

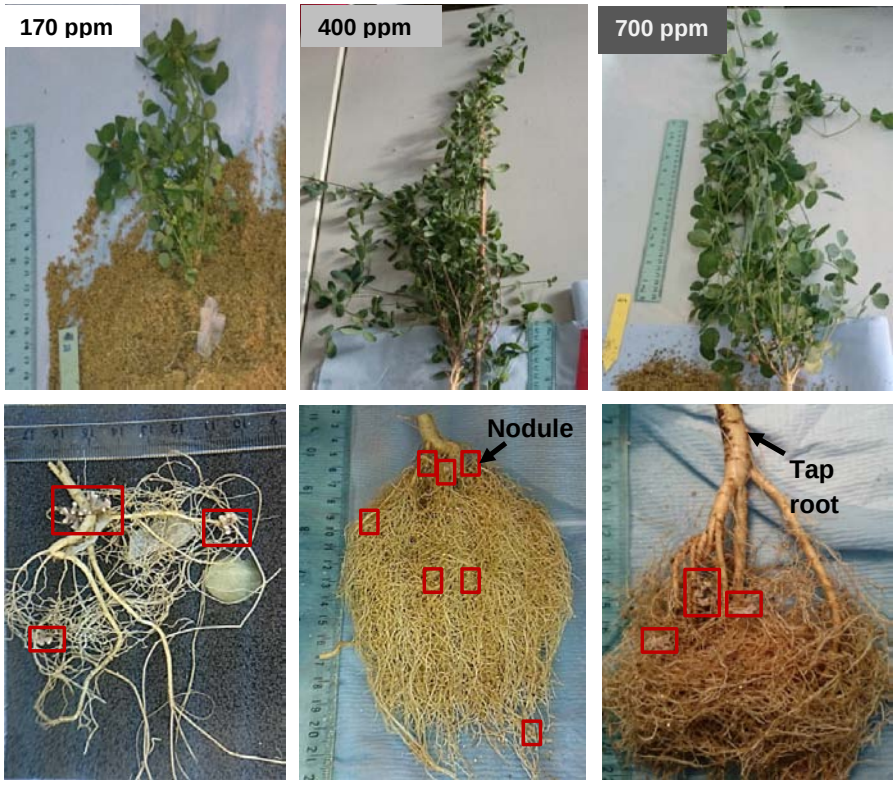

Figure 5.3. Aboveground plant size and belowground architecture and nodulation of *Medicago sativa* plants inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170,400 and 700 ppm. The red squares show few visible attached nodules.

In terms of biomass, 170 ppm plants had approximately 15-to-18-fold lower biomass than those at 400 and 700 ppm plants, respectively (Table 5.1). However, 170 ppm plants had a greater leaf area ratio (total leaf area/total plant biomass), and had a greater proportion of aboveground biomass.

Leaf and root N concentrations were similar across [CO_2] levels. Only nodules of 700 ppm plants

had a significantly lower N concentration and greater biomass compared to plants grown at 170 and 400 ppm.

Table 5.1. Whole-plant, aboveground and belowground traits of *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent means (n=3 ± 1 SE) of each CO_2 level. Different letters represent significant differences among treatment levels ($P<0.05$, honest significant differences (HSD) test, following ANOVA). *P* represents the significance level of the ANOVA.

	Growth [CO_2] concentration (ppm)			Significance
Traits	**170**	**400**	**700**	***P***
Whole-plant traits				
Total Biomass (g)	0.52 ± 0.06^{b}	7.83 ± 2.81^{ab}	9.84 ± 4.26^{a}	$P<0.05$
Plant height (cm)	32.70 ± 4.66^{b}	74.00 ± 6.12^{a}	89.13 ± 15.07^{a}	$P<0.001$
Root-to-shoot ratio	0.34 ± 0.07 ^{b}	0.60± 0.10 ^{ab}	0.81 ± 0.25 ^{a}	$P<0.05$
Leaf area:Total Biomass (m^2Kg^{-1})	24.72 ± 3.97^{a}	17.27 ± 6.18^{ab}	10.30 ± 2.47^{a}	$P<0.05$
RGR (mg g^{-1} day^{-1})	0.031 ± 0.009	0.339 ± 0.010	0.226 ± 0.058	*
Aboveground traits(AG):				
Leaf area (m^2)	0.013 ± 0.00	0.14 ± 0.10	0.10 ± 0.03	$P<0.1$
AG Biomass (g)	0.39 ± 0.05^{b}	4.86 ± 1.62^{ab}	5.67 ± 3.11^{a}	$P<0.05$
AG nitrogen (%)	3.71 ± 0.03	3.22 ± 0.02	3.10 ± 0.06	NS
Belowground traits:				
Root Biomass (g)	0.12 ± 0.02^{b}	2.85 ± 1.21^{a}	3.98 ± 1.11^{a}	$P<0.01$
Root nitrogen (%)	2.14 ± 0.01	2.14 ± 0.01	2.74 ± 0.05	NS
Nodule biomass (g)	0.01 ± 0.00^{b}	0.13 ± 0.01^{ab}	0.20 ± 0.01^{a}	$P<0.05$
Nodule nitrogen (%)	5.98 ± 0.01^{a}	6.30 ± 0.06^{a}	3.48 ± 0.13^{b}	$P<0.05$

(*)RGR was calculated from means of dry biomass between harvests. The standard error calculations reported here are further explained in Hunt *et al.* (2002).

5.3.2. Net C gain and growth

Net C assimilation and dark respiration, as well as belowground respiration, showed similar patterns of response to changes in atmospheric [CO_2] (Figure 5.4a-c). C assimilation per unit biomass (i.e., specific C assimilation rate) was significantly higher in plants grown at 170 ppm (Figure 5.4a). Similarly, specific rates of belowground respiration were approximately 11-to-24-fold greater in 170 ppm plants than in plants grown at 400 and 700 ppm, respectively (Figure 5.4c). Dark respiration rates expressed per unit biomass (above-ground respiration during night time) increased at low [CO_2] but not as much as below-ground respiration (Figure 5.4b).

The mean net C gain was 0.10±0.5 and 0.29±0.13 mg C g^{-1}DW per day for plants grown at 400 and 700 ppm treatments, respectively, but -1.34±01.67 mg C g^{-1}DW per day at low [CO_2] (Figure 5.4d).

