9-23-2019 1:30 PM

Carbon and Nitrogen Dynamics in Plants Grown at Low CO2 Conditions of the Past

Andre Galvao Duarte, *The University of Western Ontario*

Supervisor: Way, Danielle A., *The University of Western Ontario*
Co-Supervisor: Longstaffe, Fred J., *The University of Western Ontario*
A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

Abstract

For most of the recent evolutionary history of plants, atmospheric CO_2 concentrations have been far below modern values. However, little is known about how plants grown in a low CO_2 environment balance their carbon demand for growth while meeting their requirement for nitrogen acquisition, the establishment of mycorrhizal fungal associations, and the production of defense compounds. Here, I investigated how low CO_2 affects *Elymus canadensis* and *Picea mariana* by comparing their growth at low and current CO_2 concentrations. I found that reduced N availability exacerbated low CO_2 effects on growth, and reduced stomatal index and N isotope composition, indicating that the use of these variables as paleo-indicators can be influenced by N availability. Mycorrhizal association was enhanced in low-CO_2 plants, and increased root N but decreased root $\delta^{15}N$, which could skew the interpretation of $\delta^{15}N$ when comparing non- and mycorrhizal ancient plant samples. Growth at low CO_2 decreased the formation and size of resin ducts and terpene production, suggesting that plants growing at past CO_2 conditions had reduced capacity to chemically defend against herbivory.

Keywords

Plant physiology, photosynthesis, plant growth, sub-ambient CO_2, nitrogen isotopes, carbon isotopes, mycorrhizae, resin duct, terpene.

Summary for Lay Audience

Carbon dioxide (CO_2) is an atmospheric gas essential to plant life as plants use CO_2 in photosynthesis to produce the energy necessary for growth. In the past, atmospheric CO_2 concentrations were far below the levels observed today, which limited plant growth. By studying plants grown at the low CO_2 concentrations of the past we can learn about how plants thrived in an environment where carbon resources were limiting, and recreate ancient dynamics between plants and their environment. In my thesis, I compared the performance of Canada wild rye (*Elymus canadensis*) and black spruce (*Picea mariana*) grown at modern CO_2 concentrations and CO_2 conditions that occurred 20,000 years ago, when CO_2 concentrations were less than half the levels observed today. I investigated how plants growing under such past conditions were affected by the availability of nitrogen, a soil nutrient essential for plants. I also compared the association between plants and fungi when grown at low and ambient CO_2 conditions. Lastly, I examined whether plants growing at low CO_2 conditions would be able to form compounds that are used as defense against herbivory. I found that nitrogen availability and association with fungi alter certain plant traits that are used in the study of ancient plant samples, and thus the present use of these traits may require reconsideration. I also found that plants growing at the low CO_2 conditions of the past associated more closely with fungi, which helped increase plant access of nitrogen. Finally, I observed that low CO_2-grown plants not only had reduced growth but also had a decreased ability to form defensive compounds called terpenes. In other words, plants in the past had a lower capacity to defend against parasites and herbivores compared to modern plants.

Co-Authorship Statement

All data chapters are co-authored with my supervisors Professors Danielle A. Way and Fred J. Longstaffe, who provided funding for all projects, contributed to study design, and helped edit all chapters. A version of Chapter 2 is under revision in the journal *Functional Plant Biology*.

Acknowledgments

I would like to express my profound gratitude to my supervisors Professors. Danielle A. Way and Fred J. Longstaffe for their mentorship and support. I also would like to thank my advisors Professors. Hugh Henry and Brent Sinclair for their guidance. I am also grateful for the support and companionship I received throughout the years from the Way lab's and LSIS' members. I also would like to acknowledge the financial support from the Natural Sciences and Engineering Research Council of Canada and from the Conselho Nacional de Desenvolvimento Científico e Tecnológico through the program Sciences without Borders.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

3-PGA = 3-phosphoglycerate

A_{400} = net CO_2 assimilation rates measured at 400 ppm CO_2

A_{growth} = net CO_2 assimilation rates measured at growth CO_2

A_{net} = net CO_2 assimilation rates

ATP = adenosine triphosphate

[C] = carbon concentration

C_i = intercellular CO_2 concentration

CO_2 = carbon dioxide

G3P = glyceraldehyde 3-phosphate

g_s = stomatal conductance

$g_{s\text{-}400}$ = stomatal conductance measured at 400 ppm CO_2

$g_{s\text{-}growth}$ = stomatal conductance measured at growth CO_2

J_{max} = maximum electron transport rates

LGM = Last Glacial Maximum

N = nitrogen

[N] = nitrogen concentration

NADPH = nicotinamide adenine dinucleotide phosphate

NH_4^+ = ammonium

NO_3^- = nitrate

O_2 = molecular oxygen

P680 = photosystem II primary donor

P700 = photosystem I primary donor

PG = phosphoglycolate

PSI = photosystem I

PSII = photosystem II

R_{dark} = dark respiration measured at 400 ppm CO_2

RuBP = ribulose-1,5-bisphosphate.

Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

$SI_{abaxial}$ = abaxial stomatal index

$SI_{adaxial}$ = adaxial stomatal index

SLA = specific leaf area

V_{cmax} = maximum Rubisco carboxylation rates

$\delta^{13}C$ = stable carbon isotope composition

$\delta^{13}C_{chamber}$ = stable carbon isotope composition of air CO_2 inside the growth chamber

$\delta^{13}C_{leaf}$ = stable carbon isotope composition of leaves

$\delta^{13}C_{root}$ = stable carbon isotope composition of roots

$\delta^{15}N$ = stable nitrogen isotope composition

$\delta^{15}N_{AC}$ = stable nitrogen isotope composition of plants grown at ambient CO_2

$\delta^{15}N_{LC}$ = stable nitrogen isotope composition of plants grown at low CO_2

$\delta^{15}N_{leaf}$ = stable nitrogen isotope composition of leaves

$\delta^{15}N_{root}$ = stable nitrogen isotope composition of roots

$\Delta^{15}N_{leaf}$ = the difference in the stable nitrogen isotope composition of leaves between ambient CO_2- and low CO_2-grown plants ($\delta^{15}N_{AC}$ - $\delta^{15}N_{LC}$)

$\Delta^{15}N_{root}$ = the difference in the stable nitrogen isotope composition of roots between ambient CO_2- and low CO_2-grown plants ($\delta^{15}N_{AC}$ - $\delta^{15}N_{LC}$)

Chapter 1

1 General Introduction

Carbon dioxide (CO_2) is the substrate required for photosynthetic production of carbohydrates, the basic form of energy used by all living organisms (Farquhar and Sharkey, 2003; Stick, 2001). As a major limitation for photosynthesis and plant growth, changes in CO_2 availability, can have significant effects on plant productivity and evolution (Bazzaz and Bazzaz, 1996; Cowling and Sykes, 1999). For instance, the C_4 photosynthetic pathway, which concentrates CO_2 at the site of carboxylation, may have evolved as a mechanism to enhance photosynthetic rates during low CO_2 periods (Sage, 2004). In recent decades, CO_2 emissions have been accelerating at a global scale due to fossil-fuel burning and land use change (Andres *et al.*, 2012; Hofmann *et al.*, 2006; Keeling, 1973; Raupach *et al.*, 2007). However, as shown in Figure 1.1, CO_2 concentrations have been below 270 ppm for the millennia before the Industrial Revolution; concentrations were as low as 180 ppm by the end of the Last Glacial Maximum, about 20,000 years ago (Augustin *et al.*, 2004; Petit *et al.*, 1999).

Despite the prevalence of low CO_2 conditions over the recent evolutionary history of plants, studies investigating plant responses to low atmospheric CO_2 concentrations are fewer than those examining the effects of future higher CO_2 levels. Nevertheless, the literature provides several instances where modern plants exposed to low CO_2 concentrations of the past had their fitness significantly altered when compared to those grown at ambient CO_2 concentrations. For instance, growth at low CO_2 decreases photosynthetic rates and, consequently, biomass production (Agüera *et al.*, 2006; Cowling and Sage, 1998; Polley *et al.*, 1993; Sage, 1995; Sage and Cowling, 1999; Temme *et al.*, 2013), increases time to flowering, and hinders reproduction (Tissue *et al.*, 1995; Ward *et al.*, 2000; Ward and Strain, 1997). Low CO_2 may have even influenced the timing of when agriculture became feasible, as biomass production was too low to support the level of productivity required for the successful establishment of agriculture

(Sage, 1995). Therefore, a strong foundational knowledge of plant responses to the low CO_2 concentrations of the past is needed to enhance our understanding of plant evolutionary patterns and net primary productivity over geologic time scales (Gerhart and Ward, 2010).

While previous studies have established that growth at low CO_2 conditions limits plant development, advancing low CO_2 studies into a more ecologically relevant context requires the consideration of other biotic and abiotic environmental factors influencing plant growth. For instance, nitrogen (N) is considered one of the most growth-limiting nutrient in many ecosystems, both currently and in the past (Canfield *et al.*, 2010; Falkowski, 1997). However, no studies so far have investigated how plants grown in low CO_2 environments meet their N requirements, despite the high variability of forms and quantity of nitrogen that are naturally available to plants (Stevenson *et al.*, 1999; Wardle, 1992). Similarly, the interaction between plants and mycorrhizal fungi has been an important phenomenon across geological time scales (Cairney, 2000; Smith and Read, 2008). While much is uncertain about how this symbiosis will respond to future high CO_2 levels (Mohan *et al.*, 2014), even less is known about how the exchange of nutrients between plants and fungi functioned in past low CO_2 environments. In addition, considering that growth at low CO_2 concentrations limits primary metabolic functions that sustain growth, it is unlikely that plants growing under such conditions could allocate considerable resources to secondary metabolites, such as defense compounds, in a manner similar to modern plants (War *et al.*, 2012). This assumption, however, has never been tested.

Such explorations are not only important to expand our understanding of plant responses to temporal changes in CO_2 levels, but can also offer new insights to paleo-inferences based on fossil plant samples dating from low CO_2 periods, including anatomical features used for plant fossil identification and the isotope composition of plant tissues.

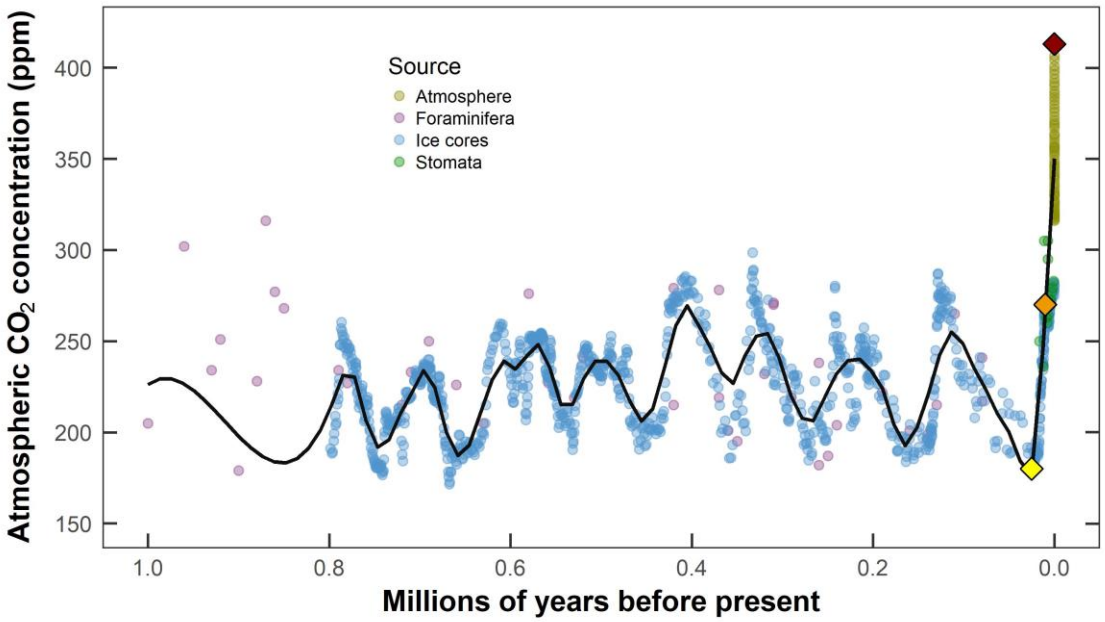

Figure 1.1. Atmospheric CO_2 concentrations over the last million years. Data since 1960 have been directly measured by The Earth System Research Laboratory in The National Oceanic and Atmospheric Administration's Office (NOAA); prior to that, CO_2 concentrations are derived from a range of paleo-indicators (pink, foraminifera; blue, ice cores; green, stomata). Specific timepoints are marked by diamonds: yellow, Last Glacial Maximum; orange, Industrial Revolution; red, present time. Data extracted from Beerling and Chaloner (1994); Lüthi *et al*. (2008); Monnin *et al*. (2001); NOAA/ESRL; Petit *et al*. (1999).

This thesis contains the projects I developed to answer the gaps in the literature outlined above. Chapter 2 describes the effects that different N sources have on plant growth, photosynthesis, and N isotope composition in *Elymus canadensis* grown at low CO_2 concentrations. In Chapter 3, the association between *E. canadensis* and the mycorrhizal fungus *Glomus intrarradices* at low CO_2 conditions is investigated, including growth parameters, fungal colonization, and carbon (C) and N isotope compositions. Chapter 4 describes how *Picea mariana* seedlings grown at ambient and sub-ambient CO_2 concentrations differ in their ability to allocate resources to defense components, such as resin ducts and terpenes. The choice of species for each experiment is discussed within the context of each chapter. Below, the effects of growth at low CO_2 concentrations on plant C and N dynamics and C and N isotope composition of plant tissues are reviewed. The functioning of the plant-mycorrhizal symbiosis, and possible low CO_2 effects on this association, are then discussed, followed by a summary of our current understanding of the effects of changing growth CO_2 concentrations on the production of plant defense compounds.

1.1 Carbon and nitrogen dynamics in plants

Nitrogen and carbon metabolism in plants are intrinsically related since C-skeletons and reductants necessary for N assimilation originate from the major C fluxes (*i.e.*, photosynthesis, photorespiration, and respiration), while N is an essential constituent of metabolic functions responsible for building the photosynthetic apparatus and sustaining growth (Nunes-Nesi *et al.*, 2010; Tegeder and Masclaux-Daubresse, 2017). Thus, by modulating rates of C exchange, growth CO_2 concentrations also determine the ability of plants to assimilate N.

1.1.1 Low CO_2 effects on carbon fluxes

Growth CO_2 concentrations have direct impacts on C cycling in plants by influencing the rates of the major leaf C fluxes: photosynthesis, photorespiration, and respiration. CO_2 is the substrate for photosynthesis, which is the incorporation of C from CO_2 by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) into plant tissues using light energy (Farquhar and von Caemmerer, 1982). CO_2 availability, and consequently the ratio of CO_2/O_2, also regulates photorespiration, which is a biochemical process in plants where Rubisco, due to its dual function as both a carboxylase and an oxygenase, fixes molecular oxygen (O_2) and releases previously fixed CO_2 (Peterhansel and Maurino, 2011). In turn, the availability of photosynthetically produced carbohydrates is the energy source for respiration. Respiration is the energy-conserving process that generates the adenosine triphosphate (ATP) needed for cell maintenance and growth, and is common to all living organisms (Millar *et al.*, 2011). These three processes are discussed in more detail below.

1.1.1.1 Photosynthesis

As shown in Figure 1.2, photosynthesis begins with the absorption of a photon that excites a specialized chlorophyll, P680, leading to the ejection of an electron from P680 (Taiz and Zeiger, 2010). This electron is transported through the photosynthetic electron transport chain to the final electron acceptor ($NADP^+$) via a series of redox reactions that include the absorption of a second photon by the antenna system of photosystem I, producing the reductant NADPH. Along the way, a proton gradient is created across the thylakoid membrane and this gradient generates ATP via an ATP synthase. Much of the energy and reducing power created in photosynthetic electron transport is used in the chloroplast stroma in the Calvin-Benson cycle. In this cycle, CO_2 is fixed to ribulose-1,5-bisphosphate (RuBP) by the enzyme Rubisco, producing 3-phosphoglycerate (PGA). This PGA is converted to 1,3-bisphosphoglycerate (which requires ATP), and then reduced to glyceraldehyde 3-phosphate (G3P), a reaction that consumes NADPH. Some

of the G3P is used to regenerate RuBP (a process that also uses ATP), while the rest is used to make glucose and other sugars.

Photosynthesis measurements are usually reported as net CO_2 assimilation rates (A_{net}), which consists of total CO_2 assimilation from photosynthesis minus rates of CO_2 lost from photorespiration and that of respired CO_2 (Rabinowitch, 1951). In the Farquhar, von Caemmerer, and Berry (1980) biochemical model of photosynthesis, A_{net} is limited by either Rubisco limitations, RuBP-regeneration limitations, or triose phosphate utilization (TPU) limitations (Figure 1.3). Depending on the leaf internal CO_2 concentration and temperature, different biochemical limitations will limit A_{net} (Busch and Sage, 2017; Farquhar *et al.*, 1980; Sage and Kubien, 2007). At low CO_2 concentrations, Rubisco limitations prevail because there is insufficient CO_2 to saturate Rubisco carboxylation rates, but Rubisco oxygenation rates are high (*i.e.*, photorespiration), assuming that the supply of RuBP is saturating. Thus, photosynthesis is limited by the maximum carboxylation rate of Rubisco (V_{cmax}). But as CO_2 substrate availability increases at higher CO_2 concentrations, photorespiration is increasingly suppressed. Under these conditions, either the ability of electron transport to support the regeneration of RuBP or other reactions within the Calvin Cycle itself become limiting for photosynthesis, such that photosynthesis is limited by the maximum electron transport rate (J_{max}) (Farquhar *et al.*, 1980). At very high CO_2 concentrations, the ability to convert triose phosphates into sucrose and starch, and thereby to release inorganic phosphorous, puts an upper ceiling on A_{net} (Sharkey, 1985).

With limited substrate for photosynthesis, growth at low CO_2 greatly decreases A_{net} (Polley *et al.*, 1993; Sage, 1995; Tissue *et al.*, 1995), and consequently biomass production (Agüera *et al.*, 2006; Li *et al.*, 2014; Sage and Coleman, 2001; Temme *et al.*, 2015). In the experiments reviewed by Sage (1995), C_3 species (such as wheat, oat, bean, and rice) had a photosynthetic performance between 25 and 50 % lower at 200 ppm CO_2 than at 360 ppm. Cowling and Sage (1998) found that total biomass declined up to 77 % when *Phaseolus vulgaris* was grown at 200 ppm in comparison to 380 ppm CO_2. A recent meta-analysis by Temme *et al.* (2013) found that a 50 % reduction in growth CO_2 decreased A_{net} by 38 % and biomass by 47 %. However, while the decrease in A_{net} is well-

established, it is still difficult to generalize how V_{cmax} and J_{max} respond to growth at low CO_2 conditions. The literature presents few examples where these variables were measured in low CO_2-grown plants, and the few results are highly variable, with increases in the rate of both parameters (Ripley *et al.*, 2013), increased V_{cmax} but not J_{max} (Anderson *et al.*, 2001), or no change in V_{cmax} and J_{max} between low CO_2- and ambient CO_2-grown plants (Cunniff *et al.*, 2017).

In order to cope with the low photosynthetic rates imposed by their C-limited environment, low CO_2-grown plants commonly develop mechanisms to enhance CO_2 uptake. These include increased stomatal density and aperture to increase the influx of CO_2 through the stomata (Elliott-Kingston *et al.*, 2016; Franks *et al.*, 2012), and higher expression of Rubisco and its activating enzyme, Rubisco activase, in order to enhance carboxylation rates (Sage and Coleman, 2001). Despite these adjustments, however, the decrease in productivity in low CO_2-grown plants is ubiquitous across low CO_2 studies (Ehleringer *et al.* 2005; Cunniff *et al.* 2017; Temme *et al.* 2013).

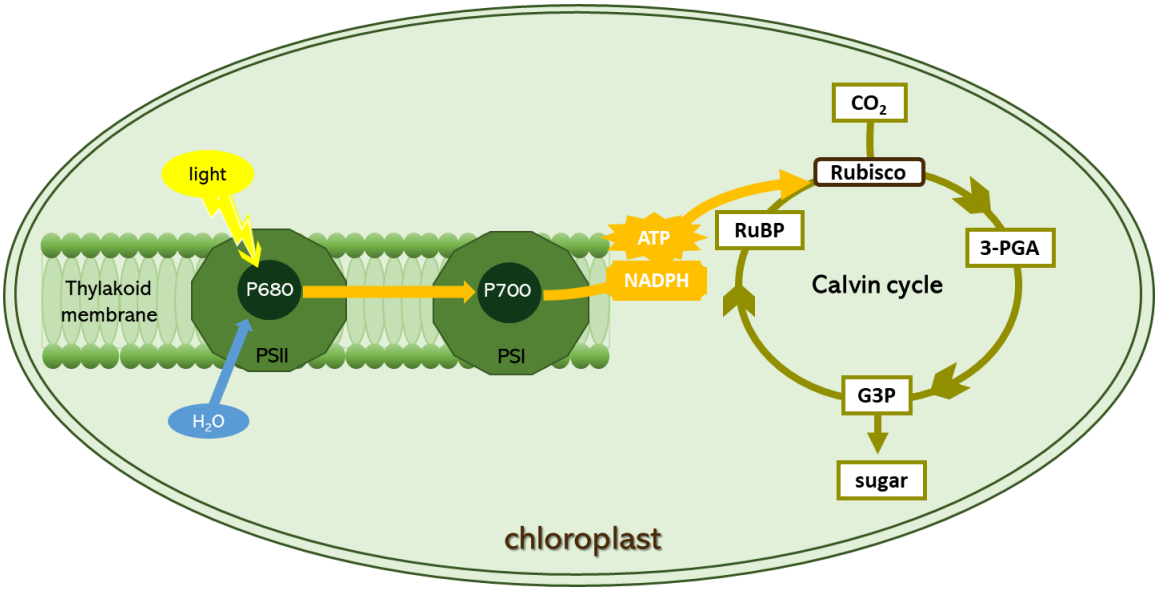

Figure 1.2. Simplified schematic of photosynthesis. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; PSII, photosystem II; P680, photosystem II primary donor; PSI, photosystem I; P700, photosystem I primary donor; NADPH, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate; 3-PGA, 3-phosphoglycerate; G3P, glyceraldehyde 3-phosphate; RuBP, ribulose-1,5-bisphosphate. Figure modified from Taiz and Zeiger (2010).

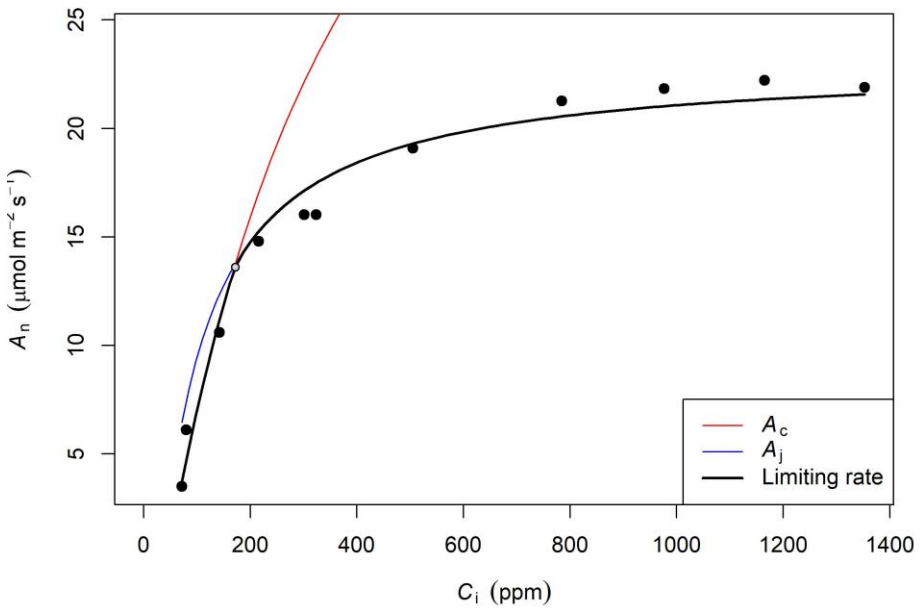

Figure 1.3. Photosynthetic CO_2 response curve based on the Farquhar, Berry and von Caemmerer (1980) biochemical model of photosynthesis. The measurement of net CO_2 assimilation rates (A_{net}) across a range of intercellular CO_2 concentrations (C_i) is used to derive the biochemical limitation of A_{net} from the maximum carboxylation rates of Rubisco (V_{cmax}, A_c, red line), and the maximum electron transport rates (J_{max}, A_j, blue line). The limiting rate (black line) is determined as the minimum A_{net} from the two biochemical limitations, which fits the measured observations. Data from *Elymus canadensis*, as described in Chapter 2.

1.1.1.2 Photorespiration

While Rubisco can fix CO_2 in photosynthesis, Rubisco is a dual function enzyme that can both carboxylate and oxygenate RuBP (Spreitzer and Salvucci, 2002). As shown in Figure 1.4, in contrast to the two molecules of PGA produced in a carboxylation event, oxygenation of RuBP by Rubisco produces one molecule of PGA and one of 2-phosphoglycolate (PG). As PG is toxic if it accumulates in plant cells (Zelitch *et al.*, 2009), it must be processed via photorespiration. The PG is converted to glycolate, which is shuttled to the peroxisome, where it is converted to glyoxylate and then (with the addition of NH_2) into glycine. This glycine is converted to serine in the mitochondria, releasing a molecule each of CO_2 and NH_4, and consuming $NADH_2$. The serine is shuttled back to the peroxisome where it is converted to pyruvate and then glycerate, with the latter reaction consuming more $NADH_2$. The glycerate can then be phosphorylated to form PGA in the chloroplast, at the cost of an ATP, while another ATP is used in photorespiration to produce the glutamate needed to provide NH_2 for the production of glycine. Therefore, overall, photorespiration consumes ATP and reducing power, while releasing previously fixed CO_2.

Photorespiration has long been considered a wasteful process that reduces the overall energy efficiency of C assimilation (Andrews, 1978; Maurino and Peterhansel, 2010; Spreitzer and Salvucci, 2002). However, recent studies have shown that photorespiration can have an important role in nitrogen assimilation by providing reductant for nitrate (NO_3^-) reduction (Bloom *et al.*, 2014; Busch *et al.*, 2018; Rachmilevitch *et al.*, 2004): as photorespiration rates increase at low CO_2, NO_3^- assimilation may be facilitated in low CO_2- compared to ambient CO_2-grown plants (see Figure 1.5 and Section 1.1.2).

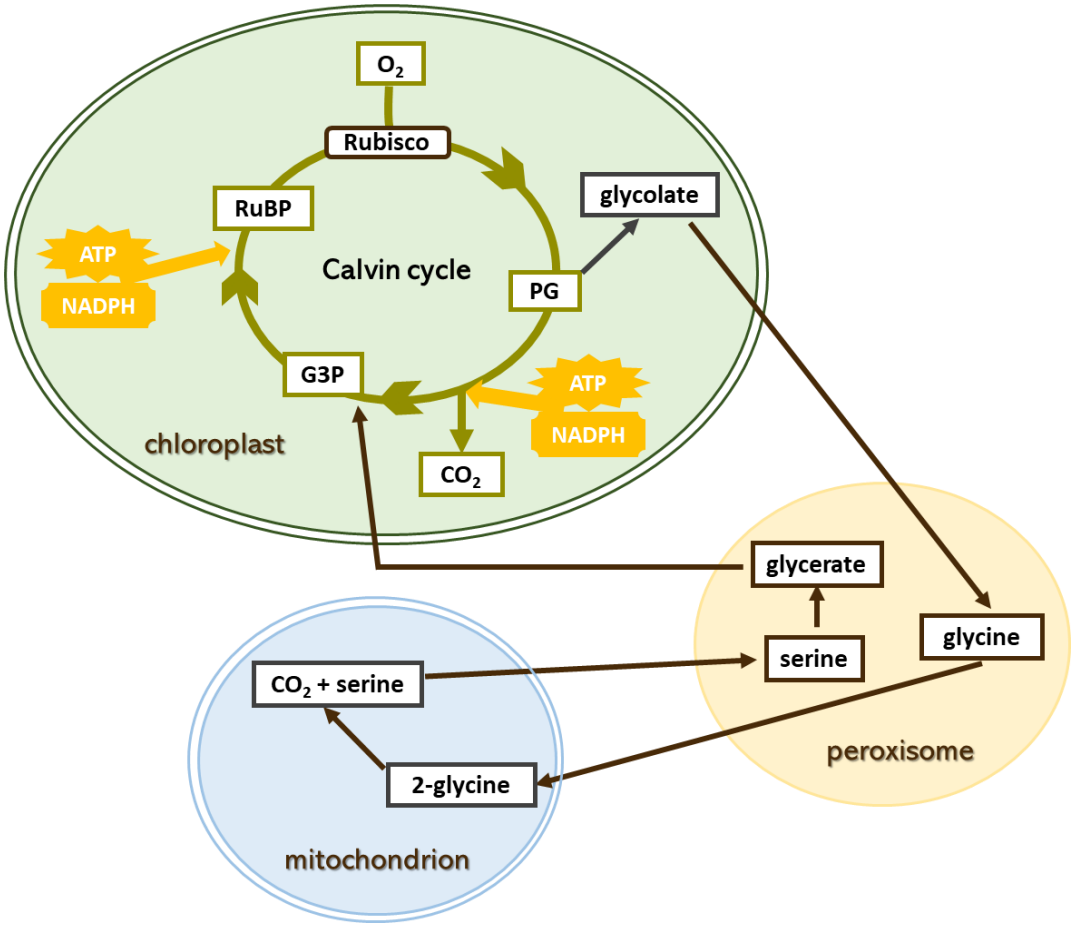

Figure 1.4. Photorespiratory pathway. Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; PG, phosphoglycolate; G3P, glyceraldehyde 3-phosphate; RuBP, ribulose-1,5-bisphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; ATP, adenosine triphosphate. Figure modified from Taiz and Zeiger (2010).

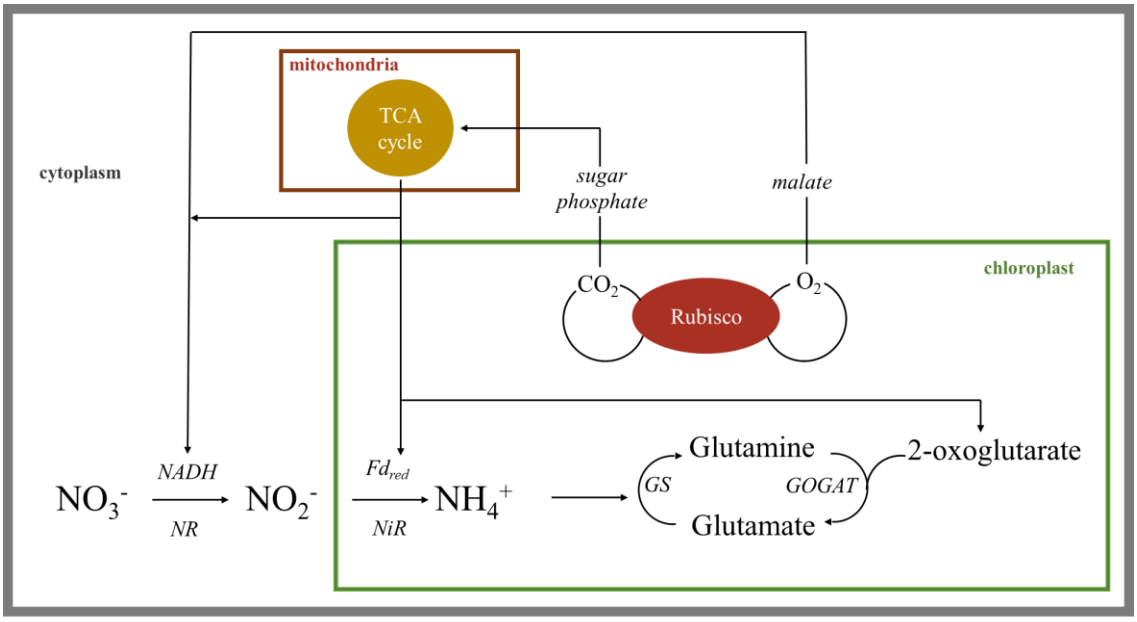

Figure 1.5. Simplified schematic of enzymes and substrates involved in inorganic N conversion that are derived from the photosynthetic and photorespiratory pathways. Nitrate (NO_3^-) is converted to nitrite (NO_2^-) by nitrate reductase (*NR*) using nicotinamide adenine dinucleotide (*NADH*). Nitrite reductase (*NiR*), using reduced ferredoxin (Fd_{red}) as an electron donor, reduces NO_2^- to ammonium (NH_4^+). NH_4^+ is then converted to glutamine by glutamine synthase (*GS*), while glutamine 2-oxoglutarate aminotransferase (*GOGAT*) converts glutamine and 2-oxoglutarate into glutamate. *NADH*, Fd_{red}, and 2-oxoglutarate are produced during the tricarboxylic acid (TCA) cycle, which is fueled by sugar phosphates produced during photosynthetic CO_2 assimilation by Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Malate derived from photorespiration (O_2 fixation by Rubisco) generates NADH and can also be used during the reduction of NO_3^- to NO_2^- in the cytoplasm. Figure derived from Andrews *et al.* (2013), Dutilleul *et al*. (2005), Krapp (2015), Nunes-Nesi *et al*. (2010), and Xu *et al.* (2012).

1.1.1.3 Respiration

Respiration involves the breakdown of glucose formed via photosynthesis to produce ATP to fuel plant metabolism (Plaxton and Podestá, 2006). Glucose is initially broken into pyruvate (glycolysis), which is oxidized to form acetyl-CoA, releasing a molecule of CO_2. The acetyl-CoA then enters the tricarboxylic acid pathway, where it is oxidized to CO_2, producing reductants for use in the mitochondrial electron transport chain. Oxidation of these reductants (and succinate, which is also created in the Citric Acid cycle) generates a proton gradient across the inner membrane of the mitochondria that drives ATP synthesis. Both respiration and photorespiration consume O_2 and release CO_2, but mitochondrial respiration occurs in both the light and in the dark, unlike photorespiration, which operates only in the light.

Possible effects of growth at low CO_2 conditions on respiration, however, have been scarcely explored. Decreased respiration rates in low CO_2-grown plants may be expected due to reduced carbohydrate availability and a lower demand for respiratory activity under a low growth rate environment. Indeed, this hypothesis aligns with the findings of Gonzàlez-Meler *et al.* (2009), who observed decreased respiration rates in *Arabidopsis thaliana* grown at 200 ppm compared to 370 ppm. Cowling and Sage (1998), however, found that respiration rates in low CO_2-grown *Phaseolus vulgaris* varied depending on growth temperature. Since these two studies seem to be the only available works that directly measured respiration in plants grown from seed at low CO_2 conditions, more data is required before a better understanding of respiratory regulation in low CO_2-grown plants can be built.

1.1.2 Nitrogen assimilation in plants

Nitrogen is a highly mobile element circulating between the atmosphere, soil and living organisms, and is involved in the formation of major macromolecules in plants, such as proteins, nucleic acids, and chlorophyll (Krapp, 2015; Mengel *et al.*, 2001). Nitrogen has been a major limitation on plant productivity in many terrestrial ecosystems on geological

timescales (Canfield *et al.* 2010; Falkowski 1997). Nitrogen is particularly limiting in temperate and higher latitude ecosystems where nitrification rates are low (Galloway *et al.*, 2004), and currently also limits plant yield in most agricultural systems (Robertson and Vitousek, 2009). Nitrogen is available for plant uptake in organic forms, mostly as amino acids, and as inorganic compounds, mainly as nitrate (NO_3^-) and ammonium (NH_4^+) (Lipson and Nasholm, 2001; Nacry *et al.*, 2013). Which N form is more readily available to plants depends on soil properties, such as grain size, pH, and ion exchange capacity, as well the soil microbiota (Stevenson *et al.*, 1999; Wardle, 1992).

A simplified schematic of NO_3^- reduction is shown in Figure 1.5. First, NO_3^- is reduced to nitrite (NO_2^-) by nitrate reductase (NR), using NADH; NO_2^- is then converted to NH_4^+ by nitrite reductase (NiR) (Foyer and Noctor 2006). NH_4^+ that has been converted from NO_3^- or taken up from the soil is then assimilated via the glutamine synthetase (GS)/glutamine 2-oxoglutarate aminotransferase (GOGAT) cycle into glutamine and glutamate (Krapp 2015). Levels of cytoplasmic NADH depend on photorespiration rates, since malate is exported during photorespiration from the chloroplast to the peroxisome, where it generates NADH (Rachmilevitch *et al.*, 2004). Increased cytoplasmatic NADH levels at low CO_2 could then enhance NO_3^- reduction, which contrasts with elevated CO_2 studies where decreased photorespiration rates have been shown to decrease NO_3^- reduction (Bloom *et al.*, 2010; 2012).

The dynamics of N uptake and assimilation from different N sources may be different between plants grown at past low CO_2 levels in comparison to modern plants, since photosynthetic rates decrease while photorespiration increases at low CO_2 growth conditions. First, meeting the energy requirements for N assimilation at low CO_2 becomes more challenging due to the limited carbohydrate supply (Pace *et al*., 1990; Sage and Coleman, 2001). Second, the photorespiratory process, which is enhanced at low CO_2, can provide an additional source of reductant availability for the reduction of NO_3^- (Busch *et al.*, 2018; Rachmilevitch *et al.*, 2004).

1.1.3 Stable Carbon and Nitrogen isotope compositions in plants

Isotopes are atoms with the same number of protons and electrons but different numbers of neutrons. They are denoted based on their atomic mass, for example, in ^{12}C the number 12 represents the sum of 6 neutrons and 6 protons. Stable isotopes are those that are energetically stable and do not decay, meaning they are not radioactive (Sharp, 2007). Since isotopic differences are exceedingly small, isotopic compositions (δ) are expressed in parts per thousand deviation from an accepted standard as:

$$\delta(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right)$$

where R is the ratio between the heavy to light isotope for the sample (R_{sample}) and the standard ($R_{standard}$) (Sharp, 2007).

The natural abundance of the heavier and rarer C and N stable isotopes (^{13}C and ^{15}N, respectively) relative to the more abundant isotopes ^{12}C and ^{14}N, defined as $\delta^{13}C$ and $\delta^{15}N$, are commonly used indicators with several applications in plant biology and paleoecology (Breecker, 2017; Farquhar *et al.*, 1989; Michener and Lajtha, 2007). The primary characterization of C isotope composition in plant tissues is based on the discrimination against ^{13}C that occurs during photosynthesis. Molecules of ^{13}C-containing CO_2 diffuse more slowly than $^{12}CO_2$ molecules, and the enzyme Rubisco further discriminates against ^{13}C during photosynthesis (Farquhar *et al.*, 1982, 1989). The resulting $\delta^{13}C$ of plant tissues is thus dependent on CO_2 supply through the stomata and enzymatic demand for CO_2 (Cernusak *et al.*, 2013; Farquhar *et al.*, 1982; Michener and Lajtha, 2007).

Some of the many applications of plant $\delta^{13}C$ include the use of leaf $\delta^{13}C$ to identify photosynthetic pathways, *i.e.*, C_3, C_4, or CAM (Farquhar *et al.*, 1989). In C_3 photosynthesis, the diffusion of CO_2 through the stomata has an apparent fractionation of about 4.4 ‰ and Rubisco discriminates against ^{13}C by about 29 ‰. The competition between these two processes produces an average $\delta^{13}C$ of about −27 ‰ for C_3 plants utilizing atmospheric CO_2 with $\delta^{13}C$ of about −8 ‰ (Sharp, 2007). C_4 photosynthesis, however, is initially catalyzed by a different enzyme, phosphoenolpyruvate (PEP)

carboxylase, which has a discrimination of approximately −6 ‰ during CO_2 fixation (Farquhar, 1983). PEP carboxylase then produces C_4 compounds that are catabolized in the bundle sheath cells, resulting in the release of CO_2, which accumulates around Rubisco, suppressing photorespiration and stimulating photosynthesis (Ehleringer and Pearcy, 1983). A small amount of ^{13}C-enriched CO_2 leaks from the bundle sheath, which slightly lowers the $\delta^{13}C$ of the CO_2 left for fixation. These processes, coupled with the initial diffusive fractionation of ~4.4 ‰ for atmospheric CO_2 entering the stomata, causes C_4 plants to have a more positive $\delta^{13}C$ than C_3 species: −13 ‰ on average (Berry, 1989). The Crassulacean acid metabolism (CAM) pathway relies on the same enzymes as the C_4 pathway, but there is a temporal segregation in the activity of the enzymes involved, as well as reduced CO_2 leakage, since cells are large and succulent, and thus the average $\delta^{13}C$ in CAM plants clusters around −11 ‰ (Farquhar, 1983). However, some species use the CAM pathway facultatively, switching between C_3 and CAM photosynthesis, resulting in the $\delta^{13}C$ of facultative CAM plants ranging between −11 ‰ and −27 ‰ (Farquhar, 1983).

The $\delta^{13}C$ of tree rings can also be used to derive environmental conditions during tree growth (Lipp *et al.*, 1991; McCarroll and Loader, 2004). For example, the $\delta^{13}C$ of the cellulose found in tree rings can be used as a record of shifts in atmospheric CO_2 concentrations, changes in temperature that affect C assimilation, and rates of respiration of the surrounding vegetation (Lipp *et al.*, 1991; Mazany *et al.*, 1980). A similar use to this indicator can be applied in paleo-reconstructions, and the $\delta^{13}C$ of fossil samples can be used, for example, to derive past atmospheric CO_2 concentrations (Cui and Schubert, 2016; Penuelas and Azcon-Bieto, 1992), identify plant functional types (Brooks *et al.*, 1997; Porter *et al.*, 2017), and characterize extreme weather events that changed plant community composition in the past (Hoetzel *et al.*, 2013; Keeley and Rundel, 2005). The carbon isotope composition of other plant tissues can also be used to identify photosynthetic pathways and environmental conditions. For instance, Hoetzel *et al.* (2013) used the $\delta^{13}C$ of plant waxes to reconstruct the expansion of C_4 grasses after intense fire activity that occurred during the Pliocene (between 8 and 3 million years ago).

