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Dawn Marie Matarese  
Portland State University

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Impacts of Rhizosphere CO<sub>2</sub> on Root  
Phosphoenolpyruvate Carboxylase Activity, Root Respiration Rate  
and Rhizodeposition in *Populus spp.*

by

Dawn Marie Matarese

A thesis submitted in partial fulfillment of the  
requirements for the degree of

Master of Science  
in  
Biology

Thesis Committee:  
Todd N. Rosenstiel, Chair  
Sarah Eppley  
Stanley Hillman

Portland State University  
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## **Abstract**

Roots live in and have evolved in a high carbon dioxide (CO<sub>2</sub>) environment, yet relatively little research has been conducted on the impacts of soil dissolved inorganic carbon (DIC) on root metabolism. In this thesis, I explore the impacts of root-zone DIC on whole plant biomass accumulation, water use efficiency, and above-ground gas exchange. In addition, I explore the impacts of root-zone DIC on root processes: root PEP-Carboxylase activity, root respiration rate and root exudation of Krebs cycle organic acids.

Root-zone DIC did not impact biomass accumulation, leaf gas exchange parameters or water use efficiency under the growth conditions examined. Root-zone DIC did increase root PEP-Carboxylase activity, but decreased root respiration (both CO<sub>2</sub> production and O<sub>2</sub> consumption) and decreased organic acid exudation rates. Increase in measurement CO<sub>2</sub> partial pressure was found to cause an instantaneous decrease in root CO<sub>2</sub> production, and I provide evidence that changes in root metabolism (CO<sub>2</sub> uptake by roots) are part of the cause of this phenomenon. A hypothesized relationship between root respiration rate and Krebs cycle organic acid exudation was not supported by my data. I conclude that root-zone DIC has important impacts on critical functions of root metabolism, and should be considered as an important abiotic factor much in the same way atmospheric CO<sub>2</sub> is for leaves and whole plant biology.

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## Table Of Contents

i	Abstract
ii	Acknowledgements
iv	List of Tables
v	List of Figures
1	Chapter I Introduction
18	Chapter II Methods
30	Chapter III Results
37	Chapter IV Discussion
53	Tables and Figures
72	References

## List Of Tables

- 53      Table 1: Leaf Gas Exchange Characteristics
- 53      Table 2: Whole Plant Biomass and Water Use

## List Of Figures

- 54      Figure 1: Root PEP-C Activity
- 55      Figure 2: Malate Inhibition of Root PEP-C Activity
- 56      Figure 3: Effects of pH on Root PEP-C Activity
- 57      Figure 4: Root CO<sub>2</sub> Production
- 58      Figure 5: Root Respiration CO<sub>2</sub> Response Curve
- 59      Figure 6: Root Liquid Phase O<sub>2</sub> Consumption
- 60      Figure 7: Root Gas Phase O<sub>2</sub> Consumption
- 61      Figure 8: Variation in Individual Root Respiration
- 62      Figure 9: Root Respiratory Response to Measurement CO<sub>2</sub> concentration
- 63      Figure 10: Krebs Cycle Organic Acid Exudation: Succinate and Total
- 64      Figure 11: Krebs Cycle Organic Acid Exudation: Citrate/ $\alpha$ -Ketoglutarate, Pyruvate, Malate/Fumarate
- 65      Figure 12: Succinate Exudation and Root CO<sub>2</sub> Production
- 66      Figure 13: Succinate Exudation and Root Response to measurement CO<sub>2</sub>
- 67      Figure 14: Pyruvate Exudation and Root CO<sub>2</sub> Production
- 68      Figure 15: Pyruvate Exudation and Root Response to measurement CO<sub>2</sub>
- 69      Figure 16: Citrate/ $\alpha$ -Ketoglutarate Exudation and Root CO<sub>2</sub> Production
- 70      Figure 17: Representation of Metabolic Activities in a Root Cell associated with Root Carbon Fixation
- 71      Figure 18: Illustration of Gas-Mixer and Hydroponic Root Chamber System

## **Chapter I Introduction**

### **PLANT ROOTS AND THE RHIZOSPHERE**

Roots began to evolve during the late Silurian period, at the time that plant life moved onto land (Kenrick 2002). The development of extensive root systems accelerated when plants moved into drier areas (Fitter 2002). Early roots were rhizoids, which are little more than protrusions of the plant epidermal layer, that grew off of prostrate stems. Roots as we know them did not exist until the development of the vascular system and the bipolar growth pattern, in which the shoot has one apical meristem, growing up, and the root has another, growing downward (Kenrick 2002). These developments allowed plants to grow larger, and differentiate into other growth habits. Root systems have been evolving throughout the history of land plants, for almost 400 million years, and are an integral part of plant physiological systems.