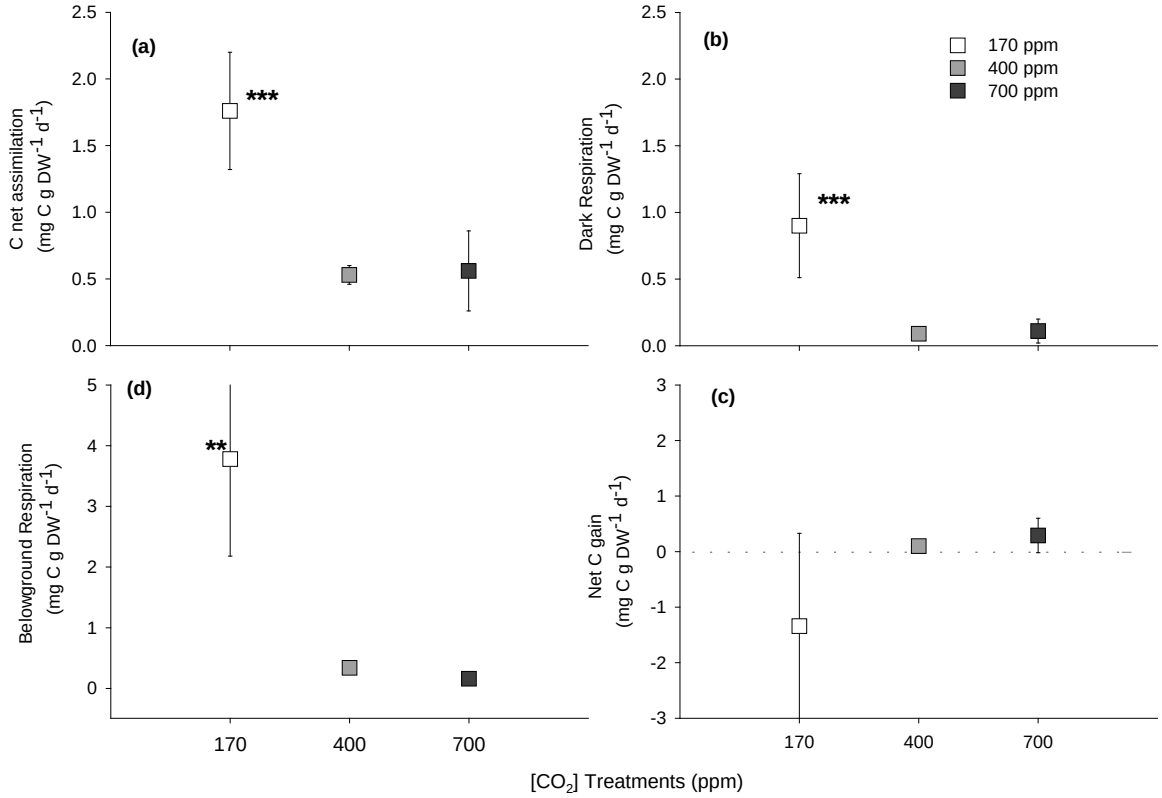

Figure 5.4. Net whole-chamber daily net C assimilation (a), above-ground dark (b), and belowground respiration (c) as well as daily net C gain (d) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170,400 and 700 ppm. Values represent means (mg C g^{-1} DWd^{-1}, ± 1 SD) based on three replicates and three days of measurements. Error bars not visible are within the limits of the symbols. Statistically significant effects of CO_2 levels are indicated by asterisks (* P<0.001; ** P<0.01).**

The highest rates of transpiration (also expressed per unit biomass) were observed in plants grown

at 170 ppm (Figure 5.5a,b). However, stomatal conductance was similar across CO_2 treatments (Figure 5.5c). Consequently, high rates of C assimilation at low daytime transpiration yielded the highest water use efficiency in plants grown at 700 ppm (Figure 5.5d).

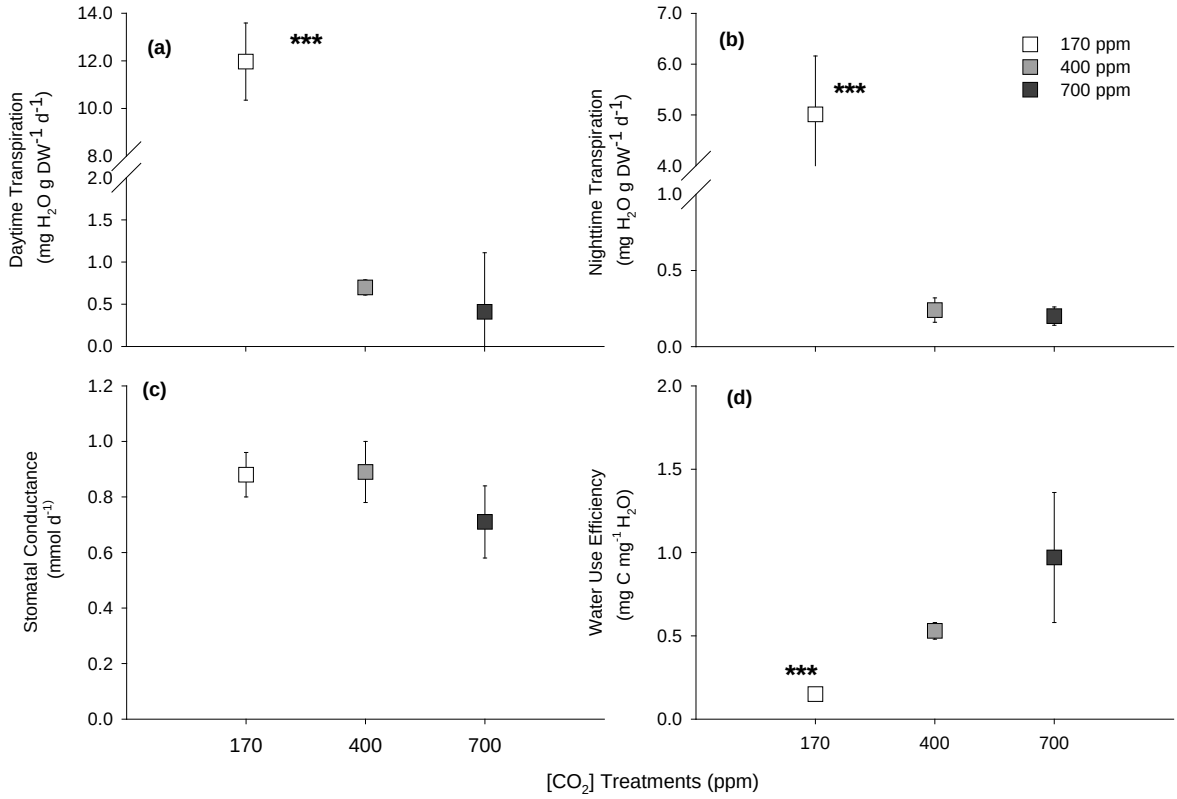

Figure 5.5. Whole-chamber daytime (a) and nighttime transpiration (b), stomatal conductance (c) and water-use efficiency (d) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2]: 170,400 and 700 ppm. Values represent means (± 1 SD) based on three replicates and three days of measurements. Error bars not visible are within the limits of the symbols. Statistically significant effects of CO_2 levels are indicated by asterisks (* P<0.001).**

While specific respiration rates of leaves were similar across CO_2 levels (Table 5.2). Root and nodule specific respiration rates of plants grown at 170 ppm were greater than in plants grown at higher [CO_2]. The most notable difference was observed in nodules. Nodules of plants grown at 170 ppm had very high specific respiration rates (620.71 ± 149.89 nmol CO_2 g^{-1} s^{-1}), whereas plants grown at 400 ppm had the lowest rate (27.51 ± 4.41 nmol CO_2 g^{-1} s^{-1}) (Table 5.2).