The $\delta^{15}N$ of different plant tissues is highly variable (from –10 to +10 ‰; Evans, 2001). Such variation results from differences in the $\delta^{15}N$ of the N sources available to plants (*e.g.*, NH_3^+ *vs* NH_4^-), which depends on the N formation process (*e.g.*, mineralization, nitrification), and, within plant tissues, are a product of enzymatic discrimination against the heavier N isotope (Hogberg, 1997). Different N sources are taken up and assimilated in plants through diverse mechanisms whose isotopic discrimination is mostly regulated by external N availability (Evans, 2001; Robinson, 2001). Nitrate reductase, the enzyme responsible for nitrate assimilation in roots and leaves, shows a barely detectable discrimination against ^{15}N; however, as nitrate availability increases in the growing medium, isotopic discrimination can proportionally shift from 0 to about –4 ‰ (Hogberg, 1997). Isotopic discrimination during ammonium assimilation can occur during the active transport of ammonium into roots cells, and enzymatic discrimination by glutamine synthetase (Yoneyama *et al.*, 1991; Evans, 2001). Discrimination also depends on ammonium availability as, for instance, Yoneyama *et al.* (1991) found that $\delta^{15}N$ in *Oryza sativa* shifted from +4.1 to +12.6 ‰ when the ammonium concentration in the growth medium increased from 1.4 to 7.3 mM.

Plant $\delta^{15}N$ has also been a valuable tool for animal paleobiologic studies in the reconstruction of ancient food webs (Boecklen *et al.*, 2011). Primary producers provide food sources with distinct $\delta^{15}N$ profiles that will be reflected in the tissues of consumers (Casey and Post, 2011). The $\delta^{15}N$ of bulk collagen of consumers usually reflects the isotopic composition of the plants being consumed, with a ^{15}N enrichment of 2 to 5 ‰ per trophic level (Garten *et al.*, 2008); the average ^{15}N-enrichment is typically ~3.4 ‰ (Minagawa and Wada, 1984; Post, 2002). Much of our current understanding of N fractionation in plants, however, derives from the functioning of N dynamics in extant species grown under modern CO_2 concentrations (Krapp, 2015). Thus, verifying potential shifts in N discrimination when plants are exposed to low growth CO_2 conditions could help validate assumptions based on $\delta^{15}N$ from plant samples dating from low-CO_2 periods of geological time.

1.2 Plant-mycorrhizal interactions

Mycorrhizal associations are defined as the mutualistic symbiotic association between soil fungi and plant roots, functioning through the reciprocal exchange of mineral and organic resources (Johnson *et al.*, 1997). Mycorrhizal fungi are classified into four types according to their morphology and common choice of host plant: arbuscular, ericoid, orchid, and ectomycorrhizae (Brundrett and Tedersoo, 2018). Arbuscular mycorrhizae (Figure 1.6) is the most abundant group, occurring in more than 73 % of all known vascular plants to form mycorrhizal associations (Brundrett, 2009). Arbuscular mycorrhizae are also the most ancient type of mycorrhizal fungi, being found in fossils estimated to be more than 460 million years old (Redecker *et al.*, 2000). The arbuscular colonization starts after the contact between the fungal hyphae and the root epidermis where the fungus grows into the root cortex and develops highly branched hyphal structures called arbuscules (Gutjahr and Parniske, 2013). The arbuscules are then surrounded by a plant-derived periarbuscular membrane that forms an interface for nutrient exchange between the plant and the fungus (Lanfranco et al., 2018).

While the arbuscular mycorrhizal association has been established to be beneficial to both the fungi and plant host, there is considerable uncertainty regarding the extent to which its benefits are maintained under varying growth CO_2 concentrations (Becklin *et al.*, 2016; Tang *et al.*, 2009). For instance, in elevated CO_2 studies, there are examples where mycorrhizal colonization and plant growth are enhanced at elevated compared to ambient CO_2 growth conditions (Lovelock *et al.*, 1996; Rillig *et al.*, 1999), while in others, mycorrhizal colonization only benefits certain plant groups (Tang *et al.*, 2009), or no change in colonization is observed (Allen *et al.*, 2005; Clark *et al.*, 2009). Similarly, the only available study that investigated mycorrhizal association in low CO_2-grown plants (Becklin *et al.*, 2016) found that association with arbuscular mycorrhizae had opposite effects on the growth of two *Taraxacum* species.

Despite the scarcity of data on mycorrhizal associations at low CO_2 conditions, it is likely that the exchange of resources in this association at low CO_2 concentrations of the past differ from the plant-fungal interaction observed in current CO_2 conditions. This

mutualism has been considered evolutionarily stable due to its bidirectional control, where plants reward the best fungal partners with more carbohydrates, while the fungi increase nutrient transfer to roots that offer more carbohydrates (Kiers *et al.*, 2011). With reduced photosynthesis and carbohydrate production caused by limited CO_2 availability, plants grown at low CO_2 conditions would have a decreased capacity to allocate C compounds to the fungi. In turn, hyphal growth and nutrient uptake and transfer, especially nitrogen and phosphorus, from the mycorrhizae to the plant might be reduced.

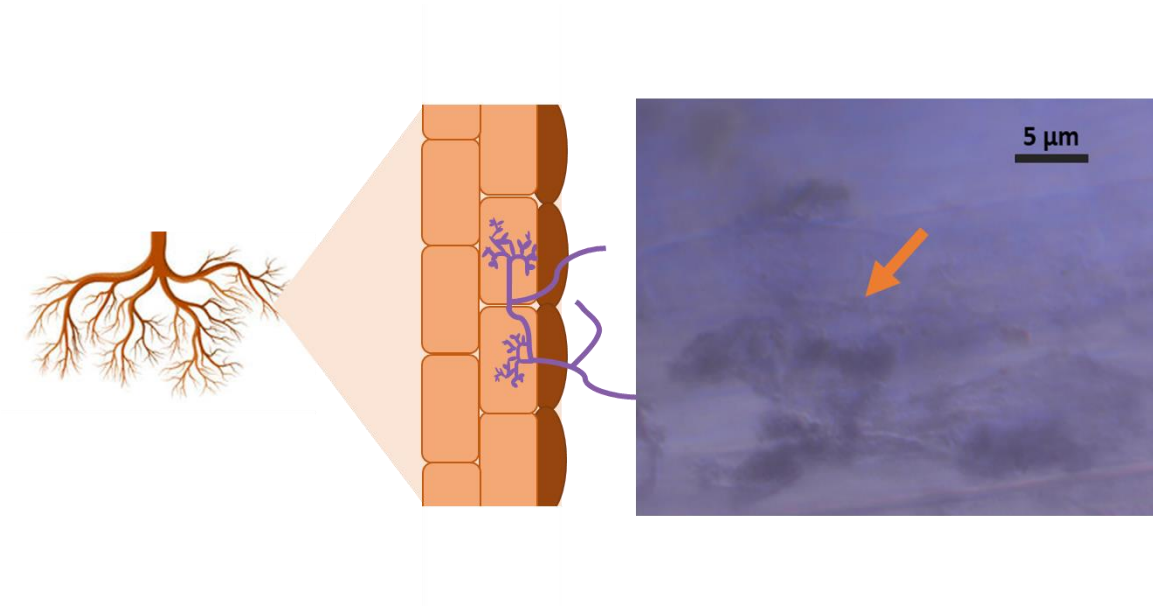

Figure 1.6. Root colonization by an arbuscular mycorrhizal fungus. Cartoon illustration of an arbuscular mycorrhizal fungi inside root cells showing the arbuscules (tree-like structures) connected to hyphae (purple lines). To the right, light microscopy showing arbuscules of *Glomus intraradices* indicated by the arrow.

As C and N dynamics between plants and fungi is influenced by changes in CO_2 availability (Alberton *et al.*, 2005; Treseder, 2004), there could be differences in the $\delta^{13}C$ and $\delta^{15}N$ between non-mycorrhizal and mycorrhizal plants growing at past low CO_2 conditions. In plants growing at ambient CO_2 conditions, arbuscular mycorrhizal association can reduce discrimination against ^{13}C by stimulating increased plant water-use efficiency (Querejeta *et al.*, 2006, 2003). Additionally, the organic N transferred from the fungus to the plant is usually depleted of ^{15}N due to enzymatic reactions within the fungi (Hobbie *et al.*, 2005). Thus, investigating plant C and N isotope compositions of mycorrhizal plants grown at past low CO_2 levels can offer new insights to interpreting isotope signals from ancient plant samples known to form mycorrhizal associations.

1.3 Effects of growth CO_2 on defense compound production

The production of defensive compounds is a major plant strategy against herbivory and parasitism (Karban and Baldwin, 2007). The production of such compounds, however, is usually highly costly to the C budget of plants (Zangerl and Bazzaz, 1992). Thus, plants must balance C investment between primary functions to sustain growth and the production of defense elements that enhance their fitness (Baldwin, 2010; Hartmann, 1996; Holopainen, 2004).

Defense compounds deter herbivory through two major mechanisms: anti-nutrition and toxicity (Chen, 2008). Anti-nutrition refers to limiting food access to herbivore, for example, by fortifying cell walls or attracting natural enemies of the attacking herbivore. Toxicity refers to the metabolites produced by plants that can disturb digestion or inhibit hormonal pathways in the herbivore (Chen, 2008). Many classes of metabolites are produced with defensive functions, including terpenes, alkenes, alcohols, esters, and other secondary compounds (Kesselmeier and Staudt, 1999; Peñuelas and Llusià, 2003). Terpenes, which are C-based compounds formed by isoprene units (C5), are the most

abundant group of volatile organic compound emitted by plants, and form the majority of the compounds present in tree resin (Holopainen, 2004; Langenheim, 1990; Pichersky and Raguso, 2018).

Resins are mixtures of terpenes and phenolic compounds produced in internal ducts or specialized glands of plants (Langenheim, 1990). Resin ducts form specialized secretory structures to store resins and contain their potential toxic effect to metabolic functions of the surrounding cells (Langenheim, 1994). In conifers, resin duct size and density can be used as indicators of resin production and overall host resistance against herbivory and pathogens (Boucher *et al.*, 2001; Martin *et al.*, 2002; Tomlin and Borden, 1997). Often, extant conifers display a fixed number and position of ducts in their needles, such that this anatomical feature is consistent within a taxonomic level (Wu and Hu, 1997), and is thus commonly used in the identification of fossil samples (Falcon-Lang *et al.*, 2016; Harland *et al.*, 2007; Manum *et al.*, 2000; Zidianakis *et al.*, 2016). However, if plants are exposed to conditions (such as low CO_2 concentrations) that limit terpene, and consequently resin production, it is unknown whether the formation of these structures would also be affected.

According to the carbon-nutrient balance hypothesis (Bryant *et al.*, 1983), allocation to defense compounds depends on C and nutrient availability and the relationship of the concentration of these elements to growth rate. This hypothesis predicts that C and N will be allocated to the production of secondary metabolites only after the main requirements for growth have been met (Hamilton *et al.*, 2001). Since this hypothesis was put forward, there has been much consideration given to possible effects of increased atmospheric CO_2 on plant defenses (Fajer *et al.*, 1992; Noctor and Mhamdi, 2017; Staudt *et al.*, 2001; Zavala *et al.*, 2012). Intuitively, conditions that favor the accumulation of non-structural carbohydrates, such as elevated CO_2, would be expected to increase defense compound concentrations (Mohan *et al.*, 2006; Novick *et al.*, 2012). In contrast, as low CO_2 concentrations constrain growth, allocation to secondary compounds may be suppressed. However, changes in the production of defense compounds induced by elevated CO_2 seem to be species-specific (Peñuelas and Estiarte, 1998; Robinson *et al.*, 2012; Zavala *et al.*, 2012), and increases in C allocation to such compounds have been shown to occur

only when water or nutrients are limiting (Lambers, 1993). So far, no studies have analyzed how low CO_2 impacts the allocation of resources to defense compounds; such studies could underpin a better understanding of plant-animal interactions in the recent evolutionary past when carbon was limiting.

1.4 A caveat in low-CO_2 studies: The possibility of plant adaptation since low-CO_2 periods

While on geological time scales low CO_2 periods seem not distant, it can be argued that since the last low CO_2 period enough time may has passed for modern genotypes to adapt to rising CO_2 conditions. Especially considering that evolutionary changes in certain plant traits may take place relatively fast: for instance, Franks et al. (2007) showed that the evolutionary changes in the flowering time in *Brassica rapa* happened over the span of seven years as adaptative shifts occurred due to changes in precipitation. Thus, since 20,000 years ago, at the end of the LGM when CO_2 was a as low as 180 ppm (Petit et al., 1999), plant species that were more physiologically plastic may have adapted traits to explore the increasing availability of atmospheric CO_2.

Furthermore, Gerhart and Ward (2010) suggested that since plants can readily alter biochemical and biophysical process (*i.e.* acclimate) to changes in growth CO_2 levels, the response of modern genotypes may not precisely represent past genotype responses to past CO_2 levels. Thus, the plant responses to growth at low CO_2 conditions discussed above may represent short-term physiological adjustments instead of the adaptive capability of plants to respond to low growth CO_2. In this context, it is important to distinguish acclimation as the short-term physiological, structural or biochemical adjustments induced by an environmental stimulus, and adaptation as the response of a species to an environmental stimulus that persists over multiple generations (Smith and Dukes, 2013). While whether plants have adapted to cope with rising CO_2 levels since the LGM is uncertain, modern plant genotypes represent in most cases the only subjects available for low CO_2 studies.

1.5 Research questions and hypothesis

There is still much to be investigated regarding how a low CO_2-world influenced plant physiological and biochemical processes, particularly since plant C and N cycling in past C-limited environments had implications for plant defense, mycorrhizal interactions and the baseline isotopic composition of ancient food webs. Based on this premise, I investigated the following research questions:

Chapter 2: Are growth and the N isotope composition of low CO_2-grown plants affected by different N-sources in a different way than plants grown at current CO_2 conditions? Considering that a low CO_2 environment may facilitate the assimilation of NO_3^- over NH_4^+, plants fertilized with NO_3^-, compared to NH_4^+-fertilized plants, may have higher growth and photosynthetic capacity due to higher N availability during its allocation to the photosynthetic apparatus. As well, by limiting soil N availability, the combined C and N limitation may cause low CO_2-grown plants to decrease discrimination against the heavier N isotope (^{15}N), resulting in a more positive N isotope composition.

Chapter 3: How does reduced C availability in low CO_2 conditions alter the exchange of resources between plants and fungi in a mycorrhizal association? I envisioned two possible contrasting scenarios: the mutualistic interaction may be enhanced, or both organisms might conserve resources and their exchange of C and N resources would decrease. In the first scenario, plants may more closely associate with mycorrhizae in order to stimulate the transfer of N from the fungi, and this extra N can then be invested in the photosynthetic apparatus, increasing photosynthetic rates and carbohydrate production that can be further transferred to the fungi. In the second scenario, plants may adopt a more conservative strategy that would reduce carbohydrate allocation to mycorrhizal fungi, thus restricting the mutualistic exchange in the association. Either scenario is likely to change the $\delta^{13}C$ and $\delta^{15}N$ of tissues of low CO_2- compared to ambient CO_2-grown plants. In the first, higher C assimilation would decrease discrimination against ^{13}C, while a higher N flow provided by the fungi would allow greater discrimination against ^{15}N. The opposite may be seen in the second scenario: the $\delta^{13}C$ of uninoculated control plants and mycorrhizal plants could be similar if there are no differences between them in C assimilation, but if C assimilation decreases in

mycorrhizal plants, plants may increase discrimination against ^{13}C. Additionally, plant roots may have to compete with the fungi for soil N in this second scenario, thus leading to reduced discrimination against ^{15}N.

Chapter 4: Is the ability to produce defense compounds reduced in low CO_2-grown plants compared to plants grown at modern CO_2 conditions? Due to reduced carbon availability, the ability of low CO_2-grown plants to photosynthetically fix carbon declines, and so does biomass. With limitations being imposed on primary functions to sustain growth, plants grown at low CO_2 could produce fewer secondary metabolites for use as defense compounds compared to plants grown at ambient CO_2 conditions.

References

Agüera, E., Ruano, D., Cabello, P., de la Haba, P., 2006. Impact of atmospheric CO_2 on growth, photosynthesis and nitrogen metabolism in cucumber (*Cucumis sativus* L.) plants. J. Plant Physiol. 163, 809–817. https://doi.org/10.1016/J.JPLPH.2005.08.010

Alberton, O., Kuyper, T.W., Gorissen, A., 2005. Taking mycocentrism seriously: Mycorrhizal fungal and plant responses to elevated CO_2. New Phytol. 167, 859–868. https://doi.org/10.1111/j.1469-8137.2005.01458.x

Allen, M.F., Klironomos, J.N., Treseder, K.K., Oechel, W.C., 2005. Responses of soil biota to elevated CO_2 in a chaparral ecosystem. Ecol. Appl. 15, 1701–1711. https://doi.org/10.1890/03-5425

Anderson, L.J., Maherali, H., Johnson, H.B., Polley, H.W., Jackson, R.B., 2001. Gas exchange and photosynthetic acclimation over subambient to elevated CO_2 in a C_3-C_4 grassland. Glob. Chang. Biol. 7, 693–707. https://doi.org/10.1046/j.1354-1013.2001.00438.x

Andres, R.J., Boden, T.A., Bréon, F.-M., Ciais, P., Davis, S., Erickson, D., Gregg, J.S., Jacobson, A., Marland, G., Miller, J., Oda, T., Olivier, J.G.J., Raupach, M.R., Rayner, P., Treanton, K., 2012. A synthesis of carbon dioxide emissions from fossil-fuel combustion. Biogeosciences 9, 1845–1871. https://doi.org/10.5194/bg-9-1845-2012

Andrews, M., Raven, J.A., Lea, P.J., 2013. Do plants need nitrate? The mechanisms by which nitrogen form affects plants. Ann. Appl. Biol. 163, 174–199. https://doi.org/10.1111/aab.12045

Andrews, T.J., 1978. Photorespiration - still unavoidable? FEBS Lett. 90, 1–9. https://doi.org/10.1016/0014-5793(78)80286-5

Augustin, L., Barbanate, C., Barnes, P.R.F., Barnola, J.M., Bigler, M., Castellano, E., Cattani, O., Chappellaz, J., Dahl-Jensen, D., Delmonte, B., et al., 2004. Eight glacial cycles from an Antarctic ice core EPICA community members. Nature 429, 623–628.

Baldwin, I.T., 2010. Plant volatiles. Curr. Biol. 20, R392–R397. https://doi.org/10.1016/j.cub.2010.02.052

Bazzaz, F.A., Bazzaz, F., 1996. Plants in changing environments: Linking physiological, population, and community eology. Cambridge University Press.

Becklin, K.M., Mullinix, G.W.R., Ward, J.K., 2016. Host plant physiology and mycorrhizal functioning shift across a glacial through future [CO_2] Gradient. Plant Physiol. 172, 789–801. https://doi.org/10.1104/pp.16.00837

Beerling, D.J., Chaloner, W.G., 1994. Atmospheric CO_2 changes since the last glacial maximum: evidence from the stomatal density record of fossil leaves. Rev. Palaeobot. Palynol. 81, 11–17. https://doi.org/10.1016/0034-6667(94)90123-6

Berry, J.A., 1989. Studies of mechanisms affecting the fractionation of carbon isotopes in photosynthesis, in: Stable Isotopes in Ecological Research. Springer, New York, NY, pp. 82–94. https://doi.org/10.1007/978-1-4612-3498-2_6

Bloom, A.J., Asensio, J.S.R., Randall, L., Rachmilevitch, S., Cousins, A.B., Carlisle, E.A., 2012. CO_2 enrichment inhibits shoot nitrate assimilation in C_3 but not C_4 plants and slows growth under nitrate in C_3 plants. Ecology 93, 355–367. https://doi.org/10.1890/11-0485.1

Bloom, A.J., Burger, M., Kimball, B., Jr, P.P., 2014. Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. Nat. Clim. Chang. 4, 477–180. https://doi.org/10.1038/NCLIMATE2183

Bloom, A J, Burger, M., Rubio Asensio, J.S., Cousins, A.B., 2010. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. Science 328, 899–903. https://doi.org/10.1126/science.1186440

Boecklen, W.J., Yarnes, C.T., Cook, B.A., James, A.C., 2011. On the use of stable isotopes in trophic ecology. Annu. Rev. Ecol. Evol. Syst. 42, 411–440. https://doi.org/10.1146/annurev-ecolsys-102209-144726

Boucher, D., Lavallée, R., Mauffette, Y., 2001. Biological performance of the white pine weevil in relation to the anatomy of the resin canal system of four different host species. Can. J. For. Res. 31, 2035–2041. https://doi.org/10.1139/cjfr-31-11-2035

Breecker, D.O., 2017. Atmospheric pCO_2 control on speleothem stable carbon isotope

compositions. Earth Planet. Sci. Lett. 458, 58–68. https://doi.org/10.1016/j.epsl.2016.10.042

Brooks, J.R., Flanagan, L.B., Buchmann, N., Ehleringer, J.R., 1997. Carbon isotope composition of boreal plants: functional grouping of life forms. Oecologia 110, 301–311. https://doi.org/10.1007/s004420050163

Brundrett, M.C., 2009. Mycorrhizal associations and other means of nutrition of vascular plants: Understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil. https://doi.org/10.1007/s11104-008-9877-9

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon / Nutrient balance of boreal plants in relation to vertebrate herbivory. OIKOS 40, 57–368.

Busch, F.A., Sage, R.F., 2017. The sensitivity of photosynthesis to O_2 and CO_2 concentration identifies strong Rubisco control above the thermal optimum. New Phytol. 213, 1036–1051. https://doi.org/10.1111/nph.14258

Busch, F.A., Sage, R.F., Farquhar, G.D., 2018. Plants increase CO_2 uptake by assimilating nitrogen via the photorespiratory pathway. Nat. Plants 4, 46–54. https://doi.org/10.1038/s41477-017-0065-x

Cairney, J.W.G., 2000. Evolution of mycorrhiza systems. Naturwissenschaften 87, 467–475. https://doi.org/10.1007/s001140050762

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of Earth's nitrogen cycle. Science 330, 192–196. https://doi.org/10.1126/science.1186120

Casey, M.M., Post, D.M., 2011. The problem of isotopic baseline: Reconstructing the diet and trophic position of fossil animals. Earth-Science Rev. 106, 131–148. https://doi.org/10.1016/J.EARSCIREV.2011.02.001

Cernusak, L.A., Ubierna, N., Winter, K., Holtum, J.A.M., Marshall, J.D., Farquhar, G.D., 2013. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. New Phytol. 200, 950–965. https://doi.org/10.1111/nph.12423

Chen, M.-S., 2008. Inducible direct plant defense against insect herbivores: A review. Insect Sci. 15, 101–114. https://doi.org/10.1111/j.1744-7917.2008.00190.x

Clark, N.M., Rillig, M.C., Nowak, R.S., 2009. Arbuscular mycorrhizal fungal abundance in the Mojave Desert: Seasonal dynamics and impacts of elevated CO_2. J. Arid Environ. 73, 834–843. https://doi.org/10.1016/J.JARIDENV.2009.03.004

Cowling, S. a, Sykes, M.T., 1999. Physiological significance of low atmospheric CO_2 for plant–climate interactions. Quat. Res. 52, 237–242. https://doi.org/10.1006/qres.1999.2065

Cowling, S. A., Sage, R.F., 1998. Interactive effects of low atmospheric CO_2 and elevated temperature on growth, photosynthesis and respiration in *Phaseolus vulgaris*. Plant, Cell Environ. 21, 427–435. https://doi.org/10.1046/j.1365-3040.1998.00290.x

Cui, Y., Schubert, B.A., 2016. Quantifying uncertainty of past pCO_2 determined from changes in C_3 plant carbon isotope fractionation. Geochim. Cosmochim. Acta 172, 127–138. https://doi.org/10.1016/J.GCA.2015.09.032

Cunniff, J., Jones, G., Charles, M., Osborne, C.P., 2017. Yield responses of wild C_3 and C_4 crop progenitors to subambient CO_2 : A test for the role of CO_2 limitation in the origin of agriculture. Glob. Chang. Biol. 23, 380–393. https://doi.org/10.1111/gcb.13473

Dutilleul, C., Lelarge, C., Prioul, J.-L., De Paepe, R., Foyer, C.H., Noctor, G., 2005. Mitochondria-driven changes in leaf NAD status exert a crucial influence on the control of nitrate assimilation and the integration of carbon and nitrogen metabolism. Plant Physiol. 139, 64–78. https://doi.org/10.1104/pp.105.066399

Ehleringer, J., Pearcy, R.W., 1983. Variation in quantum yield for CO_2 uptake among C_3 and C_4 plants. Plant Physiol. 73, 555–559. https://doi.org/https://doi.org/10.1104/pp.73.3.555

Ehleringer, J.R., Cerling, T.E., Dearing, M.D., 2005. A History of atmospheric CO_2 and its effects on plants, animals, and ecosystems, 1st ed. Springer, Salt Lake City, UT, USA.

Elliott-Kingston, C., Haworth, M., Yearsley, J.M., Batke, S.P., Lawson, T., McElwain, J.C., 2016. Does size matter? Atmospheric CO_2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO_2. Front. Plant Sci. 7, 1253. https://doi.org/10.3389/fpls.2016.01253

Evans, R.D., 2001. Physiological mechanisms influencing plant nitrogen isotope composition. Trends Plant Sci. 6, 121–126. https://doi.org/10.1016/S1360-1385(01)01889-1

Fajer, E.D., Bowers, M.D., Bazzaz, F.A., 1992. The effect of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. Am. Nat. 140, 707–723.

Falcon-Lang, H.J., Mages, V., Collinson, M., 2016. The oldest *Pinus* and its preservation by fire. Geology 44, 303–306. https://doi.org/10.1130/G37526.1

Falkowski, P.G., 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. Nature 387, 272–274. https://doi.org/10.1038/nature02381.1.

Farquhar, G., O'Leary, M., Berry, J., 1982. On the relationship between carbon isotope

discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9, 121. https://doi.org/10.1071/PP9820121

Farquhar, G.D., 1983. On the nature of carbon isotope discrimination in C_4 species. Aust. J. Plant Physiol. 10, 205–231. https://doi.org/10.1071/PP9830205

Farquhar, G.D., Ehleringer, J.R., Hubick, K.T., 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. 503–537. https://doi.org/1040-2519/89/0601-503

Farquhar, G.D., Sharkey, T.D., 2003. Stomatal conductance and photosynthesis. Annu. Rev. Plant Physiol. 33, 317–345. https://doi.org/10.1146/annurev.pp.33.060182.001533

Farquhar, G.D., von Caemmerer, S., 1982. Modelling of photosynthetic response to environmental conditions, in: Physiological Plant Ecology II. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 549–587. https://doi.org/10.1007/978-3-642-68150-9_17

Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149, 78–90. https://doi.org/10.1007/BF00386231

Foyer, C.H., Noctor, G., 2006. Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism, Vol. 12. ed. Springer Science & Business Media.

Franks, P.J., Leitch, I.J., Ruszala, E.M., Hetherington, A.M., Beerling, D.J., 2012. Physiological framework for adaptation of stomata to CO_2 from glacial to future concentrations. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367, 537–546. https://doi.org/10.1098/rstb.2011.0270

Franks, S.J., Sim, S., Weis, A.E. 2007. Rapid evolution of flowering time by an annual plant in response to a limate fluctuation. Proc. Nat. Acad. Sci. 104, 1278–1282. https:// doi/10.1073/pnas.06083791040

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R., Vorosmarty, C.J., 2004. Nitrogen cycles: Past, present, and future. Biogeochemistry 70, 226–2004. https://doi.org/10.1007/s10533-004-0370-0

Garten, C.T., Hanson, P.J., Todd, D.E., Lu, B.B., Brice, D.J., 2008. Natural ^{15}N- and ^{13}C-abundance as indicators of forest nitrogen status and soil carbon dynamics, Stable Isotopes in Ecology and Environmental Science: Second Edition. https://doi.org/10.1002/9780470691854.ch3

Gerhart, L.M., Ward, J.K., 2010. Plant responses to low [CO_2] of the past. New Phytol.

188, 674–695. https://doi.org/10.1111/j.1469-8137.2010.03441.x

Gonzàlez-Meler, M.A., Blanc-Betes, E., Flower, C.E., Ward, J.K., Gomez-Casanovas, N., 2009. Plastic and adaptive responses of plant respiration to changes in atmospheric CO_2 concentration. Physiol. Plant. 137, 473–484. https://doi.org/10.1111/j.1399-3054.2009.01262.x

Gutjahr, C., Parniske, M., 2013. Cell and developmental biology of arbuscular mycorrhiza symbiosis. Annu. Rev. Cell Dev. Biol. 29, 593–617. https://doi.org/10.1146/annurev-cellbio-101512-122413

Hamilton, J.G., Zangerl, A.R., DeLucia, E.H., Berenbaum, M.R., 2001. The carbon-nutrient balance hypothesis: Its rise and fall. Ecol. Lett. 4, 86–95. https://doi.org/10.1046/j.1461-0248.2001.00192.x

Harland, M., Francis, J.E., Brentnall, S.J., Beerling, D.J., 2007. Cretaceous (*Albian-Aptian*) conifer wood from Northern Hemisphere high latitudes: Forest composition and palaeoclimate. Rev. Paleobotany Play. 143, 167–196. https://doi.org/10.1016/j.revpalbo.2006.07.005

Hartmann, T., 1996. Diversity and variability of plant secondary metabolism: a mechanistic view, in: Proceedings of the 9th International Symposium on Insect-Plant Relationships. Springer Netherlands, Dordrecht, pp. 177–188. https://doi.org/10.1007/978-94-009-1720-0_42

Hobbie, E.A., Ari, A.E., Ae, J., Trappe, J., 2005. Foliar and fungal ^{15}N:^{14}N ratios reflect development of mycorrhizae and nitrogen supply during primary succession: testing analytical models. Oecologia 146, 258–268. https://doi.org/10.1007/s00442-005-0208-z

Hoetzel, S., Dupont, L., Schefuß, E., Rommerskirchen, F., Wefer, G., 2013. The role of fire in Miocene to Pliocene C_4 grassland and ecosystem evolution. Nat. Geosci. 6, 1027–1030. https://doi.org/10.1038/ngeo1984

Hofmann, D.J., Butler, J.H., Dlugokencky, E.J., Elkins, J.W., Masarie, K., Montzka, S.A., Tans, P., 2006. The role of carbon dioxide in climate forcing from 1979 to 2004: introduction of the Annual Greenhouse Gas Index. Tellus 58B, 614–619. https://doi.org/10.1111/j.1600-0889.2006.00201.x

Hogberg, P., 1997. ^{15}N natural abundance in soil-plant systems. New Phytol. 137, 179–203. https://doi.org/10.1046/j.1469-8137.1997.00808.x

Holopainen, J.K., 2004. Multiple functions of inducible plant volatiles. Trends Plant Sci. 9, 529–533. https://doi.org/10.1016/J.TPLANTS.2004.09.006

Holopainen, J.K., Himanen, S.J., Yuan, J.S., Chen, F., Stewart, C.N., 2013. Ecological functions of terpenoids in changing climates, in: Natural Products: Phytochemistry, Botany, and Metabolism of Alkaloids, Phenolics and Terpenes. pp. 2913–2940.

https://doi.org/10.1007/978-3-642-22144-6_129

Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytolist 135, 575–585.

Karban, R., Baldwin, I.T., 2007. Induced responses to herbivory. University of Chicago Press.

Keeley, J.E., Rundel, P.W., 2005. Fire and the Miocene expansion of C_4 grasslands. Ecol. Lett. 8, 683–690. https://doi.org/10.1111/j.1461-0248.2005.00767.x

Keeling, C.D., 1979. The Suess Effect: ^{13}Carbon-^{14}Carbon Interrelations. Environ. Int. 2, 229–300. https://doi.org/10.1016/0160-4120(79)90005-9

Keeling, C.D., 1973. Industrial production of carbon dioxide from fossil fuels and limestone. https://doi.org/10.3402/tellusa.v25i2.9652

Kesselmeier, J., Staudt, M., 1999. Biogenic Volatile Organic Compounds (VOC): An overview on emission, physiology and ecology. J. Atmos. Chem. 33, 23–88.

Kiers, E.T., Duhamel, M., Beesetty, Y., Mensah, J.A., Franken, O., Verbruggen, E., Fellbaum, C.R., Kowalchuk, G.A., Hart, M.M., Bago, A., Palmer, M., West, S.A., Vandenkoornhuyse, P., Jansa, J., Bücking, H., 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. Science 333, 880–882. https://doi.org/10.1126/science.1208473

Krapp, A., 2015. Plant nitrogen assimilation and its regulation: a complex puzzle with missing pieces. Plant Biol. 25, 115–122. https://doi.org/doi.org/10.1016/j.pbi.2015.05.010

Lambers, H., 1993. Rising CO_2, secondary plant metabolism, plant-herbivore interactions and litter decomposition theoretical considerations. Vegetatio 104/105, 263–271. https://doi.org/10.1007/BF00048157

Lanfranco, L., Fiorilli, V., Gutjahr, C., 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. New Phytol. 220, 1031–1046. https://doi.org/10.1111/nph.15230

Langenheim, J.H., 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. J. Chem. Ecol. 20, 1223–1280.

Langenheim, J.H., 1990. Plant Resins. Am. Sci. 78, 16–24.

Li, Y., Xu, J., Haq, N.U., Zhang, H., Zhu, X.-G., 2014. Was low CO_2 a driving force of C_4 evolution: *Arabidopsis* responses to long-term low CO_2 stress. J. Exp. Bot. 65, 3657–3667. https://doi.org/10.1093/jxb/eru193

Lipp, J., Trimborn, P., Fritz, P., Moser, H., Becker, B., Frenzel, B., 1991. Stable isotopes

in tree ring cellulose and climatic change. Tellus B 43, 322–330. https://doi.org/10.1034/j.1600-0889.1991.t01-2-00005.x

Lipson, D., Nasholm, T., 2001. The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. Oecologia 128, 305–316. https://doi.org/10.1007/s004420100693

Lovelock, C.E., Kyllo, D., Winter, K., 1996. Growth responses to vesicular-arbuscular mycorrhizae and elevated CO_2 in seedlings of a tropical tree, *Beilschmiedia pendula*. Funct. Ecol. 10, 662–667.

Lüthi, D., Le Floch, M., Bereiter, B., Blunier, T., Barnola, J.-M., Siegenthaler, U., Raynaud, D., Jouzel, J., Fischer, H., Kawamura, K., Stocker, T.F., 2008. High-resolution carbon dioxide concentration record 650,000–800,000 years before present. Nature 453, 379–382. https://doi.org/10.1038/nature06949

Manum, S.B., Van Konijnenburg-Van Cittert, J.H.A., Wilde, V., 2000. *Tritaenia Maegdefrau et* Rudolf, Mesozoic '*Sciadopitys*-like' leaves in mass accumulations. Rev. Palaeobot. Palynol. 109, 255–269. https://doi.org/10.1016/S0034-6667(99)00058-5

Martin, D., Tholl, D., Gershenzon, J., Bohlmann, J., 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol. 129, 1003–1018. https://doi.org/10.1104/pp.011001

Maurino, V.G., Peterhansel, C., 2010. Photorespiration: Current status and approaches for metabolic engineering. Curr. Opin. Plant Biol. 13, 248–255. https://doi.org/10.1016/J.PBI.2010.01.006

Mazany, T., Lerman, J.C., Long, A., 1980. Carbon-13 in tree-ring cellulose as an indicator of past climates. Nature 287, 432–435. https://doi.org/10.1038/287432a0

McCarroll, D., Loader, N.J., 2004. Stable isotopes in tree rings. Quat. Sci. Rev. 23, 771–801. https://doi.org/10.1016/j.quascirev.2003.06.017

McIntyre, S., Díaz, S., Lavorel, S., Cramer, W., 1999. Plant functional types and disturbance dynamics – Introduction. J. Veg. Sci. 10, 603–608. https://doi.org/10.1111/j.1654-1103.1999.tb00103.x

Mengel, K., Kirkby, E.A., Kosegarten, H., Appel, T., 2001. Nitrogen, in: Principles of Plant Nutrition. Springer Netherlands, Dordrecht, pp. 397–434. https://doi.org/10.1007/978-94-010-1009-2_7

Michener, R., Lajtha, K., 2007. Stable isotopes in ecology and environmental science, Second Edi. ed. Blackwell Publishing.

Millar, A.H., Whelan, J., Soole, K.L., Day, D.A., 2011. Organization and regulation of

mitochondrial respiration in plants. Annu. Rev. Plant Biol. 62, 79–104. https://doi.org/10.1146/annurev-arplant-042110-103857

Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta 48, 1135–1140. https://doi.org/10.1016/0016-7037(84)90204-7

Mohan, J.E., Cowden, C.C., Baas, P., Dawadi, A., Frankson, P.T., Helmick, K., Hughes, E., Khan, S., Lang, A., Machmuller, M., Taylor, M., Witt, C.A., 2014. Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecol. 10, 3–19. https://doi.org/10.1016/j.funeco.2014.01.005

Mohan, J.E., Ziska, L.H., Schlesinger, W.H., Thomas, R.B., Sicher, R.C., George, K., Clark, J.S., 2006. Biomass and toxicity responses of poison ivy (*Toxicodendron radicans*) to elevated atmospheric CO_2. PNAS 103, 9086–9089. https://doi.org/10.1890/06-1609.1

Monnin, E., Indermühle, A., Dällenbach, A., Flückiger, J., Stauffer, B., Stocker, T.F., Raynaud, D., Barnola, J.M., 2001. Atmospheric CO_2 concentrations over the last glacial termination. Science 291, 112–4. https://doi.org/10.1126/science.291.5501.112

Nacry, P., Bouguyon, E., Gojon, A., 2013. Nitrogen acquisition by roots: Physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370, 1–29. https://doi.org/10.1007/s11104-013-1645-9

Noctor, G., Mhamdi, A., 2017. Climate change, CO_2, and defense: The metabolic, redox, and signaling perspectives. Trends Plant Sci. 22, 857–870. https://doi.org/10.1016/j.tplants.2017.07.007

Novick, K.A., Katul, G.G., McCarthy, H.R., Oren, R., 2012. Increased resin flow in mature pine trees growing under elevated CO_2 and moderate soil fertility. Tree Physiol. 32, 752–763. https://doi.org/10.1093/treephys/tpr133

Nunes-Nesi, A., Fernie, A.R., Stitt, M., 2010. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol. Plant 3, 973–996. https://doi.org/10.1093/mp/ssq049

Pace, G M, Volk, R.J., Jackson, W. a, 1990. Nitrate reduction in response to CO_2-limited photosynthesis : Relationship to carbohydrate supply and nitrate reductase activity in maize seedlings. Plant Physiol. 92, 286–92. https://doi.org/10.1104/pp.92.2.286

Peñuelas, J., Azcon-Bieto, J., 1992. Changes in leaf $\delta^{13}C$ of herbarium plant species during the last 3 centuries of CO_2 increase. Plant, Cell Environ. 15, 485–489. https://doi.org/10.1111/j.1365-3040.1992.tb01000.x

Peñuelas, J., Estiarte, M., 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? Tree 13, 20–24. https://doi.org/10.1016/S0169-5347(97)01235-

4

Peñuelas, J., Llusià, J., 2003. BVOCs: Plant defense against climate warming? Trends Plant Sci. 8, 105–109. https://doi.org/10.1016/S1360-1385(03)00008-6

Peterhansel, C., Maurino, V.G., 2011. Photorespiration redesigned. Plant Physiol. 155, 49–55. https://doi.org/10.1104/pp.110.165019

Petit, R.J., Raynaud, D., Basile, I., Chappellaz, J., Ritz, C., Delmotte, M., Legrand, M., Lorius, C., Pe, L., 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399, 429–413. https://doi.org/10.1038/20859

Pichersky, E., Raguso, R.A., 2018. Why do plants produce so many terpenoid compounds? New Phytol. 220, 692–702. https://doi.org/10.1111/nph.14178

Plaxton, W.C., Podestá, F.E., 2006. The functional organization and control of plant respiration. CRC. Crit. Rev. Plant Sci. 25, 159–198. https://doi.org/10.1080/07352680600563876

Polley, H.W., Johnson, H.B., Marino, B.D., Mayeux, H.S., 1993. Increase in C_3 plant water-use efficiency and biomass over glacial to present CO_2 concentrations. Nature 361, 61–64. https://doi.org/10.1038/361061a0

Porter, A.S., Yiotis, C., Montañ Ez, I.P., Mcelwain, J.C., 2017. Evolutionary differences in $\Delta^{13}C$ detected between spore and seed bearing plants following exposure to a range of atmospheric O_2 :CO_2 ratios; implications for paleoatmosphere reconstruction. Geochim. Cosmochim. Acta 213, 517–533. https://doi.org/10.1016/j.gca.2017.07.007

Post, D.M., 2002. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology 83, 703–718. https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2

Querejeta, J.I., Allen, M.F., Caravaca, F., Roldán, A., 2006. Differential modulation of host plant $\delta^{13}C$ and $\delta^{18}O$ by native and nonnative arbuscular mycorrhizal fungi in a semiarid environment. New Phytol. 169, 379–387. https://doi.org/10.1111/j.1469-8137.2005.01599.x

Querejeta, J.I., Barea, M.J., Allen, M.F., Caravaca, F., Roldµn, A., 2003. Differential response of $\delta^{13}C$ and water use efficiency to arbuscular mycorrhizal infection in two aridland woody plant species. Oecologia 135, 510–515. https://doi.org/10.1007/s00442-003-1209-4

Rabinowitch, E.I., 1951. Photosynthesis and related processes, Vol. 72 No. ed. LWW.