As the belowground organs of plants, roots perform two primary functions: roots obtain resources from the soil, such as water and nutrients, and they provide anchorage to the soil matrix (Fitter 2002). Other secondary functions of roots vary by species. Roots can provide storage (tubers), and act as means of propagation and dispersal (root buds and suckers) (Fitter 2002). Variation in root morphology is limited compared to what is seen in leaves, but roots do vary in diameter, color and texture, as well as in physiology, such as growth rate, longevity, transport



capacity, membrane composition, and association with symbiotic organisms (Fitter 2002, Kottke 2002).

The rhizosphere is the region of soil influenced by root activity (Neumann and Römheld 2002). The soil in the rhizosphere is different from the surrounding bulk soil firstly because of rhizodeposition, which is the release of various forms of organic carbon from roots (Jones 2009). These carbon sources attract microbial populations (Lambers 2009). Additionally, roots and microbes associating in the rhizosphere alter soil structure by creating pores, soil aggregates, and altering the flow paths of water through the soil (Angers 1998, Bundt 2001).

### ***Soil Carbon Dioxide***

Although much experimental research has focused on the role of atmospheric carbon dioxide ( $\text{CO}_2$ ) in influencing leaf physiology, little research has been conducted on roots, despite the fact that roots live in a relatively enriched  $\text{CO}_2$  environment, when compared to shoots and leaves. Root respiration and microbial respiration, including decomposition of organic material, are major contributors to the soil inorganic carbon pool. Soil respiration, which is the measure of  $\text{CO}_2$  flux out of the soil from root and microbial respiration, is 10 times larger than the flux from fossil fuel combustion, when measured on the global scale (Andrews and Schlesinger 2001). However, soil limits the diffusion of  $\text{CO}_2$ , resulting in far higher partial pressures below ground than in the atmosphere. Measurements of soil partial pressure of  $\text{CO}_2$  ( $P_{\text{CO}_2}$ ) in forests are in the range of 0.1 to 2%  $\text{CO}_2$  at depths of 15 to 70cm (1,000 to 20,000ppm). At depths of 1 to 2 meters,  $P_{\text{CO}_2}$  can be as high as 5.4% (Andrews and Schesinger 2001, Karberg 2005, Norton 2001), whereas atmospheric  $P_{\text{CO}_2}$  is currently estimated at approximately 0.038% and rising (Karberg 2005).

Understanding the dynamics of carbon dioxide in soil is further complicated by the fact that water and air in the soil environment come into equilibrium over time, and as a result, carbon dioxide dissolves and exists in different forms that are collectively known as dissolved inorganic carbon (DIC). DIC is the sum of dissolved CO<sub>2</sub> gas (CO<sub>2(aq)</sub>), carbonic acid (H<sub>2</sub>CO<sub>3</sub>\*), bicarbonate (HCO<sub>3</sub><sup>-</sup>), and carbonate (CO<sub>3</sub><sup>2-</sup>). The relative distribution between these species at any given time is primarily driven by pH and temperature, as well as additional components of soil chemistry (Karberg 2005).

### ***Movement of rhizosphere CO<sub>2</sub> into roots***

There is evidence that either bicarbonate (HCO<sub>3</sub><sup>-</sup>) or dissolved CO<sub>2</sub> gas, or perhaps both, can move into root cells. For example, roots grown at 5,000ppm CO<sub>2</sub> took up 9 times greater DIC than roots grown at 360ppm (Cramer and Richards 1999). Bicarbonate (more prominent at higher pH) requires a transporter (van der Westhuizen 1998), but CO<sub>2</sub> gas could diffuse into a cell, although it may enter via aquaporins, as well. Aquaporins are protein channels spanning cellular membranes that regulate the movement of water, and some solutes, into cells (Maurel 2008). Recent research into the role of aquaporins in plants has revealed that carbon dioxide gas is also transported via aquaporins, and that various types of aquaporins are present in root tissues (Sade 2010, Maurel 2008). This research reveals a likely pathway for rhizosphere CO<sub>2</sub> to enter root cells. Inside of cells, CO<sub>2</sub> would be subject to the same speciation into DIC forms as occurs in any aqueous medium. However, the enzyme carbonic anhydrase, which very rapidly interconverts between CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup>, has been found in root cell cytosol (Tetu 2007, Dimou 2009). In fact, root carbonic anhydrase and root PEP carboxylase (see discussion below) have been found together in

the same locations, which allows for the possibility that they are working in concert to transform cytosolic CO<sub>2</sub> into organic molecules.