Table 5.2. Tissue-specific respiration rates of incubated leaves, roots and nodules of *Medicago sativa* inoculated with *Ensifer meliloti*. Values represent means (± 1 SE, n=3) of each CO_2 level. Different letters indicate significant differences between levels ($P<0.05$, honest significant differences (HSD) test, following ANOVA). *P* represents the significance level of the ANOVA.

Specific Respiration rate (nmol CO_2 g^{-1} s^{-1})	Growth [CO_2] (ppm)			Significance
	170	400	700	*P*
Leaves	33.68 ± 15.51	22.02 ± 0.77	11.38 ± 2.09	NS
Roots	75.02 ± 14.85[a]	10.58 ± 2.55[b]	4.92 ± 1.49[b]	$P<0.01$
Nodules	620.71 ± 149.89[a]	27.51 ± 4.41[b]	77.38 ± 29.16[b]	$P<0.01$

5.3.3. NSCs

In general, starch and sucrose showed opposite trends (Figure 5.6). For example, when sucrose accumulated in nodules, starch decreased (Figure 5.6i,j), both in leaves and in roots (Figure 5.6e-h). By contrast, glucose and fructose concentrations in roots and nodules were similar across CO_2 levels.

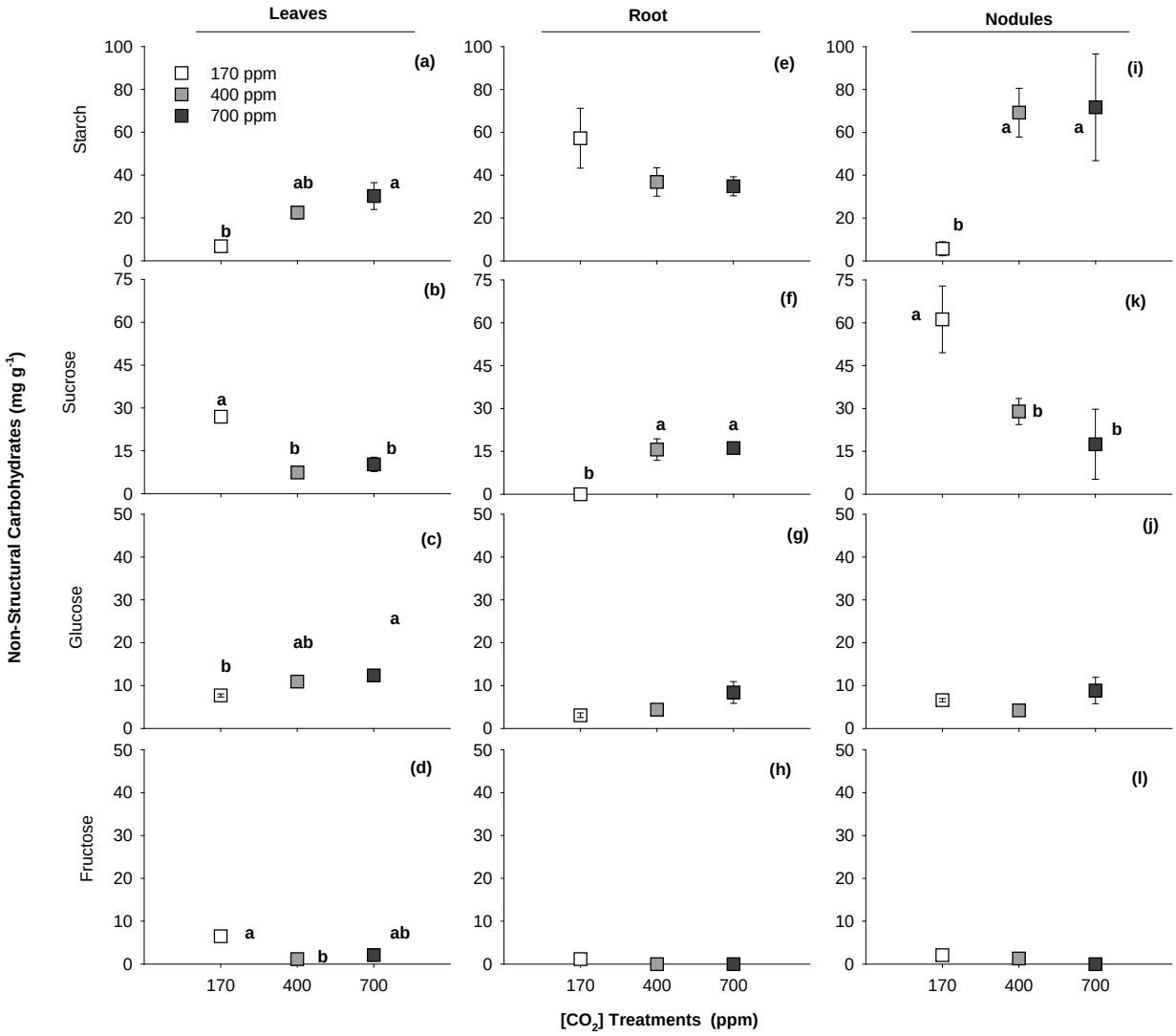

Figure 5.6. Nonstructural carbohydrate (NSC) concentrations (from top to bottom: Starch, sucrose, glucose and fructose) in leaves (a-d), roots (e-h) and nodules (i-l) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2] levels: 170, 400 and 700 ppm. Values represent means ± 1 (n=3). Different letters indicate significant differences across CO_2 levels (P<0.05) (Turkey's honest significant test (HSD) following significant ANOVA).

The $\delta^{13}C$ of respired CO_2, (calculated using eqn. 5.5; Figure 5.7) indicated that plants grown at 170 ppm had the fastest mobilization of photoassimilates between aboveground and belowground tissues(Figure 5.7c) although more labelled $^{13}CO_2$ was fixed at higher [CO_2] (Figure 5.7a).