Rachmilevitch, S., Cousins, A.B., Bloom, A.J., 2004. Nitrate assimilation in plant shoots depends on photorespiration. Proc. Natl. Acad. Sci. 101, 11506–11510

Raupach, M.R., Marland, G., Ciais, P., Le Qué Ré, C., Canadell, J.G., Klepper, G., Field, C.B., 2007. Global and regional drivers of accelerating CO_2 emissions. Proc. Natl. Acad. Sci. 104, 10288–10293

Redecker, D., Kodner, R., Graham, L.E., 2000. Glomalean fungi from the Ordovician. Science 289, 1920–1. https://doi.org/10.1126/science.289.5486.1920

Rillig, M.C., Field, C.B., Allen, M.F., 1999. Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands. Oecologia 119, 572–577

Ripley, B.S., Cunniff, J., Osborne, C.P., 2013. Photosynthetic acclimation and resource use by the C_3 and C_4 subspecies of *Alloteropsis semialata* in low CO_2 atmospheres. Glob. Chang. Biol. 19, 900–910. https://doi.org/10.1111/gcb.12091

Robertson, G.P., Vitousek, P.M., 2009. Nitrogen in agriculture: Balancing the cost of an essential resource. Annu. Rev. Environ. Resour. 34, 97–125. https://doi.org/10.1146/annurev.environ.032108.105046

Robinson, D., 2001. δ^{15}N as an integrator of the nitrogen. Trends Ecol. Evol. 16, 153–162. https://doi.org/10.1016/s0169-5347(00)02098-x

Robinson, E.A., Ryan, G.D., Newman, J.A., 2012. A meta-analytical review of the effects of elevated CO_2 on plant-arthropod interactions highlights the importance of interacting environmental and biological variables. New Phytol. 194, 321–336. https://doi.org/10.1111/j.1469-8137.2012.04074.x

Romero-Munar, A., Fernández Del-Saz, N., Ribas-Carbó, M., Flexas, J., Baraza, E., Florez-Sarasa, I., Fernie, A.R., Gulías, J., 2017. Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation. Plant. Cell Environ. https://doi.org/10.1111/pce.12902

Sage, R.F., 2004. The evolution of C_4 photosynthesis. New Phytol. 161, 341–370. https://doi.org/10.1111/j.1469-8137.2004.00974.x

Sage, R.F., 1995. Was low atmospheric CO_2 during the Pleistocene a limiting factor for the origin of agriculture? Glob. Chang. Biol. 1, 93–106. https://doi.org/10.1111/j.1365-2486.1995.tb00009.x

Sage, R.F., Coleman, J.R., 2001. Effects of low atmospheric CO_2 on plants: More than a thing of the past. Trends Plant Sci. 6, 18–24. https://doi.org/10.1016/S1360-1385(00)01813-6

Sage, Rowan F, Cowling, S.A., 1999. Implications of stress in low CO_2 atmospheres of the past: Are today's plants too conservative for a high CO_2 world?, in: Luo, Y., Mooney, H.A. (Eds.), Carbon Dioxide and Environmental Stress. Academic Press, San Diego, CA, USA, pp. 289–304.

Sage, R.F., Kubien, D.S., 2007. The temperature response of C_3 and C_4 photosynthesis. Plant. Cell Environ. 30, 1086–1106. https://doi.org/10.1111/j.1365-

Sharkey, T.D., 1985. Photosynthesis in Intact Leaves of C_3 Plants: Physics, Physiology and Rate Limitations. Bot. Rev. 51, 53–105.

Sharp, Z., 2007. Principles of stable isotope geochemistry. Pearson/Prentice Hall, Upper Saddle River, NJ.

Smith, S.E., Read, D., 2008. Mycorrhizal Symbiosis, in: Mycorrhizal Symbiosis Third Edition Introduction. Academic Press, Amsterdam, pp. 1–9

Smith, N., Dukes, J., 2013. Plant respiration and photosynthesis in global-scale models: Incorporating acclimation to temperature and CO_2. Global Ch. Bio. 19, 45-63. https://doi.org/10.1111/j.1365-2486.2012.02797.x

Spreitzer, R.J., Salvucci, M.E., 2002. Rubisco: Structure, regulatory interactions, and possibilities for a better enzyme. Annu. Rev. Plant Biol 53, 449–75. https://doi.org/10.1146/annurev.arplant.53.100301.135233

Staudt, M., Joffre, R., Rambal, S., Kesselmir, J., 2001. Effect of elevated CO_2 on monoterpene emission of young *Quercus* trees and its relation to structural and ecophysiological parameters. Tree Physiol. 21, 437–445.

Stevenson, F.J., Stevenson, E.J., Cole, M.A., 1999. Cycles of soils: Carbon, nitrogen, phosphorus, sulfur, micronutrients. John Wiley & Sons, Ltd.

Stick, R. V, 2001. Carbohydrates: The sweet molecules of life. Elsevier.

Taiz, L., Zeiger, E., 2010. Plant Physiology, Fifth edti. ed. Sinauer Associates Inc.

Tang, J., Xu, L., Chen, X., Hu, S., 2009. Interaction between C_4 barnyard grass and C_3 upland rice under elevated CO_2: Impact of mycorrhizae. Acta Oecologica 35, 227–235. https://doi.org/10.1016/J.ACTAO.2008.10.005

Tegeder, M., Masclaux-Daubresse, C., 2017. Source and sink mechanisms of nitrogen transport and use. New Phytol. https://doi.org/10.1111/nph.14876

Temme, A.A., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2013. Meta-analysis reveals profound responses of plant traits to glacial CO_2 levels. Ecol. Evol. 3, 4525–4535. https://doi.org/10.1002/ece3.836

Temme, A.A., Liu, J.C., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2015. Winners always win: Growth of a wide range of plant species from low to future high CO_2. Ecol. Evol. 5, 4949–4961. https://doi.org/10.1002/ece3.1687

Tissue, D., Griffin, K., Thomas, R., Strain, B., 1995. Effects of low and elevated CO_2 on C_3 and C_4 annuals -II. Photosynthesis and leaf biochemistry. Oecologia 101, 21–28.

Tomlin, E.S., Borden, A.H., 1997. Thin bark and high density of outer resin ducts: interrelated resistance traits in Sitka spruce against the White Pine Weevil (Coleoptera: *Curculionidae*). J. Econ. Entomol. 90, 235–239.

Treseder, K.K., 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO_2 in field studies. New Phytol. 164, 347–355. https://doi.org/10.1111/j.1469-8137.2004.01159.x

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S., Sharma, H.C., 2012. Mechanisms of plant defense against insect herbivores. Plant Signal. Behav. 7, 1306–20. https://doi.org/10.4161/psb.21663

Ward, J.K., Antonovics, J., Thomas, R.B., Strain, B.R., 2000. Is atmospheric CO_2 a selective agent on model C_3 annuals? Oecologia 123, 330–341. https://doi.org/10.1007/s004420051019

Ward, J.K., Strain, B.R., 1997. Effects of low and elevated CO_2 partial pressure on growth and reproduction of Arabidopsis thaliana from different elevations. Plant, Cell Environ. 20, 254–260. https://doi.org/10.1046/j.1365-3040.1997.d01-59.x

Wardle, D.A., 1992. A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. Biol. Rev. 67, 321–358. https://doi.org/10.1111/j.1469-185X.1992.tb00728.x

Wu, H., Hu, Z.-H., 1997. Comparative anatomy of resin ducts of the *Pinaceae*. Trees 11, 135–1143.

Xu, G., Fan, X., Miller, A.J., 2012. Plant nitrogen assimilation and use efficiency. Annu. Rev. Plant Biol 63, 153–82. https://doi.org/10.1146/annurev-arplant-042811-105532

Yoneyama, T., Omata, T., Nakata, S., Yazaki, J., 1991. Fractionation of nitrogen isotopes during the uptake and assimilation of ammonia by plants. Plant Cell Physiol. 32, 1211–1217.

Zangerl, A.R., Bazzaz, F.A., 1992. Theory and pattern in plant defense allocation, in: Plant Resistance to Herbivores and Pathogens. University of Chicago Press, Chicago, pp. 363–391.

Zavala, J. a, Nabity, P.D., Delucia, E.H., 2012. An emerging understanding of mechanisms governing insect herbivory under elevated CO_2. Annu. Rev. Entomol. 79–97. https://doi.org/10.1146/annurev-ento-120811-153544

Zelitch, I., Schultes, N.P., Peterson, R.B., Brown, P., Brutnell, T.P., 2009. High glycolate oxidase activity is required for survival of maize in normal air. Plant Physiol. 149, 195–204. https://doi.org/10.1104/pp.108.128439

Zidianakis, G., Iliopoulos, G., Zelilidis, A., Kovar-Eder, J., 2016. Pinus remains from the Pitsidia plant assemblage document coastal pine forests in southern Crete during the

late Miocene. Rev. Palaeobot. Palynol. 235, 11–30. https://doi.org/10.1016/j.revpalbo.2016.09.003

Chapter 2

2 Nitrogen fertilization influences low CO_2 effects on plant performance

2.1 Abstract

Low atmospheric CO_2 conditions prevailed for most of the recent evolutionary history of plants. While low CO_2 concentrations reduce plant growth compared to modern levels, low-CO_2 effects on plant performance may also be affected by nitrogen availability, since low leaf nitrogen decreases photosynthesis while CO_2 concentrations influence nitrogen assimilation. To investigate the influence of N availability on plant performance at low CO_2, I grew *Elymus canadensis* at ambient (~400 ppm) and sub-ambient (~180 ppm) CO_2 levels, under four N-treatments: *nitrate*-only; *ammonium*-only; a *full* and a *half* mix of nitrate and ammonium. Growth at low CO_2 decreased biomass in the *full* and *nitrate* treatments, but not in *ammonium* and *half* plants. Low CO_2 effects on photosynthetic and maximum electron transport rates were influenced by fertilization, with photosynthesis being most strongly impacted by low CO_2 in *full* plants. Low CO_2 reduced stomatal index in *half* plants, suggesting that the use of this indicator in paleo-inferences can be influenced by N availability. Under low CO_2 concentrations, *nitrate* plants discriminated more against ^{15}N while *half* plants discriminated less against ^{15}N compared to the *full* treatment, suggesting that N availability should be considered when using N isotopes as paleo-indicators.

2.2 Introduction

For most of their recent evolutionary history, plants have experienced atmospheric CO_2 concentrations far below the levels observed today. By the end of the Last Glacial

Maximum (~20 kyBP), CO_2 concentrations were as low as 180 ppm (Petit *et al.* 1999). The low CO_2 concentrations that prevailed for the last million years could have implications for modern plant physiology and ecosystem function, as plant traits that evolved in a low CO_2 world may influence plant responses to current, rapidly increasing CO_2 levels (Becklin *et al.* 2017; Gerhart and Ward 2010; Franks *et al.* 2013).

Growth at low CO_2 affects plant physiology and performance in numerous ways (Cowling and Sage, 1998; Gerhart and Ward, 2010; Sage and Cowling, 1999; Temme *et al*., 2015). Growth at low CO_2 greatly decreases net CO_2 assimilation rates (A_{net}) (Polley *et al.*, 1993; Sage, 1995; Tissue *et al.*, 1995) and biomass (Sage and Coleman, 2001; Temme *et al.*, 2015), and delays flowering time (Ward and Strain, 1997; Ward *et al.*, 2000). A meta-analysis by Temme *et al.* (2013) found that a 50 % reduction in CO_2 decreased A_{net} 38%, biomass 47 %, and water use efficiency 48 %. At low CO_2, A_{net} is reduced as Rubisco carboxylation rates are limited by substrate availability, while photorespiration rates increase, releasing previously fixed CO_2 (Ehleringer *et al.*, 2005). There is little evidence that photosynthetic capacity (*i.e.*, maximum Rubisco carboxylation rates, V_{cmax}, and maximum electron transport rates, J_{max}) is up-regulated under low CO_2 to enhance A_{net}, although few studies have investigated low CO_2 effects on these variables (but see Cunniff *et al*., 2017; Ripley *et al.* 2013; Tissue *et al.* 1995). Instead, leaves developed at low CO_2 commonly have greater stomatal size, aperture and/or density (Elliott-Kingston *et al.*, 2016; Franks *et al.*, 2012; Temme *et al.*, 2013), which enhance CO_2 uptake. Despite these stomatal adjustments, reduced A_{net} at low CO_2 concentrations decrease plant productivity (Ehleringer *et al.* 2005; Temme *et al.* 2013; Cunniff *et al.* 2017).

Reductions in A_{net} and enhanced photorespiration in plants grown at low CO_2 decrease carbohydrate production and storage (Cowling and Sykes 1999), which can reduce the capacity to assimilate nutrients such as nitrogen (Sage and Coleman 2001). Nitrogen has been a limitation on terrestrial productivity on geological timescales (Canfield *et al.* 2010; Falkowski 1997), and is currently the most limiting nutrient for plant productivity in many terrestrial ecosystems. Nitrogen assimilation and C metabolism in plants are linked, since C-skeletons and reductants necessary for N transport and conversion depend

on photosynthetically-fixed C, and are therefore influenced by photosynthetic and photorespiratory rates (Bloom *et al.*, 2012, 2014; Busch *et al.*, 2018). For example, N assimilation can be almost completed halted under CO_2 deprivation (Pace *et al.*, 1990).

Soil inorganic nitrogen is commonly available to plants as NO_3^- or NH_4^+ depending on soil properties, such as soil type, pH, and ion exchange capacity (Stevenson *et al.*, 1999; Wardle, 1992). In plants, NO_3^- is reduced to nitrite (NO_2^-) by nitrate reductase using NADH, then converted to NH_4^+ and finally to glutamine and glutamate (Foyer and Noctor, 2006). Levels of cytoplasmic NADH depend on photorespiration rates, since malate is exported during photorespiration from the chloroplast to the peroxisome, where it generates NADH (Rachmilevitch *et al.*, 2004). Photorespiration, which increases under low CO_2 conditions, can thereby enhance NO_3^- assimilation (Bloom *et al.*, 2010, 2014), while decreased photorespiration rates at elevated CO_2 reduce NO_3^- assimilation by decreasing reductant availability (Bloom *et al.*, 2014; Busch *et al.*, 2018). Hence, compared to current CO_2 levels, plants may perform proportionally better when supplied with NO_3^- than NH_4^+ in a low CO_2 environment.

Plants are usually adapted to preferentially take up either NO_3^- or NH_4^+ (Alt *et al*,. 2017; Dier *et al.*, 2017), reflecting the N form most available in their growth environment, although this preference can be altered by changes in growth CO_2 (BassiriRad *et al.* 1997). While changes in atmospheric CO_2 concentrations may not directly alter soil properties, soil-plant dynamics are likely to be affected by changes in CO_2 levels through effects on leaf carbon fluxes (namely photosynthesis, respiration, and photorespiration). Thus, past low CO_2 concentrations may have altered plant N dynamics, and consequently N cycling in ecosystems, in ways that are presently unknown.

One commonly used tool to investigate nitrogen cycling in plants and ecosystems is the analysis of the natural abundance of ^{15}N relative to ^{14}N ($\delta^{15}N$) (Robinson, 2001). Different N sources are taken up and assimilated through diverse mechanisms whose isotopic discrimination is mostly regulated by external N availability (Evans, 2001; Robinson 2001). The enzyme nitrate reductase, for instance, shows little discrimination against ^{15}N, but as NO_3^- availability increases, so does discrimination against ^{15}N

(Hogberg, 1997). Isotopic discrimination during NH_4^+ assimilation can occur as NH_4^+ is actively transported into roots cells, or through enzymatic activity (Evans, 2001; Yoneyama *et al.*, 1991). There are no data available, however, on how discrimination against ^{15}N may be shifted when plants must meet the C requirements for NO_3^- and NH_4^+ uptake and assimilation under low CO_2, *i.e.*, C-limited, conditions.

To address these questions, I grew *Elymus canadensis* (Canada wild rye, Figure 2.1) at current and sub-ambient CO_2 conditions under four different N fertilization regimes. *Elymus canadensi*s is a cool season perennial grass, and was selected because this genus is well recorded during the Last Glacial Maximum, and was likely an important resource for ancient humans (Perry and Quigg, 2011; Zazula *et al.*, 2006). Based on the premise that a low CO_2 environment may favor plants grown with NO_3^- over those grown with NH_4^+, and that low CO_2 effects may be exacerbated by low N availability, we hypothesized that: 1) low CO_2 would reduce plant growth compared to current CO_2, but growth reductions would be greatest when N availability was limited (*i.e.*, under low N or NH_4^+-only treatments); 2) photosynthetic capacity (V_{cmax} and J_{max}) would be most strongly increased by low CO_2 in the full N and NO_3 treatments, where there was sufficient N to allocate to photosynthetic proteins; 3) low CO_2 would increase stomatal index, an effect that would be greatest in treatments where low N constrains the ability to increase photosynthetic capacity, but where increasing intercellular CO_2 concentrations would increase A_{net}; and 4) reduced N availability under low CO_2 would decrease discrimination against the heavier N isotope (^{15}N), resulting in a more positive N isotope composition.

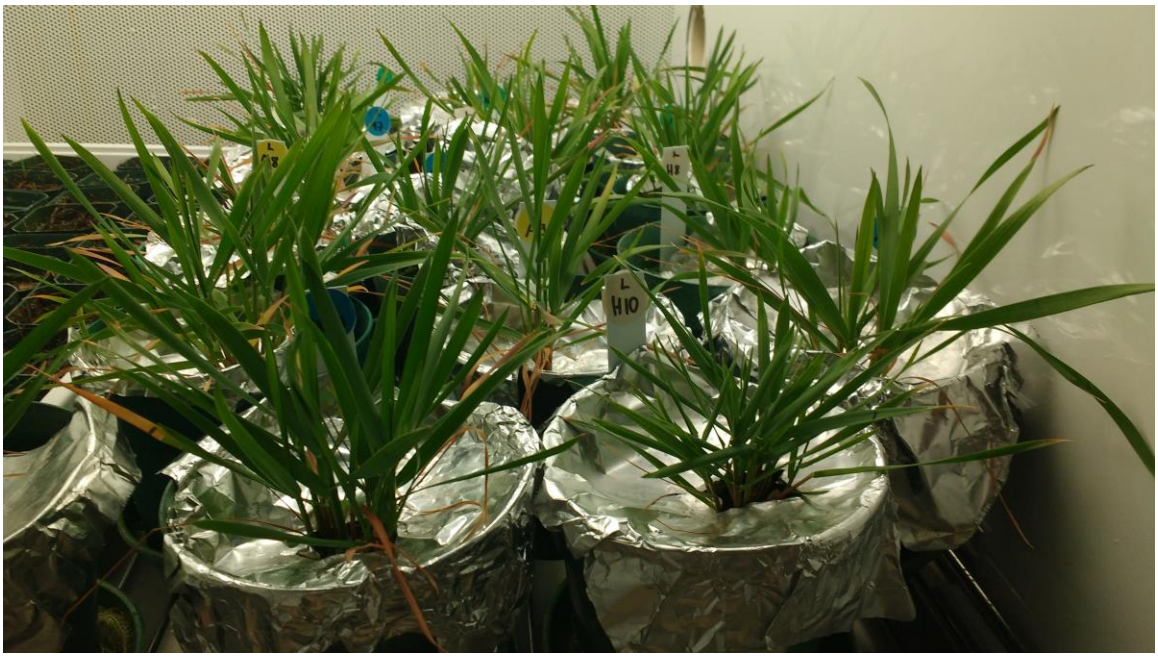

Figure 2.1 *Elymus canadensis* plants used in this experiment.

2.3 Materials and methods

2.3.1 Experimental design and treatments

Two growth chambers (GCW15, Environmental Growth Chambers, Chagrin Falls, OH, USA), one for each CO_2 treatment, were maintained at 74 ± 5 % relative humidity, an irradiance of 300 ± 28 µmol photons m^{-2} s^{-1}, a 12.5-hour photoperiod, and 16 ± 0.1 °C (means ± SE). While only one chamber per CO_2 treatment was used due to equipment constraints, environmental conditions were constantly monitored and regulated to maintain same chamber conditions for both CO_2 treatments. Average differences in relative humidity were 2 %, PAR differed by 2.35 µmol photons m^{-2} s^{-1}, and temperature by 0.19 °C between chambers. Photoperiod and temperatures emulated average growing season conditions of the last three years at the site where seeds were collected (Environment Canada, Midland WPCP Station). Each chamber contained a CO_2 control system consisting of an acrylic box filled with soda lime to absorb air CO_2, and fans attached to the boxes' lids. Fans were connected to a CO_2 analyzer (LI-820, LI-COR, Lincoln, NE, USA) coupled with a DIN process controller (Omega CN7633, Omega Engineering, Stamford, CT, USA). CO_2 concentrations averaged 401 ± 3 ppm in the ambient CO_2 chamber (AC), and 189 ± 12 ppm in the low CO_2 chamber (LC).

To minimize soil property influences on N availability, perlite was used as a growing medium. Perlite has been used previously in soilless experiments and does not affect mineralization rates in N supplementation experiments (Jasso-Chaverria *et al.*, 2005; Tyson *et al.*, 2007). Each chamber contained twenty 2 L pots planted with *E. canadensis* seeds (from Wildflower Farm, Coldwater, ON, Canada). Pots were then subdivided into four N treatments (n = 5 pots per CO_2 × fertilizer treatment): a full-strength, NO_3^--only fertilizer (*nitrate*); a full-strength NH_4^+-only fertilizer (*ammonium*); a full-strength NO_3^-/NH_4^+ mix fertilizer (*full*); and a half-strength NO_3^-/NH_4^+ mix fertilizer (*half*). The *full* treatment was a Hoagland's mix, while the *nitrate, ammonium* and *half* treatments were prepared by modifying the Hoagland's recipe (Hoagland and Arnon, 1950). Plants were watered three times per week and fertilized weekly: plants in the *nitrate, ammonium* and *full* treatments received 200 ml of water with 1.04 mol N/L, and plants in the *half*

treatment received 200 ml of water with 0.52 mol N/L. All other nutrients were similar across treatments, as was the fertilizer pH (6.58 ± 0.04). Pots were rotated weekly within each chamber to minimize intra-chamber effects.

2.3.2 Gas exchange measurements

Net CO_2 assimilation rates (A_{net}) and stomatal conductance (g_s) were measured once seedlings were 90 days old, using a portable photosynthesis system (Walz GFS-3000, Effeltrich, Germany). Five seedlings per treatment were assessed. Two fully mature leaves were equilibrated for ~10 minutes at a CO_2 concentration of 400 μmol mol^{-1}. CO_2 concentrations were then changed to 300, 180, 100, 60, 400, 600, 800, 1000, 1200, 1500, and back to 400 μmol mol^{-1}. Leaves were maintained at each CO_2 level until a stable A_{net} was achieved. Cuvette conditions included a 16 °C leaf temperature, 750 μmol s^{-1} flow rate (as recommended for the 3010-S cuvette of the Walz GFS-300), and an irradiance of 1200 μmol m^{-2} s^{-1}, which was light-saturating based on light-response curves (data not shown). After each measurement, leaves inside the cuvette were photographed and leaf area estimated using ImageJ 1.48v (Schneider *et al.*, 2012).

The response of A_{net} to intercellular CO_2 concentration (C_i) was used to derive photosynthetic capacity (Farquhar *et al.*, 1980). Values of V_{cmax} (maximum Rubisco carboxylation rates) and J_{max} (maximum electron transport rates) were parameterized in R 3.2.1 (R Core Team, 2013) using the package '*plantecophys*' (Duursma 2015).

2.3.3 Biomass

After gas exchange, plants were harvested and subsamples of roots and shoots from five plants per treatment were collected and their area was estimated from digital images (ImageJ 1.48v - Schneider *et al.*, 2012). These tissues were dried at 60 °C to a constant mass, and weighed. The ratio of leaf area to leaf dry mass was used to calculate specific leaf area (SLA).

2.3.4 Stomatal index

Three mature leaves, including leaves used in the photosynthetic measurements, were sampled from five plants per treatment to obtain a negative mould. Dental resin (President SEM High Resolution Replication Kit, TED Pella INC., Essex, United Kingdom) was spread on a 3 cm x 1 cm area on both surfaces of each leaf. A positive mould was obtained by pushing the negative resin mould onto a layer of wet clear nail varnish on a microscope slide. Using the positive moulds, stomatal index (SI) was assessed at a magnification of 20x using an optical microscope (Nikon Eclipse Ci, Nikon, Melville, NY, USA), and calculated as:

$$\text{Stomatal Index} = \frac{\text{number of stomata}}{(\text{number of stomata} + \text{number of epidermal cells})}$$

Images were taken with a Nikon DS-Ri2 camera (Nikon, Melville, NY, USA) and analyzed using NIS Elements Documentation imaging software (Nikon).

2.3.5 Isotopes, C and N concentration

For measurements of δ^{15}N, and C and N concentration, five mature leaves and a section containing entire roots (from the root crown to the tip) were harvested from each plant (n = 5), dried at 60 °C to constant mass, and ground. Isotopic abundance measurements were performed using a Delta^{Plus} isotope-ratio mass-spectrometer (Thermo Scientific, Bremen, Germany) coupled in continuous flow mode with an elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA). Sample reproducibility was ± 0.14 ‰ for δ^{15}N (17 replicates), and ± 0.1 % gDW^{-1} for %N (17 replicates). Sample reproducibility for [C] was ± 0.29 % gDW^{-1} (11 replicates). A δ^{15}N of +6.37 ± 0.1 ‰ was obtained for 30 analyses of an internal keratin standard (accepted value = +6.36 ‰).

The nitrogen isotope composition of root, and leaves was reported using the standard delta notation (δ^{15}N):

$$\delta^{15}\mathrm{N} = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right)$$

where R_{sample} was calibrated to AIR ($R_{standard}$) using international standards USGS40 and USGS41. We then compared changes in the isotope composition of leaves and roots between CO_2 treatments in each N treatment:

$$\Delta^{15}\mathrm{N}_{leaf/root} = \delta^{15}\mathrm{N}_{AC} - \delta^{15}\mathrm{N}_{LC}$$

where $\Delta^{15}\mathrm{N}$ represents the difference found in either leaves ($\Delta^{15}\mathrm{N}_{leaf}$) or roots ($\Delta^{15}\mathrm{N}_{root}$) between ambient ($\delta^{15}\mathrm{N}_{AC}$) and low CO_2 grown plants ($\delta^{15}\mathrm{N}_{LC}$).

2.3.6 Statistical analysis

Two-way ANOVAs were used to assess treatment effects of growth CO_2, fertilizer, and their interaction on all parameters except for $\Delta^{15}\mathrm{N}$ where a one-way ANOVA was used to estimate fertilization effects. Where treatment effects were significant ($p \leq 0.05$), Tukey's HSD tests were used to identify differences among treatments. Results are shown as means ± standard error. Data analysis was performed in R 3.2.1 (R Core Team 2013).

2.4 Results

2.4.1 Biomass, SLA, [C] and [N]

LC reduced total biomass by 47 %, and decreased shoot and root biomass by 40 and 51 %, respectively, in comparison to AC (Table 2.1; Fig. 2.2). While there was no $CO_2 \times$ fertilizer interaction on shoot biomass, there was for total and root biomass (Table 2.1). At AC, root biomass was 44 % lower in *half* plants than in *full* plants, but at LC, there were no differences between N treatments in root mass. *Ammonium* plants had lower root and total biomass than *full* plants at AC, while *nitrate* plants were similar to *full* plants for shoot, root and total biomass in both CO_2 treatments. Within an N treatment, the decrease in growth in LC compared to AC was greatest for the *full* and *nitrate* plants, with less of a

growth impact of LC on the *ammonium* and *half* plants (Fig. 2.2). The root/shoot ratio of LC plants was 34 % lower than AC plants, indicating a shift towards relatively more shoot biomass at low CO_2. *Nitrate* plants had lower SLA then plants from the other N treatments (Table 2.1; Fig. 2.2). Leaf [C] was unaffected by growth CO_2, while growth at LC increased leaf and root [N] (Tables 2.1 and 2.2).

Table 2.1. Analysis of variance testing growth CO_2, fertilizer treatment, and their interaction on plant traits. Specific leaf area (SLA); carbon concentration ([C]); nitrogen concentration ([N]); net CO_2 assimilation rates at growth CO_2 (A_{growth}) and a common CO_2 of 400 ppm (A_{400}); maximum Rubisco carboxylation rates (V_{cmax}); maximum electron transport rates (J_{max}); stomatal conductance at growth CO_2 ($g_{s\text{-}growth}$) and a common CO_2 of 400 ppm ($g_{s\text{-}400}$); abaxial ($SI_{abaxial}$) and adaxial ($SI_{adaxial}$) stomatal index; and the difference between the isotope composition of leaves ($\Delta^{15}N_{leaf}$) or roots ($\Delta^{15}N_{root}$) between ambient and low CO_2 plants. The *p*-values are shown with significant effects in bold ($p \leq 0.05$).

	ANOVA		
	CO_2	Fertilizer	$CO_2 \times$ Fertilizer
Total biomass	**< 0.01**	**< 0.01**	**0.05**
Shoot biomass	**< 0.01**	**< 0.01**	0.14
Root biomass	**< 0.01**	**< 0.01**	**0.05**
Root/shoot ratio	**0.03**	**< 0.01**	0.45
SLA	**< 0.01**	**0.01**	0.06
Leaf [C]	0.75	**< 0.01**	0.35
Root [C]	**< 0.01**	**0.02**	0.18
Leaf [N]	**< 0.01**	**< 0.01**	0.76
Root [N]	**< 0.01**	**< 0.01**	0.60
A_{growth}	**< 0.01**	**< 0.01**	**< 0.01**
A_{400}	0.11	**< 0.01**	**0.01**
V_{cmax}	0.67	**< 0.01**	0.35
J_{max}	0.18	**< 0.01**	**0.04**
$g_{s\text{-}growth}$	0.38	**< 0.01**	0.95
$g_{s\text{-}400}$	0.09	**0.01**	0.95
$SI_{abaxial}$	0.58	**< 0.01**	0.64
$SI_{adaxial}$	0.08	**< 0.01**	**0.02**
$\Delta^{15}N_{leaf}$		**< 0.01**	
$\Delta^{15}N_{root}$		**< 0.01**	

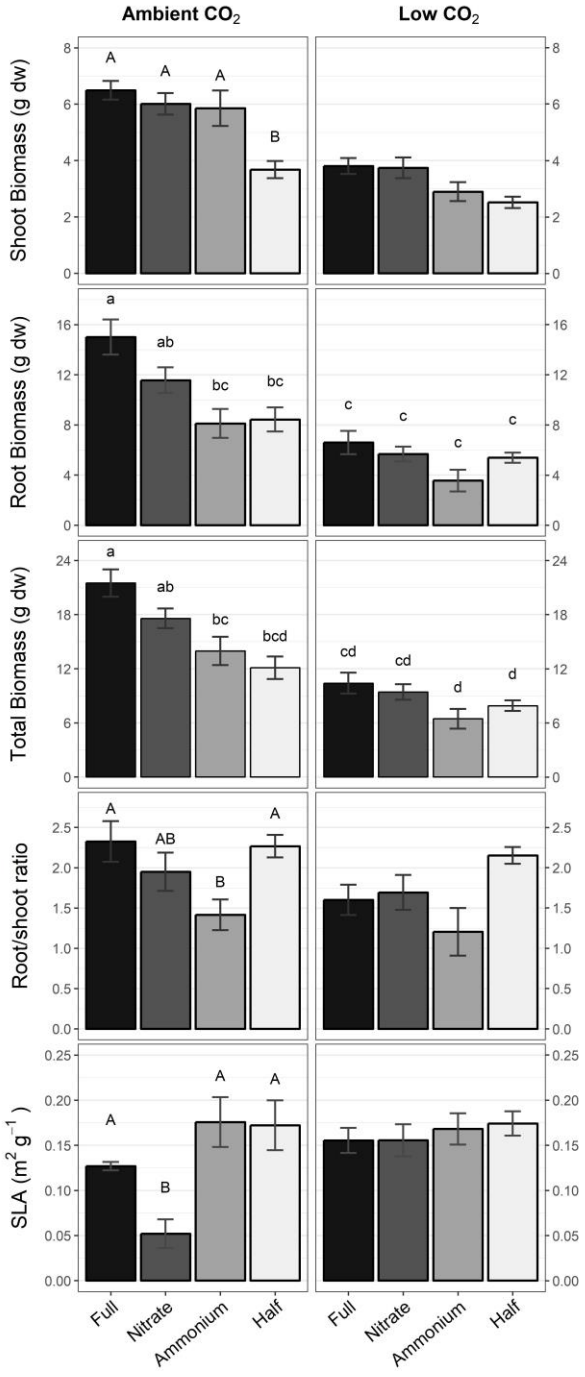

Figure 2.2. Plant biomass and specific leaf area (SLA). Shoot, root, and total dry biomass, root/shoot, and SLA of plants grown at ambient (left panels) and low CO_2 (right panels) in each fertilization treatment (*Full*, *Nitrate*, *Ammonium* and *Half*). Where the interaction term is significant ($p < 0.05$), differences between groups are represented by lower-case letters. Upper-case letters (shown only on ambient CO_2 data) represent differences between fertilizers when there is no significant interaction. Means ± SE, n=5.

Table 2.2. Carbon ([C]) and nitrogen ([N]) concentration in leaves and roots of plants grown at ambient (AC) and low CO_2 (LC) for each of the four nitrogen treatments: full-strength NO_3^-/NH_4^+ fertilizer (*Full*); full-strength, NO_3^--only fertilizer (*Nitrate*); full-strength NH_4^+-only fertilizer (A*mmonium*); and half-strength NO_3^-/NH_4^+ fertilizer (*Half*). Upper-case letters (shown only on AC) represent the fertilizer treatment effects ($p \leq 0.05$), as there is no significant interaction term. Means ± SE, n=5.

			[C] (% dry weight)	[N] (% dry weight)
Leaf	AC	*Full*	41.87 ± 0.23^{A}	3.09 ± 0.07^{A}
		Nitrate	42.16 ± 0.33^{A}	3.92 ± 0.06^{A}
		Ammonium	42.81 ± 0.21^{A}	3.85 ± 0.37^{A}
		Half	41.57 ± 0.21^{B}	2.96 ± 0.15^{B}
	LC	*Full*	42.33 ± 0.19	3.84 ± 0.16
		Nitrate	42.26 ± 0.16	4.65 ± 0.25
		Ammonium	41.47 ± 0.92	4.99 ± 0.32
		Half	41.07 ± 0.32	3.58 ± 0.15
Root	AC	*Full*	40.90 ± 0.05^{A}	0.72 ± 0.02^{B}
		Nitrate	40.44 ± 0.52^{AB}	0.85 ± 0.05^{AB}
		Ammonium	39.57 ± 0.39^{B}	1.08 ± 0.16^{A}
		Half	39.48 ± 0.27^{B}	0.67 ± 0.03^{B}
	LC	*Full*	39.65 ± 0.12	0.86 ± 0.06
		Nitrate	39.45 ± 0.18	1.24 ± 0.11
		Ammonium	39.11 ± 0.19	1.64 ± 0.34
		Half	39.51 ± 0.31	0.91 ± 0.03

2.4.2 Photosynthetic parameters

The A_{net} measured at growth CO_2 (A_{growth}) in *full* plants was 46 % lower in LC than AC plants, but there was no reduction in A_{growth} in LC compared to AC in the other N treatments (Table 2.1; Fig. 2.3). When A_{net} was measured at 400 ppm CO_2 (A_{400}), rates were highest in *full* plants at AC, but similar between *full* and *nitrate* plants in LC. *Ammonium* plants at LC had similar A_{400} as *full* and *nitrate* plants, but also similar A_{400} as *half* plants. V_{cmax} was unaffected by growth CO_2 (Table 2.1; Fig. 2.3). The V_{cmax} values in *full* and *nitrate* plants were twice that of *ammonium* and *half* plants. In contrast, J_{max} was affected by fertilization and a CO_2 x fertilizer interaction (Table 2.1; Fig. 2.3). In AC, *full* plants had the highest J_{max} values, which were similar to those of the *nitrate* plants, but higher than those of *ammonium* and *half* plants. In LC, both *full* and *nitrate* plants had higher J_{max} values than *ammonium* and *half* plants.

2.4.3 Stomatal conductance and index

Both stomatal conductance measured at growth CO_2 ($g_{s\text{-}growth}$) and at 400 ppm CO_2 ($g_{s\text{-}400}$) varied with fertilization, but was unaffected by growth CO_2 (Table 2.1; Fig. 2.4), with the lowest values in *ammonium* plants. The stomatal index of the abaxial and adaxial sides of leaves ($SI_{abaxial}$ and $SI_{adaxial}$) was unaffected by growth CO_2 (Table 2.1; Fig. 2.4), but there was a growth CO_2 $\times$ fertilizer interaction on $SI_{adaxial}$, which in *half* leaves was 23 % higher in AC compared to LC leaves.

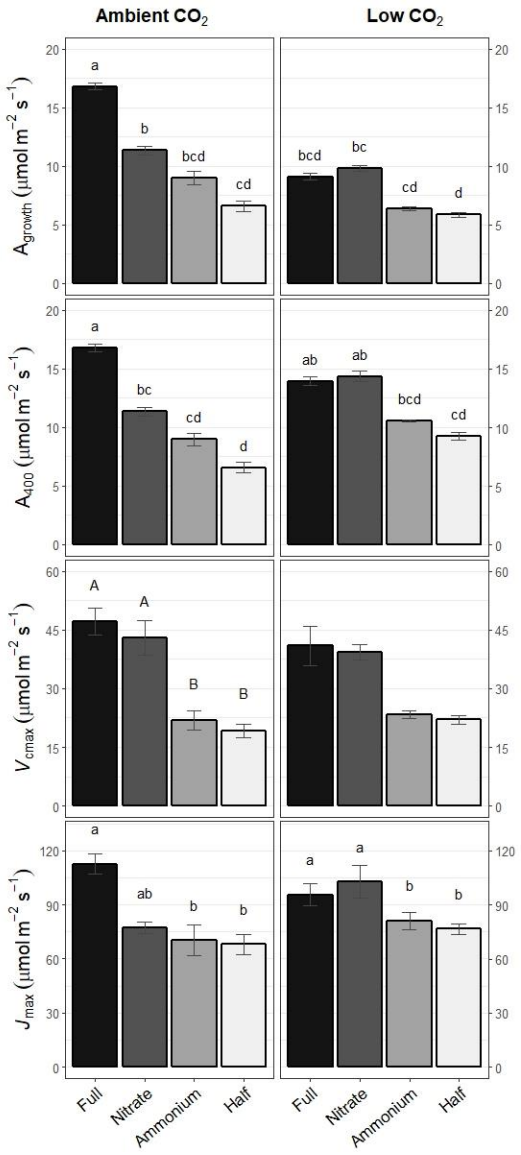

Figure 2.3. Net CO_2 assimilation (A_{net}), maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates. A_{net} measured at growth CO_2 (A_{growth}) and a common CO_2 concentration of 400 ppm (A_{400}) of plants grown at ambient (left panels) and low CO_2 (right panels) in each fertilization treatment (*Full*, *Nitrate*, *Ammonium* and *Half*). Where the interaction term was significant ($p < 0.05$), differences between groups are represented by lower-case letters. Upper-case letters (shown only on ambient CO_2 data) represent differences between fertilizers when there is no significant interaction. Means ± SE, n=5.

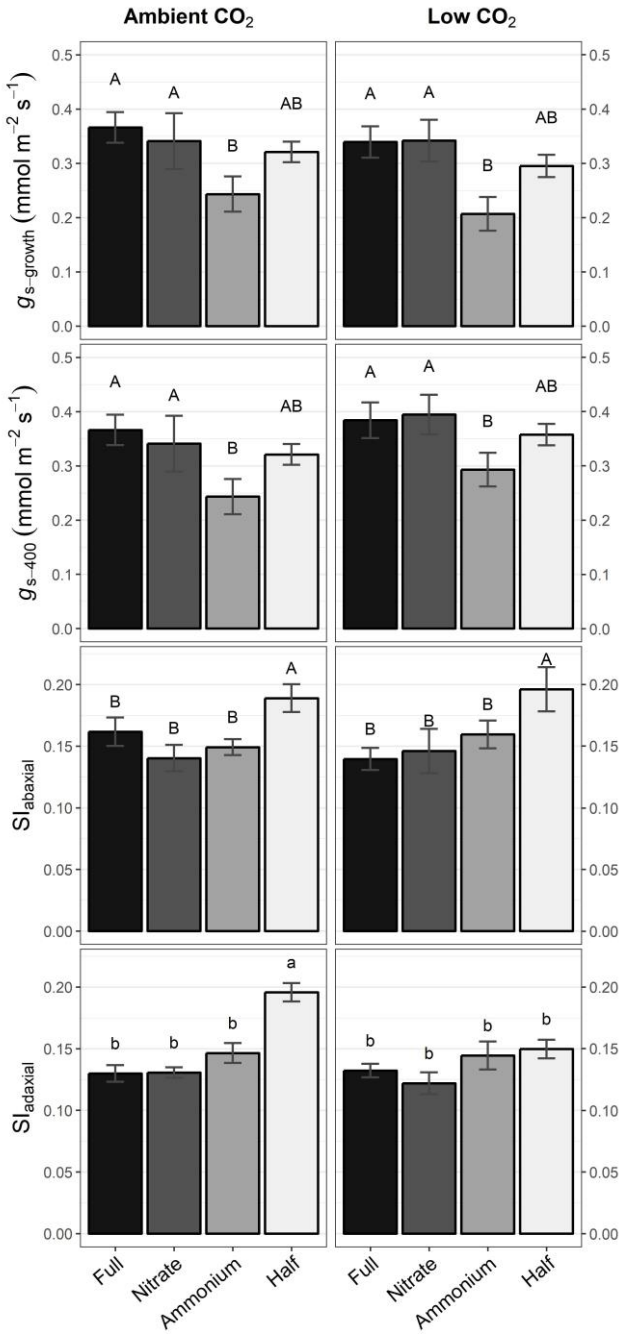

Figure 2.4. Stomatal conductance measured at growth CO_2 ($g_{s\text{-}growth}$) and a common CO_2 concentration of 400 ppm ($g_{s\text{-}400}$), and stomatal index measured on the abaxial ($SI_{abaxial}$) and adaxial ($SI_{adaxial}$) sides of the leaves. Where the interaction term was significant ($p < 0.05$), differences between groups are represented by lower-case letters. Upper-case letters (shown only on ambient CO_2 data) represent differences between fertilizers when there is no significant interaction. Means ± SE, n=5.

Nitrogen isotopes

Leaves fertilized with NO_3^--only were more depleted of ^{15}N at LC compared to AC, while leaves of *half* plants were enriched in ^{15}N at LC compared to AC (Tables 2.1 and 2.3). Values of $\Delta^{15}N_{root}$ also varied with CO_2 and fertilization, but there was no interaction (Table 2.1). Except for the *half* treatment, where $\Delta^{15}N$ was similar between the CO_2 treatments, roots were depleted of ^{15}N at LC relative to AC.

Table 2.3. Difference in nitrogen isotope composition of leaves and roots between plants grown at ambient and low CO_2 for each nitrogen treatment: full-strength NO_3^-/NH_4^+ fertilizer (*Full*); full-strength, NO_3^--only fertilizer (*Nitrate*); full-strength NH_4^+-only fertilizer (*Ammonium*); and half-strength NO_3^-/NH_4^+ fertilizer (*Half*)**.** $\Delta^{15}N$ is calculated as the difference in nitrogen isotope composition between ambient and low CO_2-grown plants ($\delta^{15}N_{AC}$ - $\delta^{15}N_{LC}$) for leaves ($\Delta^{15}N_{leaf}$) and roots ($\Delta^{15}N_{root}$). Where the interaction term was significant ($p < 0.05$), differences between groups are represented by lower-case letters. Upper-case letters represent differences between fertilizers when there is no significant interaction. Means ± SE, n = 5.