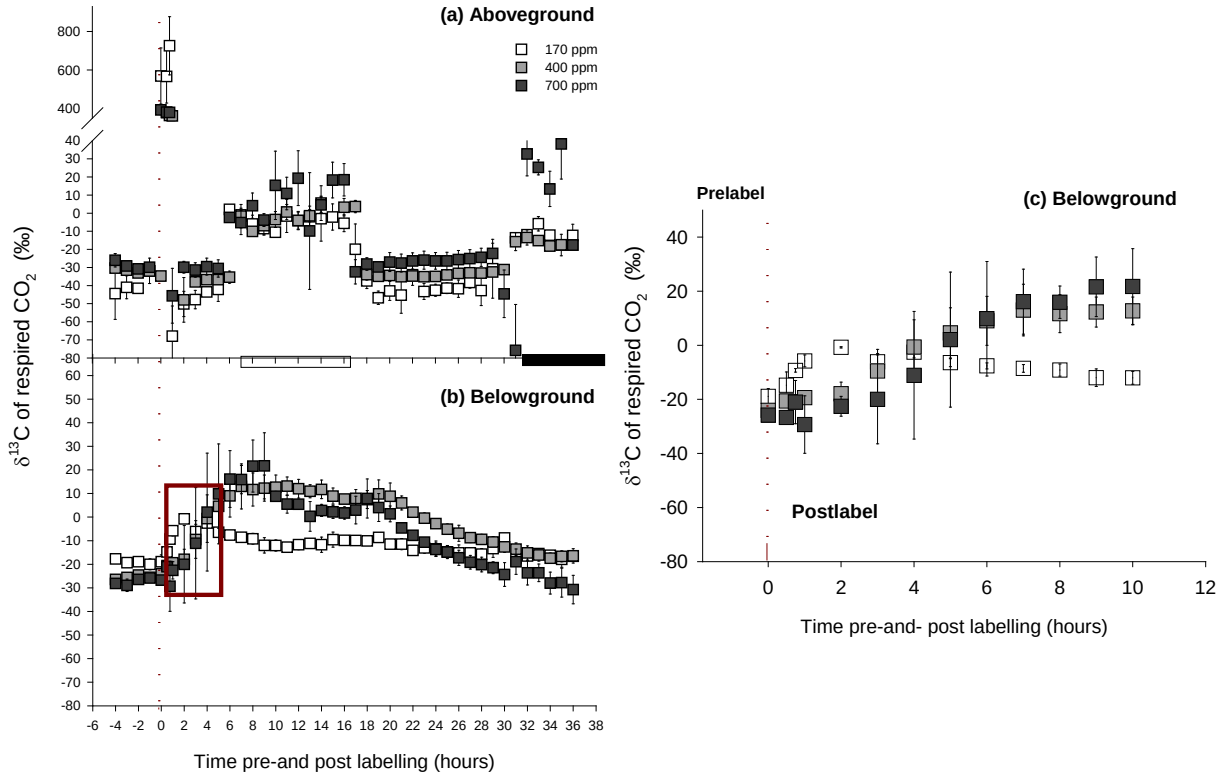

Figure 5.7. Above-and belowground $\delta^{13}C$ of respired CO_2 (Eqn. 5.5; panels a and b, respectively. Mean ± 1SD) of *Medicago sativa* inoculated with *Ensifer meliloti* grown at three atmospheric [CO_2] levels: 170, 400 and 700 ppm. The red dashed line shows the beginning of the pulse label and the night time is indicated on panel a's x-axis. Panel c shows a blow-up of the red square in panel b highlighting $\delta^{13}C$ dynamics immediately after labelling.

5.4. Discussion

5.4.1. Major findings

M. sativa plants grown under C-limitation assimilated less C, but when calculated per unit of biomass these plants allocated proportionally more C to respiration, particularly belowground, likely to maintain the ability to fix-N. Elevated [CO_2] plants increased C storage and water-use efficiency, potentially increasing their ability to withstand environmental stresses like drought.

5.4.2. Greater specific C use in nodules changed the NSC allocation pattern

Lower concentrations of NSC (both soluble sugars and starch), together with higher specific respiration rates and a faster appearance of the label in belowground respiration indicate rapid

allocation of newly-assimilated C to nodulated roots at low [CO_2]. Our results are in accord with other labelling experiments that showed the partitioning of newly-assimilated C to nodulated roots was up to 20 % in *Phaseolus vulgaris* and 40-to-60% in *G. max*. Nodule respiration depends more on new C than root respiration (Hansen *et al.*, 1992, Hansen *et al.*, 1993) and can enhance respiration rates by an order of magnitude compared to non-nodulated roots (Vessey *et al.*, 1998). The relatively higher sink strength of nodulated roots in the low [CO_2] treatment caused a negative C balance over the period of our observations. Likely, this increment in C allocation to belowground tissues was used to fuel nodule metabolism and thus N-fixation. However, plants cannot maintain a negative C balance even under low C-availability. Hence, it is likely that periods of high N-fixation activity (negative C balance) might be followed by days of less C demand in belowground tissues. Alternatively, periods of high C-assimilation (e.g., with higher light availability or lower humidity) may provide enough C supply surplus to buffer other periods of negative C balance. Such periods of lower belowground sink activity would also liberate C resources for other C-sinks like growth. C allocation to growth and biomass partitioning depend on the plant's developmental stage and changes in [CO_2] (Poorter *et al.*, 2012). At low [CO_2] plants were still at the vegetative stage, while those plants grown at higher [CO_2] treatments were already at the early flowering stage. Higher root-to-shoot-ratios and lower C-assimilation rates indicated a strong sink for photosynthates in belowground tissues.

5.4.3. Greater C cost of N fixation reduced C allocation to belowground growth

The prolonged vegetative stage observed in low [CO_2] plants could be explained by the concomitant effect of C limitation and periods of negative C balance, as mirrored by the lower RGR of these plants. Overall, in nodulated *M. sativa* plants we observed a decoupling of RGR and LAR. Hence, growth responses to variations in [CO_2] could be explained through physiological but not morphological differences (e.g., net C gain). Other studies on a variety of species (16 forbs, 6 woody and 6 grasses) showed that morphological changes (higher LAR) were unrelated to growth changes in response to CO_2 variation (Temme *et al.*, 2016).

Interestingly, C availability changed root architecture and nodule morphology in our experiment. Legumes are known to have highly plastic root systems (Mohd-Radzman *et al.*, 2013), and their root architecture can be influenced by N and C availability (Jackson *et al.*, 2008). In terms of C allocation, others have observed in non-nodulated plants that newly-assimilated C was used for the production

of fine-roots, whereas respiration was partly fueled by a storage pool (Lynch *et al.*, 2013). Based on these results it would be expected that low [CO_2] plants would have higher fine-root density, given the fast use of newly assimilated C by belowground tissues. However, the strong C limitation at low [CO_2] apparently required allocation of newly-assimilated C primarily to respiration, thereby reducing belowground growth and fine-root production.

5.4.4. Elevated [CO_2] increased C storage and water-use efficiency

Changes in the atmospheric [CO_2] level experienced by plants had a direct influence on water-use efficiency, which increased with the [CO_2] level at relatively constant VPD. However, this was not controlled by stomatal conductance and did not translate into enhanced plant growth under elevated vs. ambient [CO_2]. Similar growth between ambient and elevated [CO_2] is consistent with similar net C assimilation in these treatments. C assimilation rates may have been limited at elevated [CO_2] by light conditions in the climate chambers, as discussed in Chapter 4, and not by acclimation of the C-assimilation machinery, as has been previously observed in *M. sativa* (Sanz-Sáez *et al.*, 2010).

Higher water-use efficiency together with increased C storage under elevated [CO_2] is positively related with higher resilience to environmental stresses, like drought (Franks *et al.*, 2012). For example, Serraj (2003) observed increases in biomass in *G. max* in droughted plants grown under elevated [CO_2]. However, drought is a major limitation of N-fixation in legumes (Aranjuelo *et al.*, 2014, Nasr Esfahani *et al.*, 2014, Rogers *et al.*, 2009). Under elevated [CO_2], N-fixation limitation has been attributed to increases in ureides, amides and other amino acids in the nodules, and not to limited C- supply (Rogers *et al.*, 2009).

Elevated [CO_2] has perhaps the greatest potential to favor legumes under moderate drought. *G. max* grown under elevated CO_2 (FACE experiment) reduced stomatal conductance and thus, canopy transpiration (Leakey *et al.*, 2009a). This may help to maintain soil water content at levels above any N-fixation inhibition threshold (Rogers *et al.*, 2009), as has been observed in *M. sativa* under progressive moderate drought and subsequent recovery (Erice *et al.*, 2011).

5.5. Implications

Under changing atmospheric [CO_2], nutrient limitation is not the driving factor of growth in nodulated *M. sativa*. However, high [CO_2] caused an increase in allocation to belowground biomass including NSC. This has important implications for a forage legume like alfalfa. The usual method

of managing a forage legume like alfalfa is through harvesting of aboveground tissues at the end of the vegetative stage, followed by a subsequent regrowth of biomass. Higher root-to-shoot ratio and NSC content might increase the rate of regrowth.

CHAPTER 6

Synthesis and conclusions

6.1. C availability influenced C and N distribution in nodulated plants

The two separate studies contained in this dissertation made it possible to explore the importance of environmental factors such as drought or atmospheric [CO_2] on C allocation and overall N-gain in different rhizobium-legume associations. What is particularly novel in this thesis is that we investigated growth and performance of a nodulated common forage species across the range of Late Pleistocene low, via ambient to predicted future elevated [CO_2], which has not been reported before.

Overall, it can be concluded that nodulated plants regulate allocation of the C they assimilate to maximize their potential for growth and symbiotic N exchange in response to changes in [CO_2] or water availability.

Chapter 2 shows that *Leucaena* seedlings regulated N content via changes in their whole plant biomass, and differed in N-fixation and biomass accumulation according to the inoculated N-fixing bacteria strain. Drought caused plant growth to cease, and in seedlings inoculated with *Rhizobium tropici* plant N was slightly reduced. Such a reduction might indicate greater N use compared to resupply from N-fixation. N-fixation was probably affected due to the down-regulation of bacteroid's respiration by reduced C availability. These results showed that *R. tropici* is a better choice than *Mesorrhizobium loti* as a symbiont for *Leucaena* seedlings for forest restoration and agroforestry applications under increasingly drier conditions.

In **Chapter 3,** changes in [CO_2] affected plant development in nodulated *Medicago sativa*. C allocation was maximized under low [CO_2] thereby maximizing the potential to fix-N. However, growth rates for plants grown at ambient and elevated [CO_2] were similar. Therefore, *M. sativa* as a N-fixer might not have a competitive advantage over non-fixing plant in terms of growth under higher compared to ambient atmospheric [CO_2].

Chapter 4 shows how the respiration-to-C assimilation ratio and allocation of NSC can lead to the growth patterns described in Chapter 3. High specific respiration rates in roots were maintained by high C allocation (indicated by high NSC) to support greater C cost for N-fixation. Moreover, the

observation that there was not much difference in growth at 400 and 700 ppm [CO_2] was at least partly explained by the highest [CO_2] plants being limited by the rather low light in our growth chambers. Thus, there is the possibility that plants under elevated [CO_2] may not achieve full potential assimilation if they grow in lower light environments.

Chapter 5 reinforces allocation patterns seen over the entire period of plant growth (Chapter 3) by tracking allocation of new photosynthates directly using isotope labelling of new photosynthetic products. *M. sativa* plants grown under C-limitation assimilated less C, but when calculated per unit of biomass, these plants allocated proportionally more C to respiration, particularly belowground, likely to maintain the ability to fix-N. Elevated [CO_2] plants increased C storage and water-use efficiency, potentially increasing their ability to withstand environmental stresses like drought.

In *M. sativa* plants metabolic processes like photosynthesis and respiration were affected by changes in [CO_2], while N acquisition in such a symbiotic system was not. Under C limitation, the greater allocation of C to belowground respiration at NSC leaf expenses caused growth reduction. It seems that under obligate symbiosis, bacteroids activity prioritized C demands for N-fixation. On the other hand, elevated [CO_2] increased C storage but not growth.

Climate change can act to both amplify and constrain of N-fixation in legumes. Variations in plant-rhizobia interactions can influence the successful establishment of legume trees during early developmental stages and influence the benefit accrued from the symbiosis.

The interactive effects of C allocation and N-fixation observed in two legume species studied in this thesis showed that:

(1) Legumes have the potential to maximize the benefit of elevated [CO_2] by matching stimulated C-assimilation and NSC production with increased N-fixation.

(2) This potential can only be realized in the absence of limitations on plants growth, such as severe drought.

(3) Rising atmospheric [CO_2] greatly increases the ability to withstand moderate droughts by increasing the C storage and the C gain per unit of water loss.

These observations complement previous studies on legumes about the implications of changing environment benefits of N-fixation, especially in alfalfa, a forage legume where nodule performance

has been extensively studied (see Aranjuelo *et al.*, 2014 and references therein).

These observations, however, are contribute to the hypothesis that under elevated [CO_2] or drier scenarios, legumes like *M. sativa* or *Leucaena* will have a competitive advantage over other non N-fixing plant functional types. Both legume species studied here can establish two symbioses simultaneously, with N-fixing bacteria and with arbuscular mycorrhizal fungi. Double symbiotic nodulated mycorrhizal plants growing with limited nutrient and water availability will likely increase their resilience to changing environmental conditions. However, it was observed that light is an important limitation for *M. sativa* growth. This might negatively affect yield under elevated [CO_2], despite the fact that forage quality might improve.

6.2. Future Research

There are still key knowledge gaps in drawing general conclusions about the impact of climate change on legumes to both agriculture and the function of natural ecosystems. Despite the great economic and ecological importance of legumes, so far a large number of studies have focused only on four species: *G. max* (soybean), *Trifolium repens* (white clover), *M. truncatula* and *M. sativa* (alfalfa) and their response to drought (Aranjuelo *et al.*, 2014). Legumes are frequently exposed to varying temperature, VPD, light and, nutrient availability conditions that limit plant nodule-rhizobia interactions and effectiveness (Lira *et al.*, 2015). It is suggested to study in more detail the response of legume-rhizobia associations to these stressors, especially those that will imbalance C supply and demand, like light and temperature.

Moreover, only a single leguminous food crop (soybean) has been the subject of a fully open-air CO_2 enrichment experiment, and this study did not report the effects of elevated [CO_2] on N-fixation. Under field conditions, where nutrient limitation usually limits legume growth, more studies are needed on a larger time scale, in which combined effects of two or more environmental stressors that affect plant growth are studied, for example drought and elevated temperatures, or light and elevated temperatures. Such knowledge should be included in future research of legume performance for agricultural and environmental purposes within a changing environmental context.