	$\Delta^{15}N_{leaf}$	$\Delta^{15}N_{root}$
Full	-0.10 ± 0.19^{BC}	0.54 ± 0.22^{A}
Nitrate	1.18 ± 0.19^{A}	0.27 ± 0.22^{AB}
Ammonium	0.27 ± 0.18^{AB}	0.77 ± 0.15^{A}
Half	-0.79 ± 0.26^{C}	-0.10 ± 0.29^{B}

2.5 Discussion

I hypothesized that growth at low CO_2 combined with NH_4^+-only or low N fertilization would decrease plant growth, photosynthetic capacity, and discrimination against ^{15}N, while increasing stomatal index, when compared to low CO_2 plants given a full-strength NH_4^+-NO_3^- or NO_3^--only fertilizer. While the results are more complex than my hypotheses, I did find that the effect of low CO_2 on many plant traits was altered by the source or quantity of N available.

2.5.1 Decrease in growth at low CO_2

The results do not support my first hypothesis that growth in *ammonium* and *half* plants would be reduced more strongly by LC than *full* or *nitrate* plants. Instead, plants that had the lowest growth in AC (*ammonium* and *half*) had only relatively small decreases in total biomass between AC and LC conditions, while *full* plants had a 51 % decrease in growth. This implies that low N availability constrains the growth response to a 220 ppm rise in CO_2 concentration. While there is a large body of work showing that low N availability limits plant responses to high CO_2 (*e.g.*, LeBauer and Treseder, 2008; Norby *et al.*, 2010; Reich *et al.*, 2006), to our knowledge, this is the first work to show that low N conditions also limit plant growth responses to changes in CO_2 levels that occurred over the past 20,000 years.

Compared to AC plants, LC plants had higher leaf [N], which commonly reflects greater investment in photosynthetic machinery to enhance C uptake (Ellsworth *et al.*, 2004). As noted below, however, there was little evidence that higher [N] in LC plants was used to increase photosynthetic capacity. Examining how the N treatments affected growth within a CO_2 treatment allows us to establish the direct impact of changes in N source and amount on plant traits. Unsurprisingly, *half* plants had lower growth than *full* plants at AC. We can also compare the growth of *nitrate* and *ammonium* plants to *full* plants, which were the control fertilizer treatment. In AC, *nitrate* plants were similar to the *full* treatment in shoot, root and total biomass, as well as root/shoot ratio, while *ammonium*

plants had lower root and total biomass than *full* plants, and a lower root/shoot ratio. Since perlite was chosen to minimize changes in N availability due to physical and chemical properties of the growing media, taken together, these results indicate that *E. canadensis* is likely a nitrate-preferring species. But, although this was true at AC, there was little evidence of an N source preference at LC. At LC, shoot and total biomass was similar across all four N treatments, and while *ammonium* plants had a lower root/shoot ratio than the *full* plants, their root/shoot was similar to that of the *nitrate* plants. Thus, carbon limitation was a stronger determinant than N source for plant growth. Overall, plants responded to C limitation by increasing allocation to shoots and reducing leaf thickness or density (*i.e.*, increasing SLA), regardless of fertilization regime.

2.5.2 Reductions in photosynthetic performance at low CO_2 depend on N availability

I hypothesized that LC plants would up-regulate V_{cmax} and J_{max}, with the strongest up-regulation in the *full* and *nitrate* treatments. But growth CO_2 did not affect V_{cmax} or J_{max}. Instead, V_{cmax} varied with fertilization, with *full* and *nitrate* plants having higher V_{cmax} than the *ammonium* and *half* plants. Values of J_{max} in *full* and *nitrate* plants were also higher than those of *ammonium* and *half* plants at LC, although *nitrate* plants had similar J_{max} values to the other N treatments in AC. The availability of N therefore appears to have been a stronger determinant of photosynthetic capacity than CO_2 availability. Differences in V_{cmax} and J_{max}, however, are not correlated with leaf [N] *per se*: while *ammonium* and *half* plants both had equally low photosynthetic capacity, *ammonium* plants had higher leaf [N] than *half* plants, with leaf [N] similar to the *full* and *nitrate* leaves that had high V_{cmax} and J_{max}. Although the down-regulation response of V_{cmax} to high CO_2 is well known (Ellsworth *et al,.* 2004; Griffin *et al.*, 2000; Tissue *et al.*, 1999), it is still hard to generalize how low CO_2 impacts photosynthetic capacity. Tissue *et al.* (1995) found that V_{cmax} was 23% lower in *Abutilon theophrasti* grown at low CO_2 than at current CO_2, a result linked to decreased Rubisco content and activation state. Ripley *et al.* (2013) observed an up-regulation of photosynthetic capacity in C_3 species grown at

~180 ppm CO_2, which was linked to increased leaf [N]. Future low CO_2 studies should therefore work to enhance our understanding of photosynthetic physiology in C-limited conditions.

Considering how N source and availability influenced V_{cmax} and J_{max}, it is not surprising that A_{growth}, and its response to LC, also varied with fertilization. I observed a strong decline in A_{growth} in LC compared to AC in *full* plants (85 %), but there was no reduction in A_{growth} under LC in the other N treatments. The lack of a change in A_{growth} between AC and LC in the *nitrate*, *ammonium* and *half* treatments implies that plants either experienced N limitations for synthesizing photosynthetic enzymes and pigments, or that nutrient availability limited growth potential, which in turn reduced photosynthesis (Fatichi *et al.*, 2014). Given that LC actually increased leaf [N] and had no effect on V_{cmax} (and only a minor impact on J_{max}), the data support the growth limitation hypothesis, indicating that low N availability can constrain growth and photosynthetic responses to low and current CO_2 conditions. Most studies on the effects of low CO_2 on plants (reviewed in Gerhart and Ward, 2010; Temme *et al.*, 2013) do not consider that plant growth is often limited by N availability in the field (LeBauer and Treseder, 2008). Based on these results, examinations of plant responses to changing CO_2 in the geologic past should consider the influence of nutrient limitations, rather than being conducted under N-replete conditions.

There was no increase in g_s from either long-term anatomical shifts or short-term stomatal behavior to help mitigate the low C availability at LC. The SI was similar in most N treatments, and g_s was unresponsive to growth CO_2 concentrations across N treatments. While plants grown at LC can increase g_s to enhance CO_2 uptake (Pinto *et al.*, 2014; Ripley *et al.*, 2013), no change in g_s was observed between AC and LC plants. Given that water loss increases as g_s increases, *E. canadensis* may be forgoing enhanced CO_2 uptake to prevent excess water loss.

2.5.3 $SI_{adaxial}$ decreased at low CO_2 when N was limiting

Contrary to my third hypothesis, growth at low CO_2 did not increase SI. Although the relationship between SI and growth CO_2 concentration is generally inverse (Beerling and Royer, 2002), changes in SI in response to growth CO_2 can be highly variable. In historical collections, Beerling (2005) found a decrease in SI as CO_2 rose from 280 to 360 ppm, but SI increased proportionally to growth CO_2 in *Solanum dimidiatum* grown between 200 to 550 ppm CO_2 (Maherali *et al.*, 2002). While reviewing SI values published for 176 C_3 species, Royer (2001) observed that SI was inversely correlated to CO_2 concentrations in only 36 % but in 94 % of experimental and fossil studies, respectively. This variety of responses indicates that growth CO_2 effects on SI from experimental manipulations with current genotypes may not be very reliable for predicting SI responses to CO_2 on evolutionary scales, a common concern in low CO_2 studies (Ward and Strain, 1997). While environmental conditions such as irradiance, temperature and humidity are possible confounding factors when using SI to infer growth CO_2 (Royer, 2001), the results indicate that N availability can also influence SI. I found that SI was similar between AC and LC plants except when plants were N-limited (*i.e.*, *half* plants). The reduction in SI in plants under C- and N-limitation may be related to the observation that *half* plants also had low A_{growth} and V_{cmax}. In this case, the benefit from enhanced CO_2 influx through stomata may not outweigh potential increases in water loss, given that photosynthetic capacity was low.

2.5.4 C and N availability regulates N isotopic discrimination

Roots of LC plants discriminated more against ^{15}N when compared to AC, which may be a direct effect of reduced root biomass in LC plants. As LC had reduced root growth in comparison to AC plants, their N requirement would be reduced and more readily met by preferentially using the lighter isotope from the available N pool.

In leaves, NO_3^- fertilization caused a depletion of ^{15}N in LC plants compared to AC plants (1.2 ‰), while this difference was much smaller when the only N source was NH_4^+ (0.3 ‰). NO_3^- can be assimilated in both roots and shoots, while NH_4^+ is mostly assimilated in roots (Hachiya and Sakakibara 2016). Thus, plants fertilized with only NO_3^- are more depleted of ^{15}N compared to NH_4^+-fed plants since NO_3^- available for assimilation in leaves can originate from a NO_3^- pool that was previously exposed to assimilation in the roots (Evans *et al.*, 1996). In addition, the enzymes involved in the initial steps of NO_3^- assimilation discriminate against the heavier N isotope (Tcherkez and Farquhar 2006); growth at LC, where C availability (and consequently energy) was limited, increased this discrimination. Thus, the preference for the lighter N isotope in LC *nitrate* plants was probably the result of reduced N requirements for metabolism. In contrast, growth at LC decreased discrimination against ^{15}N in leaves of *half* plants, in agreement with our fourth hypothesis. N assimilation processes should discriminate less against the heavier N isotope when N availability is low (Evans, 2001), since plants must enhance N uptake from a limited external N pool. Hence, as *half* plants were already C-limited, N-limitation further decreased the discrimination against ^{15}N.

Given the diversity of N sources available to plants and the variability in N availability on temporal and spatial scales (Canfield *et al.*, 2010; LeBauer and Treseder, 2008), interpretations of the N isotope composition of plant tissues should consider factors influencing the origin of assimilated N. Information on soil properties and environmental conditions could represent a starting point to derive the major N transformations (*e.g.*, mineralization, nitrification) determining possible N forms that are most bioavailable (Amundson *et al.*, 2003; Weil and Brady 2016). Integrating potential N sources into studies of plant N isotope signals not only provides a more holistic view of plant N cycling, but could also help interpret paleoecology inferences based on N isotope signals, such as reconstruction of past trophic interactions. Plant $\delta^{15}N$ is used in paleobiology studies to identify animal diet and trophic position (Boecklen *et al.*, 2011), as the N isotope signature in consumer bulk collagen usually reflects the isotope composition of the plants consumed at the base of the food web (Garten *et al.*, 2008). An enrichment of 3.4 ‰ in $\delta^{15}N$ is commonly considered to be the standard difference between trophic levels (Minagawa and Wada, 1984; Post, 2002). Our results show a 2 ‰ difference

between leaves of *nitrate* and *half* plants, a difference that could potentially skew the interpretation of $\delta^{15}N$ in paleo-animal tissues if the vegetation at the base of the food web came from plants that were either N-limited or had only NO_3^- available.

2.5.5 NO_3^- *vs* NH_4^+ as N sources at low CO_2

While we were unable to measure photorespiration directly, photorespiration rates increase at low CO_2 concentrations (Ehleringer *et al.*, 2005; Farquhar and von Caemmerer, 1982) and can provide reductant for nitrate conversion (Bloom *et al.*, 2010, 2014). In our study, *nitrate* plants had higher photosynthetic capacity and stomatal conductance than *ammonium* plants, which allowed total biomass of *nitrate* plants to be closer to *full* than *ammonium* plants, while *ammonium* plants were mostly similar to the *half* treatment. While studies covering a wider range of NO_3^- and NH_4^+-preferring species are needed, our observations are consistent with the hypothesis that in the low CO_2 period before the Industrial Revolution, where CO_2 concentrations were 180-270 ppm *(*Petit *et al.*, 1999; Monnin *et al.*, 2001), NO_3^--preferring species such as *E. canadensis* may have had some advantage over species preferentially using NH_4^+. Such observations complement those from elevated CO_2 studies where plants performed better with NH_4^+ than NO_3, highlighting that the ability to use NO_3^- or NH_4^+ is likely to change in a high CO_2 future (Bloom *et al*., 2010, 2012; Cheng et al., 2012).

2.6 References

Alt, D.S., Doyle, J.W., Malladi, A., 2017. Nitrogen-source preference in blueberry (*Vaccinium sp.*): Enhanced shoot nitrogen assimilation in response to direct supply of nitrate. J. Plant Physiol. 216, 79–87. https://doi.org/10.1016/j.jplph.2017.05.014

Amundson, R., Austin, A.T., Schuur, E.A.G., Yoo, K., Matzek, V., Kendall, C., Uebersax, A., Brenner, D., Baisden, W.T., 2003. Global patterns of the isotopic composition of soil and plant nitrogen. Global Biogeochem. Cycles 17. https://doi.org/10.1029/2002GB001903

BassiriRad, H., Griffin, K.L., Reynolds, J.F., Strain, B.R., 1997. Changes in root NH_4^+ and NO_3^- absorption rates of loblolly and ponderosa pine in response to CO_2 enrichment. Plant Soil 190, 1–9. https://doi.org/10.1023/A:1004206624311

Becklin, K.M., Walker, S.M., Way, D.A., Ward, J.K., 2017. CO_2 studies remain key to understanding a future world. New Phytol. 214, 34–40. https://doi.org/10.1111/nph.14336

Beerling, D.J., 2005. Evolutionary responses of land plants to atmospheric CO_2, in: A History of Atmospheric CO_2 and Its Effects on Plants, Animals, and Ecosystmes. Springer, pp. 114–132.

Beerling, D.J., Royer, D.L., 2002. Reading a CO_2 signal from fossil stomata. New Phytol. 153, 387–397. https://doi.org/10.1046/j.0028-646X.2001.00335.x

Bloom, A.J., Asensio, J.S.R., Randall, L., Rachmilevitch, S., Cousins, A.B., Carlisle, E.A., 2012. CO_2 enrichment inhibits shoot nitrate assimilation in C_3 but not C_4 plants and slows growth under nitrate in C_3 plants. Ecology 93, 355–367. https://doi.org/10.1890/11-0485.1

Bloom, A. J., Burger, M., Asensio, J.S.R., Cousins, A.B., Zhou, L., Shew, H.D., Rufty, T.W., Hu, S., 2010. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and Arabidopsis. Science. 328, 899–903. https://doi.org/10.1126/science.1186440

Bloom, A.J., Burger, M., Kimball, B., Jr, P.P., 2014. Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. Nat. Clim. Chang. 4, 477–180. https://doi.org/10.1038/NCLIMATE2183

Boecklen, W.J., Yarnes, C.T., Cook, B.A., James, A.C., 2011. On the use of stable isotopes in trophic ecology. Annu. Rev. Ecol. Evol. Syst. 42, 411–440. https://doi.org/10.1146/annurev-ecolsys-102209-144726

Busch, F.A., Sage, R.F., Farquhar, G.D., 2018. Plants increase CO_2 uptake by assimilating nitrogen via the photorespiratory pathway. Nat. Plants 4, 46–54. https://doi.org/10.1038/s41477-017-0065-x

Canfield, D.E., Glazer, A.N., Falkowski, P.G., 2010. The evolution and future of Earth's nitrogen cycle. Science. 330, 192–196. https://doi.org/10.1126/science.1186120

Cheng, L., Booker, F.L., Tu, C., Burkey, K.O., Zhou, L., Shew, H.D., Rufty, T.W., Hu, S., 2012. Arbuscular mycorrhizal fungi increase organic carbon decomposition under elevated CO_2. Science. 337, 1084–1087. https://doi.org/10.5061/dryad.b7f53

Cowling, S. a, Sykes, M.T., 1999. Physiological significance of low atmospheric CO_2 for plant–climate interactions. Quat. Res. 52, 237–242. https://doi.org/10.1006/qres.1999.2065

Cowling, S. A., Sage, R.F., 1998. Interactive effects of low atmospheric CO_2 and

elevated temperature on growth, photosynthesis and respiration in *Phaseolus vulgaris*. Plant, Cell Environ. 21, 427–435. https://doi.org/10.1046/j.1365-3040.1998.00290.x

Cunniff, J., Jones, G., Charles, M., Osborne, C.P., 2017. Yield responses of wild C_3 and C_4 crop progenitors to subambient CO_2: A test for the role of CO_2 limitation in the origin of agriculture. Glob. Chang. Biol. 23, 380–393. https://doi.org/10.1111/gcb.13473

Dier, M., Meinen, R., Erbs, M., Kollhorst, L., Baillie, C.-K., Kaufholdt, D., Kücke, M., Weigel, H.-J., Zörb, C., Hänsch, R., Manderscheid, R., 2017. Effects of Free Air Carbon Dioxide Enrichment (FACE) on nitrogen assimilation and growth of winter wheat under nitrate and ammonium fertilization. Glob. Chang. Biol. 24, e40-e54. https://doi.org/10.1111/gcb.13819

Duursma, R.A., 2015. Plantecophys - An R package for analysing and modelling leaf gas exchange data. PLoS One 10, e0143346. https://doi.org/10.1371/journal.pone.0143346

Ehleringer, J.R., Cerling, T.E., Dearing, M.D., 2005. A History of atmospheric CO_2 and its effects on plants, animals, and ecosystems, 1st ed. Springer, Salt Lake City, UT, USA.

Elliott-Kingston, C., Haworth, M., Yearsley, J.M., Batke, S.P., Lawson, T., McElwain, J.C., 2016. Does size matter? Atmospheric CO_2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO_2. Front. Plant Sci. 7, 1253. https://doi.org/10.3389/fpls.2016.01253

Ellsworth, D.S., Reich, P.B., Naumburg, E.S., Koch, G.W., Kubiske, M.E., Smith, S.D., 2004. Photosynthesis, carboxylation and leaf nitrogen responses of 16 species to elevated pCO_2 across four free-air CO_2 enrichment experiments in forest, grassland and desert. Glob. Chang. Biol. 10, 2121–2138. https://doi.org/10.1111/j.1365-2486.2004.00867.x

Evans, R.D., 2001. Physiological mechanisms influencing plant nitrogen isotope composition. Trends Plant Sci. 6, 121–126. https://doi.org/10.1016/S1360-1385(01)01889-1

Evans, R.D., Bloom, A.J., Sukrapanna, S.S., Ehleringer, J.R., 1996. Nitrogen isotope composition of tomato (*Lycopersicon esculentum* Mill. cv. T-5) grown under ammonium or nitrate nutrition. Plant, Cell Environ. 19, 1317–1323. https://doi.org/10.1111/j.1365-3040.1996.tb00010.x

Falkowski, P.G., 1997. Evolution of the nitrogen cycle and its influence on the biological sequestration of CO_2 in the ocean. Nature 387, 272–274. https://doi.org/10.1038/nature02381.1.

Farquhar, G., O'Leary, M., Berry, J., 1982. On the relationship between carbon isotope

discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9, 121. https://doi.org/10.1071/PP9820121

Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149, 78–90. https://doi.org/10.1007/BF00386231

Fatichi, S., Leuzinger, S., Körner, C., 2014. Moving beyond photosynthesis: from carbon source to sink-driven vegetation modeling. New Phytol. 201, 1086–1095. https://doi.org/10.1111/nph.12614

Fogel, R., 1988. Interactions among soil biota in coniferous ecosystems. Agric. Ecosyst. Environ. 24, 69–85. https://doi.org/10.1016/0167-8809(88)90057-6

Foyer, C.H., Noctor, G., 2006. Photosynthetic nitrogen assimilation and associated carbon and respiratory metabolism, Vol. 12. ed. Springer Science & Business Media.

Franks, P.J., Adams, M.A., Amthor, J.S., Barbour, M.M., Berry, J.A., Ellsworth, D.S., Farquhar, G.D., Ghannoum, O., Lloyd, J., McDowell, N., Norby, R.J., Tissue, D.T., von Caemmerer, S., 2013. Sensitivity of plants to changing atmospheric CO_2 concentration: From the geological past to the next century. New Phytol. 197, 1077–1094. https://doi.org/10.1111/nph.12104

Franks, P.J., Leitch, I.J., Ruszala, E.M., Hetherington, A.M., Beerling, D.J., 2012. Physiological framework for adaptation of stomata to CO_2 from glacial to future concentrations. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367, 537–546. https://doi.org/10.1098/rstb.2011.0270

Garten, C.T., Hanson, P.J., Todd, D.E., Lu, B.B., Brice, D.J., 2008. Natural ^{15}N- and ^{13}C-abundance as indicators of forest nitrogen status and soil carbon dynamics, Stable Isotopes in Ecology and Environmental Science: Second Edition. https://doi.org/10.1002/9780470691854.ch3

Gavito, M.E., Jakobsen, I., Mikkelsen, T.N., Mora, F., 2019. Direct evidence for modulation of photosynthesis by an arbuscular mycorrhiza-induced carbon sink strength. New Phytol. nph.15806. https://doi.org/10.1111/nph.15806

Gerhart, L.M., Ward, J.K., 2010. Plant responses to low [CO_2] of the past. New Phytol. 188, 674–695. https://doi.org/10.1111/j.1469-8137.2010.03441.x

Griffin, K.L., Tissue, D.T., Turnbull, M.H., Whitehead, D., 2000. The onset of photosynthetic acclimation to elevated CO_2 partial pressure in field-grown *Pinus radiata* D. Don. after 4 years. Plant, Cell Environ. 23, 1089–1098. https://doi.org/10.1046/j.1365-3040.2000.00622.x

Hachiya, T., Sakakibara, H., 2016. Interactions between nitrate and ammonium in their uptake, allocation, assimilation, and signaling in plants. J. Exp. Bot. 68, erw449.

https://doi.org/10.1093/jxb/erw449

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil, 2nd edit. ed. Circular. California agricultural experiment stations.

Hogberg, P., 1997. ^{15}N natural abundance in soil-plant systems. New Phytol. 137, 179–203. https://doi.org/10.1046/j.1469-8137.1997.00808.x

Jasso-Chaverria, C., Hochmuth, G.J., Hochmuth, R.C., Sargent, S.A., 2005. Fruit yield, size and color responses of two greenhouse cucumber types to nitrogen fertilization in perlite soilless culture. Horttechnology 15, 565–571.

LeBauer, David S., Treseder, K.K., 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally ditributed. Ecology 89, 371–379. https://doi.org/10.1890/06-2057.1

Maherali, H., Oberle, B., Stevens, P.F., Cornwell, W.K., Mcglinn, D.J., 2016. Mutualism persistence and abandonment during the evolution of the mycorrhizal symbiosis. Am. Nat. 188, E113–E125. https://doi.org/10.5061/dryad.n8bm9

Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta 48, 1135–1140. https://doi.org/10.1016/0016-7037(84)90204-7

Monnin, E., Indermühle, A., Dällenbach, A., Flückiger, J., Stauffer, B., Stocker, T.F., Raynaud, D., Barnola, J.M., 2001. Atmospheric CO_2 concentrations over the last glacial termination. Science 291, 112–4. https://doi.org/10.1126/science.291.5501.112

Norby, R.J., Warren, J.M., Iversen, C.M., Medlyn, B.E., McMurtrie, R.E., 2010. CO_2 enhancement of forest productivity constrained by limited nitrogen availability. Proc. Natl. Acad. Sci. U. S. A. 107, 19368–73. https://doi.org/10.1073/pnas.1006463107

Pace, G M, Volk, R.J., Jackson, W. a, 1990. Nitrate reduction in response to CO_2-limited photosynthesis: Relationship to carbohydrate supply and nitrate reductase activity in maize seedlings. Plant Physiol. 92, 286–92. https://doi.org/10.1104/pp.92.2.286

Perry, L., Michael Quigg, J., 2011. Starch remains and stone boiling in the Texas Panhandle part II: Identifying Wildrye (*Elymus* spp.). Plains Anthropol. 56, 109–119. https://doi.org/10.1179/pan.2011.011

Petit, R.J., Raynaud, D., Basile, I., Chappellaz, J., Ritz, C., Delmotte, M., Legrand, M., Lorius, C., Pe, L., 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399, 429–413. https://doi.org/10.1038/20859

Pinto, H., Sharwood, R.E., Tissue, D.T., Ghannoum, O., 2014. Photosynthesis of C_3, C_3–

C_4, and C_4 grasses at glacial CO_2. J. Exp. Bot. 65, 3669–3681. https://doi.org/10.1093/jxb/eru155

Polley, H.W., Johnson, H.B., Marino, B.D., Mayeux, H.S., 1993. Increase in C_3 plant water-use efficiency and biomass over glacial to present CO_2 concentrations. Nature 361, 61–64. https://doi.org/10.1038/361061a0

Post, D.M., 2002. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology 83, 703–718. https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2

R Core Team, 2013. R: A Language and Environment for Statistical Computing.

Rachmilevitch, S., Cousins, A.B., Bloom, A.J., 2004. Nitrate assimilation in plant shoots depends on photorespiration. Proc. Natl. Acad. Sci. 101, 11506–11510.

Reich, P.B., Hobbie, S.E., Lee, T., Ellsworth, D.S., West, J.B., Tilman, D., Knops, J.M.H., Naeem, S., Trost, J., 2006. Nitrogen limitation constrains sustainability of ecosystem response to CO_2. Nature 440, 922–925. https://doi.org/10.1038/nature04486

Ripley, B.S., Cunniff, J., Osborne, C.P., 2013. Photosynthetic acclimation and resource use by the C_3 and C_4 subspecies of *Alloteropsis semialata* in low CO_2 atmospheres. Glob. Chang. Biol. 19, 900–910. https://doi.org/10.1111/gcb.12091

Robinson, D., 2001. δ^{15}N as an integrator of the nitrogen. Trends Ecol. Evol. 16, 153–162. https://doi.org/10.1016/s0169-5347(00)02098-x

Royer, D.L., 2001. Stomatal density and stomatal index as indicators of paleoatmospheric CO_2 concentration. Rev. Palaeobot. Palynol. 114, 1–28. https://doi.org/10.1016/S0034-6667(00)00074-9

Sage, R.F., 1995. Was low atmospheric CO_2 during the Pleistocene a limiting factor for the origin of agriculture? Glob. Chang. Biol. 1, 93–106. https://doi.org/10.1111/j.1365-2486.1995.tb00009.x

Sage, Rowan F, Cowling, S.A., 1999. Implications of stress in low CO_2 atmospheres of the past: Are today's plants too conservative for a high CO_2 world? in: Luo, Y., Mooney, H.A. (Eds.), Carbon Dioxide and Environmental Stress. Academic Press, San Diego, CA, USA, pp. 289–304.

Schneider, C. a, Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

Stevenson, F.J., Stevenson, E.J., Cole, M.A., 1999. Cycles of soils: carbon, nitrogen, phosphorus, sulfur, micronutrients. John Wiley & Sons, Ltd.

Tcherkez, G., Farquhar, G.D., 2006. Viewpoint : Isotopic fractionation by plant nitrate

reductase, twenty years later. Funct. Plant Biol. 33, 531. https://doi.org/10.1071/FP05284

Temme, A.A., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2013. Meta-analysis reveals profound responses of plant traits to glacial CO_2 levels. Ecol. Evol. 3, 4525–4535. https://doi.org/10.1002/ece3.836

Temme, A.A., Liu, J.C., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2015. Winners always win: Growth of a wide range of plant species from low to future high CO_2. Ecol. Evol. 5, 4949–4961. https://doi.org/10.1002/ece3.1687

Tissue, D., Griffin, K., Thomas, R., Strain, B., 1995. Effects of low and elevated CO_2 on C_3 and C_4 annuals -II. Photosynthesis and leaf biochemistry. Oecologia 101, 21–28.

Tyson, R. V., Simonne, E.H., Davis, M., Lamb, E.M., White, J.M., Treadwell, D.D., 2007. Effect of nutrient solution, nitrate-nitrogen concentration, and pH on nitrification rate in perlite medium. J. Plant Nutr. 30, 901–913. https://doi.org/10.1080/15226510701375101

Ward, J.K., Antonovics, J., Thomas, R.B., Strain, B.R., 2000. Is atmospheric CO_2 a selective agent on model C_3 annuals? Oecologia 123, 330–341. https://doi.org/10.1007/s004420051019

Ward, J.K., Strain, B.R., 1997. Effects of low and elevated CO_2 partial pressure on growth and reproduction of *Arabidopsis thaliana* from different elevations. Plant, Cell Environ. 20, 254–260. https://doi.org/10.1046/j.1365-3040.1997.d01-59.x

Wardle, D.A., 1992. A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. Biol. Rev. 67, 321–358. https://doi.org/10.1111/j.1469-185X.1992.tb00728.x

Yoneyama, T., Handley, L.L., Scrimgeour, C.M., Fisher, D.B., Raven, J.A., 1997. Variations of the natural abundances of nitrogen and carbon isotopes in *Triticum aestivum*, with special reference to phloem and xylem exudates. New Phytol. 137, 205–213.

Yoneyama, T., Omata, T., Nakata, S., Yazaki, J., 1991. Fractionation of nitrogen isotopes during the uptake and assimilation of ammonia by plants. Plant Cell Physiol. 32, 1211–1217.

Zazula, Grant D., Schweger, C.E., Beaudoin, A.B., McCourt, G.H., 2006. Macrofossil and pollen evidence for full-glacial steppe within an ecological mosaic along the Bluefish River, eastern Beringia. Quat. Int. 142–143, 2–19. https://doi.org/10.1016/J.QUAINT.2005.03.010

Zazula, G. D., Telka, A.M., Harington, C.R., Schweger, C.E., Mathewes, R.W., 2006. New Spruce (*Picea* spp.) macrofossils from Yukon territory: Implications for late Pleistocene refugia in Eastern Beringia. Arctic 59, 391–400.

Chapter 3

3 Plant-mycorrhizal interactions differ between past low CO_2 conditions and current CO_2 concentrations

3.1 Abstract

The association between plants and arbuscular mycorrhizal fungi has been one of the most important symbioses on earth in the recent geological past. While much is uncertain regarding the impact of future elevated CO_2 on plant-mycorrhizal interactions, even less is known about how this association functioned in the past, when CO_2 concentrations were far below modern values. Similarly, whether mycorrhizal associations can shift plant C and N isotope composition at low CO_2 conditions of the past is currently unknown despite the importance of isotope data in paleo-reconstructions. To address these knowledge gaps, I grew *Elymus canadensis* at CO_2 conditions representative of the Last Glacial Maximum (180 ppm), pre-Industrial Revolution (270 ppm), and current levels (400 ppm) with and without association with *Glomus intraradices*. I found that the mycorrhizal association decreased root biomass and [C] of leaves and roots across all CO_2 concentrations, but net photosynthetic rates increased with growth CO_2 more strongly in control than mycorrhizal plants. Plants grown at 180 ppm CO_2 relied on the mycorrhizae to enhance N assimilation, resulting in a ^{15}N-depletion of 2.7‰ in mycorrhizal *versus* control plants, a shift in N isotope composition that could confound trophic level discrimination. Our results show that a mycorrhizal association that does not enhance N assimilation and leads to reduced photosynthetic rates at current CO_2 levels does not have the same effects at the low CO_2 conditions of the past. Additionally, paleo-reconstructions of ancient food webs based on δ^{15}N can be affected by whether an animal consumed leaves and(or) roots of mycorrhizal plants.

3.2 Introduction

The symbiosis between plants and arbuscular mycorrhizal fungi is arguably one of the most important and ancient symbioses on earth, having evolved more than 460 million years ago (Cairney, 2000; Smith and Read, 2008). Approximately 80 % of all known land plants can establish mycorrhizal associations, with about 74 % of angiosperms species being known to associate with arbuscular mycorrhizae (Brundrett, 2009; Wang and Qiu, 2006). In this association, the arbuscular fungi increase plant access to soil nutrients, especially phosphorus and nitrogen, by acquiring nutrients with their hyphae, extending the range of root foraging (Govindarajulu *et al.*, 2005; Smith and Read, 2008). In exchange, plants provide the fungi with carbohydrates, where up to 20% of photosynthetically-fixed carbon can be transferred to the mycorrhizae (Jakobsen and Rosendahl, 1990; Wright *et al.*, 1998a). Although this association is commonly seen as beneficial to plant development and growth (Van Der Heijden, 2004; Wright *et al.*, 1998b), its mutualistic benefits are uncertain in future climatic scenarios, where atmospheric CO_2 concentrations are predicted to continue rising above current levels (Mohan *et al.*, 2014). Studies investigating plant-mycorrhizal interactions at elevated CO_2 have found conflicting results. In some studies, arbuscular mycorrhizal colonization increases under high CO_2 (Lovelock *et al.*, 1996; Rillig *et al.*, 1999), in others, colonization varies depending on species and growth environment (Gamper *et al.*, 2004; Klironomos *et al.*, 1998; Rillig *et al.*, 1998; Tang *et al.*, 2009), and yet in others, mycorrhizal colonization is unaffected by CO_2 enrichment (Allen *et al.*, 2005; Clark *et al.*, 2009). This variability in responses regarding enhanced or decreased mycorrhizal association is also observed in ectomycorrhizal fungi colonizing plants grown at elevated CO_2 (Lukac *et al.*, 2003; Parrent *et al.*, 2006; Parrent and Vilgalys, 2007).

While the impact of elevated CO_2 on plant-mycorrhizal interactions is unclear, it is even less certain how this association functioned in the recent geological past, when CO_2 concentrations were far lower than modern values (Petit *et al.*, 1999). For ~400,000 years before the Industrial Revolution, CO_2 concentrations were below 270 ppm (Augustin *et al.*, 2004), with CO_2 concentrations as low as 180 ppm by the end of the Last Glacial Maximum, about 20,000 years ago (Petit *et al.*, 1999). Such circumstances raise the

question of whether plant traits that evolved under low CO_2 conditions may still influence how plants respond to current increasing CO_2 concentrations (Becklin *et al.*, 2017; Franks *et al.*, 2013; Gerhart and Ward, 2010). As low CO_2 availability reduces photosynthesis and carbohydrate production (Cowling and Sykes, 1999; Tissue *et al.*, 1995), the capacity of mycorrhizal plants to allocate carbon to the fungi may have been reduced in the past. The association could potentially shift from mutualist to parasitic if the C costs of the association to plants exceeds the net benefit being provided by fungal nutrient uptake (Johnson *et al.*, 1997). Becklin *et al.* (2016) present the only study that investigated mycorrhizal association across a range of CO_2 concentrations, including sub-ambient CO_2, and found that the arbuscular mycorrhizal association stimulated growth in *Taraxacum officinale* at elevated CO_2, but suppressed it at low CO_2. In the same study, however, *Taraxacum ceratophorum* responded positively to the mycorrhizal association at all CO_2 concentrations.

The scarcity of data on the subject, and the variable effects of CO_2 on mycorrhizal colonization, not only challenges our understanding of plant-fungal interactions in the past, but also has implications for paleobiology studies that reconstruct ancient environmental dynamics based on plant material through techniques such as stable isotopes. Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope compositions of ancient plant samples are commonly used as indicators to identify plant physiological features such as photosynthetic pathway and net CO_2 assimilation rates, as well as to derive past environmental conditions and establish the isotopic baseline of food webs (Boecklen *et al.*, 2011; Cui and Schubert, 2016; Hare *et al.*, 2018; Reichgelt and D'Andrea, 2019; Tahmasebi *et al.*, 2018, 2017). Arbuscular mycorrhizal association shifts the $\delta^{13}C$ of plants grown at current CO_2 conditions by reducing discrimination against ^{13}C, an effect that has been associated with increased water use efficiency (Querejeta *et al.*, 2003, 2006). Mycorrhizal association also changes the N isotope composition of plants tissues, as the organic N transferred from the fungus to the plant is depleted of ^{15}N (Hobbie *et al.*, 2005). This depletion mostly occurs due to enzymatic reactions within the fungi producing ^{15}N-depleted amino acids, especially arginine, which are transferred to the plant host (Cruz *et al.*, 2007; Hobbie *et al.*, 1999; Hobbie and Högberg, 2012). While only one study observed that low CO_2 effects on plant growth can be influenced by

mycorrhizal association (Becklin *et al.*, 2016), the degree to which mycorrhizal associations can shift plant isotope composition at low CO_2 conditions of the past is currently unknown.

When grown under C-limited conditions, one scenario is that plants may adopt a more conservative C-use strategy that would result in reduced carbohydrate allocation to mycorrhizal fungi, thus restricting the mutualistic benefits of the association. In this scenario discrimination against ^{13}C in roots of mycorrhizal low CO_2-grown plants may not be different from non-mycorrhizal plants, as the carbohydrate flow to the roots would not be enhanced by the presence of the mycorrhizae. The $\delta^{15}N$ of mycorrhizal plants would also be similar to non-mycorrhizal plants, as a weaker mycorrhizal association would limit the N provided to the plant host by the fungi, or discrimination against ^{15}N may decrease in mycorrhizal compared to non-mycorrhizal plants if the symbionts compete for N.

In a second scenario, under low CO_2 conditions, plants may more closely associate with mycorrhizae, thus enhancing access to soil N. The extra N can then be invested in the photosynthetic apparatus, increasing photosynthetic rates and carbohydrate production in this C-limited environment. If the second scenario were to happen however, discrimination against ^{13}C may be less than observed in ambient CO_2-grown plants, as C assimilation would be up-regulated in low CO_2 plants to meet the demand for growth and C allocation to the fungi. In this case, plant $\delta^{15}N$ may become more negative than non-mycorrhizal plants grown at low CO_2, as a greater provision of N by the fungi allows for greater discrimination against ^{15}N.

To test these hypotheses, I grew *Elymus canadensis* at CO_2 conditions representative of the Last Glacial Maximum (~180 ppm), pre-Industrial Revolution (~270 ppm), and current levels (~400 ppm). *Elymus canadensi*s was chosen since the presence of this genus is well-recorded during the LGM and was likely an important resource for ancient humans (Zazula *et al.*, 2006; Perry and Quigg 2011). We then inoculated *E. canadensis* with *Glomus intraradices*, which is a model fungus in arbuscular mycorrhizal research

and known to be highly effective in mobilizing N (Govindarajulu *et al.*, 2005; Miller and Cramer, 2004).

3.3 Methods

3.3.1 Experimental design and treatments

The experiment was replicated twice in time using three growth chambers (GCW15, Environmental Growth Chambers, Chagrin Falls, OH, USA). In each chamber, CO_2 concentrations were maintained to represent current ambient CO_2 (AC, ~400 ppm), pre-industrial low CO_2 (LC, ~270 ppm) and CO_2 levels like those at the end of The Last Glacial maximum (GC, ~180 ppm). Air CO_2 levels in the chambers were regulated by a CO_2 scrubbing system. The system comprised an acrylic box filled with soda lime to absorb CO_2 from the air. A CO_2 analyzer (LI-820, LI-COR, Lincoln, NE, USA) coupled with a DIN process controller (Omega CN7633, Omega Engineering, Stamford, CT, USA) was connected to fans attached to the box's lid. CO_2 concentrations in the chambers averaged 400.1 ± 0.6 ppm, 269.3 ± 1.1, and 180.9 ± 1.0 (means ± standard deviation) for the AC, LC and GC treatments, respectively. Chambers were maintained at a relative humidity of 74 ± 5%, photosynthetically active radiation (PAR) of 300 ± 28 µmol photons m^{-2} s^{-1}, photoperiod of 12.5 hours, and air temperature of 16 ± 0.1 °C (means ± SE). Day length and temperature were chosen based on the average growing season conditions of the last three years at the site where the seeds were harvested (Environment Canada, Midland WPCP Station).

In each replicate experiment, twelve 2 L pots filled with sterilized soil (Pro-Mix, Premier Tech Ltda., Quebec, Canada) were placed in each chamber and planted with *Elymus canadensis* seeds (obtained from Wildflower Farm, Coldwater, ON, Canada - 44°654'241" N, 79°558'969" E). Once plants were 30 days old, the seedlings were removed from the pots and weighed. Half of the seedlings in each chamber were then inoculated with the arbuscular mycorrhizal fungus *Glomus intraradices*, and all plants were replanted in their original pots. The other half of the plants were not inoculated and

served as control. The fungal strain was provided by the Agriculture and Agri-Food Canada (AAFC) branch at Ottawa from the Glomeromycetes in vitro Collection (GINCO). The fungus was propagated on modified Strullu-Romand medium (Declerck *et al.*, 1998). Pots were rotated weekly within each chamber, and plants were watered three times per week and fertilized weekly with a half-strength Hoagland's recipe (Hoagland and Arnon, 1950). Once the first replicate of the experiment was finished, the experiment was repeated, rotating the CO_2 treatments between the growth chambers.

3.3.2 Biomass

Ninety days after inoculation, plants were harvested and weighed to assess changes in fresh biomass after inoculation. Relative growth rate (RGR) was then calculated as (Hoffmann and Poorter, 2002):

$$RGR = \frac{ln(W_2) - ln(W_1)}{t_2 - t_1}$$

where W_1 is the fresh weight on the day the plants were inoculated ($t_1 = 1$), and W_2 is the fresh weight at harvest ($t_2 = 90$). All plants were then divided into roots and shoots to determine the masses of these separate tissues and dried at 60 °C to a constant mass, and weighed.

3.3.3 Fungal colonization

After fresh biomass was assessed, two-centimeter sections of twelve fine roots from both the control and mycorrhizal treatments (12 roots from 12 plants) were analyzed for fungal infection using the grid line method (Giovannetti and Mosse, 1980). Before staining, roots were cleared as per Nylund *et al.* (1982) through consecutive baths in KOH, bleach in H_2O_2, HCl, and deionized water. The roots were then stained with 0.05 % Trypan blue diluted in lactophenol (Phillips and Hayman, 1970). Presence and absence of infection

was recorded and expressed as a percentage (Giovannetti and Mosse, 1980). The control plants were also assessed to ensure that accidental inoculation did not occur.

3.3.4 Isotopes, C and N concentration

For measurements of $\delta^{13}C$, $\delta^{15}N$, and C and N concentrations, fully mature leaves and sections containing entire roots (from the root crown to the tip) were harvested from each plant, dried at 60 °C to constant mass, and ground. The isotopic abundance was measured using a Delta V^{Plus} isotope-ratio mass-spectrometer (IRMS; Thermo Scientific, Bremen, Germany) coupled in continuous flow mode with an elemental analyzer (Costech Analytical Technologies, Valencia, CA, USA). The C and N isotope results from the plant material are presented in the usual delta notation ($\delta^{13}C$ and $\delta^{15}N$) calibrated to VPDB and AIR, respectively:

$$\delta(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{5tandard}}\right)$$

where R is the ratio between the heavy to light isotope for the sample (R_{sample}) and the standard ($R_{standard}$).