Summary

Climate projections suggest a significant increase in the atmospheric concentration of CO_2 ($[CO_2]$) as well as an increase in the frequency and severity of climate extremes, such as droughts and heatwaves. Those changes may have profound implications for plant functions and the balance of plant carbon (C). The plant's C balance represents the difference between net C-assimilation and numerous plant-C sinks, including respiration, growth, defense, and storage.

Nodulated plants represent a special case in terms of C balance. Legumes have the ability to use a portion of the assimilated C to obtain nitrogen (N) through symbiosis with N-fixing nodule inducing bacteria, known as rhizobia. Changes in C availability may influence N-fixation in these root nodules. Rising $[CO_2]$ may stimulate N-fixation by increasing C-assimilation rates and higher plant carbohydrate content. On the contrary, limiting growing conditions like the reduction of water availability causes C-assimilation inhibition and decreases substrates available for bacterial respiration, potentially limiting N-fixation. A large number of studies analyzing legume performance has been conducted specifically focusing on the nodules, without considering the rest of the plant. This makes it difficult to investigate mutual regulatory effects. Studies on plant C allocation to bacteroids and N returns to the plant are a matter of great concern, because N-fixing plants may actively change plant C allocation to maintain tissue N content and positive growth rates under changes in C availability.

The aim of the work presented in this thesis is to improve our understanding of how changes in environmental conditions affect the balance between C allocation and N-fixation in nodulated plants. Such knowledge is of critical importance to understand and predict the physiological responses of legumes to rising atmospheric $[CO_2]$. To do so, manipulative experiments were utilized to investigate the importance of environmental factors such as drought and atmospheric $[CO_2]$ on the C allocation and overall N gain in different rhizobium-legume associations. A unique feature of this study is that legume plants were forced into an obligate symbiotic relationship, i.e., plants had to allocate C to N-fixing bacteria to secure their N supply. The N-fixing bacteria used in this thesis produced indeterminate nodules. Therefore, growth and development of both plants and rhizobia were entirely controlled by the C and N trade-off between them.

The following section outlines the main aims of the studies that were conducted for this thesis.

In our first study the main aim was to:

1. Evaluate the influence of different rhizobium strains on overall plant biomass and total C and N content in plant tissues and determine how these parameters change during a short but intense drought.

In the second study, through a series of experiments C allocation and N-fixation were studied under manipulated [CO_2] conditions, the main aims were to:

2. Understand shifts in plant C allocation to growth but also changes in overall plant N-gain as a response to changes in atmospheric [CO_2].
3. Identify the influence of atmospheric [CO_2] on leaf-level physiological processes as photosynthesis and respiration as well as light inhibition of leaf respiration and photorespiration, but also total N and amino acids concentrations and non-structural carbohydrates in plant leaf and root tissues.
4. Investigate, using a whole-plant approach, how different atmospheric [CO_2] influences allocation to other sinks like respiration as well as storage, and if potential differences in C allocation patterns could be explained by the C cost of symbiotic N-fixation.

In the first study, the effect of rhizobium strains on plant development and drought response for a common tropical legume plant were examined. The study compared early growth and stoichiometric parameters of *Leucaena leucocephala* seedlings inoculated with *Mesorrhizobium loti* or *Rhizobium tropici*. The results indicate that *Leucaena* inoculated with *R. tropici*, fixed more N and gained more biomass than seedlings associated with *M. loti*. Although both associations ceased fixing N during drought, *R. tropici* seedlings had on average longer roots, and their nodules accumulated more proline, suggesting that seedlings inoculated with *R. tropici* perform better are better adapted to cope better with drought conditions.

In the second study, plant C supply was manipulated by growing *Medicago sativa* L. associated with *Ensifer meliloti* (*Sinorhizobium meliloti*) under controlled atmospheric [CO_2] conditions. It was investigated how atmospheric [CO_2] (late Pleistocene, 170 ppm; ambient, 400 ppm and projected future, 700 ppm) influenced plant C assimilation, growth and development and how this, in turn, affected C-N exchanges between plants and rhizobia. The timing of seedling emergence and establishment of nodulation was similar across all [CO_2] levels. In *M. sativa* plants, biomass

production and plant growth, but not N content increased with increasing atmospheric [CO_2] at different developmental stages. C allocation to growth and N-fixation changed in a way to maintain functional tissue stoichiometry at different developmental stages, to maintain sufficient N concentration for plant growth independent of atmospheric [CO_2].

During the vegetative stage, leaf-level gas exchange measurements were performed to investigate mesophyll and biochemical limitations on C-assimilation and on respiration. C-assimilation responded to biochemical limitations, rather than stomatal limitations. Low [CO_2] plants were limited by the carboxylation capacity of RuBisCO (1,5,-bisphosphate carboxylase enzyme), and not by RuBP regeneration as seen when leaves were exposed to higher [CO_2] during measurement of photosynthesis parameters. Light inhibited respiration at low and ambient [CO_2], and the changes in respiration rates, in both leaves and roots suggest that nodulated *M. sativa* regulated the tissue maintenance cost (i.e., respiration per unit of N), as well as C allocation to respiration supply and to growth. These results suggest that acclimation responses of plant C balance to long-term exposure to sub-atmospheric and elevated [CO_2] are driven by changes in C allocation. High specific rates of respiration in low [CO_2] roots are associated with high nonstructural carbohydrates (NSC), indicating plants are allocating a greater proportion of the C they fix to fuel belowground respiration, which suggest that more C needs to be expended to maintain plant N levels.

It was also investigated how whole-plant C balance and C allocation patterns varied in 16-17 week old nodulated *Medicago sativa* L. plants. This was investigated through a stable isotope label to trace the fate of newly fixed-C over a 72 h period, using a chamber design that allowed to partition aboveground and belowground fluxes. Overall, higher [CO_2] levels resulted in a higher biomass yield compared to those grown at 170 ppm. However, when expressed per gram of dry biomass, rates of net C assimilation and belowground respiration were highest at low [CO_2]. N concentrations in plant tissues were maintained under atmospheric [CO_2] changes. Concentrations of NSC were highest in leaves of plants grown at 700 ppm, but NSC in nodules were constant across all [CO_2] treatments. The tracking of the label $^{13}CO_2$ supplied to the plants demonstrated faster use of new photosynthetic products to support belowground respiration in low [CO_2] plants, while plants grown at higher [CO_2], respired the C that was fixed over a period of several days. These results suggest that sink organs like roots and nodules are fed with newly-assimilated NSCs from leaves to support respiration. Especially in 170 ppm plants, this causes a major respiratory loss of newly assimilated C, which in turn reduces C allocation to growth. Moreover, plant metabolic processes like

photosynthesis and respiration were affected by changes in [CO_2], while N acquisition in such a symbiotic system was not.

Overall, it can be concluded that nodulated plants regulate the allocation of C to maximize their potential for growth and symbiotic N exchanges in response to changes in [CO_2] and water availability. The interactive effects of C allocation and N-fixation observed in two legume species studied in this thesis showed that:

(1) Legumes have the potential to maximize the benefit of elevated [CO_2] by matching stimulated C-assimilation and NSC production with increased N-fixation.

(2) This potential can only be realized in the absence of limitations on plants growth, such as severe drought.

(3) Rising atmospheric [CO_2] greatly increases the ability to withstand moderate droughts by increasing the C storage and the water-use efficiency.

Zusammenfassung

Gemäß aktuellen Klimaprognosen ist von einem deutlichen Anstieg der atmosphärischen CO_2-Konzentration ($[CO_2]$) auszugehen. Zudem werden Klimaextreme wie Dürren und Hitzewellen an Häufigkeit und Intensität zunehmen. Diese Veränderungen können sich tiefgreifend auf die Funktionsweise und die Kohlenstoffbilanz von Pflanzen auswirken. Letztere stellt dabei die Differenz zwischen Netto-Assimilation und zahlreichen Kohlenstoffsenken, unter anderem Atmung, Wachstum, Verteidigung und Speicherung im System Pflanze dar.

Rhizobien-assoziierte Pflanzen nehmen eine Sonderstellung in Bezug auf ihre Kohlenstoffbilanz ein. Leguminosen haben die Fähigkeit, einen Teil des assimilierten Kohlestoffes (C) zu verwenden, um Stickstoff (N) durch die Symbiose mit Knöllchen-bildenden, N-fixierenden Bakterien (Rhizobien) zu gewinnen. Änderungen in der C-Verfügbarkeit können die N-Fixierung in den Wurzelknöllchen beeinflussen. Als Reaktion auf eine zunehmende $[CO_2]$ in der Atmosphäre können sich die C-Assimilationsrate sowie der Kohlenhydratgehalt in der Pflanze erhöhen, was eine Steigerung der N-Fixierung durch Bakterien zur Folge haben kann. Im Gegenzug können wachstumslimitierende Faktoren, wie eine reduzierte Wasserverfügbarkeit, die C-Assimilation hemmen und dadurch die Substratverfügbarkeit für bakterielle Atmung verringern, was möglicherweise die N-Fixierung einschränkt. Das Gros der Studien über das Wachstumsverhalten von Leguminosen ist speziell auf die Knöllchen fokussiert, wobei der übrige Teil der Pflanze vernachlässigt wird und damit die Beurteilung vonvon wechselseitigen regulatorischen Effekten erschwert wird. Untersuchungen zur C-Allokation von der Pflanze zu den mit ihr assoziierten Bakterioiden sowie zum gegenläufigen N-Fluss sind von großer Bedeutung, da N-fixierende Pflanzen die C Allokation aktiv ändern könnten, um den N-Gehalt im Gewebe und damit positive Wachstumsraten unter veränderter C-Verfügbarkeit aufrecht zu erhalten.

Das Ziel dieser Doktorarbeit ist es, unser Verständnis über den Einfluss von veränderten Umweltbedingungen auf das Gleichgewicht zwischen C Allokation und N-Fixierung in Rhizobien-assoziierten Pflanzen zu verbessern. Dieses Wissen ist von entscheidender Bedeutung für das Verständnis und die Vorhersage der physiologischen Reaktionen von Leguminosen auf einen Anstieg atmosphärischer $[CO_2]$. Zu diesem Zweck wurden manipulative Experimente durchgeführt, um die Bedeutung von Umweltfaktoren wie Trockenheit und atmosphärischen $[CO_2]$ auf die C-

Allokation sowie die Zunahme des Gesamtstickstoffs in verschiedenen Rhizobium-Leguminosen-Assoziationen zu untersuchen. Eine Besonderheit dieser Studie ist, dass Leguminosen in eine obligate symbiotische Beziehung gezwungen wurden, das heißt, die Pflanzen waren gezwungen, C zu den assoziierten N-fixierenden Bakterien zu verlagern, um ihre N-Versorgung zu sichern. Die N-fixierenden Bakterien die für die Untersuchungen im Rahmen dieser Arbeit genutzt wurden, produzierten nicht-determinierte Knöllchen. Daher war das Wachstum und die Entwicklung von sowohl Pflanze als auch Rhizobien vollständig durch den wechselseitigen C- und N-Austausch gesteuert.

Im Folgenden werden die wesentlichen Ziele der Studien, die für diese Arbeit durchgeführt wurden, erläutert.

Ziel unserer ersten Studie war es:

1. Den Einfluss verschiedener Rhizobienstämme auf die Gesamtpflanzenbiomasse und den C- sowie N-Gehalt in verschiedenen Pflanzenteilen zu bestimmen und zu untersuchen, wie sich diese Parameter während einer kurzen aber intensiven Trockenperiode ändern.

Im Rahmen einer zweiten Studie wurde mittels einer Reihe von Experimenten die C-Allokation und N-Fixierung unter manipulierten [CO_2] Bedingungen untersucht. Dabei waren die wichtigsten Ziele:

2. Änderungen bei der Allokation von C zum Zwecke des Pflanzenwachstums aber auch die Zunahme des N-Gehaltes als Reaktion auf variierende atmosphärische [CO_2] zu verstehen.

3. Den Einfluss der atmosphärischen [CO_2] auf physiologische Prozesse auf Blattebene, wie Photosynthese und Atmung sowie lichtinduzierte Hemmung der Atmung und Photorespiration, aber auch auf Gesamtstickstoffgehalt, Konzentrationen von Aminosäuren und nicht-strukturelle Kohlenhydrate in Blatt- und Wurzelgewebe zu identifizieren.

4. Zu untersuchen, wie sich verschiedene atmosphärische [CO_2] auf die C-Verteilung auf andere Senken wie Atmung und Speicherung in der gesamten Pflanze auswirken und ob potentielle Unterschiede im C Verteilungsverhalten durch die für die symbiotische N-Fixierung investierte C-Menge erklärt werden können.

In der ersten Studie wurde die Wirkung von Rhizobienstämmen auf die Entwicklung und die

Reaktion auf Trockenstress für eine in den Tropen verbreitete Leguminose untersucht. Es wurden das frühe Wachstum sowie stöchiometrische Parameter von Sämlingen der Weißkopfmimose (*Leucaena leucocephala*), die mit *Mesorrhizobium loti* oder *Rhizobium tropici* beimpft wurden, verglichen. Die Ergebnisse zeigen, dass *Leucaena* die mit *R. tropici* beimpft wurde, mehr N fixierte und mehr Biomasse produzierte als die mit *M. Loti* assoziierten Sämlinge. Obwohl beide Varianten die N-Fixierung unter Trockenstress einstellten, waren das Wurzellängenwachstum und auch der Prolingehalt der Knöllchen bei mit *R. tropici* beimpften Sämlingen im Durchschnitt höher. Das deutet darauf hin, dass mit *R. tropici* inokulierte Sämlinge besser an Trockenheit angepasst sind.