The $\delta^{13}C$ of all tissue samples were adjusted to a common value for the atmospheric CO_2 (see Appendix C). The measured $\delta^{13}C$ of the chamber CO_2 varied slightly from chamber to chamber, likely because of fractionation during interaction with the soda lime used to control CO_2 levels. The current global average $\delta^{13}C$ of atmospheric CO_2 (–8.4‰, NOAA/ESRL, https://www.esrl.noaa.gov/gmd/dv/data/) was used as the common value. The difference between the common and measured value for each chamber/treatment was used to adjust the measured $\delta^{13}C$ of each plant tissue. This approach is similar to the use of the Suess correction (Keeling, 1979) to adjust the carbon isotope composition of modern plants to pre-Industrial Revolution (AD 1850) values.

The C and N elemental and isotope analyses of the plant tissues were performed in separate analytical sessions, and for both, the standards USGS40 (glutamic acid; accepted mass fraction of C = 40.80 ‰, accepted mass fraction of N = 9.52 ‰; accepted $\delta^{13}C$ = –

26.39 ‰, accepted $\delta^{15}N$ = –4.52 ‰) and USGS41a (glutamic acid; accepted $\delta^{13}C$ = +36.55 ‰, accepted $\delta^{15}N$ = +47.55 ‰) were used. Sample reproducibility was ± 0.07 ‰ for $\delta^{15}N$ and ± 0.05 % gDW^{-1} for N concentration (26 replicates). Sample reproducibility for $\delta^{13}C$ was ± 0.05 ‰ and for C concentration, ± 2.37 % gDW^{-1} (30 replicates). Concerning tests for accuracy, a $\delta^{13}C$ of –24.01 ± 0.02 ‰ (accepted value = –24.04 ‰), and a $\delta^{15}N$ of +6.40 ± 0.02 ‰ (accepted value = +6.40 ‰) were obtained for 77 analyses of an internal laboratory keratin standard.

To collect chamber-air CO_2 in the chambers, septum-sealed vials were first evacuated and then opened and left open in the chambers overnight; the vials were then sealed the next morning just before measurements. The C isotope composition of atmospheric CO_2 was measured using a GasBench II (Thermo Scientific, Bremen, Germany) connected in continuous flow mode to a Delta V^{Plus} XL IRMS (Thermo Scientific, Bremen, Germany), using an autosampler (Combi Pal - CTC Anaytics Gmbh, Switzerland) to deliver the atmospheric CO_2 from the septum-sealed sampling vials to the GasBench II. Because of the low CO_2 concentration of CO_2 in the chamber-air samples for chambers having 180 ppm CO_2, the gas was cryogenically frozen as it was collected from the GasBench II for delivery to the IRMS. This allowed sufficient CO_2 to be collected for reliable isotopic analysis. The standard CO_2 against which the sample CO_2 was compared was also diluted to concentrations similar to the samples. Sample reproducibility was ± 0.31 ‰ for 38 replicates, and a $\delta^{13}C$ of –10.34 ± 0.04 ‰ (accepted value = –10.40 ‰) was obtained for 44 analyses of carbon dioxide carbon isotope reference gas (Ozteck Trading Corporation, Safford, AZ, USA).

3.3.5 Gas exchange measurements

Net CO_2 assimilation rates at growth CO_2 (A_{growth}) and dark respiration at 400 ppm (R_{dark}) were measured 90 days after the fungal inoculation using a portable photosynthesis system (Walz GFS-3000, Effeltrich, Germany). A_{growth} was measured on two fully mature leaves at the base of the blade crown of each plant. Cuvette conditions included a 16 °C leaf temperature, 750 µmol s^{-1} flow rate, and light conditions of 1200 µmol m^{-2} s^{-1}, which

was established as light-saturated based on light-response curves (data not shown); A_{growth} was measured once gas exchange was stable under the cuvette conditions. After the A_{growth} measurements, CO_2 in the cuvette was changed, when necessary, to 400 ppm, lights were turned off, and plants were dark-acclimated for at least 20 minutes until a stable R_{dark} was achieved for R_{dark} measurements.

3.3.6 Statistical analysis

The effect of growth CO_2, fungal inoculation and their interaction was tested with two-way ANOVAs. Where treatment effects were significant ($p \leq 0.05$), Tukey's HSD was used to identify differences among the treatments. Results are shown as means ± standard error. Data analysis was performed in R 3.2.1 (R Core Team, 2013).

3.4 Results

CO_2 effects were observed in both leaf, root, and total biomass at harvest, with higher biomass in AC plants compared to plants grown at sub-ambient CO_2. While there was no inoculation effect on leaf biomass, root biomass was on average 19 % lower in the mycorrhizal treatment (Table 3.1, Figure 3.1). The RGR 90 days after fungal inoculation in the mycorrhizal treatment showed that plants in each CO_2 treatment grew at the same rate as non-inoculated plants (Figure 3.1). In the mycorrhizal treatment, root colonization was negatively correlated with growth CO_2. Root colonization by the mycorrhizal fungi was 35.09 ± 2.79 % (means ± sd) in AC plants, 45.65 ± 6.42 % of LC roots were colonized, and 63.58 ± 2.16 % of GC roots were colonized by the mycorrhizae. Thus, root colonization was 30 % and 81 % higher in LC and GC plants, respectively, compared to AC plants. Colonization was not observed in the control treatment.

While $[C]_{leaf}$ varied with growth CO_2, with lower CO_2 concentrations resulting in higher $[C]_{leaf}$ compared to ambient CO_2, $[C]_{root}$ was unaffected by growth CO_2 (Table 3.1, Figure 3.2). In addition, regardless of growth CO_2, the association with mycorrhizae decreased

[C] in both leaves and roots by ~10 % compared to control plants. Association with mycorrhizal fungi lowered the carbon isotope composition values in leaves of LC and GC plants, but not AC plants in comparison to leaves of control plants (Table 3.1, Figure 3.2, Appendix B). This increased discrimination against ^{13}C in sub-ambient CO_2-grown plants resulted in more negative $\delta^{13}C$ in leaves of GC and LC plants (a reduction of 1 ‰ and 3.4 ‰, respectively; Figure 3.2). While mycorrhizal association also resulted in more negative $\delta^{13}C$ in roots, this effect varied depending on growth CO_2. The decrease in $\delta^{13}C$ values in mycorrhizal compared to control plants was small and non-significant in GC plants, but significant in both LC (2.5 ‰) and AC plants (1.2 ‰).

Table 3.1. Analysis of variance results testing growth CO_2, mycorrhizal inoculation, and their interaction term on plant traits. Carbon isotope signature of leaves ($\delta^{13}C_{leaf}$) and roots ($\delta^{13}C_{root}$), and carbon concentration in leaves and roots ($[C]_{leaf}$, $[C]_{root}$, respectively); nitrogen isotope composition of leaves ($\delta^{15}N_{leaf}$) and roots ($\delta^{15}N_{root}$) and nitrogen concentration in leaves and roots ($[N]_{leaf}$, $[N]_{root}$, respectively); net CO_2 assimilation rates at growth CO_2 (A_{growth}); dark respiration measured at 400 ppm CO_2 (R_{dark}). The *p*-values are shown with significant effects highlighted in bold ($p \leq 0.05$).

	$[CO_2]$	Inoculation	$[CO_2]$ x inoculation
Leaf biomass	**<0.001**	0.086	0.239
Root biomass	**0.004**	**0.029**	0.403
Total biomass	**<0.001**	**0.010**	0.127
Relative growth rate	0.120	0.807	0.182
Root colonization	**<0.001**		
$\delta^{13}C_{leaf}$	**<0.001**	**<0.001**	**<0.001**
$\delta^{13}C_{root}$	0.073	**<0.001**	**0.011**
$[C]_{leaf}$	**0.048**	**<0.001**	0.503
$[C]_{root}$	0.584	**<0.001**	0.233
$\delta^{15}N_{leaf}$	**0.002**	0.098	0.134
$\delta^{15}N_{root}$	**0.028**	0.158	**<0.001**
$[N]_{leaf}$	**<0.001**	**0.022**	**0.005**
$[N]_{root}$	**0.002**	**<0.001**	**0.007**
A_{growth}	**<0.001**	0.173	**0.007**
R_{dark}	**<0.001**	**0.008**	**0.036**

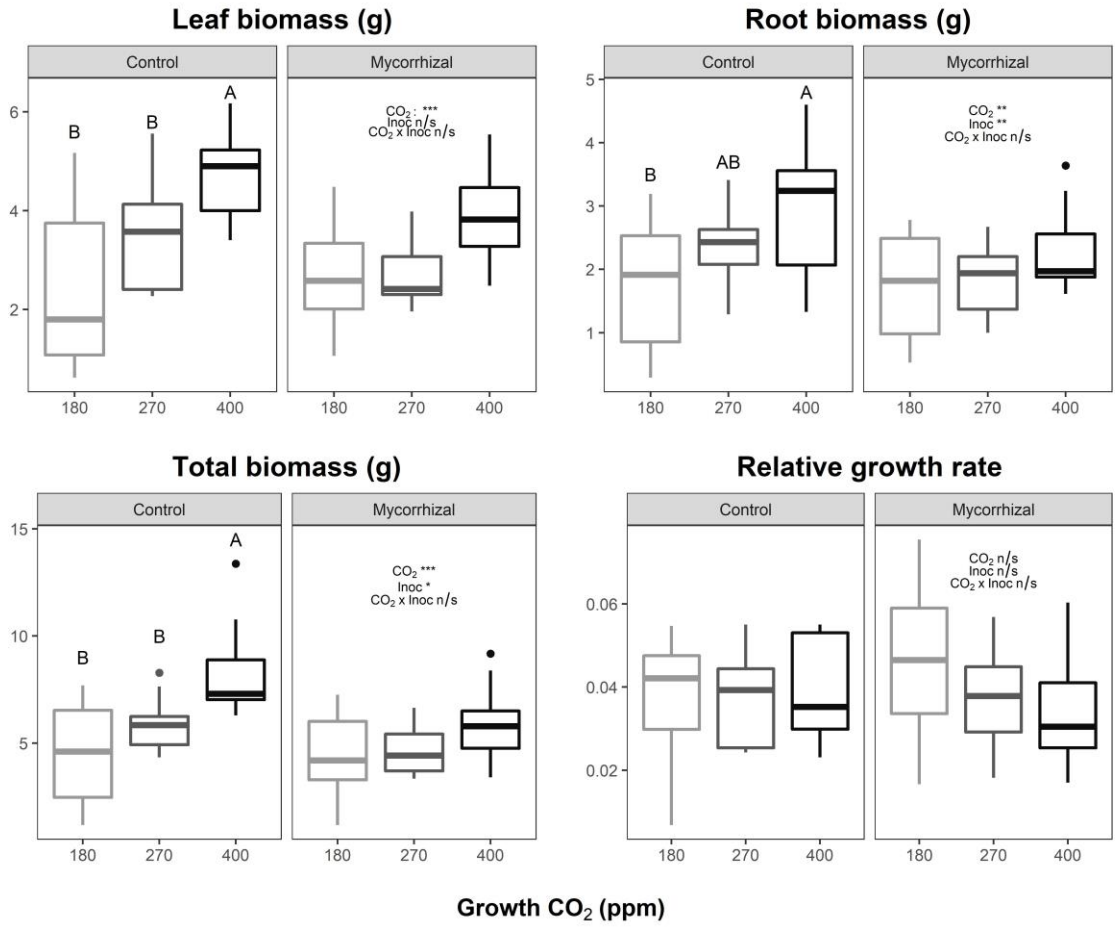

Figure 3.1. Leaf, root, and total biomass, and relative growth rate. Upper-case letters (shown only for the control data) represent differences between growth CO_2 when there is no significant interaction term; levels of significance for CO_2 and inoculation effects and their interaction term are given as: * if $p \leq 0.05$, ** if $p \leq 0.01$, and *** if $p \leq 0.001$. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 12.

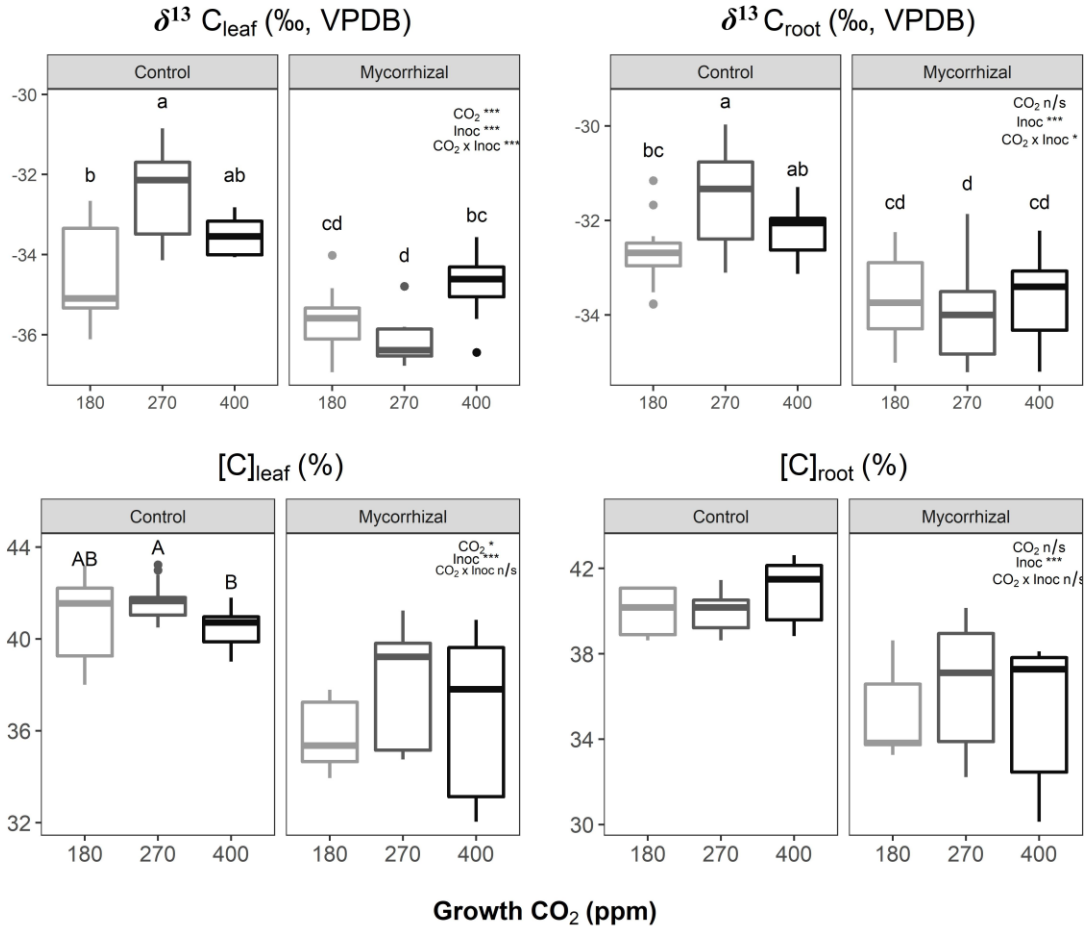

Figure 3.2. Variations in carbon isotope composition and concentration. Carbon isotope signature of leaves ($\delta^{13}C_{leaf}$) and roots ($\delta^{13}C_{root}$), and leaf and root C concentration ($[C]_{leaf}$ and $[C]_{root}$) Levels of significance for CO_2 and inoculation effects and their interaction term are given as: * if $p \leq 0.05$, ** if $p \leq 0.01$, and *** if $p \leq 0.001$. Where the interaction term was significant, differences between groups are represented by lower-case letters. Upper-case letters (shown only for the control data) represent differences between growth CO_2 concentrations when there is no significant interaction term. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 12.

Association with mycorrhizae in GC plants resulted in increased [N] in both leaves and roots in comparison to control plants and the other growth CO_2 treatments (Table 3.1, Figure 3.3). While LC and AC plants had similar [N] in the control and mycorrhizal treatments, mycorrhizal GC plants had 16 % and 49 % higher [N] in leaves and roots, respectively, in comparison to control GC plants. While $\delta^{15}N_{leaf}$ was unaffected by the mycorrhizal treatment, $\delta^{15}N_{root}$ was influenced by the interaction between growth CO_2 and fungal colonization (Figure 3.3). This effect was particularly strong in roots of GC plants, where there was a depletion of ^{15}N by 2.7 ‰ in mycorrhizal compared to control plants.

Net photosynthetic rates increased with growth CO_2 (A_{growth}) as A_{growth} was higher in AC and LC than GC plants (Table 3.1, Figure 3.4). The interaction between mycorrhizal colonization and growth CO_2 affected A_{growth}, such that rising CO_2 stimulated A_{growth} more strongly in control than mycorrhizal plants. Effects of the interaction between growth CO_2 and mycorrhizal association in dark respiration (R_{dark}) were mainly present in AC plants, where R_{dark} rates were 53 % lower in mycorrhizal than control plants. (Figure 3.4).

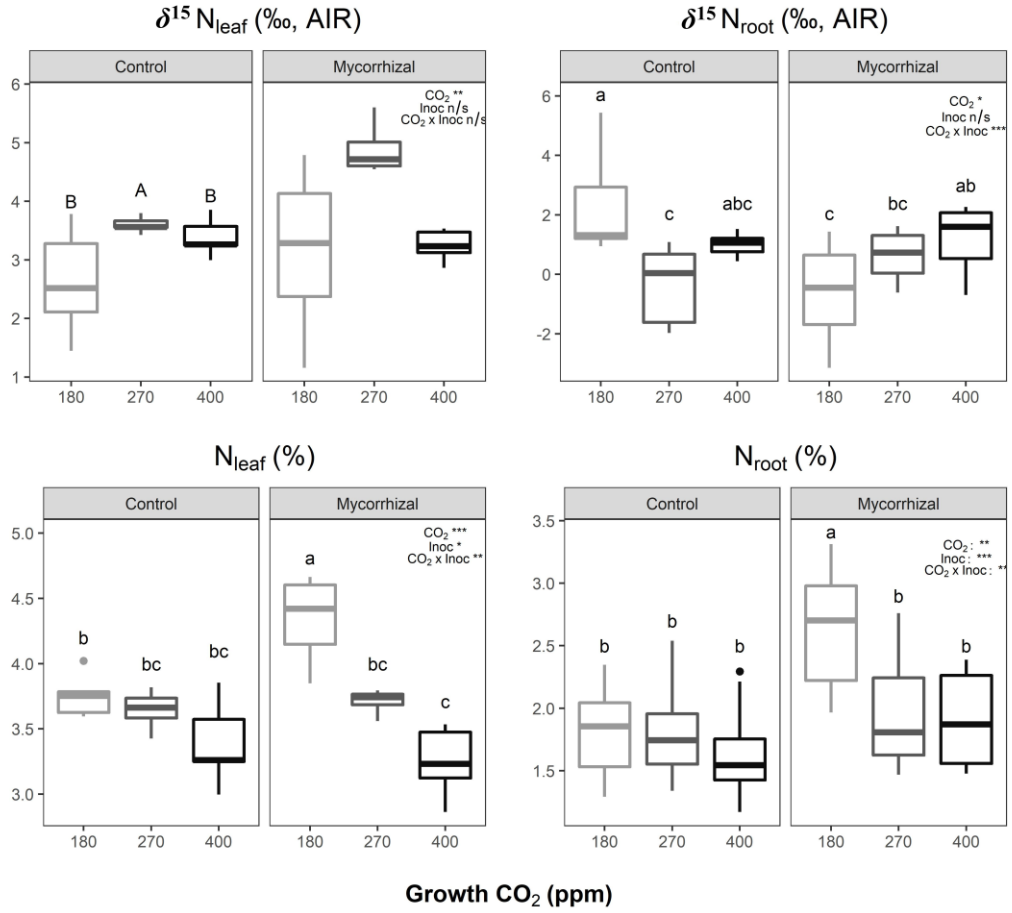

Figure 3.3. Nitrogen isotope composition and nitrogen concentration. N isotope composition of leaves ($\delta^{15}N_{leaf}$) and roots ($\delta^{15}N_{root}$), and leaf and root N concentrations ($[N]_{leaf}$ and $[N]_{root}$). Levels of significance for CO_2 and inoculation effects and their interaction term are given as: * if $p \leq 0.05$, ** if $p \leq 0.01$, and *** if $p \leq 0.001$. Where the interaction term was significant, differences between groups are represented by lower-case letters. Upper-case letters (shown only on the control data) represent differences between growth CO_2 concentrations when there is no significant interaction term. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 12.

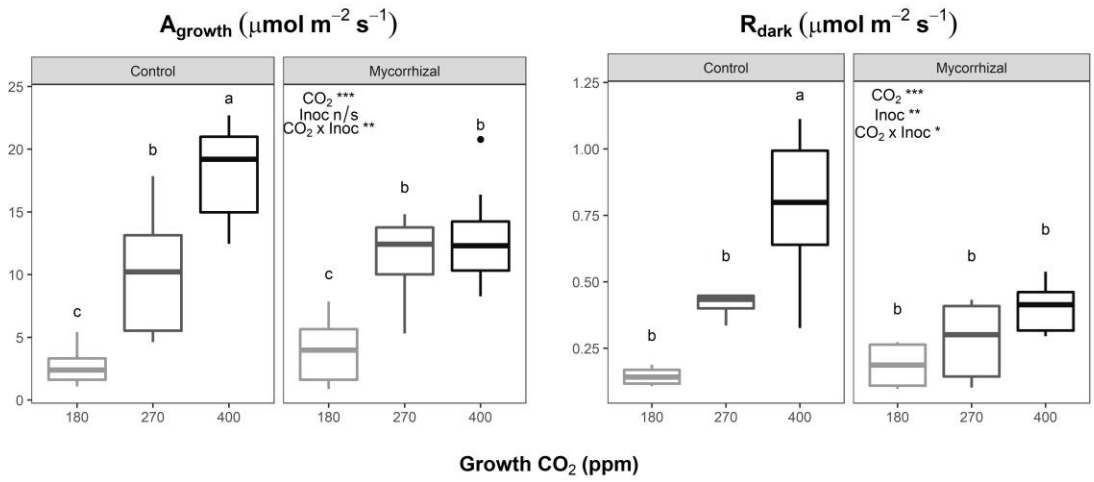

Figure 3.4. Net CO_2 assimilation (A) and dark respiration (R_{dark}) rates. A measured at growth CO_2 (A_{growth}) and dark respiration measured at 400 ppm (R_{dark}). Levels of significance for CO_2 and inoculation effects and their interaction term are given as: * if $p \leq 0.05$, ** if $p \leq 0.01$, and *** if $p \leq 0.001$. Differences between groups are represented by lower-case letters considering a significant interaction term for $p \leq 0.05$. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n=12.

3.5 Discussion

I investigated whether low CO_2 conditions of the past alter mycorrhizal colonization, and the impact of mycorrhizal association on plant growth, leaf carbon fluxes, and tissue C and N isotope composition. I found that the mycorrhizal association increased N access for *E. canadensis* grown at 180 ppm: GC plants with mycorrhizae had higher leaf and root [N] than control GC plants, despite having similar leaf and root mass. The stronger impact of mycorrhizae on the N economy of GC plants was associated with a greater degree of root colonization. There was no evidence, however, that the higher colonization rates in GC plants reduced plant carbon availability (as might be expected with a stronger C sink) compared to mycorrhizal LC and AC plants. In contrast, mycorrhizal inoculation dampened the effect of rising CO_2 on leaf gas exchange and plant growth, such that A_{growth} and R_{dark} were similar between LC and AC mycorrhizal plants, as were growth increments between all CO_2 levels in mycorrhizal plants.

3.5.1 GC plants associated with mycorrhizae benefited from higher [N]

The establishment of mycorrhizal associations has been shown to enhance N assimilation in certain plant species (*e.g.*, He *et al.*, 2003; Hodge and Fitter, 2010; Tobar *et al.*, 1994), and the carbon supplied by the plant host can trigger changes in fungal gene expression that stimulate N uptake and transport by the mycorrhizal fungi (Fellbaum *et al.*, 2012). Furthermore, enhanced N uptake through mycorrhizal associations can be a strategy to ameliorate environmental stresses such as low soil fertility (Karst *et al.*, 2008) and water stress (Saia *et al.*, 2014; Tobar *et al.*, 1994). For instance, Subramanian and Charest (1998) found that under drought stress, the activity of key enzymes involved in N assimilation was higher in *Zea mays* colonized by *G. intraradices* than in non-inoculated plants. In the same way, our data indicate that *E. canadensis* relied on *G. intraradices* to enhance N assimilation as a strategy to cope with the C-limitation being imposed by the growth environment. Mycorrhizal association reduced plant C investment in root tissue, as both root biomass and $[C]_{root}$ decreased in mycorrhizal plants in all CO_2 treatments.

Since mycorrhizal fungi complement root functions with regard to accessing soil nutrients (Newsham *et al.*, 1995; Van Der Heijden, 2004), plants appear to have allocated C to the fungi in exchange for these functions at the expense of root growth (Cui and Caldwell, 1996; Rapparini *et al.*, 1994; Saravesi *et al.*, 2014; Smith and Gianinazzi-Pearson, 1990). However, GC plants seem to have benefited the most from this exchange in terms of N provisioning. Both $[N]_{root}$ and $[N]_{leaf}$ in GC plants were higher in mycorrhizal compared to control plants, while $[N]_{root}$ and $[N]_{leaf}$ in LC and AC plants were unaltered by mycorrhizal inoculation. This is also supported by the observation that GC plants had the highest percentage of colonized roots among the CO_2 treatments. Conversely, LC and AC plants, grown in environments where C was less limiting than that experienced by GC plants, did not benefit from increased N uptake through the mycorrhizal association. Under less stressful growth conditions where the necessary soil nutrients are readily available, such as the well-fertilized soils used in this experiment, the mycorrhizal association can present little benefit for plant growth; growth rates can be even reduced due to the carbon demand of the mycorrhizal fungi (Eissenstat *et al.*, 1993; Smith *et al.*, 2009).

3.5.2 AC plants showed little benefit from mycorrhizae

Mycorrhizal association did not promote greater N acquisition or biomass increment in AC plants, and it actually reduced leaf carbon fluxes, such that A_{growth} and R_{dark} were lower in AC mycorrhizal than AC control plants. The interaction between growth CO_2 and inoculation influenced both A_{growth} and R_{dark}. While this response to growth CO_2 is expected given the differences in the availability of substrate for photosynthesis, the plateau in A_{growth} in mycorrhizal AC plants shows that *E. canadensis* contrasts with many species where photosynthesis is stimulated by mycorrhizal association in plants grown at ambient or elevated CO_2 (Amaya-Carpio *et al.*, 2009; Augé *et al.*, 2016; Gavito *et al.*, 2019; Kaschuk *et al.*, 2009; Miller *et al.*, 2002). How much mycorrhizae influence R_{dark} is less well-studied, although comparisons of R_{dark} between mycorrhizal and non-mycorrhizal plants have shown that differences may or may not occur depending on

species, stage growth, and interactions with other environmental conditions (Colpaert *et al.*, 1996; Del-Saz *et al.*, 2017; Fahey *et al.*, 2016; Romero-Munar *et al.*, 2017).

Nevertheless, our results show that a mycorrhizal association that does not enhance plant N tissue concentrations and that leads to reduced A_{growth} and R_{dark} under current CO_2 levels (*i.e.*, AC plants) need not have the same effects at low CO_2 conditions. Thus, the benefits for plants from this mutualistic association may have varied over time as CO_2 levels in the atmosphere increased, while potentially affecting plant N dynamics and carbon fluxes.

3.5.3 Mycorrhizal association shifts C and N isotope compositions at low CO_2

The use of C and N isotope composition of plant tissues has several applications, including investigating plant development in past environments and providing a baseline for reconstructing ancient food webs (Boecklen *et al*., 2011; Casey and Post, 2011; Hare *et al*., 2018; Lipp *et al*., 1991; Tahmasebi *et al*., 2018, 2017). Similar to previous reports (Badeck *et al.*, 2005; Cernusak *et al.*, 2009; Pardo *et al.*, 2013), we found that the C isotope composition of leaves and roots differ in their isotopic composition, with leaves having more negative $\delta^{13}C$. At low CO_2 conditions, however, the mycorrhizal association generally altered this difference by increasing the discrimination against ^{13}C and ^{15}N in leaves and roots.

The observation that discrimination against ^{13}C was unchanged in roots of mycorrhizal GC plants compared to the other CO_2 treatments is most likely the result of the higher degree of association that GC plants established with the mycorrhizal fungi. Since this association is based on C and nutrient exchange between the two symbiotic organisms, the enhanced N access provided by the fungi to the plants would stimulate larger exports of C-based compounds from the plant to the fungus. Thus, plants would use more of the C resources available, including ^{13}C. By comparison, leaves of mycorrhizal GC plants presented a higher discrimination against ^{13}C, and consequently a more negative C

isotope signature, in comparison to non-mycorrhizal GC plants. As leaves of mycorrhizal GC plants did not show enhanced growth, and consequently C use for biomass production, there was no reduction in discrimination against ^{13}C. Therefore, how the presence of the mycorrhizae differently affected $\delta^{13}C$ signals in roots and leaves adds to the complexity when using C isotopes to identify photosynthetic pathways (*i.e.*, C_3, C_4, CAM) and past climate conditions such as temperature, CO_2 levels, and light intensity (Cernusak *et al.*, 2013; Grocke, 2007; O'Leary, 1981). In addition, the average $\delta^{13}C$ in roots and leaves of *E. canadensis* was –33.7 ‰, which is notably more negative than the average $\delta^{13}C$ values for C_3 species of –27 ‰ (Michener and Lajtha, 2007). However, $\delta^{13}C$ values in C_3 species can range between –37 ‰ and –20 ‰ (Sharp, 2007), and previous works have found similar low $\delta^{13}C$ values for this species (Still *et al*., 2003; Weremijewicz *et al*., 2018).

Plant $\delta^{15}N$ is commonly used in paleobiology studies to identify animal diet and trophic position (Boecklen *et al.*, 2011), and although isotope trophic discrimination factors can vary depending on the organisms being studied, an enrichment of 3.4 ‰ in $\delta^{15}N$ is considered the standard difference between trophic levels (Minagawa and Wada, 1984; Post, 2002). There are instances, however, where the trophic discrimination factor between plants and primary consumers is relatively small or there is a depletion of ^{15}N (*e.g*., Perkins *et al.*, 2014; Post, 2002; Yoneyama *et al.*, 1997). For example, Holá *et al.* (2015) found that the N trophic discrimination factor between *Zea mays* and *Sus scrofa* was –2.3 ‰. Thus, the depletion of ^{15}N found in roots of mycorrhizal GC plants (–2.7 ‰) compared to control GC plants shows that mycorrhizal fungi can change the N isotope composition of roots to an extent that could be mistaken for a trophic level change in an animal whose diet largely comprised such tissues. This depletion of ^{15}N in mycorrhizal GC plants is not unexpected considering that plants discriminate more against ^{15}N when the N pool available is relatively large (Evans, 2001), but to our knowledge, this has never been demonstrated before.

While colonization by the mycorrhizae decreased root $\delta^{15}N$ in GC plants, $\delta^{15}N_{leaf}$ was unchanged by the mycorrhizal association. Despite enhanced [N] in both leaves and roots of mycorrhizal GC plants, different from roots, there was no increase in discrimination

against ^{15}N in leaves. It is possible that, in an effort to mitigate the effects of their C-limiting environment, GC plants may have drawn more widely on the greater N pool available in leaves to invest in the photosynthetic apparatus. GC plants, however, did not present enhanced CO_2 assimilation, as A_{growth} was similar in both non- and mycorrhizal plants. Thus, plants in both treatments may have been operating at the maximum photosynthetic capacity allowed at such CO_2-limited environment. Besides, even if not photosynthetically active, structures such as Rubisco and other proteins can serve as N storages for the greater N pool present in the leaves (Cooke and Weih, 2005; Millard, 1988; Staswick, 1994).

There are three major implications of these observations. First, paleo-reconstructions based on $\delta^{15}N$ can be confounded if there is no knowledge whether an animal consumed either or both of leaves and roots of mycorrhizal plants. Second, low-CO_2 ecosystems may have had more extensive mycorrhizal associations than seen in current CO_2 levels. Third, mycorrhizal plants growing at low CO_2 conditions of the past may have presented a higher nutritional value to herbivores due to their higher [N]. Such observations warrant future studies of possible herbivore preference for mycorrhizal plants in the past and its implication for N transfer between trophic levels and resulting N isotope composition.

We suggest that future studies using C and N isotope of ancient plant material should consider whether the species being studied commonly forms mycorrhizal associations, and if possible, examine the material for structural fungal vestiges. Several lists identifying mycorrhizal plant species are available (Brundrett, 2009, 2002; Öpik *et al.*, 2006; Wang and Qiu, 2006), as well as several records and methods of fungal identification in fossilized plant samples (*e.g.*, Allen *et al.*, 2006; Krings *et al.*, 2013; Strullu-Derrien and Strullu, 2007; Taylor *et al.*, 2015). Finally, the significance of mycorrhizal associations within this context might be time- and species-dependent, as plant-fungal associations may have been established and abandoned over time (Maherali *et al.*, 2016).

3.6 References

Allen, M.F., Klironomos, J.N., Treseder, K.K., Oechel, W.C., 2005. Responses of soil biota to elevated CO_2 in a chaparral ecosystem. Ecol. Appl. 15, 1701–1711. https://doi.org/10.1890/03-5425

Allen, N., Nordlander, M., Mcgonigle, T., Basinger, J., Kaminskyj, S., 2006. Arbuscular mycorrhizae on Axel Heiberg Island (80°N) and at Saskatoon (52°N) Canada. Can. J. Bot. 84, 1094–1100.

Amaya-Carpio, L., Davies, F.T., Fox, T., He, C., 2009. Arbuscular mycorrhizal fungi and organic fertilizer influence photosynthesis, root phosphatase activity, nutrition, and growth of *Ipomoea carnea* ssp. *fistulosa*. Photoshenthica 47, 1–10.

Augé, R.M., Toler, H.D., Saxton, A.M., 2016. Mycorrhizal stimulation of leaf gas exchange in relation to root colonization, shoot size, leaf phosphorus and nitrogen: a quantitative analysis of the literature using meta-regression. Front. Plant Sci. 7, 1084. https://doi.org/10.3389/fpls.2016.01084

Augustin, L., Barbanate, C., Barnes, P.R.F., Barnola, J.M., Bigler, M., Castellano, E., Cattani, O., Chappellaz, J., Dahl-Jensen, D., Delmonte, B., et al., 2004. Eight glacial cycles from an Antarctic ice core EPICA community members. Nature 429, 623–628.

Badeck, F.-W., Tcherkez, G., Nogués, S., Piel, C., Ghashghaie, J., 2005. Post-photosynthetic fractionation of stable carbon isotopes between plant organs—a widespread phenomenon. Rapid Commun. Mass Spectrom. 19, 1381–1391. https://doi.org/10.1002/rcm.1912

Becklin, K.M., Mullinix, G.W.R., Ward, J.K., 2016. Host plant physiology and mycorrhizal functioning shift across a glacial through future [CO_2] Gradient. Plant Physiol. 172, 789–801. https://doi.org/10.1104/pp.16.00837

Becklin, K.M., Walker, S.M., Way, D.A., Ward, J.K., 2017. CO_2 studies remain key to understanding a future world. New Phytol. 214, 34–40. https://doi.org/10.1111/nph.14336

Boecklen, W.J., Yarnes, C.T., Cook, B.A., James, A.C., 2011. On the use of stable isotopes in trophic ecology. Annu. Rev. Ecol. Evol. Syst. 42, 411–440. https://doi.org/10.1146/annurev-ecolsys-102209-144726

Brundrett, M.C., 2009. Mycorrhizal associations and other means of nutrition of vascular plants: Understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320, 37-77. https://doi.org/10.1007/s11104-008-9877-9

Brundrett, M.C., 2002. Coevolution of roots and mycorrhizas of land plants. New Phytol. 154, 275–304. https://doi.org/10.1046/j.1469-8137.2002.00397.x

Cairney, J.W.G., 2000. Evolution of mycorrhiza systems. Naturwissenschaften 87, 467–475. https://doi.org/10.1007/s001140050762

Casey, M.M., Post, D.M., 2011. The problem of isotopic baseline: Reconstructing the diet and trophic position of fossil animals. Earth-Science Rev. 106, 131–148. https://doi.org/10.1016/J.EARSCIREV.2011.02.001

Cernusak, L.A., Tcherkez, G., Keitel, C., Cornwell, W.K., Santiago, L.S., Knohl, A., Barbour, M.M., Williams, D.G., Reich, P.B., Ellsworth, D.S., Dawson, T.E., Griffiths, H.G., Farquhar, G.D., Wright, I.J., 2009. Why are non-photosynthetic tissues generally ^{13}C enriched compared with leaves in C_3 plants? Review and synthesis of current hypotheses. Funct. Plant Biol. 36, 199. https://doi.org/10.1071/FP08216

Cernusak, L.A., Ubierna, N., Winter, K., Holtum, J.A.M., Marshall, J.D., Farquhar, G.D., 2013. Environmental and physiological determinants of carbon isotope discrimination in terrestrial plants. New Phytol. 200, 950–965. https://doi.org/10.1111/nph.12423

Clark, N.M., Rillig, M.C., Nowak, R.S., 2009. Arbuscular mycorrhizal fungal abundance in the Mojave Desert: Seasonal dynamics and impacts of elevated CO_2. J. Arid Environ. 73, 834–843. https://doi.org/10.1016/J.JARIDENV.2009.03.004

Colpaert, J. V, van Laere, A., van Assche, J.A., 1996. Carbon and nitrogen allocation in ectomycorrhizal and non-mycorrhizal *Pinus sylvestris* L. seedlings. Tree Physiol. 16, 787–793.

Cooke, J.E.K., Weih, M., 2005. Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. New Phytol. 167, 19–30. https://doi.org/10.1111/j.1469-8137.2005.01451.x

Cowling, S. a, Sykes, M.T., 1999. Physiological significance of low atmospheric CO_2 for plant–climate interactions. Quat. Res. 52, 237–242. https://doi.org/10.1006/qres.1999.2065

Cowling, S. A., Sage, R.F., 1998. Interactive effects of low atmospheric CO_2 and elevated temperature on growth, photosynthesis and respiration in *Phaseolus vulgaris*. Plant, Cell Environ. 21, 427–435. https://doi.org/10.1046/j.1365-3040.1998.00290.x

Cruz, C., Egsgaard, H., Trujillo, C., Ambus, P., Requena, N., Martins-Loução, M.A., Jakobsen, I., 2007. Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhizal fungi. Plant Physiol. 144, 782–792. https://doi.org/10.1104/pp.101.3.865

Cui, M., Caldwell, M.M., 1996. Facilitation of plant phosphate acquisition by arbuscular mycotrhizas from enriched soil patches. I. Roots and hyphae exploiting the same soil volume. New Phytol. 133, 453–460. https://doi.org/10.1111/j.1469-

8137.1996.tb01912.x

Cui, Y., Schubert, B.A., 2016. Quantifying uncertainty of past pCO_2 determined from changes in C_3 plant carbon isotope fractionation. Geochim. Cosmochim. Acta 172, 127–138. https://doi.org/10.1016/J.GCA.2015.09.032

Declerck, S., Strullu, D.G., Plenchette, C., 1998. Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: A proposed methodology for germplasm collection. Mycologia 90, 579. https://doi.org/10.2307/3761216

Del-Saz, N.F., Romero-Munar, A., Alonso, D., Aroca, R., Baraza, E., Flexas, J., Ribas-Carbo, M., 2017. Respiratory ATP cost and benefit of arbuscular mycorrhizal symbiosis with *Nicotiana tabacum* at different growth stages and under salinity. J. Plant Physiol. 218, 243–248. https://doi.org/10.1016/j.jplph.2017.08.012

Eissenstat, D.M., Graham, J.H., Syvertsen, J.P., Drouillard, D.L., 1993. Carbon economy of sour orange in relation to mycorrhizal colonization and phosphorus status. Ann. Bot. 71, 1–10. https://doi.org/10.1006/ANBO.1993.1001

Evans, R.D., 2001. Physiological mechanisms influencing plant nitrogen isotope composition. Trends Plant Sci. 6, 121–126. https://doi.org/10.1016/S1360-1385(01)01889-1

Fahey, C., Winter, K., Slot, M., Kitajima, K., 2016. Influence of arbuscular mycorrhizal colonization on whole-plant respiration and thermal acclimation of tropical tree seedlings. Ecol. Evol. 6, 859–870. https://doi.org/10.1002/ece3.1952

Fellbaum, C.R., Gachomo, E.W., Beesetty, Y., Choudhari, S., Strahan, G.D., Pfeffer, P.E., Kiers, E.T., Bücking, H., 2012. Carbon availability triggers fungal nitrogen uptake and transport in arbuscular mycorrhizal symbiosis. Proc. Natl. Acad. Sci. 109, 2666–2671. https://doi.org/10.1073/PNAS.1118650109

Franks, P.J., Adams, M.A., Amthor, J.S., Barbour, M.M., Berry, J.A., Ellsworth, D.S., Farquhar, G.D., Ghannoum, O., Lloyd, J., McDowell, N., Norby, R.J., Tissue, D.T., von Caemmerer, S., 2013. Sensitivity of plants to changing atmospheric CO_2 concentration: From the geological past to the next century. New Phytol. 197, 1077–1094. https://doi.org/10.1111/nph.12104

Gamper, H., Peter, M., Jansa, J., Luscher, A., Hartwig, U.A., Leuchtmann, A., 2004. Arbuscular mycorrhizal fungi benefit from 7 years of free air CO_2 enrichment in well-fertilized grass and legume monocultures. Glob. Chang. Biol. 10, 189–199. https://doi.org/10.1111/j.1529-8817.2003.00734.x

Gavito, M.E., Jakobsen, I., Mikkelsen, T.N., Mora, F., 2019. Direct evidence for modulation of photosynthesis by an arbuscular mycorrhiza-induced carbon sink strength. New Phytol. nph.15806. https://doi.org/10.1111/nph.15806

Gerhart, L.M., Ward, J.K., 2010. Plant responses to low [CO_2] of the past. New Phytol.

188, 674–695. https://doi.org/10.1111/j.1469-8137.2010.03441.x

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring mycorrhizal infections in roots. New Phytol. 84, 489–500. https://doi.org/10.1111/j.1469-8137.1980.tb04556.x

Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., Bücking, H., Lammers, P.J., Shachar-Hill, Y., 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435, 819–823. https://doi.org/10.1038/nature03610

Grocke, D.R., 2007. Carbon-isotope analyses of fossil plants as a chemostratigraphic and palaeoenvironmental tool. Lethaia 31, 1–13. https://doi.org/10.1111/j.1502-3931.1998.tb00482.x

Hare, V.J., Loftus, E., Jeffrey, A., Ramsey, C.B., 2018. Atmospheric CO_2 effect on stable carbon isotope composition of terrestrial fossil archives. Nat. Commun. 9, 252. https://doi.org/10.1038/s41467-017-02691-x

He, X.-H., Critchley, C., Bledsoe, C., 2003. Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). CRC. Crit. Rev. Plant Sci. 22, 531–567. https://doi.org/10.1080/713608315

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil, 2nd edit. ed. Circular. California agricultural experiment stations.

Hobbie, E.A., Ari, A.E., Ae, J., Trappe, J., 2005. Foliar and fungal ^{15}N:^{14}N ratios reflect development of mycorrhizae and nitrogen supply during primary succession: testing analytical models. Oecologia 146, 258–268. https://doi.org/10.1007/s00442-005-0208-z

Hobbie, E.A., Högberg, P., 2012. Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. New Phytol. 196, 367–382. https://doi.org/10.1111/j.1469-8137.2012.04300.x

Hobbie, E.A., Macko, S.A., Shugart, H.H., 1999. Insights into nitrogen and carbon dynamics of ectomycorrhizal and saprotrophic fungi from isotopic evidence. Oecologia 118, 353–360. https://doi.org/10.1007/s004420050736

Hodge, A., Fitter, A.H., 2010. Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. Proc. Natl. Acad. Sci. 107, 13754–13759. https://doi.org/10.1073/pnas.1005874107

Holá, M., Ježek, M., Kušta, T., Košatová, M., 2015. Trophic discrimination factors of stable carbon and nitrogen isotopes in hair of corn fed wild boar. PLoS One 10, e0125042. https://doi.org/10.1371/journal.pone.0125042

Jakobsen, I., Rosendahl, L., 1990. Carbon flow into soil and external hyphae from roots

of mycorrhizal cucumber plants, New Phytol.

Johnson, N.C., Graham, J.H., Smith, F.A., 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytolist 135, 575–585.

Karst, J., Marczak, L., Jones, M.D., Turkington, R., 2008. The mutualism–parasitism continuum in ectomycorrhizas: a quantitative assessment using meta-analysis. Ecology 89, 1032–1042. https://doi.org/10.1890/07-0823.1

Kaschuk, G., Kuyper, T.W., Leffelaar, P.A., Hungria, M., Giller, K.E., 2009. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? Soil Biol. Biochem. 41, 1233–1244. https://doi.org/10.1016/J.SOILBIO.2009.03.005

Klironomos, J.N., Ursic, M., Rillig, M., Allen, M.F., 1998. Interspecific differences in the response of arbuscular mycorrhizal fungi to *Artemisia tridentata* grown under elevated atmospheric CO_2. New Phytol. 138, 599–605. https://doi.org/10.1046/j.1469-8137.1998.00141.x

Krings, M., Taylor, T.N., Dotzler, N., 2013. Fossil evidence of the zygomycetous fungi. Persoonia 30, 1–10. https://doi.org/10.3767/003158513X664819

Lipp, J., Trimborn, P., Fritz, P., Moser, H., Becker, B., Frenzel, B., 1991. Stable isotopes in tree ring cellulose and climatic change. Tellus B 43, 322–330. https://doi.org/10.1034/j.1600-0889.1991.t01-2-00005.x

Lovelock, C.E., Kyllo, D., Winter, K., 1996. Growth responses to vesicular-arbuscular mycorrhizae and elevated CO_2 in seedlings of a tropical tree, *Beilschmiedia pendula*. Funct. Ecol. 10, 662–667.

Lukac, M., Calfapietra, C., Godbold, D.L., 2003. Production, turnover and mycorrhizal colonization of root systems of three *Populus* species grown under elevated CO_2 (POPFACE). Glob. Chang. Biol. 9, 838–848. https://doi.org/10.1046/j.1365-2486.2003.00582.x

Maherali, H., Oberle, B., Stevens, P.F., Cornwell, W.K., Mcglinn, D.J., 2016. Mutualism persistence and abandonment during the evolution of the mycorrhizal symbiosis. Am. Nat. 188, E113–E125. https://doi.org/10.5061/dryad.n8bm9

Millard, P., 1988. The accumulation and storage of nitrogen by herbaceous plants, Plant, Cell and Environment 11, 1-8.

Miller, R.M., Miller, S.P., Jastrow, J.D., Rivetta, C.B., 2002. Mycorrhizal mediated feedbacks influence net carbon gain and nutrient uptake in *Andropogon gerardii*. New Phytol. 155, 149–162. https://doi.org/10.1046/j.1469-8137.2002.00429.x

Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta

48, 1135–1140. https://doi.org/10.1016/0016-7037(84)90204-7

Mohan, J.E., Cowden, C.C., Baas, P., Dawadi, A., Frankson, P.T., Helmick, K., Hughes, E., Khan, S., Lang, A., Machmuller, M., Taylor, M., Witt, C.A., 2014. Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecol. 10, 3–19. https://doi.org/10.1016/j.funeco.2014.01.005

Newsham, K.K., Fitter, A.H., Watkinson, A.R., 1995. Multi-functionality and biodiversity in arbuscular mycorrhizas. Trends Ecol. Evol. 10, 407–411. https://doi.org/10.1016/S0169-5347(00)89157-0

Nylund, J.-E., Kasimir, A., Arveby, A.S., Unestam, T., 1982. Simple diagnosis of ectomycorrhiza formation and demonstration of the architecture of the Hartig net by means of a clearing technique. For. Pathol. 12, 103–107. https://doi.org/10.1111/j.1439-0329.1982.tb01379.x

O'Leary, M.H., 1981. Carbon isotope fractionation in plants. Phytochemistry 20, 553–567.

Öpik, M., Moora, M., Liira, J., Zobel, M., 2006. Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J. Ecol. 94, 778–790. https://doi.org/10.1111/j.1365-2745.2006.01136.x

Pardo, L.H., Semaoune, P., Schaberg, P.G., Eagar, C., Sebilo, M., 2013. Patterns in δ^{15}N in roots, stems, and leaves of sugar maple and American beech seedlings, saplings, and mature trees. Biogeochemistry 112, 275–291. https://doi.org/10.1007/s10533-012-9724-1

Parrent, J.L., Morris, W.F., Vilgalys, R., 2006. CO_2-enrichment and nutrient availability alter ectomycorrhizal fungal communities. Ecology 87, 2278–2287. https://doi.org/10.1890/0012-9658(2006)87[2278:CANAAE]2.0.CO;2

Parrent, J.L., Vilgalys, R., 2007. Biomass and compositional responses of ectomycorrhizal fungal hyphae to elevated CO_2 and nitrogen fertilization. New Phytol. 176, 164–174. https://doi.org/10.1111/j.1469-8137.2007.02155.x

Perkins, M.J., Robbie, A.M., van Frank, F.J., Kelly, S.D., Rees, G., Bearhop, S., 2014. Aplication of nitrogen and carbon stable isotopes (δ^{15}N and δ^{13}C) to quantify food chain length and trophic structure. PLoS One 9, e93281.

Perry, L., Michael Quigg, J., 2011. Starch remains and stone boiling in the Texas Panhandle part II: Identifying Wildrye (*Elymus* spp.). Plains Anthropol. 56, 109–119. https://doi.org/10.1179/pan.2011.011

Petit, R.J., Raynaud, D., Basile, I., Chappellaz, J., Ritz, C., Delmotte, M., Legrand, M., Lorius, C., Pe, L., 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399, 429–413. https://doi.org/10.1038/20859

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 158-IN18.

Post, D.M., 2002. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology 83, 703–718. https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2

Querejeta, J.I., Allen, M.F., Caravaca, F., Roldán, A., 2006. Differential modulation of host plant $\delta^{13}C$ and $\delta^{18}O$ by native and nonnative arbuscular mycorrhizal fungi in a semiarid environment. New Phytol. 169, 379–387. https://doi.org/10.1111/j.1469-8137.2005.01599.x

R Core Team, 2013. R: A Language and Environment for Statistical Computing.

Rapparini, F., Baraldi, R., Bertazza, G., Branzanti, B., Predieri, S., 1994. Vesicular-arbuscular mycorrhizal inoculation of micropropagated fruit trees. J. Hortic. Sci. 69, 1101–1109. https://doi.org/10.1080/00221589.1994.11516551

Reichgelt, T., D'Andrea, W.J., 2019. Plant carbon assimilation rates in atmospheric CO_2 reconstructions. New Phytol. nph.15914. https://doi.org/10.1111/nph.15914

Rillig, M.C., Allen, M.F., Klironomos, J.N., Chiariello, N.R., Field, C.B., 1998. Plant species-specific changes in root-inhabiting fungi in a California annual grassland: responses to elevated CO_2 and nutrients. Oecologia 113, 252–259. https://doi.org/10.1007/s004420050376

Rillig, M.C., Field, C.B., Allen, M.F., 1999. Soil biota responses to long-term atmospheric CO_2 enrichment in two California annual grasslands. Oecologia 119, 572–577

Romero-Munar, A., Fernández Del-Saz, N., Ribas-Carbó, M., Flexas, J., Baraza, E., Florez-Sarasa, I., Fernie, A.R., Gulías, J., 2017. Arbuscular mycorrhizal symbiosis with *Arundo donax* decreases root respiration and increases both photosynthesis and plant biomass accumulation. Plant. Cell Environ. https://doi.org/10.1111/pce.12902

Saia, S., Amato, G., Frenda, A.S., Giambalvo, D., Ruisi, P., 2014. Influence of arbuscular mycorrhizae on biomass production and nitrogen fixation of Berseem clover plants subjected to water stress. PLoS One 9, e90738. https://doi.org/10.1371/journal.pone.0090738

Saravesi, K., Ruotsalainen, A.L., Cahill, J.F., 2014. Contrasting impacts of defoliation on root colonization by arbuscular mycorrhizal and dark septate endophytic fungi of *Medicago sativa*. Mycorrhiza 24, 239–245. https://doi.org/10.1007/s00572-013-0536-5

Smith, F.A., Grace, E.J., Smith, S.E., 2009. More than a carbon economy: Nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. New

Phytol. 182, 347–358. https://doi.org/10.1111/j.1469-8137.2008.02753.x

Smith, S., Gianinazzi-Pearson, V., 1990. Phosphate aptake and arbuscular activity in mycorrhizal *Allium cepa* L.: Effects of photon irradiance and phosphate nutrition. Funct. Plant Biol. 17, 177. https://doi.org/10.1071/PP9900177

Smith, S.E., Read, D., 2008. Mycorrhizal Symbiosis, in: Mycorrhizal Symbiosis Third Edition Introduction. Academic Press, Amsterdam, pp. 1–9

Staswick, P.E., 1994. Storage proteins of vegetative tissues. Annu. Rev. Plant Physiol. Plant Mol. BioIogy 45, 303–325.

Strullu-Derrien, C., Strullu, D.-G., 2007. Mycorrhization of fossil and living plants. Comptes Rendus Palevol 6, 483–494. https://doi.org/10.1016/J.CRPV.2007.09.006

Subramanian, K.S., Charest, C., 1998. Arbuscular mycorrhizae and nitrogen assimilation in maize after drought and recovery. Physiol. Plant. 102, 285–296. https://doi.org/10.1034/j.1399-3054.1998.1020217.x

Tahmasebi, F., Longstaffe, F.J., Zazula, G., 2018. Nitrogen isotopes suggest a change in nitrogen dynamics between the Late Pleistocene and modern time in Yukon, Canada. PLoS One 13, e0192713. https://doi.org/10.1371/journal.pone.0192713

Tahmasebi, F., Longstaffe, F.J., Zazula, G., Bennett, B., 2017. Nitrogen and carbon isotopic dynamics of subarctic soils and plants in southern Yukon Territory and its implications for paleoecological and paleodietary studies. PLoS One 12, e0183016. https://doi.org/10.1371/journal.pone.0183016

Tang, J., Xu, L., Chen, X., Hu, S., 2009. Interaction between C_4 barnyard grass and C_3 upland rice under elevated CO_2: Impact of mycorrhizae. Acta Oecologica 35, 227–235. https://doi.org/10.1016/J.ACTAO.2008.10.005

Taylor, T.N., Krings, M., Taylor, E.L., 2015. Fungal diversity in the fossil record, in: Systematics and Evolution. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 259–278. https://doi.org/10.1007/978-3-662-46011-5_10

Tissue, D., Griffin, K., Thomas, R., Strain, B., 1995. Effects of low and elevated CO_2 on C_3 and C_4 annuals -II. Photosynthesis and leaf biochemistry. Oecologia 101, 21–28

Tobar, R., Azcon, R., Barea, J.M., 1994. Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. New Phytol. 126, 119–122. https://doi.org/10.1111/j.1469-8137.1994.tb07536.x

Van Der Heijden, M.G.A., 2004. Arbuscular mycorrhizal fungi as support systems for seedling establishment in grassland. Ecol. Lett. 7, 293–303. https://doi.org/10.1111/j.1461-0248.2004.00577.x

Wright, D.P., Read, D.J., Scholes, J.D., 1998a. Mycorrhizal sink strength influences whole plant carbon balance of *Trifolium repens* L. Plant. Cell Environ. 21, 881–891. https://doi.org/10.1046/j.1365-3040.1998.00351.x

Wright, D.P., Scholes, J.D., Read, D.J., 1998b. Effects of VA mycorrhizal colonization on photosynthesis and biomass production of *Trifolium repens* L. Plant, Cell Environ. 21, 209–216. https://doi.org/10.1046/j.1365-3040.1998.00280.x

Yoneyama, T., Handley, L.L., Scrimgeour, C.M., Fisher, D.B., Raven, J.A., 1997. Variations of the natural abundances of nitrogen and carbon isotopes in *Triticum aestivum*, with special reference to phloem and xylem exudates. New Phytol. 137, 205–213

Zazula, Grant D., Schweger, C.E., Beaudoin, A.B., McCourt, G.H., 2006. Macrofossil and pollen evidence for full-glacial steppe within an ecological mosaic along the Bluefish River, eastern Beringia. Quat. Int. 142–143, 2–19. https://doi.org/10.1016/J.QUAINT.2005.03.010

Chapter 4

4 Low growth CO_2 concentrations of the past decrease plant chemical defense production

4.1 Abstract

Growth at the low atmospheric CO_2 concentrations that dominated most of the recent geological past is known to decrease plant growth. It is unknown, however, how plants balanced carbon investment between primary functions to sustain growth and the production of secondary metabolites, such as defense compounds. Here, we present the first investigation contrasting plant growth and formation of chemical defense components (resin ducts and terpenes) between plants grown at current (400 ppm) and low CO_2 concentrations representative of the Last Glacial Maximum (180 ppm). *Picea mariana* grown at low CO_2 conditions had reduced biomass as well as lower photosynthetic and respiratory rates in comparison to ambient CO_2-grown plants. Needles of low-CO_2 grown plants had reduced resin duct area, and in some cases, lacked one or both ducts. Growth at C-limited conditions also decreased the production of the major monoterpenes produced by *P. mariana*, particularly α-pinene. Thus, plants growing at past low CO_2 conditions may have had a reduced capability to deter herbivory compared to modern plants. Furthermore, the absence of resin ducts poses new complexities to using this indicator in paleo-reconstructions, as the structural characteristics of resin ducts and their number are commonly used in species identification of conifer fossils.

4.2 Introduction

Plants must balance carbon investment between primary functions to sustain growth and the production of secondary metabolites, such as defense compounds, which enhance

plant fitness (Baldwin, 2010; Hartmann, 1996; Holopainen, 2004). Carbon, and thus energy, availability for these important functions depends directly on leaf carbon fluxes (*i.e.*, photosynthesis, respiration, and photorespiration), and is therefore influenced by atmospheric CO_2 concentrations (Drake *et al.*, 1997; Dusenge *et al.*, 2018; Williams *et al.*, 2018). Since atmospheric CO_2 levels are predicted to increase in the near future (Ciais *et al.*, 2013), considerable effort has been put into understanding the effects of elevated CO_2 on plant growth and chemical defense (*e.g.*, Mohan *et al.*, 2006; Noctor and Mhamdi, 2017; Penuelas *et al.*, 1997; Zavala *et al.*, 2012). However, throughout the recent evolutionary history of plants, CO_2 concentrations have been far below the levels observed today, being as low as 180 ppm 20,000 years ago by the end of the Last Glacial Maximum (LGM; Petit *et al.*, 1999). These low CO_2 conditions that prevailed for the last millennia may still influence how plants respond to future changes in CO_2 levels (Becklin et al., 2017; Gerhart and Ward, 2010; Sage and Cowling, 1999).

By reducing substrate availability, low CO_2 environments decrease photosynthesis, while enhancing photorespiration (Sage and Coleman, 2001). Low photosynthetic rates limit plant growth and plants grown at low CO_2 have reduced biomass compared to plants grown at current CO_2 conditions (Ripley *et al.*, 2013; Sage and Cowling, 1999; Temme *et al.*, 2013). How changes in growth CO_2 affect the production of defense compounds, however, is far from fully understood. Plants grown at elevated CO_2 have shown increased (Coley *et al.*, 2002; Penuelas *et al.*, 1997), decreased (Sallas *et al.*, 2003; Vannette and Hunter, 2011), or unchanged (Constable *et al.*, 1999; Heyworth *et al.*, 1998) defense compound production compared to ambient CO_2-grown plants. While these studies provide insight into how plants may respond to future climates, so far, no studies have investigated how the production of defense compounds is affected by growth at the low CO_2 conditions of the past. Models developed by Schurgers *et al.* (2009) predicted that plant emissions of terpenes and other volatile organic compounds may have risen since the LGM as vegetation cover changed with increasing temperature and CO_2. This hypothesis, however, has not yet been tested.

Low carbon availability could suppress the production of carbon-based compounds such as terpenes, the largest family of natural plant chemicals (Langenheim, 1994), which

have antibacterial (Dorman and Deans, 2000), antifungal (Cheng *et al.*, 2004), and insect-deterrent properties (Boulogne *et al.*, 2012). As terpenes form the majority of compounds found in tree resin (Langenheim, 1990), a decline in terpene synthesis at low CO_2 might also reduce resin production and alter resin duct structure in conifers. Resin ducts are specialized anatomical structures that accumulate resin terpenes, and in conifers, their density and size can serve as indicators of chemical defense production and host resistance against herbivory and pathogens (Boucher *et al.*, 2001; Martin *et al.*, 2002; Tomlin and Borden, 1997). Resin ducts are also used to identify plant fossil samples (Falcon-Lang *et al.*, 2016; Manum *et al.*, 2000; Zidianakis *et al.*, 2016), and differences in these structures between plants grown at modern and low CO_2 levels of the past could therefore have implication for paleo-ecological inferences. For instance, Zazula *et al.* (2006) identified conifer needles more than 20,000 years-old as belonging to ancient *Picea mariana* or *P. glauca* based on whether the fossil needles contained one or two resin ducts. Furthermore, investigating the ability of plants to produce defense compounds at low CO_2 conditions could also inform us of plant susceptibility to herbivory in ancient environments. Changes in CO_2 concentrations have been hypothesized to contribute to faunal extinctions (Graham and Lundelius Jr, 1984; Guthrie, 1984, 2003), but whether rising CO_2 since the LGM could have increased plant chemical defense traits and thereby impacted herbivores by increasing food toxicity is unclear.

To evaluate how growth at past low CO_2 concentrations alters carbon allocation to growth and defense, we grew black spruce (*Picea mariana*) in current CO_2 conditions and CO_2 levels found at the end of the LGM. Black spruce was present, and has persisted, in North American forests since the LGM (Prentice et al., 1991; G. D. Zazula et al., 2006). As carbon availability is reduced at low CO_2, we assessed changes in biomass and photosynthetic potential, as we expected a decline in the ability of low CO_2-grown plants to fix carbon via photosynthesis. We hypothesized that decreased photosynthesis would limit terpene defense production, which would be reflected in reduced resin duct area in low CO_2 needles compared to ambient CO_2-grown plants.

4.3 Methods

4.3.1 Experimental design and plant material

Four growth chambers (GCW15, Environmental Growth Chambers, Chagrin Falls, OH, USA) were divided into two CO_2 treatments: in two chambers, CO_2 concentrations were maintained at current, ambient conditions (AC), and the other two chambers were set at low CO_2 levels (LC). CO_2 concentrations averaged 400 ± 0.6 ppm in AC chambers and 184 ± 1.1 ppm in LC chambers (means ± SE). CO_2 levels in the chambers were regulated by a CO_2 scrubbing system consisting of an acrylic box filled with soda lime to absorb CO_2 from the air. Fans were attached to the box's lid and controlled by a CO_2 analyzer (LI-820, LI-COR, NB, USA) coupled with a DIN process controller (Omega CN7633, Omega Engineering, QC, Canada). Environmental conditions in the growth chambers were maintained at a relative humidity of 73 ± 0.3 %, photosynthetically active radiation (PAR) of 284 ± 0.2 µmol photons m^{-2} s^{-1}, a photoperiod of 14 hours, day air temperature of 21 ± 0.1 °C, and night temperatures of 12 ± 0.1 °C (means ± SE). Day length and air temperatures were chosen to emulate average growing season conditions of the last three years at the site where seeds were collected (obtained from Environment Canada, Gore Bay Climate Station).

Black spruce (*Picea mariana)* seeds were obtained from the Canadian National Tree Seed Centre (from seed zone 27; 46.250000 °E, -82.50000 °N) and sown in 2-gallon pots filled with soil (Pro-Mix BX Mycorrhizae, Premier Tech Horticulture, Rivière-du-Loup, QC, Canada). Each chamber contained eighteen pots that were rotated weekly to minimize intra-chamber effects. Plants were watered three times per week (200 ml per pot) and fertilized weekly using a half-strength Hoagland's recipe (Hoagland and Arnon, 1950). Once the plants had grown for six months, I measured gas exchange, biomass, leaf microscopy, and terpene composition.

4.3.2 Gas exchange measurements

Net CO_2 assimilation rates (A_{net}) at growth CO_2 (A_{growth}) and at 400 ppm CO_2 (A_{400}), as well as stomatal conductance at growth CO_2 and at 400 ppm CO_2 ($g_{s\text{-}growth}$ and $g_{s\text{-}400}$, respectively), were measured on 5-6 seedlings per chamber per replicate using a portable photosynthesis system (Walz GFS-3000, Effeltrich, Germany). The response of A_{net} to intercellular CO_2 concentrations (C_i) was also measured to derive maximum Rubisco carboxylation rates (V_{cmax}) and maximum electron transport rates (J_{max}), following the photosynthetic model of Farquhar *et al.* (1980). Response curves were initiated after equilibrating the leaves for approximately 10 minutes at a CO_2 concentration of 400 µmol mol^{-1}, which was then changed to 300, 180, 100, 60, 400, 600, 800, 1000, 1200, 1500, and back to 400 µmol mol^{-1}. Before being measured, leaves were maintained at each CO_2 level until a stable A_{net} was achieved. Cuvette conditions included a 21 °C leaf temperature, 750 µmol s^{-1} flow rate, a relative humidity of 60 %, and an irradiance of 1400 µmol m^{-2} s^{-1}, which was established as light-saturated based on light-response curves (data not shown). Gas exchange values were corrected for the actual leaf area measured by harvesting the needles inside the cuvette, photographing them, and estimating their projected-leaf area using ImageJ 1.48v (Schneider *et al.*, 2012). All gas exchange data are presented on a projected leaf area basis. Values of V_{cmax} and J_{max} were calculated in R 3.2.1 (R Core Team, 2013) using the package '*plantecophys*' (Duursma, 2015). Once the CO_2 response curves were finished, the cuvette lights were switched off, while maintaining the remaining cuvette conditions, and plants were left to acclimate to dark conditions for at least 20 minutes until a stable A_{net} was achieved in order to measure dark respiration (R_d).

4.3.3 Biomass

Needles, stems, and roots of 5-6 seedlings per chamber per replicate were separated and dried at 60 °C until a constant mass was achieved. Needle surface area was estimated

from digital images (ImageJ 1.48v - Schneider *et al*., 2012). The ratio of leaf area to leaf dry weight was used to calculate specific leaf area (SLA).

4.3.4 Resin duct microscopy

At harvest, a subsample of needles was immediately fixed for microscopy analysis of resin ducts from 5-6 seedlings per chamber per replicate. In small plants from the LC treatment that had few needles, all needles were harvested, while in seedlings with abundant needles, needles were randomly harvested at different heights along the main stem. Needles were stored in 4 % paraformaldehyde buffered with 0.2 M phosphate containing 0.3 M sucrose, and refrigerated. After a week, needles were rinsed in phosphate buffer, and trimmed using a razor blade such that only the middle section (approximately 5 mm in length) of the needle remained. Samples were processed by soaking in 95 % ethanol and 5 % methanol solutions, and fixed with Paraplast X-tra wax (Sigma-Aldrich, Oakville, ON).

The paraffin-embedded needles were trimmed into 10 μm-thick sections using a rotary microtone and mounted on Superfrost Plus poly-L-lysine-coated slides (Thermo Fisher Scientific, Waltham, MA, USA). Slides were deparaffinized in a series of xylene baths, rehydrated, and equilibrated to Invitrogen phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA, USA). Samples were stained with a 0.001 % solution of Streptavidin Alexa Fluor 633 fluorescent stain (Thermo Fisher Scientific, Waltham, MA, USA) and a Calcofluor White/Evans blue dye counterstain (Sigma Aldrich, Oakville, ON). Visualization and estimation of resin duct lumen area were done using an AxioImager Z1 Upright Fluorescence Microscope (Carl Zeiss Ltd., North York, ON).

4.3.5 Monoterpene and diterpene analysis

Fully mature needles were collected from all seedlings (5-6 seedlings per chamber per replicate), immediately frozen in liquid nitrogen and stored at -80 °C until analysis. For extraction, needles were cut with a razor blade into ~2 mm pieces, and extracted with

methyl-tert-butyl ether MTBE, containing 5 µg mL^{-1} dichlorodehydroabietic acid and 50 µg mL^{-1} isobutyl benzene as internal standards, for 16 h at room temperature. After extraction, needle tissue was removed by centrifugation, and the extract was split in two and transferred into a vial for monoterpene analysis and a vial for diterpene analysis as described by Robert *et al.*, (2010). The monoterpene fraction was washed with 0.3 mL of 100 ammonium carbonate $(NH_4)_2CO_3$, (pH 8.0). The diterpene fraction was derivatized with 2 mM trimethylsilyldiazomethane in diethyl ether (Sigma Aldrich, Oakville, ON).

Monoterpenes were analyzed on an Agilent HP-5 column (5 % phenyl methyl siloxane, diameter = 250 µm, length = 30 m, and film thickness = 0.25 µm) at 0.8 mL min^{-1} He on an Agilent 6890A system GC, Agilent 7683 series GC Sampler, and an Agilent 5973 Mass Selective Detector at 70 eV. The GC temperature program was as follows: 40 °C for 4 min, increase at 6 °C min^{-1} to 90 °C, followed by increase at 9 °C min^{-1} to 310 °C and hold for 4 min with split injections at a ratio 5:1 an injector held at 240 °C. Diterpenes were analyzed using an Agilent AT-1000 column at 0.9 mL min^{-1} helium on an Agilent 7890A system GC, Agilent GC Sampler 80, and a 7000A GC/MS triple quad M5975C inert XL MSD with triple axis detector at 70 eV. The GC temperature program was as follows: 150 °C for 1 min, increase at 0.6 °C min^{-1} to 180 °C, 25 °C min^{-1} to 240 °C, hold for 5 min with pulsed splitless injector held at 250 °C.

Integration of the peaks from the GC-MS was performed using Hewlett Packard Chemstation software. Relative amounts of monoterpenes and diterpenes were calculated by comparing the integrated peak area with the area of the internal standards, followed by normalization by the amount of tissue used in each extraction. Identification of terpenes was based on comparison of retention times and mass spectra with authentic standards or with mass spectra in the Wiley library.

4.3.6 Statistical analysis

Growth, gas exchange parameters, resin duct area, total mono and diterpene abundances, as well as individual terpene abundance were analyzed with a one-way ANOVA to assess growth CO_2 effects. The effect of growth CO_2 on the presence and absence of resin ducts

was analyzed using a binominal logistic regression. Results are shown as means ± standard error. Data analysis was performed in R 3.2.1 (R Core Team, 2013).

4.4 Results

4.4.1 Biomass

Growth at low CO_2 greatly decreased plant biomass. The above- and below-ground biomass of LC plants were reduced 72 and 74 %, respectively, leading to a 73 % decrease in total biomass in comparison to AC plants (Figure 4.1 and 4.2). LC plants increased allocation to leaves in relation to root biomass as the shoot/root ratio was 55 % greater in LC compared to AC plants (Figure 4.2). LC plants also invested in greater leaf area in relation to leaf mass, resulting in a 58 % increase in SLA in comparison to AC plants (Figure 4.2). The needle area of AC plants was also smaller than needles of AC plants by 59 % on average (Figure 4.2).

Figure 4.1 *Picea mariana* plants harvested at the end of the experiment. On the left side, a plant grown at ambient CO_2 conditions, and on the right, a low-CO_2 grown plant.

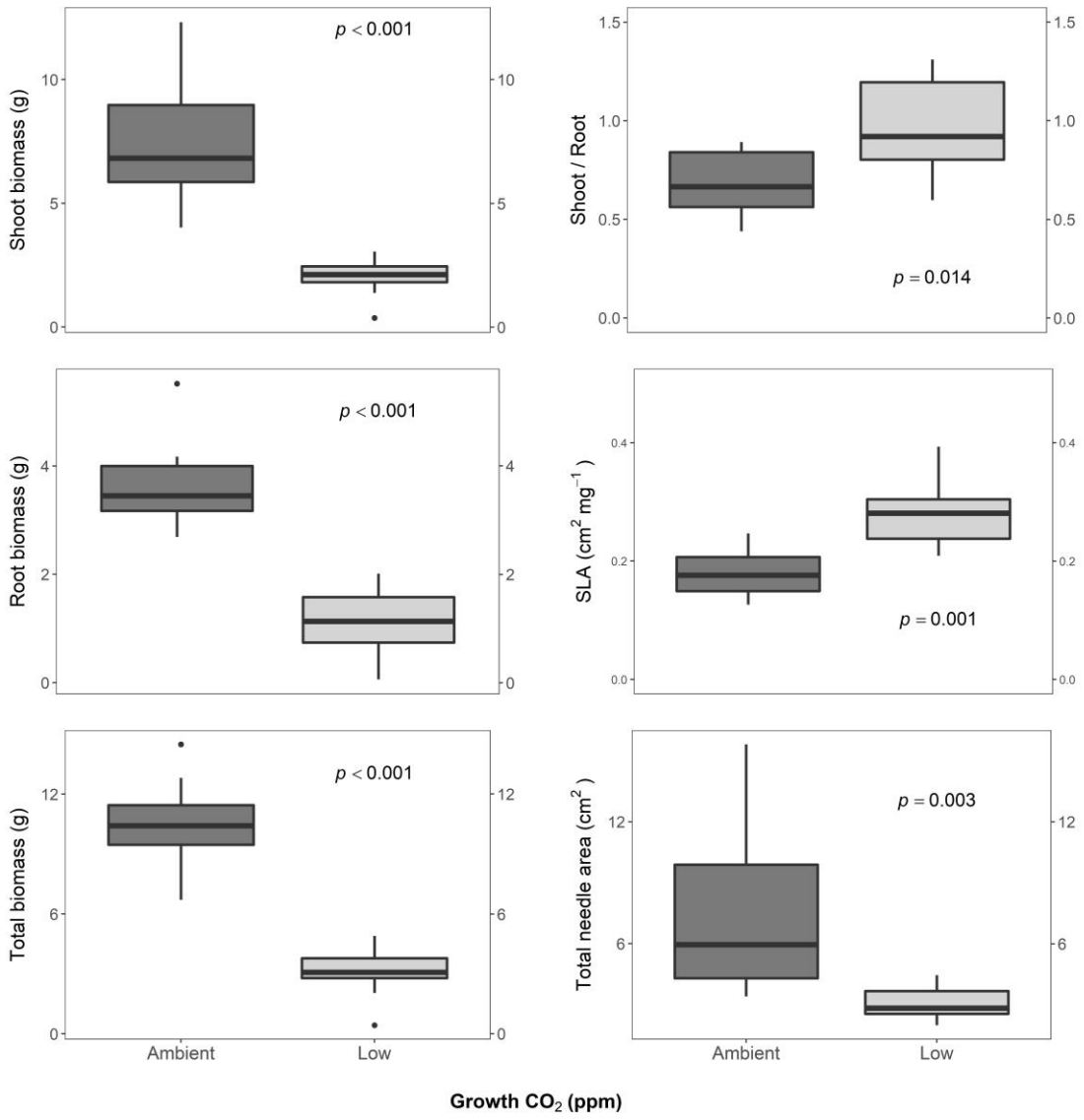

Figure 4.2. Plant biomass, Specific Leaf Area (SLA), and total leaf area: Shoot, root, and total biomass, shoot/root ratio, SLA, and total needle area of plants grown at ambient and low CO_2. The *p*-values indicate growth CO_2 effects on the variables measured, considering a $p \leq 0.05$ as significant. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 10-12.

4.4.2 Gas-exchange parameters

The A_{net} measured at growth CO_2 (A_{growth}) of LC plants was almost half of those in AC plants (Figure 4.3), although values of A_{400} were similar in LC and AC plants (Figure 4.2). Stomatal conductance, either measured at growth CO_2 ($g_{s\text{-}growth}$) or at a common CO_2 of 400 ppm ($g_{s\text{-}400}$), did not differ between the CO_2 treatments (Figure 4.3). Growth CO_2 had no effect on V_{cmax} (Figure 4.4), but J_{max} was 38 % lower in LC plants in comparison to AC plants (Figure 4.4). However, the V_{cmax} / J_{max} ratio was 29 % higher in LC compared to AC plants. The LC plants had 72 % lower R_d than AC plants (Figure 4.4), and the A_{growth}/R_d ratio was twice that of AC plants.

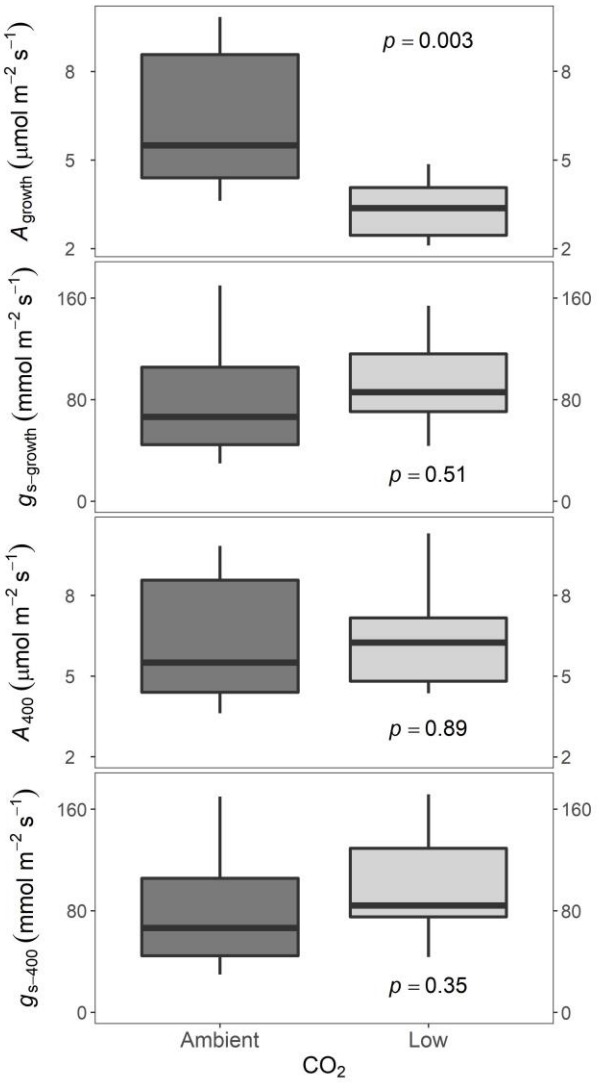

Figure 4.3. Net CO_2 assimilation rates (A) and stomatal conductance (g_s) measured at growth CO_2 (A_{growth} and $g_{s\text{-}growth}$) and at a common CO_2 concentration of 400 ppm (A_{400} and $g_{s\text{-}400}$). The p-values indicate growth CO_2 effects on the variables measured, considering a $p \leq 0.05$ as significant. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n= 10-12.

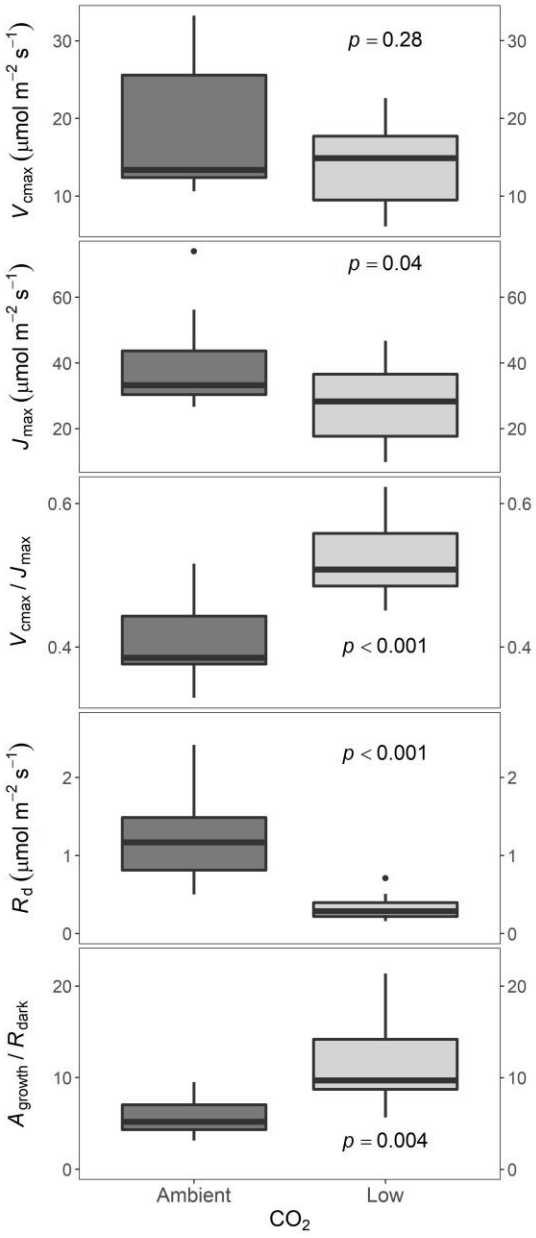

Figure 4.4. Maximum carboxylation (V_{cmax}) and electron transport (J_{max}) rates, the ratio between V_{cmax} and J_{max}, dark respiration (R_d), and the ratio between A_{growth} and R_d. The *p*-values indicate growth CO_2 effects on the variables measured, considering a $p \leq 0.05$ as significant. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 10-12.

4.4.3 Resin duct anatomy

While both resin ducts were present in all needles of AC plants, 34 % of the needles from LC plants lacked either one or both resin ducts (Figure 4.5). The lumen area of the resin ducts in needles from LC plants that had ducts were ~50 % smaller than those in AC plants (Figure 4.6). We assessed whether this reduction in lumen area at LC was caused by a reduction in needle cross-sectional area (*i.e.*, whether resin ducts represented a similar proportion of the needle cross-sectional area in both CO_2 treatment). When the resin duct lumen area was considered in relation to the needle cross-sectional area, resin ducts of LC plants were still 54 % smaller than resin ducts from AC plants (Figure 4.6).

4.4.4 Mono and diterpenes

The most abundant monoterpenes observed were bornyl cetate, camphene and α-pinene. The diterpene profile was dominated by dehydroabietic acid, levopimaric acid, and palustric acid (Figure 4.7). Monoterpene abundance was lower in LC compared to AC plants ($p = 0.009$), with this difference being mostly driven by decreases in bornyl cetate and α-pinene (Figure 4.7). However, the normalized peak area of diterpenes was unaffected by the CO_2 treatment ($p = 0.37$).

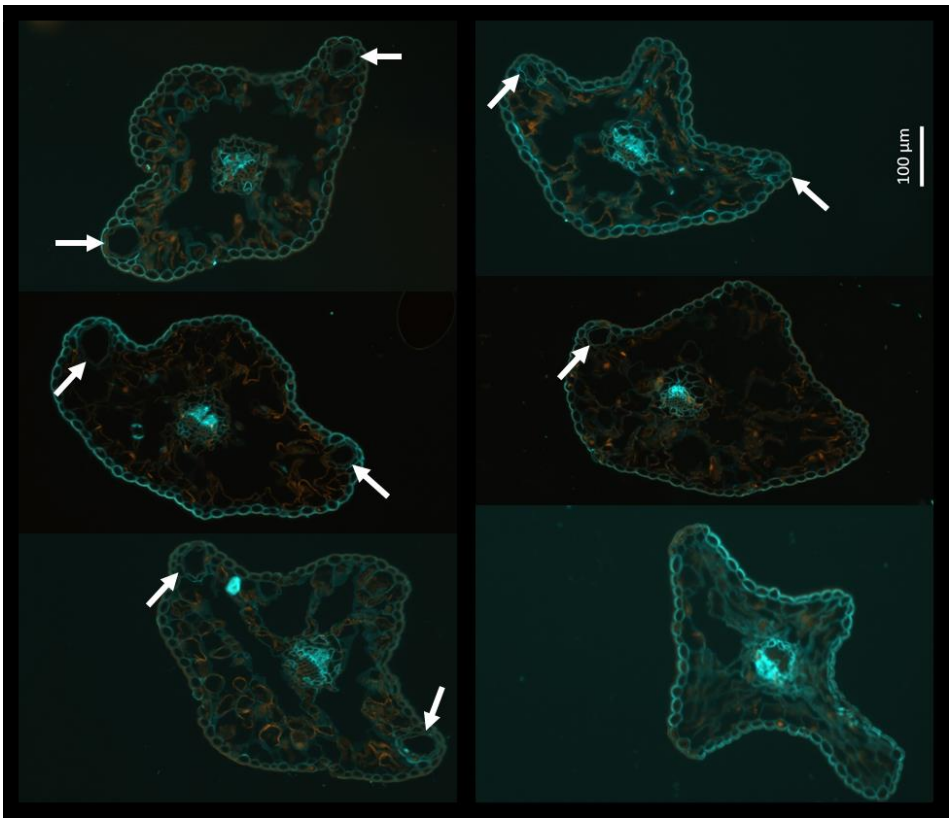

Figure 4.5. Needle cross-sections. Sections on the left side were obtained from needles of ambient CO_2-grown plants where both resin ducts are present. On the right side, needle cross-sections from low CO_2-grown plants display examples of where both resin ducts are present (upper), only one duct can be seen (middle), or a needle lacking both ducts (lower). Fluorescence microscopy at 100 µm.