In der zweiten Studie wurde die C-Zufuhr für mit *Ensifer meliloti* (*Sinorhizobium meliloti*) assoziierter Luzerne (*Medicago sativa L.*) manipuliert, indem sie unter kontrollierten atmosphärischen [CO_2] Bedingungen angezogen wurde. In diesem Versuch sollte eruiert werden, wie die atmosphärische [CO_2] (Spätpleistozän, 170 ppm; rezent, 400 ppm und prognostiziert, 700 ppm) die C Assimilation, das Wachstum und die Entwicklung der Pflanze beeinflusst, und wie sich dies wiederum auf den C-N Austausch zwischen Pflanzen und Rhizobien auswirkt. Das Auflaufverhalten der Sämlinge und die Etablierung der Knöllchen waren in allen [CO_2]-Varianten ähnlich. Mit zunehmender atmosphärischer [CO_2] stieg jedoch die Biomasseproduktion und das Pflanzenwachstum, aber nicht der N-Gehalt, bei verschiedenen Entwicklungsstadien der *M. sativa*-Pflanzen. Die für Wachstum und N-Fixierung aufgewendete C-Menge veränderte sich während der verschiedenen Entwicklungsstadien derart, dass unabhängig von der atmosphärischen [CO_2] ein funktionelles Verhältnis im Pflanzengewebe geschaffen wurde, das eine ausreichende N-Verfügbarkeit zur Aufrechterhaltung des Pflanzenwachstums ermöglichte.

Während der Wachstumsphase wurden Gasaustauschmessungen auf Blattebene durchgeführt um Limitierungen auf die C Assimilation und Atmung zu untersuchen, die durch das Mesophyll oder biochemische Aspekte hervorgerufen werden. Dabei zeigte sich, dass die Aufnahme von C eher durch biochemische als durch Stomata-basierte Aspekte begrenzt ist. So war unter niedriger [CO_2] die Carboxylierungskapazität von RuBisCO (1,5,-bisphosphate Carboxylase Enzym) limitierend und nicht die RuBP-Regeneration, was durch Behandeln der Blätter mit höheren [CO_2] während der Messung der Photosynthese-Parameter gezeigt wurde. Licht-inhibierte Atmung bei niedrigen sowie rezenten [CO_2] und die Änderung der Atmungsraten in sowohl Blättern als auch Wurzeln, legen nahe, dass Rhizobien-assoziierte *M. sativa* die Erhaltungskosten für das Pflanzengewebe (Atmung

pro Einheit N) und die C- Allokation zugunsten von Atmung und Wachstum regulieren konnten. Diese Ergebnisse legen nahe, dass die flexible Allokation von C die Anpassungsreaktionen der C Bilanz auf die langfristige Exposition gegenüber subatmosphärischen und erhöhten [CO_2] steuert. Hohe spezifische Wurzelatmungsraten unter niedriger [CO_2] sind mit hohen Konzentrationen nicht-struktureller Kohlenhydrate (NSC) verknüpft, was darauf hinweist, dass die Pflanzen einen größeren Anteil des fixierten C für die unterirdische Atmung aufwenden. Das wiederum bedeutet, dass mehr C aufgewendet werden muss um einen bestimmten N-Gehalt aufrechtzuerhalten. Es wurde auch untersucht, wie die C-Bilanz und C-Allokationsmuster in 16 bis 17 Wochen alten Rhizobien-assoziierten *Medicago sativa L.* Pflanzen variieren. Eine Stabilisotopenmarkierung ermöglichte es, den Abbau von neu aufgenommenem C über einen Zeitraum von 72 h zu verfolgen. Dafür wurde ein Messaufbau verwenden, durch den eine Unterscheidung in ober- und unterirdische Flüsse möglich war. Im Vergleich zu den Erträgen bei 170 ppm führten höhere [CO_2] generell zu einer höheren Biomasse. Auf Gramm Trockenbiomasse umgerechnet, waren die Netto-C-Assimilationsraten sowie die Bodenatmungsraten bei niedrigen [CO_2] am höchsten. Die N-Gehalte im Pflanzengewebe wurden trotz der Änderungen atmosphärischer [CO_2] beibehalten. Die Konzentrationen von NSC waren in den Blättern von Pflanzen bei 700 ppm am höchsten, in den Knöllchen hingegen unter allen [CO_2] konstant. Durch das Labeln der Pflanzen mit $^{13}CO_2$ konnte nachgewiesen werden, dass unter niedriger [CO_2] neue Photosyntheseprodukte schneller für die Bodenatmung genutzt wurden. Dahingegen veratmeten bei höheren [CO_2] angezogene Pflanzen C, der über einen Zeitraum von mehreren Tagen fixiert worden war. Diese Ergebnisse legen nahe, dass Wurzeln und Knöllchen als C-Senken mit frisch assimilierten NSCs versorgt werden, um die Atmung zu unterstützen. Besonders in den 170 ppm-inkubierten Pflanzen verursacht dies einen großen Verlust von frisch assimiliertem C zugunsten der Atmung, was den für das Wachstum bereitstehenden Pool reduziert. Zusätzlich waren pflanzliche Stoffwechselprozesse wie Photosynthese und Atmung von den Veränderungen in der [CO_2] betroffen, während es der N-Gewinn in einem derartigen symbiotischen System nicht war.

Insgesamt kann gefolgert werden, dass Rhizobien-assoziierte Pflanzen die Verteilung von C regulieren können, um ihr Potential für Wachstum und symbiotischen N-Austausch in Reaktion auf die Veränderungen der [CO_2] und Wasserverfügbarkeit zu maximieren. Dies sind die Auswirkungen auf die Leguminosen in einem Zukunftsszenario einer sich ändernden Umwelt:

(1) Leguminosen haben das Potenzial, den Vorteil der erhöhten [CO_2] zu maximieren, indem sie auf die stimulierte C Assimilation mit erhöhter N_2-Fixierung reagieren.

(2) dieses Potential kann nur ausgenutzt werden, wenn keine wachstumsbeschränkenden Faktoren wie Trockenstress bestehen.

(3) steigende atmosphärische [CO_2] erhöht die Fähigkeit, durch die Steigerung der C-Speicherung und dem damit gegebenen C Gewinn pro Einheit Wasserverlust, kurze Dürreperioden zu überstehen.

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Author Contributions to the Manuscript

Manuscript 1:

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Conceived and designed the experiments: Gabriela Pereyra, Susan Trumbore, Henrik Hartmann and Waldemar Ziegler; performed the experiments: Gabriela Pereyra and Waldemar Ziegler; wrote the paper together: Gabriela Pereyra, Henrik Hartmann, Beate Michalzik and Susan Trumbore.

Editorial note:

The publication and manuscripts were edited to obtain a common style and format in this dissertation. Editorial modifications included fond, layout of tables, numbering of chapters, figures and table. Moreover, a common citation style was used though the chapters and it was located at the end of the dissertation.

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Gabriela Pereyra

Selbständigkeitserklärung

Ich erkläre, dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittle, persönlichen Mitteilungen und Quellen angefertigt habe.

Jena, 8 Mai 2016

Gabriela Alejandra Pereyra Ojeda