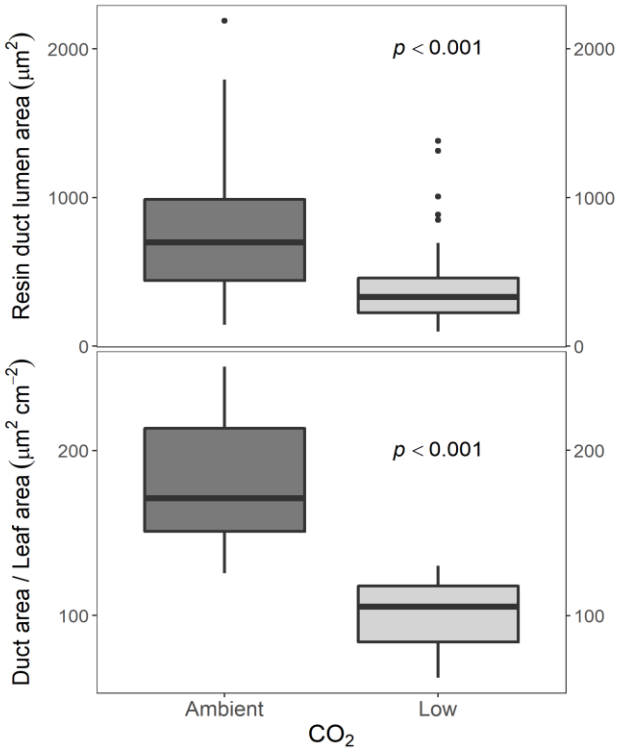

Figure 4.6. Resin duct lumen area and the ratio of resin duct area per total leaf area. The *p*-values indicate growth CO_2 effects on the variables measured, considering a $p \leq 0.05$ as significant. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 10-12.

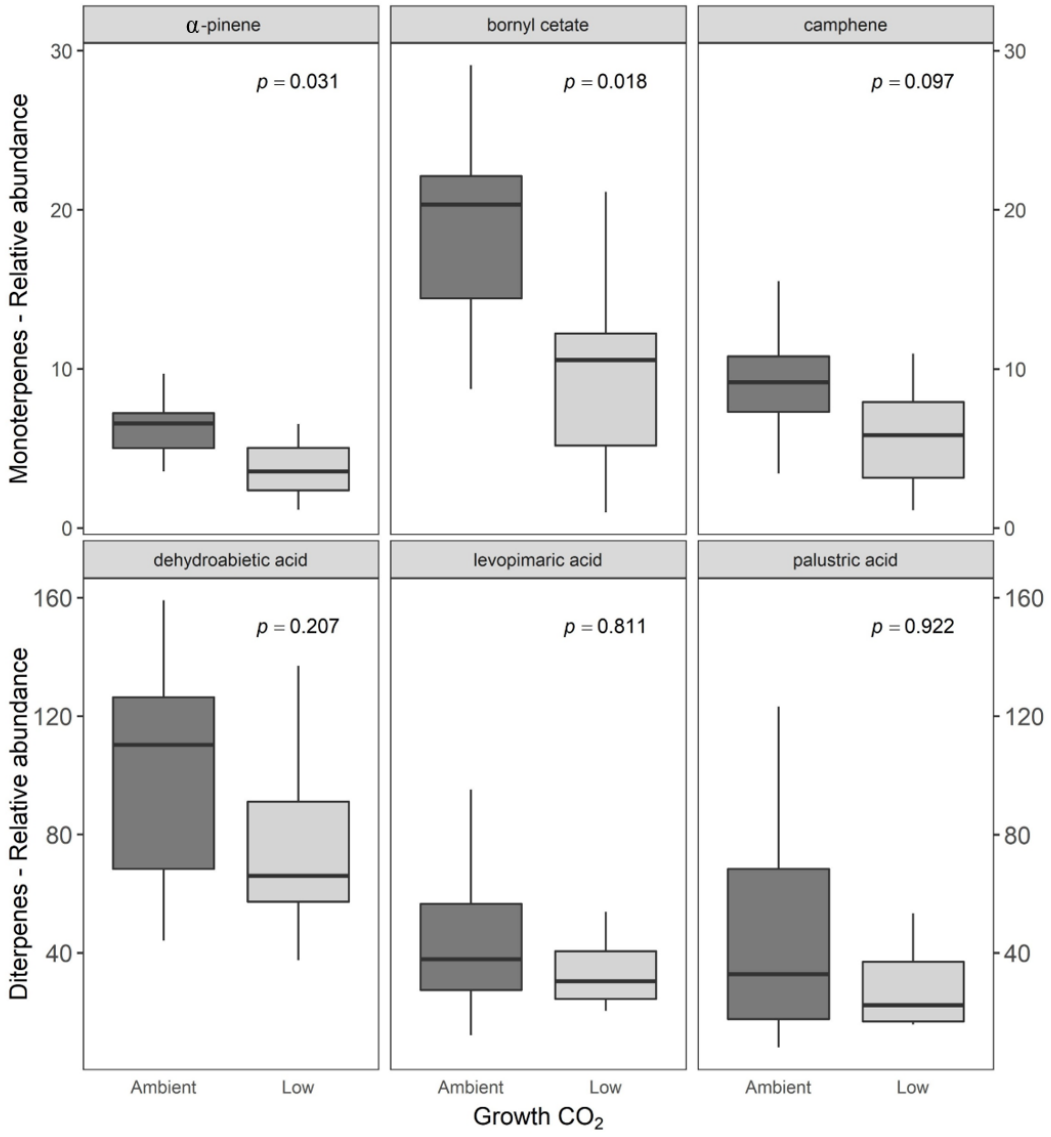

Figure 4.7. Relative abundance of mono and diterpenes measured to an internal standard. The central box plot rectangle spans the first to the third quartile segmented by the median; the whisker below is the minimum, and the whisker above the box, the maximum; points represent observations above 1.5 times the likely range of variation; n = 10-12.

4.5 Discussion

Growth in C-limited environments, such as past low CO_2 conditions, can constrain primary metabolism, with direct effects on plant growth. We tested whether low CO_2 effects would also suppress secondary metabolite production, specifically defense compound production. Indeed, not only was growth reduced, but resin duct size and terpene production were also reduced in plants grown at low CO_2 compared to ambient CO_2-grown plants.

4.5.1 Plant growth and carbon fluxes

Net photosynthetic rates at growth CO_2 were, unsurprisingly, greatly decreased in LC plants as a result of reduced CO_2 availability for photosynthesis. Reduced C assimilation in turn suppressed the growth of LC plants, an effect commonly observed in plants grown at sub-ambient CO_2 (Dippery *et al.*, 1995; Ehleringer *et al.*, 2005; Sage, 1995). The low C environment experienced by LC plants also stimulated greater allocation to leaves in relation to root biomass to increase total leaf area per tree for photosynthesis, resulting in higher SLA in LC compared to AC plants. The very low respiration rates of LC plants are likely related to both low C availability for supporting respiration, but also the low demand for respiratory products under a low growth rate environment. This is further supported by an increase in the ratio between photosynthetic rates and respiration in LC compared to AC plants, and is also in line with the findings of Gonzàlez-Meler *et al.* (2009), who observed decreased respiratory energy demand for biomass maintenance in *Arabidopsis thaliana* grown at 200 ppm compared to 370 ppm.

Contrary to previous studies that found increased g_s in C_3 plants grown at low [CO_2] (Ehleringer *et al.*, 2005; Temme *et al.*, 2013; Ward *et al.*, 1999), I did not observe an up-regulation of g_s, although this mechanism would enhance C assimilation in LC leaves. Since high g_s results in increased water loss, *P. mariana* may prioritize preventing water loss over increasing carbon uptake. Instead, the strategy adopted by LC plants to cope with their C-limited conditions relied more on enhancing carboxylation by Rubisco, as

observed in the higher V_{cmax} / J_{max} ratio found in LC plants. Few studies have investigated photosynthetic capacity in plants grown at low CO_2 conditions, and the results are highly variable: growth at low CO_2 decreased both V_{cmax} and J_{max} (Ripley *et al.*, 2013), increased V_{cmax} but not J_{max} (Anderson *et al.*, 2001), or had little effect on either parameter (Cunniff *et al.*, 2017). Interestingly, these studies were conducted on herbaceous species, but *P. mariana* is an evergreen tree and may therefore respond differently to C limitation, as responses to environmental stress can vary depending on plant functional types (Kuiper *et al.*, 2014; McIntyre *et al.*, 1999). Thus, a better understanding of photosynthetic potential in plants grown at past low CO_2 conditions requires further investigation across a range of species.

4.5.2 Resin duct and terpene production

We present the first observations of reduced chemical defense capability in plants grown at the low CO_2 conditions of the past, which have implications for our understanding of CO_2 effects on plant defense across temporal scales, as well as plant function in ancient environments. We observed reduced resin duct area, or their complete absence, in the needles of LC plants, an effect that can be attributed to the reduced C assimilation and availability in LC plants. Reductions in resin duct size may be associated with decreased terpene production, since resin ducts collect terpenes and other compounds to form oleoresin, the major constitutive chemical defense in conifers (Keeling and Bohlmann, 2006). For example, Bjorkman *et al.* (1991) observed that the concentration of resin acids in Scots pine was more limited by the size of the resin ducts than the availability of substrate for resin synthesis. Furthermore, in cases analogous to the adverse growth conditions experienced by LC plants, constitutive resin duct area as well as resin flow have been shown to decline under unfavorable environmental conditions (Lombardero *et al.*, 2000; Lorio *et al.*, 1995; Lorio and Hodges, 1968; Slack *et al.*, 2016).

A monoterpene such as α-pinene commonly accounts for a substantial part of the total terpene concentration in evergreens (Geron *et al.*, 2000; Juuti *et al.*, 1990), and is active in many defense mechanisms (Kegge and Pierik, 2010; Penuelas *et al.*, 2005). Similarly,

dehydroabietic acid is one of the most common diterpenes found in conifer oleoresins (Trapp and Croteau, 2001). While monoterpene relative abundance decreased in LC compared to AC plants, diterpene abundance was unresponsive to growth CO_2. This observation, however, is not unexpected considering that these two terpene groups can function independently. Monoterpene and diterpene production are modulated by monoterpene synthase and diterpene synthase, respectively, (Funk *et al.*, 1994), and in defense responses, monoterpene synthase is activated earlier and is more active than the diterpene synthase (Steele *et al.*, 1998). Additionally, concentrations of mono and diterpenes can be differently affected when exposed to adverse environmental conditions, as seen with salinity and drought stress (Alonso *et al.*, 2015; Tounekti *et al.*, 2011).

While the carbon-nutrient balance hypothesis proposed by Bryant *et al.* (1983) predicted an increase in the production of carbon-based secondary compounds, such as terpenes, under environmental conditions that increase non-structural carbohydrate pools, often that does not seem to be the case for terpene production in elevated CO_2 studies. Most studies found terpene levels to be either unresponsive to growth at high CO_2 levels or terpene concentrations and emission are reduced in elevated CO_2- compared to ambient CO_2-grown plants (Loreto *et al.*, 2001; Peñuelas and Estiarte, 1998; Sallas *et al.*, 2003; Snow *et al.*, 2003; Williams *et al.*, 1994). Lerdau *et al.* (1995) proposed that reduced terpene production in elevated CO_2-grown plants could be a result of C allocation to secondary pathways competing with sinks when shoot growth is stimulated by high CO_2 concentrations and non-limiting nutrient availability. A similar mechanism likely caused the decrease in monoterpene production we observed in *P. mariana* in terms of competing pathways for C allocation, as C was preferentially directed to primary functions to sustain growth under C-limiting conditions. However, while the mechanism of C partitioning limiting terpene production at elevated and sub-ambient CO_2 conditions may be the same, its function in resource allocation and resulting terpene profile is different. This is exemplified by the case of certain plant species increasing the production of specific terpenes at elevated CO_2 even though overall terpene production declines. Both Heyworth *et al.* (1998) and Sallas *et al.* (2001) observed an increase in α-pinene in plants grown at elevated CO_2 compared to ambient CO_2-grown plants, while total terpene concentration was lower in elevated CO_2-grown plants. In such cases, the

difference in concentration between the various terpenes are postulated to be under a strong genetic control that may not be readily modified by environmental variables (White, 1984). It has also been suggested that monoterpene emission at elevated CO_2 could be uncoupled from photosynthesis (Holopainen *et al.*, 2013), and that augmented substrate availability at elevated CO_2 is a necessary but insufficient trigger (*i.e.*, the need of hormonal or direct physical cues) to activate secondary metabolic pathways (Fajer *et al.*, 1992).

The slower growth and reduced metabolic rates of LC plants (including variables such as biomass, photosynthesis, respiration) are direct effects of growth at C-limited conditions that prevented the allocation of resources to the production of secondary compounds, resulting in the decrease of the major terpenes produced by *P. mariana*. Physiological adjustments caused by changes in growth CO_2 are commonly greater in plants grown at low CO_2 conditions in relation to modern CO_2 levels than in elevated CO_2-grown plants compared to plants grown at current CO_2 conditions (Gerhart and Ward, 2010). Thus, the temporal continuum of atmospheric CO_2 levels influencing the ability of plants to produce defense compounds since the LGM may have shifted from a C-limiting effect to phenotypical variability of resource allocation when C is not limiting.

The absence of resin ducts and reduction of monoterpenes observed in LC plants poses new complexities to using these indicators in paleo-reconstructions. Resin ducts and terpenes have many paleobiology applications. For example, the isotopic composition of terpenes found in sediments are used in paleoenvironmental reconstructions (Huang *et al.*, 2013) and can help identify diet and feeding mode of herbivores (Danner *et al.*, 2017), while the wall thickness of epithelial cells surrounding ducts is considered a key feature for fossil diagnosis (Wu and Hu, 1997). Resin duct presence or absence, and the number of ducts and their area, have been commonly used for species assignment of plant fossils (Meijer, 2000; Obst *et al.*, 1991; Weng and Jackson, 1999). My findings, however, bring into the question the reliability of this feature when characterizing fossil samples dating from sub-ambient CO_2 periods. Furthermore, it also raises the question whether other commonly used anatomical features, such as parenchyma rays and the characteristics of vascular tissue cells, may also be affected by growth at past low CO_2

concentrations and thus deviate from patterns observed in extant species; such issues need to be addressed in future low CO_2 studies. The adverse effects that conifer resins can have on herbivores could have played an important role in altering trophic interactions in the past. For instance, it has been suggested that the migration of spruce in North America at the end of the Pleistocene may have exposed the now extinct mastodons to a food source containing resins that those animals were not adapted to digest (Metcalfe *et al.*, 2013). Our results show that a conifer species grown at CO_2 levels of that time was able to produce resin compounds, but not to the same extent as plants grown at modern CO_2 conditions.

4.6 References

Alonso, R., Berli, F.J., Bottini, R., Piccoli, P., 2015. Acclimation mechanisms elicited by sprayed abscisic acid, solar UV-B and water deficit in leaf tissues of field-grown grapevines. Plant Physiol. Biochem. 91, 56–60. https://doi.org/10.1016/j.plaphy.2015.03.011

Anderson, L.J., Maherali, H., Johnson, H.B., Polley, H.W., Jackson, R.B., 2001. Gas exchange and photosynthetic acclimation over subambient to elevated CO_2 in a C_3-C_4 grassland. Glob. Chang. Biol. 7, 693–707. https://doi.org/10.1046/j.1354-1013.2001.00438.x

Baldwin, I.T., 2010. Plant volatiles. Curr. Biol. 20, R392–R397. https://doi.org/10.1016/j.cub.2010.02.052

Becklin, K.M., Walker, S.M., Way, D.A., Ward, J.K., 2017. CO_2 studies remain key to understanding a future world. New Phytol. 214, 34–40. https://doi.org/10.1111/nph.14336

Björkman, C., Larsson, S., Gref, R., 1991. Effects of nitrogen fertilization on pine needle chemistry and sawfly performance. Oecologia 86, 202–209.

Boucher, D., Lavallée, R., Mauffette, Y., 2001. Biological performance of the white pine weevil in relation to the anatomy of the resin canal system of four different host species. Can. J. For. Res. 31, 2035–2041. https://doi.org/10.1139/cjfr-31-11-2035

Boulogne, I., Petit, P., Ozier-Lafontaine, H., Desfontaines, L., Loranger-Merciris, G., 2012. Insecticidal and antifungal chemicals produced by plants: a review. Environ. Chem. Lett. 10, 325–347. https://doi.org/10.1007/s10311-012-0359-1

Bryant, J.P., Chapin, F.S., Klein, D.R., 1983. Carbon / Nutrient balance of boreal plants in relation to vertebrate herbivory. OIKOS 40, 57–368.

Cheng, S.-S., Wu, C.-L., Chang, H.-T., Kao, Y.-T., Chang, S.-T., 2004. Antitermitic and antifungal activities of essential oil of *Calocedrus formosana* leaf and its composition. J. Chem. Ecol. 30, 1957-1967.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloaway, J., Heimann, M., Jones, C., 2013. Climate change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernamental Panel on Climate Change, in: Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M. (Eds.)

Coley, P.D., Massa, M., Lovelock, C.E., Winter, K., 2002. Effects of elevated CO_2 on foliar chemistry of saplings of nine species of tropical tree. Oecologia 133, 62–69. https://doi.org/10.1007/s00442-002-1005-6

Constable, J.V.H., Litvak, M.E., Greenberg, J.P., Monson, R.K., 1999. Monoterpene emission from coniferous trees in response to elevated CO_2 concentration and climate warming. Glob. Chang. Biol. 5, 252–267. https://doi.org/10.1046/j.1365-2486.1999.00212.x

Cunniff, J., Jones, G., Charles, M., Osborne, C.P., 2017. Yield responses of wild C_3 and C_4 crop progenitors to subambient CO_2: a test for the role of CO_2 limitation in the origin of agriculture. Glob. Chang. Biol. 23, 380–393. https://doi.org/10.1111/gcb.13473

Danner, H., Desurmont, G.A., Cristescu, S.M., van Dam, N.M., 2017. Herbivore-induced plant volatiles accurately predict history of coexistence, diet breadth, and feeding mode of herbivores. New Phytol. 726-738 https://doi.org/10.1111/nph.14428

Dippery, J.K., Tissue, D.T., Thomas, R.B., Strain, B.R., 1995. Effects of low and elevated CO_2 on C_3 and C_4 annuals. Oecologia 101, 13–20. https://doi.org/10.1007/BF00328894

Dorman, H.J.D., Deans, S.G., 2000. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88, 308–316. https://doi.org/10.1046/j.1365-2672.2000.00969.x

Drake, B.G., Gonzàlez-Meler, M.A., Long, S.P., 1997. More efficient plants: A Consequence of rising atmospheric CO_2? Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 609–639. https://doi.org/10.1146/annurev.arplant.48.1.609

Dusenge, M.E., Duarte, A.G., Way, D.A., 2018. Plant carbon metabolism and climate change: elevated CO_2 and temperature impacts on photosynthesis, photorespiration and respiration. New Phytol. 2017. https://doi.org/10.1111/nph.15283

Duursma, R.A., 2015. Plantecophys - An R package for analysing and modelling leaf gas

exchange data. PLoS One 10, e0143346. https://doi.org/10.1371/journal.pone.0143346

Ehleringer, J.R., Cerling, T.E., Dearing, M.D., 2005. A History of atmospheric CO_2 and its effects on plants, animals, and ecosystems, 1st ed. Springer, Salt Lake City, UT, USA.

Fajer, E.D., Bowers, M.D., Bazzaz, F.A., 1992. The effect of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in *Plantago*: a test of the carbon/nutrient balance hypothesis. Am. Nat. 140, 707–723

Falcon-Lang, H.J., Mages, V., Collinson, M., 2016. The oldest *Pinus* and its preservation by fire. Geology 44, 303–306. https://doi.org/10.1130/G37526.1

Farquhar, G.D., von Caemmerer, S., Berry, J.A., 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. Planta 149, 78–90. https://doi.org/10.1007/BF00386231

Funk, C., Lewinsohn, E., Vogel, B.S., Steele, C. 1, Croteau, R., 1994. Regulation of oleoresinosis in grand fir (*Abies grandis*) - Coordinate induction of monoterpene and diterpene cyclases and two cytochrome P450-dependent diterpenoid hydroxylases by stem wounding. Plant Physiol. 106, 999–1005

Gerhart, L.M., Ward, J.K., 2010. Plant responses to low [CO_2] of the past. New Phytol. 188, 674–695. https://doi.org/10.1111/j.1469-8137.2010.03441.x

Geron, C., Rasmussen, R., R. Arnts, R., Guenther, A., 2000. A review and synthesis of monoterpene speciation from forests in the United States. Atmos. Environ. 34, 1761–1781. https://doi.org/10.1016/S1352-2310(99)00364-7

Gonzàlez-Meler, M.A., Blanc-Betes, E., Flower, C.E., Ward, J.K., Gomez-Casanovas, N., 2009. Plastic and adaptive responses of plant respiration to changes in atmospheric CO_2 concentration. Physiol. Plant. 137, 473–484. https://doi.org/10.1111/j.1399-3054.2009.01262.x

Graham, R.W., Lundelius Jr, E.L., 1984. Coevolutionary disequilibrium and Pleistocene extinctions, in: Quaternary Extinctions: A Prehistoric Revolution. pp. 223–249

Guthrie, R.D., 2003. Rapid body size decline in Alaskan Pleistocene horses before extinction. Lett. to Nat. 426, 169–171. https://doi.org/10.1038/nature02070

Guthrie, R.D., 1984. Mosaics, allelochemics and nutrients: An ecological theory of late Pleistocene megafaunal extinctions., in: Quaternary Extinctions: A Prehistoric Revolution. pp. 259–298

Hartmann, T., 1996. Diversity and variability of plant secondary metabolism: a mechanistic view, in: Proceedings of the 9th International Symposium on Insect-Plant Relationships. Springer Netherlands, Dordrecht, pp. 177–188.

https://doi.org/10.1007/978-94-009-1720-0_42

Heyworth, C.J., Iason, G.R., Temperton, V., Jarvis, P.G., Duncan, A.J., 1998. The effect of elevated CO_2 concentration and nutrient supply on carbon-based plant secondary metabolites in *Pinus sylvestris* L. Oecologia 115, 344–350

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil, 2nd edit. ed. Circular. California agricultural experiment stations.

Holopainen, J.K., 2004. Multiple functions of inducible plant volatiles. Trends Plant Sci. 9, 529–533. https://doi.org/10.1016/J.TPLANTS.2004.09.006

Huang, X., Xue, J., Wang, X., Meyers, P.A., Huang, J., Xie, S., 2013. Paleoclimate influence on early diagenesis of plant triterpenes in the Dajiuhu peatland, central China. Geochim. Cosmochim. Acta 123, 106–119. https://doi.org/10.1016/j.gca.2013.09.017

Juuti, S., Arey, J., Atkinson, R., 1990. Monoterpene emission rate measurements from a Monterey pine. J. Geophys. Res. 95, 7515. https://doi.org/10.1029/JD095iD06p07515

Keeling, C.I., Bohlmann, J., 2006. Diterpene resin acids in conifers. Phytochemistry 67, 2415–2423. https://doi.org/10.1016/j.phytochem.2006.08.019

Kegge, W., Pierik, R., 2010. Biogenic volatile organic compounds and plant competition. Trends Plant Sci. 15, 126–132. https://doi.org/10.1016/j.tplants.2009.11.007

Kuiper, J.J., Mooij, W.M., Bragazza, L., Robroek, B.J.M., 2014. Plant functional types define magnitude of drought response in peatland CO_2 exchange. Ecology. 95, 123-131

Lambers, H., 1993. Rising CO_2, secondary plant metabolism, plant-herbivore interactions and litter decomposition theoretical considerations. Vegetatio 104/105, 263–271. https://doi.org/10.1007/BF00048157

Langenheim, J.H., 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. J. Chem. Ecol. 20, 1223–1280

Langenheim, J.H., 1990. Plant Resins. Am. Sci. 78, 16–24

Lerdau, M., Matson, P., Fall, R., Monson, R., 1995. Ecological controls over monoterpene emissions from Douglas-Fir (*Pseudotsuga Menziesii*). Ecology 76, 2640–2647. https://doi.org/10.2307/2265834

Lombardero, M.J., Ayres, M.P., Lorio Jr, P.L., Ruel, J.J., 2000. Environmental effects on constitutive and inducible resin defences of Pinus taeda. Ecol. Lett. 3, 329–339. https://doi.org/10.1046/j.1461-0248.2000.00163.x

Loreto, F., Fischbach, R.J., Schnitzler, J.-P., Ciccioli, P., Brancaleoni, E., Calfapietra, C., Seufert, G., 2001. Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO_2 concentrations. Glob. Chang. Biol. 7, 709–717. https://doi.org/10.1046/j.1354-1013.2001.00442.x

Lorio, P.L., Hodges, J.D., 1968. Microsite effects on oleoresin exudation pressure of large loblolly pines. Ecology 49, 1207–1210. https://doi.org/10.2307/1934519

Lorio, P.L., Stephen, F.M., Paine, T.D., 1995. Environment and ontogeny modify loblolly pine response to induced acute water deficits and bark beetle attack. For. Ecol. Manage. 73, 97–110. https://doi.org/10.1016/0378-1127(94)03500-V

Manum, S.B., Van Konijnenburg-Van Cittert, J.H.A., Wilde, V., 2000. *Tritaenia Maegdefrau* et Rudolf, Mesozoic '*Sciadopitys*-like' leaves in mass accumulations. Rev. Palaeobot. Palynol. 109, 255–269. https://doi.org/10.1016/S0034-6667(99)00058-5

Martin, D., Tholl, D., Gershenzon, J., Bohlmann, J., 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiol. 129, 1003–1018. https://doi.org/10.1104/pp.011001

Maurino, V.G., Peterhansel, C., 2010. Photorespiration: Current status and approaches for metabolic engineering. Curr. Opin. Plant Biol. 13, 248–255. https://doi.org/10.1016/J.PBI.2010.01.006

McIntyre, S., Díaz, S., Lavorel, S., Cramer, W., 1999. Plant functional types and disturbance dynamics – Introduction. J. Veg. Sci. 10, 603–608. https://doi.org/10.1111/j.1654-1103.1999.tb00103.x

Meijer, J.J.F., 2000. Fossil woods from the Late Cretaceous Aachen Formation. Rev. Palaeobot. Palynol. 112, 297–336. https://doi.org/10.1016/S0034-6667(00)00007-5

Metcalfe, J.Z., Longstaffe, F.J., Hodgins, G., 2013. Proboscideans and paleoenvironments of the Pleistocene Great Lakes: Landscape, vegetation, and stable isotopes. Quat. Sci. Rev. 76, 102–113. https://doi.org/10.1016/j.quascirev.2013.07.004

Mohan, J.E., Ziska, L.H., Schlesinger, W.H., Thomas, R.B., Sicher, R.C., George, K., Clark, J.S., 2006. Biomass and toxicity responses of poison ivy (*Toxicodendron radicans*) to elevated atmospheric CO_2. PNAS 103, 9086–9089. https://doi.org/10.1890/06-1609.1

Noctor, G., Mhamdi, A., 2017. Climate change, CO_2, and defense: The metabolic, redox, and signaling perspectives. Trends Plant Sci. 22, 857–870. https://doi.org/10.1016/j.tplants.2017.07.007

Obst, J.R., Mcmillan, N.J., Blanchette, R.A., Christensen, D.J., Faix, O., Han, J.S.,

Kuster, T.A., Landucci, L.L., Newman, R.H., Pettersen, R.C., Schwandt, V.H., Wesolowski, M.F., 1991. Characterization of Canadian Arctic fossil woods, Bulletin.

Peñuelas, J., Estiarte, M., 1998. Can elevated CO_2 affect secondary metabolism and ecosystem function? Tree 13, 20–24. https://doi.org/10.1016/S0169-5347(97)01235-4

Peñuelas, J., Estiarte, M., LLusia, J., 1997. Carbon-based secondary compounds at elevated CO_2. Photosynthetica 33, 313–316

Peñuelas, J., Llusia, J., Asensio, D., Munne-bosch, S., 2005. Linking isoprene with plant thermotolerance, antioxidants and monoterpene emissions. Plant, Cell Environ. 28, 278–286. https://doi.org/10.1111/j.1365-3040.2004.01250.x

Petit, R.J., Raynaud, D., Basile, I., Chappellaz, J., Ritz, C., Delmotte, M., Legrand, M., Lorius, C., Pe, L., 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399, 429–413. https://doi.org/10.1038/20859

Prentice, I.C., Bartlein, P.J., Webb, T., 1991. Vegetation and climate change in Eastern North America since the Last Glacial Maximum. Ecology 72, 2038–2056. https://doi.org/10.2307/1941558

R Core Team, 2013. R: A Language and Environment for Statistical Computing

Ripley, B.S., Cunniff, J., Osborne, C.P., 2013. Photosynthetic acclimation and resource use by the C_3 and C_4 subspecies of *Alloteropsis semialata* in low CO_2 atmospheres. Glob. Chang. Biol. 19, 900–910. https://doi.org/10.1111/gcb.12091

Robert, J.A., Madilao, L.L., White, R., Yanchuk, A., King, J., Bohlmann, J., 2010. Terpenoid metabolite profiling in Sitka spruce identifies association of dehydroabietic acid, (+)-3-carene, and terpinolene with resistance against white pine weevil. Botany 88, 810–820. https://doi.org/10.1139/B10-049

Sage, R.F., 1995. Was low atmospheric CO_2 during the Pleistocene a limiting factor for the origin of agriculture? Glob. Chang. Biol. 1, 93–106. https://doi.org/10.1111/j.1365-2486.1995.tb00009.x

Sage, R.F., Coleman, J.R., 2001. Effects of low atmospheric CO_2 on plants: More than a thing of the past. Trends Plant Sci. 6, 18–24. https://doi.org/10.1016/S1360-1385(00)01813-6

Sage, Rowan F, Cowling, S.A., 1999. Implications of stress in low CO_2 atmospheres of the past: Are today's plants too conservative for a high CO_2 world?, in: Luo, Y., Mooney, H.A. (Eds.), Carbon Dioxide and Environmental Stress. Academic Press, San Diego, CA, USA, pp. 289–304

Sallas, L., Kainulainen, P., Utriainen, J., Holopainen, T., Holopainen, J.K., 2001. The influence of elevated O_3 and CO_2 concentrations on secondary metabolites of Scots pine (*Pinus sylvestris* L.) seedlings. Glob. Chang. Biol. 7, 303–311. https://doi.org/10.1046/j.1365-2486.2001.00408.x

Sallas, L., Luomala, E.-M., Utriainen, J., Kainulainen, P., Hopolainen, J.K., 2003. Contrasting effects of elevated carbon dioxide concentration and temperature on Rubisco activity, chlorophyll fluorescence, needle ultrastructure and secondary metabolites in conifer seedlings. Tree Physiol. 23, 97–108. https://doi.org/https://doi.org/10.1093/treephys/23.2.97

Schneider, C. a, Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089

Schurgers, G., Hickler, T., Miller, P.A., Arneth, A., 2009. European emissions of isoprene and monoterpenes from the Last Glacial Maximum to present. Biogeosciences 6, 2779-2797

Slack, A.W., Zeibig-Kichas, N.E., Kane, J.M., Varner, J.M., 2016. Contingent resistance in longleaf pine (*Pinus palustris*) growth and defense 10 years following smoldering fires. For. Ecol. Manage. 364, 130–138. https://doi.org/10.1016/j.foreco.2016.01.014

Snow, M.D., Bard, R.R., Olszyk, D.M., Minster, L.M., Hager, A.N., Tingey, D.T., 2003. Monoterpene levels in needles of Douglas fir exposed to elevated CO_2 and temperature. Physiol. Plant. 117, 352–358. https://doi.org/10.1034/j.1399-3054.2003.00035.x

Steele, C.L., Katoh, S., Rg Bohlmann, J., Croteau, R., 1998. Regulation of oleoresinosis in grand fir (*Abies grandis*) - Differential transcriptional control of monoterpene, sesquiterpene, and diterpene synthase genes in response to wounding. Plant Physiol. 116, 1497–1504

Stockey, R.A., Rothwell, G.W., Addy, H.D., Currah, R.S., 2001. Mycorrhizal association of the extinct conifer *Metasequoia milleri*. Mycology 105, 202–205. https://doi.org/10.1017/S0953756200003221

Temme, A.A., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2013. Meta-analysis reveals profound responses of plant traits to glacial CO_2 levels. Ecol. Evol. 3, 4525–4535. https://doi.org/10.1002/ece3.836

Tomlin, E.S., Borden, A.H., 1997. Thin bark and high density of outer resin ducts: interrelated resistance traits in Sitka spruce against the White Pine Weevil (Coleoptera: *Curculionidae*). J. Econ. Entomol. 90, 235–239

Tounekti, T., Vadel, A.M., Ennajeh, M., Khemira, H., Munné-Bosch, S., 2011. Ionic interactions and salinity affect monoterpene and phenolic diterpene composition in rosemary (*Rosmarinus officinalis*). J. Plant Nutr. Soil Sci. 174, 504–514. https://doi.org/10.1002/jpln.201000213

Trapp, S., Croteau, R., 2001. Defensive resin biosynthesis in conifers. Annu. Rev. Plant Physiol. Plant Mol. Biol 52, 689–724

Vannette, R.L., Hunter, M.D., 2011. Genetic variation in expression of defense phenotype may mediate evolutionary adaptation of *Asclepias syriaca* to elevated CO_2. Glob. Chang. Biol. 17, 1277–1288. https://doi.org/10.1111/j.1365-2486.2010.02316.x

Ward, J.K., Tissue, D.T., Thomas, R.B., Strain, B.R., 1999. Comparative responses of model C_3 and C_4 plants to drought in low and elevated CO_2. Glob. Chang. Biol. 5, 857–867. https://doi.org/10.1046/j.1365-2486.1999.00270.x

Weng, C., Jackson, S.T., 1999. Late Glacial and Holocene vegetation history and paleoclimate of the Kaibab Plateau, Arizona. Palaeogeogr. Palaeoclimatol. Palaeoecol. 153, 179–201. https://doi.org/10.1016/S0031-0182(99)00070-X

White, E.E., 1984. Mode of genetic control of monoterpenes in folliage of controlled crosses of *Pinus contorta*. Silvae Genet

Williams, A., Pétriacq, P., Schwarzenbacher, R.E., Beerling, D.J., Ton, J., 2018. Mechanisms of glacial-to-future atmospheric CO_2 effects on plant immunity. New Phytol. https://doi.org/10.1111/nph.15018

Williams, R.S., Lincoln, D.E., Thomas, R.B., 1994. Loblolly pine grown under elevated CO_2 affects early instar pine sawfly performance. Oecologia 98, 64–71

Wu, H., Hu, Z.-H., 1997. Comparative anatomy of resin ducts of the *Pinaceae*. Trees 11, 135–1143

Zavala, J. a, Nabity, P.D., Delucia, E.H., 2012. An emerging understanding of mechanisms governing insect herbivory under elevated CO_2. Annu. Rev. Entomol. 79–97. https://doi.org/10.1146/annurev-ento-120811-153544

Zazula, Grant D., Schweger, C.E., Beaudoin, A.B., McCourt, G.H., 2006. Macrofossil and pollen evidence for full-glacial steppe within an ecological mosaic along the Bluefish River, eastern Beringia. Quat. Int. 142–143, 2–19. https://doi.org/10.1016/J.QUAINT.2005.03.010

Zazula, G. D., Telka, A.M., Harington, C.R., Schweger, C.E., Mathewes, R.W., 2006. New Spruce (*Picea* spp.) macrofossils from Yukon territory: Implications for late Pleistocene refugia in Eastern Beringia. Arctic 59, 391–400

Zidianakis, G., Iliopoulos, G., Zelilidis, A., Kovar-Eder, J., 2016. Pinus remains from the Pitsidia plant assemblage document coastal pine forests in southern Crete during the late Miocene. Rev. Palaeobot. Palynol. 235, 11–30. https://doi.org/10.1016/j.revpalbo.2016.09.003

Chapter 5

5 General discussion

While atmospheric CO_2 concentrations are currently rising above 400 ppm, plants have experienced CO_2 levels below 270 ppm for the past millennia, and levels as low as 180 ppm at the end of the Last Glacial Maximum (Petit *et al.*, 1999). Physiological traits developed during this period when growth was limited by CO_2 availability could have legacy effects on plant responses to modern and future rapidly increasing CO_2 concentrations (Becklin *et al.*, 2017; Gerhart and Ward, 2010; Sage and Coleman, 2001; Sage and Cowling, 1999). Despite the prevalence of low CO_2 conditions in the modern history of plants, however, the available literature on the subject is not only scarce but lacks the basic complexity surrounding plant growth in natural environments: interactions with other abiotic and biotic factors. Addressing such gaps is not only necessary to extend the temporal resolution through which we investigate plant responses to changes in growth CO_2, but can also help generate more accurate inferences based on fossil plant samples dating from low CO_2 periods.

The contribution of this thesis to expanding our understanding of plant development at low CO_2 conditions of the past included investigating how growth at low CO_2 interacts with N availability, since N is one of the most important and limiting nutrients for plant growth. I also assessed the interaction between low CO_2-grown plants and mycorrhizal fungi, which is arguably plants' most important living partner in natural ecosystems. I then further explored how plants balance their growth in C-limited environments by investigating their ability to allocate resources to secondary metabolites such as defense compounds.

In Chapter 2, I investigated whether growth and N isotope composition of low CO_2-grown plants were differently affected by different N sources. I found that the overall growth among the different N treatments was similar at low growth CO_2, but several of the variables measured, including $\delta^{15}N$, responded differently to the combined effects of low CO_2 and different N sources. In Chapter 3, I assessed how reduced C availability at

low CO_2 conditions alter the exchange of resources between plants and fungi in mycorrhizal association. Results showed that plants grown at 180 ppm had a higher degree of association with the mycorrhizal fungi and benefited from higher N acquisition, which decreased the $\delta^{15}N$ in roots of mycorrhizal plants. In Chapter 4, I explored whether the ability to produce defense compounds were reduced in low CO_2-grown plants compared to plants grown at modern CO_2 conditions. Indeed, growth at low CO_2 decreased the production of terpenes, which was accompanied by a reduction in the ability of needles from low CO_2-grown plants to form resin ducts. As discussed below, put together, these results have several implications for our understating of growth, photosynthesis, and C and N isotope compositions in plants grown during past low-CO_2 environments. Also, as shown in Figure 5.1, the interaction between the environmental factors influencing growth at low CO_2 concentrations discussed in this thesis can affect plant physiological traits in diverse ways.

5.1 Plant growth and N acquisition at low CO_2

As expected in plants grown at low CO_2 conditions, there was a strong decline in biomass production in both *Elymus canadensis* and *Picea mariana* grown at sub-ambient CO_2 compared to ambient CO_2 concentrations, and a higher allocation of biomass to leaves in relation to roots. To enhance CO_2 uptake when CO_2 availability is limited, low CO_2-grown plants favor the production of photosynthetic tissues. My work further establishes that root growth in plants grown at past low CO_2 concentrations is reduced compared to plants grown at modern conditions. In addition, I offer a new insight to the plant-soil nutrient dynamics at low CO_2 conditions: roots may have explored less soil, but did not necessarily have reduced access to soil N if they could form mycorrhizal associations. I found that *E. canadensis* grown at low CO_2 had a higher percentage of roots colonized with the mycorrhizal fungus *Glomus intraradices*, and benefited from increased root N compared to non-mycorrhizal plants grown at low CO_2.

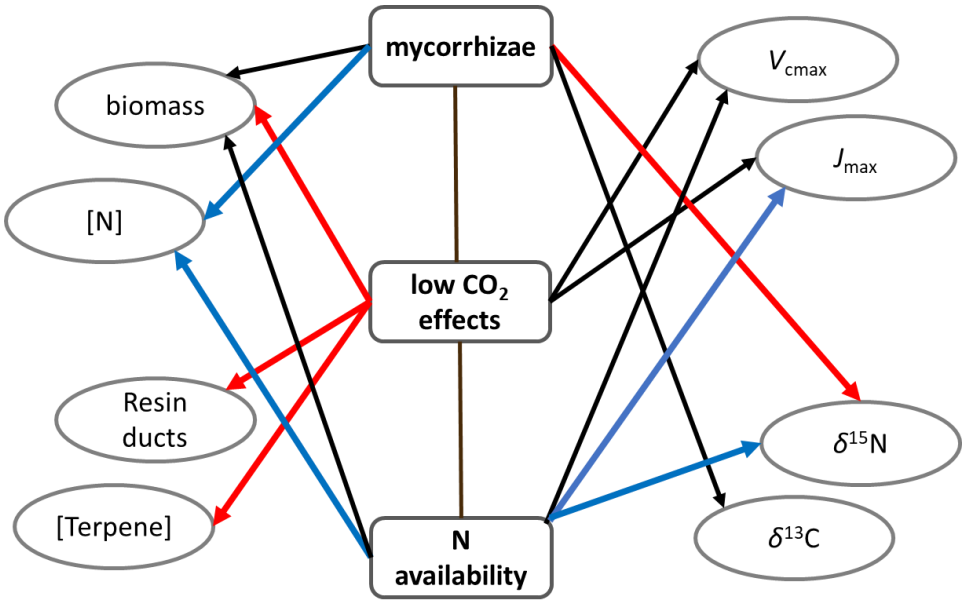

Figure 5.1. Low CO_2 effects and their interaction with mycorrhizal association and N availability on plant traits. The color of the arrows indicates whether the value of the measured parameter increased (blue), decreased (red), or was unaffected (black).

More experiments with different species of plants and fungi are necessary to ascertain if establishing a stronger association with mycorrhizal fungi was a widespread strategy adopted by plants growing at low CO_2 levels of the past. Nevertheless, my observation of an enhanced mycorrhizal association in low CO_2-grown plants is the first empirical assessment of a possible strategy for enhancing soil nutrient uptake in C-limited conditions of the past. In this regard, the next step should be to investigate phosphorus (P) uptake by mycorrhizal plants at low CO_2, since arbuscular mycorrhizal fungi also have a prominent role in mobilizing P to the host plant (Bolan, 1991).

Another aspect of plant N acquisition at low CO_2 conditions that I explored was the range in N sources available for uptake. I expected changes in plant growth and the ability to assimilate N at low CO_2 to vary depending on the N source available, due to the influence that decreased C assimilation and enhanced photorespiration have on N assimilation (Bloom *et al.,* 2010, 2014; Busch *et al.,* 2018). While photosynthetic parameters in *E. canadensis* fertilized with NO_3^- had a performance closer to fully fertilized plants in comparison to plants that received NH_4^+-only or a half-fertilization, changes in biomass or N concentration among the different N treatments were minimal. These results showed that low CO_2 limits growth and N assimilation regardless of the N source available, unless, as discussed above, N acquisition is facilitated by an extrinsic source such as mycorrhizal association. Nonetheless, mycorrhizal interactions at low CO_2 conditions exposed to different N sources should be investigated and may provide surprising results. Certain species of plants (Alt *et al.*, 2017; Dier *et al.*, 2017) and mycorrhizal fungi (Breuninger *et al.,* 2004; Chambers *et al.,* 1980) thrive better when specific N forms are available. For example, we observed enhanced cooperation between *E. canadensis* and *G. intraradices*, but if only NH_4^+ is available, and both symbionts are NO_3^--preferring species, the symbiosis could become detrimental if the symbionts were to compete for N resources.

5.2 Decreases in photosynthetic rates at low CO_2

As CO_2 is the substrate for photosynthesis, photosynthetic rates in low CO_2-grown plants is reduced in comparison to plants grown at current CO_2 conditions. This effect was observed in both *E. canadensis* and *P. mariana*, and it is a common occurrence across all plant functional types (Dalling *et al.*, 2016; Sage and Coleman, 2001; Sage and Cowling, 1999; Temme *et al*., 2013). One strategy observed in low CO_2-grown plants to compensate for low CO_2 availability is increased stomatal conductance to allow a higher influx of CO_2 through the stoma (Ehleringer *et al.*, 2005; Temme *et al.*, 2013; Ward *et al.*, 1999). I found, however, that neither *E. canadensis* nor *P. mariana* adopted this strategy; one possible explanation for this finding is that enhanced stomatal conductance can also result in increased water loss. Nevertheless, the observation that the number of stomata produced by *E. canadensis* was affected by growth at low CO_2 and reduced N availability indicates that stomatal anatomy may change even if conductance rates are not altered. This finding is particularly relevant when using stomatal anatomy and density as paleo-indicators to infer past low CO_2 concentrations.

Another aspect of photosynthetic gas exchange that has been poorly explored in low CO_2 studies is changes in maximum carboxylation rates by Rubisco (V_{cmax}) and maximum electron transport rates (J_{max}). To our knowledge, only four studies have directly measured V_{cmax} and J_{max} in plants grown from seed at low CO_2, and only herbaceous species have been measured. The scarcity of data makes it difficult to form generalized assumptions, but it seems that V_{cmax} may be more responsive in herbaceous species grown at low CO_2 than J_{max}. Tissue *et al.* (1995) observed increased V_{cmax} rates in *Abutilon theophrasti*, while a decrease in V_{cmax} in *Solanum dimidiatum* was found by Anderson *et al.* (2001), but in both cases J_{max} was unaffected. Both parameters, however, decreased in *Alloteropsis semialata* (Ripley *et al.*, 2013), while both were unaffected in *Hordeum spontaneum* and *Triticum boeoticum* (Cunniff *et al.*, 2017). It is therefore particularly interesting that I found for *P. mariana*, an evergreen tree, that J_{max} was lower in low CO_2 compared to ambient CO_2-grown plants, while V_{cmax} was unaffected. Thus, future low CO_2 studies examining V_{cmax} and J_{max} in different plant functional types are necessary to

establish a more solid baseline of changes in photosynthetic physiology for low CO_2 conditions of the past.

5.3 Mycorrhizae and N availability influence C and N isotope compositions at low CO_2

I presented here the first C and N isotope measurements of plant tissues from plants associated with a mycorrhizal fungus grown at low CO_2 conditions. The mycorrhizal association was stimulated in low CO_2-grown plants compared to plants grown at ambient conditions, which resulted in increased discrimination against ^{13}C in leaves and against ^{15}N in roots. The shift in $\delta^{13}C$ by –2.7 ‰ when comparing mycorrhizal to non-mycorrhizal plants grown at low CO_2 is substantial, to the extent that it could confound inferences based on this indicator. For example, Beerling and Jolley (1998) used the decline in $\delta^{13}C$ of about –3‰ in fossil pollen from *Inaperturopollenites hiatus*, an extinct conifer species, dating from the Palaeocene/Eocene transition (between 58 and 55 Mya) as a proxy to confirm a spike in atmospheric $^{12}CO_2$ that occurred during that period. The association between conifers and mycorrhizal fungi is widespread in extant species (Björkman, 1970; Fogel, 1988; Opik *et al.*, 2008), and is well recorded in extinct species as well (Bomfleur *et al.*, 2013; Schwendemann *et al.*, 2011; Strullu-Derrien *et al.*, 2018). For instance, Stockey *et al.* (2001) observed that the arbuscular mycorrhizal structures present in the 48.7 My-old extinct conifer *Metasequoia milleri* are similar to those present in modern conifers. Thus, if the functioning of this association in ancient species is similar to modern genotypes, the $\delta^{13}C$ of ancient plants could have become more negative if they acquired the ability to establish mycorrhizal associations. That said, the ability to establish mycorrhizal association can be abandoned and/or gained over time (Maherali *et al.*, 2016), which adds complexity to the consideration of this potential effect.

The mycorrhizal association also decreased root $\delta^{15}N$ by –2.7 ‰ in mycorrhizal compared to non-mycorrhizal plants grown at low CO_2. Another observation that

influenced $\delta^{15}N$ when comparing low CO_2- to ambient CO_2-grown plants was the source and availability of N for plant uptake. Fertilization with only NO_3^- decreased leaf $\delta^{15}N$ by 1.2 ‰, while plants that received only a half-fertilization were enriched in ^{15}N by 0.8 ‰, creating a difference of 2 ‰ between these two N treatments. Although it is uncertain if these changes in isotope discrimination can be additive, in a hypothetical scenario where mycorrhizal plants also experience N shortage, the shift in N isotope composition could result in a decrease of –3.5 ‰ in $\delta^{15}N$. Such relatively large shifts in $\delta^{15}N$ can skew interpretations of this indicator, for example, when reconstructing food webs dating from low CO_2 periods, especially considering that the standard discrimination against ^{15}N between trophic levels is 3.4 ‰ (Minagawa and Wada, 1984; Post, 2002). Adding the potential presence of a mycorrhizal association to paleo-reconstruction models may also help elucidate abnormal $\delta^{15}N$ found in ancient herbivores. For instance, the $\delta^{15}N$ in bulk collagen remnants from the woolly mammoth (*Mammuthus primigenius*), dating from the end of the Pleistocene (about 11,700 ya), was distinct from other herbivores, but similar to the carnivores that lived at the same area as the mammoth (Szpak *et al.*, 2010). It has been suggested that the unexpected ^{15}N-enrichment could be attributed to an isotopically distinct food source (Schwartz-Narbonne *et al.,* 2015). In contrast, Drucker *et al.* (2003) observed that the $\delta^{15}N$ in red deer (*Cervus elaphus*) collagen found at Rochedane (France) decreased over the Terminal Pleistocene-Early Holocene (13,000 to 8,000 ya) by more than –6 ‰. The authors concluded that changes in environmental conditions, such as increased rainfall, could not explain this variation, but hypothesized that variations in the $\delta^{15}N$ of the plants consumed caused by changes in N acquisition during that period may have affected the $\delta^{15}N$ of the red deer.

Thus, I advocate that incorporating possible N sources available for uptake and the possibility of mycorrhizal associations into interpretation of C and N isotope compositions of ancient plant samples will enhance the ecological accuracy of prediction based on these indicators. In this case, soil characteristics can be used to determine which N conversion process are more likely (Amundson *et al.*, 2003; Brady and Weil 2013), and the presence of mycorrhizae can be established by examining the fossils themselves (*e.g.*, Allen *et al.*, 2006; Strullu-Derrien and Strullu, 2007; Krings *et al.*, 2013; Taylor *et*

al., 2015) or determining whether the species commonly establishes mycorrhizal associations (Brundrett, 2009, 2002; Öpik *et al.*, 2006; Wang and Qiu, 2006).

Here I investigated only one biotic (mycorrhizal fungi) and one abiotic (N availability) environmental factor individually. Including other environmental variables and their interactions could provide new insights to how we use isotopes in paleo-ecological reconstructions. For instance, modern plants experiencing water stress are usually more enriched in ^{15}N than well-watered plants (Raimanová and Haberle, 2010; Yousfi *et al.*, 2012) and this enrichment can be accentuated by growth at elevated CO_2 (Serret *et al.*, 2018). However, we have no information if growth under low CO_2 conditions of the past can also intensify changes in $\delta^{15}N$ of plants under water stress.

5.4 Paleoecology implications of mycorrhizal associations and reduced chemical defense

The observation that plants grown at low CO_2 increase the N concentration of their roots when associated with mycorrhizae, without reducing their C content, suggests that mycorrhizal plants in the past may have presented higher nutritional value to herbivores. There are reports of modern primary consumers benefiting from higher nutrient access by feeding on mycorrhizal plants (Currie *et al.*, 2011; Gange *et al.*, 1999), and such a preference in the past may have been more common if certain herbivores were able to recognize this higher nutritional value. Thus, identifying whether producers were mycorrhizal species could help improve the characterization of nutrient transfer between trophic levels.

Another effect caused by growth at low CO_2 observed for the first time in this thesis involved the anatomy of resin ducts in a conifer. Resin ducts in needles of low CO_2-grown plants were smaller, or in some cases, absent. Thus, it is not surprising that terpene production also declined, especially the major monoterpenes that constitute the resin of *P. mariana*. There is uncertainty regarding the extent to which terpene production will be affected by CO_2 concentrations above current concentrations (Loreto *et al.*, 2001;

Penuelas *et al.*, 1997; Williams *et al.*, 1994). As low CO_2 restricts primary metabolic functions, however, it is likely that concentrations of secondary metabolites, such as terpenes, will decline under these conditions. If most plants growing in the past at low CO_2 conditions had a reduced capability to chemically defend themselves against herbivores, certain relationships between producers and primary consumers in the past probably differed from modern ones. Certain food sources may have been more palatable at the end of the LGM than they currently are, and their palatability may have decreased as CO_2 increased. Such observations support a current hypothesis that the expansion of spruce in North America at the end of the LGM exposed the mastodon to toxins present in needle resins that those animals were not adapted to digest (Metcalfe *et al.*, 2013). As CO_2 concentrations have been rising steadily since the LGM, spruce trees may have slowly increased their resin production, which may have contributed to the extinction of this mega-herbivore.

5.5 Future work

In order to achieve a better characterization of photosynthetic performance under C-limited conditions of the past, future work should investigate the photosynthetic physiology of species from different functional types, especially photosynthetic capacity, with measurements of V_{cmax} and J_{max} in less explored PFTs such as tropical species. Nutrient acquisition at past low CO_2 concentrations is also poorly characterized. While I assessed N acquisition at low CO_2, the impact of a low CO_2 environment on other essential nutrients such as P should also be investigated. Such investigations should take into account the importance of mycorrhizal association to the dynamics of soil nutrient uptake. In this regard, investigating other mycorrhizal types, such as ectomycorrhizae, is also necessary to confirm if mycorrhizal associations ubiquitously benefit nutrient acquisition in low CO_2-grown plants.

Growing different species of mycorrhizal plants, as well as plants with different N-preferences, at past low CO_2 conditions can also help improve the characterization of

ancient food webs and nutrient transfer between trophic levels, especially when using C and N isotope compositions. Discrimination against ^{15}N in *E. canadensis* grown at low CO_2 changed depending on the N source available, but it is unknow how the $\delta^{15}N$ of a NH_4^+-preferring species could change if only NO_3^- is available, especially in an environment where NO_3^- reduction is potentially facilitated by higher photorespiration. Adding further to this scenario, the presence of a mycorrhizal fungus would likely change $\delta^{15}N$ from its source to the final signal in plant tissues. Whether discrimination against ^{15}N would increase or decrease, however, is uncertain; it is unclear if the symbiosis would enhance cooperation at low CO_2 or become detrimental if the symbionts have to compete for N resources.

Considering that some needles of *P. mariana* were unable to form resin ducts, investigating the leaf anatomy of plants grown at low CO_2 is also necessary to assess which anatomical features used in the identification of leaf fossils from low CO_2 periods are a reliable indicator of species identity. Further explorations of whether C allocation to secondary metabolic functions is the main cause inhibiting resin duct formation should also be carried out. For instance, the combined effects of C-limitation at low CO_2 and reduced soil N availability may constrict the ability of plants to produce N-based defense compounds (*i.e.*, alkaloids) but not necessarily the formation of resin ducts compared to low CO_2-grown plants that are not N limited.

Lastly, a major caveat in low CO_2 studies is whether modern plant genotypes can adequately represent the response of ancient genotypes to past low CO_2 levels. One possibility to verify if the behavior of modern plants differs or not from their ancient relatives growing at low CO_2 concentrations would involve examining preserved ancient plants, or even ‘resuscitating’ ancient species when feasible. For instance, using seeds and fruits excavated from fossil burrows preserved in the Siberian permafrost dating from 30,000 years ago, Yashina *et al.* (2012) was able to grow healthy, sexually reproducing plants of *Silene stenophylla*. That said, while ancient species may be the ideal subject in low CO_2 studies, using modern genotypes is the most accessible, if not the only, available option. Besides, as shown in this thesis, modern plants still present the capacity to acclimate to sub-ambient CO_2 conditions.

5.6 References

Allen, N., Nordlander, M., Mcgonigle, T., Basinger, J., Kaminskyj, S., 2006. Arbuscular mycorrhizae on Axel Heiberg Island (80°N) and at Saskatoon (52°N) Canada. Can. J. Bot. 84, 1094–1100

Alt, D.S., Doyle, J.W., Malladi, A., 2017. Nitrogen-source preference in blueberry (*Vaccinium sp.*): Enhanced shoot nitrogen assimilation in response to direct supply of nitrate. J. Plant Physiol. 216, 79–87. https://doi.org/10.1016/j.jplph.2017.05.014

Amundson, R., Austin, A.T., Schuur, E.A.G., Yoo, K., Matzek, V., Kendall, C., Uebersax, A., Brenner, D., Baisden, W.T., 2003. Global patterns of the isotopic composition of soil and plant nitrogen. Global Biogeochem. Cycles 17. https://doi.org/10.1029/2002GB001903

Anderson, L.J., Maherali, H., Johnson, H.B., Polley, H.W., Jackson, R.B., 2001. Gas exchange and photosynthetic acclimation over subambient to elevated CO_2 in a C_3-C_4 grassland. Glob. Chang. Biol. 7, 693–707. https://doi.org/10.1046/j.1354-1013.2001.00438.x

Becklin, K.M., Walker, S.M., Way, D.A., Ward, J.K., 2017. CO_2 studies remain key to understanding a future world. New Phytol. 214, 34–40. https://doi.org/10.1111/nph.14336

Beerling, D.J., Jolley, D.W., 1998. Fossil plants record an atmospheric $^{12}CO_2$ and temperature spike across the Paleocene-Eocene transition in NW Europe. J. Geol. Soc. London. 155, 591–594.

Björkman, E., 1970. Forest tree mycorrhiza — The conditions for its formation and the significance for tree growth and afforestation. Plant Soil 32, 589–610. https://doi.org/10.1007/BF01372897

Bloom, A.J., Burger, M., Asensio, J.S.R., Cousins, A.B., Zhou, L., Shew, H.D., Rufty, T.W., Hu, S., 2010. Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. Science. 328, 899–903. https://doi.org/10.1126/science.1186440

Bloom, A.J., Burger, M., Kimball, B.A., Pinter, Jr, P.J., 2014. Nitrate assimilation is inhibited by elevated CO_2 in field-grown wheat. Nat. Clim. Chang. 4, 477–480. https://doi.org/10.1038/nclimate2183

Bolan, N.S., 1991. A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil 134, 189–207

Bomfleur, B., Decombeix, A.-L., Escapa, I.H., Schwendemann, A.B., Axsmith, B., 2013. Whole-Plant Concept and Environment Reconstruction of a *Telemachus* Conifer (Voltziales) from the Triassic of Antarctica. Int. J. Plant Sci. 174, 425–444. https://doi.org/10.1086/668686

Breuninger, M., Trujillo, C.G., Serrano, E., Fischer, R., Requena, N., 2004. Different nitrogen sources modulate activity but not expression of glutamine synthetase in arbuscular mycorrhizal fungi. Fungal Genet. Biol. 41, 542–552. https://doi.org/10.1016/J.FGB.2004.01.003

Brundrett, M.C., 2009. Mycorrhizal associations and other means of nutrition of vascular plants: Understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. Plant Soil 320, 37-77. https://doi.org/10.1007/s11104-008-9877-9

Brundrett, M.C., 2002. Coevolution of roots and mycorrhizas of land plants. New Phytol. 154, 275–304. https://doi.org/10.1046/j.1469-8137.2002.00397.x

Busch, F.A., Sage, R.F., Farquhar, G.D., 2018. Plants increase CO_2 uptake by assimilating nitrogen via the photorespiratory pathway. Nat. Plants 4, 46–54. https://doi.org/10.1038/s41477-017-0065-x

Chambers, C.A., Smith, S.E., Smith, F.A., 1980. Effects of ammonium and nitrate ions on mycorrhizal infection, nodulation and growth of *Trifolium subterraneum*. New Phytol. 85, 47–62

Cowling, S.A., Sage, R.F., 1998. Interactive effects of low atmospheric CO_2 and elevated temperature on growth, photosynthesis and respiration in *Phaseolus vulgaris*. Plant, Cell Environ. 21, 427–435. https://doi.org/10.1046/j.1365-3040.1998.00290.x

Cunniff, J., Jones, G., Charles, M., Osborne, C.P., 2017. Yield responses of wild C_3 and C_4 crop progenitors to subambient CO_2: A test for the role of CO_2 limitation in the origin of agriculture. Glob. Chang. Biol. 23, 380–393. https://doi.org/10.1111/gcb.13473

Currie, A.F., Murray, P.J., Gange, A.C., 2011. Is a specialist root-feeding insect affected by arbuscular mycorrhizal fungi? Appl. Soil Ecol. 47, 77–83. https://doi.org/10.1016/J.APSOIL.2010.12.002

Dalling, J.W., Cernusak, L.A., Winter, K., Aranda, J., Garcia, M., Virgo, A., Cheesman, A.W., Baresch, A., Jaramillo, C., Turner, B.L., 2016. Two tropical conifers show strong growth and water-use efficiency responses to altered CO_2 concentration. Ann. Bot. mcw162. https://doi.org/10.1093/aob/mcw162

Dier, M., Meinen, R., Erbs, M., Kollhorst, L., Baillie, C.-K., Kaufholdt, D., Kücke, M., Weigel, H.-J., Zörb, C., Hänsch, R., Manderscheid, R., 2017. Effects of Free Air Carbon Dioxide Enrichment (FACE) on nitrogen assimilation and growth of winter wheat under nitrate and ammonium fertilization. Glob. Chang. Biol. 24, e40-e54. https://doi.org/10.1111/gcb.13819

Drucker, D., Bocherens, H., Bridault, A., Billiou, D., 2003. Carbon and nitrogen isotopic composition of red deer (*Cervus elaphus*) collagen as a tool for tracking palaeoenvironmental change during the Late-Glacial and Early Holocene in the

northern Jura (France). Palaeogeogr. Palaeoclimatol. Palaeoecol. 195, 375–388. https://doi.org/10.1016/S0031-0182(03)00366-3

Ehleringer, J.R., Cerling, T.E., Dearing, M.D., 2005. A History of atmospheric CO_2 and its effects on plants, animals, and ecosystems, 1st ed. Springer, Salt Lake City, UT, USA

Fogel, R., 1988. Interactions among soil biota in coniferous ecosystems. Agric. Ecosyst. Environ. 24, 69–85. https://doi.org/10.1016/0167-8809(88)90057-6

Gange, A.C., Bower, E., Brown, V.K., 1999. Positive effects of an arbuscular mycorrhizal fungus on aphid life history traits. Oecologia 120, 123–131. https://doi.org/10.1007/s004420050840

Gerhart, L.M., Ward, J.K., 2010. Plant responses to low [CO_2] of the past. New Phytol. 188, 674–695. https://doi.org/10.1111/j.1469-8137.2010.03441.x

Krings, M., Taylor, T.N., Dotzler, N., 2013. Fossil evidence of the zygomycetous fungi. Persoonia 30, 1–10. https://doi.org/10.3767/003158513X664819

Loreto, F., Fischbach, R.J., Schnitzler, J.-P., Ciccioli, P., Brancaleoni, E., Calfapietra, C., Seufert, G., 2001. Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak *Quercus ilex* L. grown at elevated CO_2 concentrations. Glob. Chang. Biol. 7, 709–717. https://doi.org/10.1046/j.1354-1013.2001.00442.x

Maherali, H., Oberle, B., Stevens, P.F., Cornwell, W.K., Mcglinn, D.J., 2016. Mutualism persistence and abandonment during the evolution of the mycorrhizal symbiosis. Am. Nat. 188, E113–E125. https://doi.org/10.5061/dryad.n8bm9

Metcalfe, J.Z., Longstaffe, F.J., Hodgins, G., 2013. Proboscideans and paleoenvironments of the Pleistocene Great Lakes: Landscape, vegetation, and stable isotopes. Quat. Sci. Rev. 76, 102–113. https://doi.org/10.1016/j.quascirev.2013.07.004

Minagawa, M., Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta 48, 1135–1140. https://doi.org/10.1016/0016-7037(84)90204-7

Öpik, M., Moora, M., Liira, J., Zobel, M., 2006. Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J. Ecol. 94, 778–790. https://doi.org/10.1111/j.1365-2745.2006.01136.x

Öpik, M., Moora, M., Zobel, M., Saks, lle, Wheatley, R., Wright, F., Daniell, T., 2008. High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. New Phytol. 179, 867–876. https://doi.org/10.1111/j.1469-8137.2008.02515.x

Peñuelas, J., Estiarte, M., LLusia, J., 1997. Carbon-based secondary compounds at

elevated CO_2. Photosynthetica 33, 313–316

Petit, R.J., Raynaud, D., Basile, I., Chappellaz, J., Ritz, C., Delmotte, M., Legrand, M., Lorius, C., Pe, L., 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. Nature 399, 429–413. https://doi.org/10.1038/20859

Post, D.M., 2002. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. Ecology 83, 703–718. https://doi.org/10.1890/0012-9658(2002)083[0703:USITET]2.0.CO;2

Raimanová, I., Haberle, J., 2010. The effects of differentiated water supply after anthesis and nitrogen fertilization on δ^{15}N of wheat grain. Rapid Commun. Mass Spectrom. 24, 3567–3577. https://doi.org/10.1002/rcm

Ripley, B.S., Cunniff, J., Osborne, C.P., 2013. Photosynthetic acclimation and resource use by the C_3 and C_4 subspecies of *Alloteropsis semialata* in low CO_2 atmospheres. Glob. Chang. Biol. 19, 900–910. https://doi.org/10.1111/gcb.12091

Sage, R.F., 1995. Was low atmospheric CO_2 during the Pleistocene a limiting factor for the origin of agriculture? Glob. Chang. Biol. 1, 93–106. https://doi.org/10.1111/j.1365-2486.1995.tb00009.x

Sage, R.F., Coleman, J.R., 2001. Effects of low atmospheric CO_2 on plants: More than a thing of the past. Trends Plant Sci. 6, 18–24. https://doi.org/10.1016/S1360-1385(00)01813-6

Sage, R.F., Cowling, S.A., 1999. Implications of stress in low CO_2 atmospheres of the past: Are today's plants too conservative for a high CO_2 world?, in: Luo, Y., Mooney, H.A. (Eds.), Carbon Dioxide and Environmental Stress. Academic Press, San Diego, CA, USA, pp. 289–304

Schwartz-Narbonne, R., Longstaffe, F.J., Metcalfe, J.Z., Zazula, G., 2015. Solving the woolly mammoth conundrum: amino acid δ^{15}N-enrichment suggests a distinct forage or habitat. Sci. Rep. 5, 9791. https://doi.org/10.1038/srep09791

Schwendemann, A.B., Decombeix, A., Taylor, T.N., Taylor, E.L., Krings, M., 2011. Morphological and functional stasis in mycorrhizal root nodules as exhibited by a Triassic conifer. Proc. Natl. Acad. Sci. 108, 13630–13634. https://doi.org/10.1073/pnas.97.8.4086

Serret, M.D., Yousfi, S., Vicente, R., Piñero, M.C., Otálora-Alcón, G., del Amor, F.M., Araus, J.L., 2018. Interactive effects of CO_2 concentration and water regime on stable isotope signatures, nitrogen assimilation and growth in sweet pepper. Front. Plant Sci. 8, 2180. https://doi.org/10.3389/fpls.2017.02180

Stockey, R.A., Rothwell, G.W., Addy, H.D., Currah, R.S., 2001. Mycorrhizal association of the extinct conifer *Metasequoia milleri*. Mycology 105, 202–205.

https://doi.org/10.1017/S0953756200003221

Strullu-Derrien, C., Selosse, M.-A., Kenrick, P., Martin, F.M., 2018. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. New Phytol. https://doi.org/10.1111/nph.15076

Strullu-Derrien, C., Strullu, D.-G., 2007. Mycorrhization of fossil and living plants. Comptes Rendus Palevol 6, 483–494. https://doi.org/10.1016/J.CRPV.2007.09.006

Szpak, P., Gröcke, D.R., Debruyne, R., MacPhee, R.D.E., Guthrie, R.D., Froese, D., Zazula, G.D., Patterson, W.P., Poinar, H.N., 2010. Regional differences in bone collagen $\delta^{13}C$ and $\delta^{15}N$ of Pleistocene mammoths: Implications for paleoecology of the mammoth steppe. Palaeogeogr. Palaeoclimatol. Palaeoecol. 286, 88–96. https://doi.org/10.1016/j.palaeo.2009.12.009

Taylor, T.N., Krings, M., Taylor, E.L., 2015. Fungal diversity in the fossil record, in: Systematics and Evolution. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 259–278. https://doi.org/10.1007/978-3-662-46011-5_10

Temme, A.A., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2013. Meta-analysis reveals profound responses of plant traits to glacial CO_2 levels. Ecol. Evol. 3, 4525–4535. https://doi.org/10.1002/ece3.836

Temme, A.A., Liu, J.C., Cornwell, W.K., Cornelissen, J.H.C., Aerts, R., 2015. Winners always win: Growth of a wide range of plant species from low to future high CO_2. Ecol. Evol. 5, 4949–4961. https://doi.org/10.1002/ece3.1687

Tissue, D., Griffin, K., Thomas, R., Strain, B., 1995. Effects of low and elevated CO_2 on C_3 and C_4 annuals -II. Photosynthesis and leaf biochemistry. Oecologia 101, 21–28.

Wang, B., Qiu, Y.L., 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16, 299–363. https://doi.org/10.1007/s00572-005-0033-6

Ward, J.K., Tissue, D.T., Thomas, R.B., Strain, B.R., 1999. Comparative responses of model C_3 and C_4 plants to drought in low and elevated CO_2. Glob. Chang. Biol. 5, 857–867. https://doi.org/10.1046/j.1365-2486.1999.00270.x

Weil, R.R., Brady, N.C., 2016. The nature and properties of soils, 15th ed. Pearson

Williams, R.S., Lincoln, D.E., Thomas, R.B., 1994. Loblolly pine grown under elevated CO_2 affects early instar pine sawfly performance. Oecologia 98, 64–71

Yashina, S., Gubin, S., Maksimovich, S., Yashina, A., Gakhova, E., Gilichinsky, D., 2012. Regeneration of whole fertile plants from 30,000-y-old fruit tissue buried in Siberian permafrost. Proc. Natl. Acad. Sci. U. S. A. 109, 4008–13. https://doi.org/10.1073/pnas.1118386109

Yousfi, S., Serret, M.D., Márquez, A.J., Voltas, J., Araus, J.L., 2012. Combined use of $\delta^{13}C$, $\delta^{18}O$ and $\delta^{15}N$ tracks nitrogen metabolism and genotypic adaptation of durum wheat to salinity and water deficit. New Phytol. 194, 230–244. https://doi.org/10.1111/j.1469-

Appendices

Appendix A. Modified Hoagland's recipe for each fertilization regime. A. full-strength NO_3^-/NH_4^+ mix fertilizer (*full*); **B.** full-strength, NO_3^--only fertilizer (*nitrate*); **C.** a full-strength NH_4^+-only fertilizer (*ammonium*); **D.** half-strength NO_3^-/NH_4^+ mix fertilizer (*half*). The concentration of the stock solution of each compound (mol), based on its molecular weight (g/mol), was calculated for one liter of water (g/L) and adjusted in order to achieve the desired concentration of each element (mol/L).

A.

full	Mol. Weight (g/mol)	Stock solution (mol)	Stock solution (g/L)	Final stock solution (mL)	Element (mol/L)														
					N	K	Ca	P	S	Mg	Na	Cl	B	Mn	Zn	Cu	Mo	Co	Fe
$NH_4H_2PO_4$	115.08	1	115.08	1	0.12			0.26											
NH_4Cl	53.49	1	53.49	2.95	0.77							1.93							
KCl	74	1	74	3		1.58						1.42							
$Ca(NO_3)_2$. $4(H_2O)$	236.16	1	236.16	2	0.12		0.34												
$MgSO_4$	246.49	1	246.49	0.5					0.06	0.05									
$CoCl_2$	237.95	0.001	0.24	0.5								0.07						0.12	
H_2BO_3	61.84	0.025	1.55	0.5									0.08						
$MnSO_4$	169.01	0.002	0.34	0.5					0.09					0.16					
$ZnSO_4$	287.55	0.002	0.58	0.5					0.05						0.11				
$CuSO_4$	249.71	0.0005	0.12	0.5					0.06							0.13			
MoN_2O_4	241.95	0.0005	0.12	0.5	0.029												0.20		
Fe - EDTA	346.08	0.02	6.92	0.5															0.08
NaH_2PO_4	138	0.005	0.69	1				0.22			0.16								
Final concentration of the element (mol/L)					1.04	1.58	0.34	0.48	0.28	0.05	0.16	3.42	0.08	0.16	0.11	0.13	0.20	0.12	0.08

B.

nitrate	Mol. Weight (g/mol)	Stock solution (mol)	Stock solution (g/L)	Final stock solution (mL)	Element (mol/L)														
					N	K	Ca	P	S	Mg	Na	Cl	B	Mn	Zn	Cu	Mo	Co	Fe
KNO_3	101.1	1	101.10	2.9	0.40	1.12													
$NaNO_3$	84.99	1	84.99	3	0.49														
KH_2PO_4	136.09	1	136.09	1.6		0.46		0.35											
$Ca(NO_3)_2.\ 4(H_2O)$	236.16	1	236.16	2	0.12		0.34												
$MgSO_4.\ 7(H_2O)$	246.49	1	246.49	0.5					0.06	0.05									
NaH_2PO_4	138	0.005	0.69	0.5				0.11			0.08								
$CoCl_{2.}\ 6(H_2O)$	237.95	0.001	0.24	0.5								0.07						0.12	
H_2BO_3	61.84	0.025	1.55	0.5									0.08						
$MnSO_4.\ (H_2O)$	169.01	0.002	0.34	0.5					0.09					0.16					
$ZnSO_4.\ 7(H_2O)$	287.55	0.002	0.58	0.5					0.06						0.11				
$CuSO_4.\ 5(H_2O)$	249.71	0.0005	0.12	0.5					0.06							0.13			
$MoN_2O_4.\ 2(H_2O)$	241.95	0.0005	0.12	0.5	0.03												0.20		
Fe - EDTA	346.08	0.02	6.92	0.5															0.08
Final concentration of the element (mol/L)					1.04	1.58	0.34	0.46	0.28	0.05	0.08	0.07	0.08	0.16	0.11	0.13	0.20	0.12	0.08

C.

ammonium	Mol. Weight (g/mol)	Stock solution (mol)	Stock solution (g/L)	Final stock solution (mL)	Element (mol/L)													
					N	K	Ca	P	S	Mg	Na	Cl	B	Mn	Zn	Cu	Mo	Co	Fe
$NH_4H_2PO_4$	115.08	1	115.08	1.4	0.17			0.36											
NH_4Cl	53.49	1	53.49	3.2	0.84							2.09							
KCl	74	1	74	3		1.58						1.42							
$CaCl_2$	110.98	1	110.98	0.95			0.34					0.30							
$MgSO_4$	246.49	1	246.49	0.5					0.06	0.05									
NaH_2PO_4	138	0.005	0.69	0.5				0.11			0.08								
$CoCl_2$	237.95	0.001	0.23795	0.5								0.07						0.12	
H_2BO_3	61.84	0.025	1.546	0.5									0.08						
$MnSO_4$	169.01	0.002	0.33802	0.5					0.09					0.16					
$ZnSO_4$	287.55	0.002	0.5751	0.5					0.06						0.11				
$CuSO_4$	249.71	0.0005	0.124855	0.5					0.06							0.13			
MoN_2O_4	241.95	0.0005	0.120975	0.5	0.03												0.20		
Fe - EDTA	346.08	0.02	6.9216	0.5															0.08
Final concentration of the element (mol/L)					1.04	1.58	0.34	0.47	0.28	0.05	0.08	3.89	0.08	0.16	0.11	0.13	0.20	0.12	0.08

D.

half	Mol. Weight (g/mol)	Stock solution (mol)	Stock solution (g/L)	Final stock solution (mL)	Element (mol/L)													
					N	K	Ca	P	S	Mg	Na	Cl	B	Mn	Zn	Cu	Mo	Co	Fe
$NH_4H_2PO_4$	115.08	1	115.08	1	0.12			0.26											
NH_4Cl	53.49	1	53.49	0.9	0.18							0.59							
KNO_3	101.1	1	101.10	1	0.19	0.54													
KCl	74	2.3	74	1		1.03						1.09							
$CaCl_2$	110.98	1	110.98	0.95			0.34					0.30							
$MgSO_4$	246.49	1	246.49	0.5					0.06	0.05									
$CoCl_2$	237.95	0.001	0.24	0.5								0.07						0.12	
H_2BO_3	61.84	0.025	1.55	0.5									0.08						
$MnSO_4$	169.01	0.002	0.34	0.5					0.09					0.16					
$ZnSO_4$	287.55	0.002	0.58	0.5					0.06						0.11				
$CuSO_4$	249.71	0.0005	0.12	0.5					0.06							0.13			
MoN_2O_4	241.95	0.0005	0.12	0.5	0.03												0.20		
Fe - EDTA	346.08	0.02	6.92	0.5															0.08
NaH_2PO4	138	0.005	0.69	1				0.22			0.16								
Final concentration of the element (mol/L)					0.53	1.57	0.34	0.48	0.28	0.05	0.16	2	0.08	0.16	0.11	0.13	0.20	0.12	0.08

Appendix B. Carbon isotope composition of leaves ($\delta^{13}C_{leaf}$) and roots ($\delta^{13}C_{root}$) in each inoculation and growth CO_2 treatment, adjusted to a common value (–8.4‰) for atmospheric CO_2 in the growth chambers. Means ± SE, n = 12.

		$\delta^{13}C$ (‰, VPDB)					
Inoculation	**CO_2 (ppm)**	***Measured* $\delta^{13}C_{leaf}$**	***Measured* $\delta^{13}C_{root}$**	***Measured CO_2* $\delta^{13}C_{chamber}$**	***Correction to –8.4‰***	***Adjusted* $\delta^{13}C_{leaf}$**	***Adjusted* $\delta^{13}C_{root}$**
control	180	–34.91 ± 0.33	–33.01 ± 0.22	–8.72 ± 0.39	0.32 ± 0.01	–34.59 ± 0.33	–32.69 ± 0.22
	270	–31.91 ± 0.34	–30.84 ± 0.30	–7.80 ± 0.41	–0.60 ± 0.01	–32.51 ± 0.34	–31.44 ± 0.30
	400	–36.37 ± 0.14	–35.03 ± 0.17	–11.23 ± 0.13	2.83 ± 0. 01	–33.54 ± 0.14	–32.20 ± 0.17
mycorrhizae	180	–35.93 ± 0.29	–33.96 ± 0.33	–8.72 ± 0.39	0.32 ± 0.01	–35.61 ± 0.29	–33.64 ± 0.33
	270	–35.30 ± 0.32	–33.36 ± 0.35	–7.80 ± 0.41	–0.60 ± 0. 01	–35.90 ± 0.32	–33.96 ± 0.35
	400	–37.57 ± 0.24	–36.25 ± 0.36	–11.23 ± 0.13	2.83 ± 0. 01	–34.74 ± 0.24	–33.42 ± 0.36

6 CV

André Galvao Duarte

Education

2015 – present: **Ph.D. in plant ecophysiology and climate change** (expected August 2019)**,** University of Western Ontario, Department of Biology, London, ON, Canada.

2013 – 2015: **M.Sc. in Environmental Sciences,** Albert-Ludwigs-Universität Freiburg, Freiburg im Breisgau, Germany.

2003 – 2006: **B.Sc./Teaching degree in Biology,** Universidade do Estado de Minas Gerais, Passos, Brazil.

Publications

Duarte, A. G.; Katata, G., Hoshika, Y.; Hossain, M.; Kreuzwieser, J.; Arneth, A.; Ruehr, N. K. (2016). Immediate and potential long-term effects of consecutive heat waves on the photosynthetic performance and water balance in Douglas-fir. *Journal of Plant Physiology*, *205*, 57-66.

Dusenge, M. E.*; **Duarte, A. G.***; Way, D. A. (2018). Plant carbon metabolism and climate change: elevated CO_2 and temperature impacts on photosynthesis, photorespiration and respiration. *New Phytologist*, *221*, 32-49. (* authors contributed equally to the work)

In revision

Duarte, A. G.; Longstaffe, F. J.; Way, D. A. Nitrogen source and availability influence low CO_2 effects on plant performance. *Functional Plant Biology*.

In prep

Duarte, A. G.; Longstaffe, F. J.; Way, D. A. The balance between plant growth and defense compound production at low CO_2 concentrations of the past.

Duarte, A. G.; Longstaffe, F. J.; Way, D. A. Plant-mycorrhizal interaction at past low CO_2 concentrations: effects on growth and C and N isotope signature.

Book chapter in review

Duarte, A. G.; Dusenge, M. E.; McDonald, S.; Bennett, K.; Lemon, K.; Radford, J.; Way, D. A. Photosynthetic acclimation to temperature and CO_2: the role of leaf N. *Springer*.

Work experience

2015 – present: **Graduate Teaching Assistant,** University of Western Ontario, London, Canada. Lab instructor for the courses Biology for Sciences I and II, Plant as human resources, and Plant ecophysiology.

Aug. – Nov. 2014: **Research Assistant,** Institute of Meteorology and Climate Research, Karlsruhe Institute of Technology, Garmisch-Partenkirchen, Germany. Measurements and data analysis in a greenhouse experiment on the effects of elevated temperature on plant growth.

May 2014: **Student Assistant,** Faculty of Environment and Natural Resources, Albert-Ludwigs-Universität Freiburg, Germany. Translation and organization of documents related to projects of biodiversity conservation in the Amazonian floodplains.

Jul. 2007 – Aug. 2013: **Services Manager,** Banco do Brazil, Brazil. Coordination of customer service, inventory, hiring of service providers.

Conferences

André G. Duarte, Fred J. Longstaffe, Danielle A. Way (2019). Do I grow or do I fight: Plant growth and defense compound production at past low [CO_2]. Gordon Research Conference – CO_2 Assimilation in Plants: from Genome to Biome, Newry, ME, USA.

André G. Duarte, Fred J. Longstaffe, Danielle A. Way (2018). Nitrogen source and availability alter the effect of low CO_2 on growth, photosynthesis, and N dynamics. Plant Biology: Joint meeting of the Canadian Society of Plant Biologists & the American Society of Plant Biologists, Montreal, QC.

André G. Duarte, Fred J. Longstaffe, Danielle A. Way (2018). Do I grow or do I fight: Plant growth and defense compound production at past low [CO_2]. Canadian Society of Plant Biologists, Easter Regional Meeting, London, ON.

André G. Duarte, Fred J Longstaffe, Danielle A Way. (2017) Nitrogen source and availability alter the effect of low CO_2 on growth, photosynthesis, and N dynamics. Fallona Interdisciplinary Showcase, London, ON.

André G. Duarte, Fred J. Longstaffe, Danielle A. Way (2017). Nitrogen source and availability alter the effect of low CO_2 on growth, photosynthesis, and N dynamics. Canadian Society of Plant Biologists and the Canadian Society for Horticultural Science, Annual Meeting, Vancouver, BC.

André G. Duarte, Fred J. Longstaffe, Danielle A. Way (2016). Nitrogen source and availability alter the effect of low CO_2 on growth, photosynthesis, and N dynamics. Canadian Society of Plant Biologists, Easter Regional Meeting, Burlington, ON.

Nadine Ruehr, **André G. Duarte**, Almut Arneth (2016). Beyond the extreme: Recovery dynamics following heat and drought stress in trees. AGU Fall Meeting. San Francisco, USA.

André G. Duarte; Katata, Genki; Ruehr, Nadine (2015). Effects of heat waves on photosynthetic performance in Douglas-fir (*Pseudotzuga mensinensis*). Canadian Society of Plant Biologists, Easter Regional Meeting, Toronto, ON.

Scholarships and awards

PhD Scholarship – Science without borders, Conselho Nacional de Desenvolviment e Pesquisa, $90.000, 00

Poster Award, Fallona Interdisciplinary Research Showcase, $2,500.00

Travel Award, Western University – Biology Department, $400.00

Georg H. Duff Travel Bursary, Canadian Society of Plant Biologists, $75.00

Service and outreach

Councilor in the Environmental and Ecological Planning Advisory Committee of the city of London, 2019

Representative of the Faculty of Science in the Graduate Education Council of the School of Graduate and Post-Doctoral Studies at Western University, 2016 – 2018

Chairperson of the Society of Biology Graduate Students, Western University, 2016 – 2017

Member of the Fundraising committee of the Biology Graduate Research Forum 2016 at Western University