### ***Root carbon fixation***

Considering that there are high carbon dioxide concentrations below ground in the vicinity of roots, and that leaves and stems above ground uptake CO<sub>2</sub> for photosynthesis, it is unsurprising that roots also have mechanisms for fixing CO<sub>2</sub>, however this possibility is rarely considered in studies of whole plant physiology. There is considerable evidence that roots can take up rhizospheric carbon and incorporate it directly into organic molecules [Vuorinen 1992, Cramer et al 1993, Johnson 1994, 1996]. Numerous root carbon fixation studies have measured the distribution of labeled carbon (NaH<sup>14</sup>CO<sub>3</sub>, H<sup>13</sup>CO<sub>3</sub><sup>-</sup>) supplied to the roots. There are several key findings of these studies worth presenting: 1) There is direct evidence of incorporation of rhizosphere carbon into plant tissues, as the labeled carbon exposed only to roots is later measured as organic acids, amino acids, sugars and proteins in whole plant tissues. Chang and Roberts (1992) estimated that 40% of cytoplasmic bicarbonate had originated outside of the cell. 2) Inorganic carbon taken up by roots is preferentially made into organic acids and amino acids (Ting and Dugger, Johnson 1994). In addition, a few studies have reported that sugars and proteins had also been synthesized from root-derived carbon (Vuorinen 1992, Johnson 1996). Finally, Ford et al (2007) found <sup>13</sup>C label from roots distributed throughout the plant when label was applied in a 4-6 week pulse experiment. 3) To date, all studies support the role of the Phosphoenolpyruvate carboxylase enzyme (PEP-C) as the key carboxylase in the root-mediated carbon fixation process (Ting and Dugger, Chang 1992, Edwards 1998). In a very early <sup>14</sup>C study, the label was first found in oxaloacetate, the product of

PEP-C (Ting & Dugger 1967). Additional evidence of PEP-C's role in root carbon fixation is provided by Johnson et al (1994), who measured elevated PEP-C activity and content in roots that also had assimilated more  $^{14}\text{C}$  label.

## **MANY FUNCTIONS OF PHOSPHOENOLPYRUVATE CARBOXYLASE (PEP-C)**

Phosphoenolpyruvate carboxylase is most well known for its role in  $\text{C}_4$  photosynthesis. However, forms of PEP-C are found in plant tissues besides leaves, including stems, fruit, and roots (Berveiller and Damesin 1998, Dong 1998). PEP-C catalyzes the formation of oxaloacetate ( $\text{OAA}^-$ ) from phosphoenolpyruvate (PEP) and  $\text{HCO}_3^-$  with  $\text{Mg}^{2+}$  as an obligate cofactor (Latzko & Kelly 1983) (Figure 17).

### ***Regulation of Cytosolic pH***

In addition to its well-characterized role in  $\text{C}_4$  photosynthesis, the ubiquitous PEP-C enzyme is hypothesized to have several non-photosynthetic roles in plants as well:

Controversially, PEP-C has been thought to regulate cytosolic pH, in combination with Malate Dehydrogenase (MDH) and Malic Enzyme (Guern 1983). Britto & Kronzucker (2005) provide evidence that the pH stat model (above) is not consistent with observed activities of PEP-C. They argue that so many other metabolites appear to have some regulatory effect on the enzyme (PEPC activity is strongly allosterically modulated) it seems unlikely that it could also function in pH regulation. It is much more likely that the inverse is true; that pH impacts PEP-C activity *in vivo*, as it does other metabolic processes in the cell (Plaxton and Podesta 2006). Britto & Kronzucker also

suggest that the connection between malate pools and PEP-C activity has little to do with pH regulation, and more to do with the demand for anaplerotic carbon (2005). Chang and Roberts (1992) note that PEP-C activity in roots has been observed under contradictory metabolite conditions (that inhibitors and activators of PEP-C may vary in different circumstances). Despite the role of PEP-C in root metabolism, the regulation of root PEP-C *in vivo* is still not fully understood (Jeanneau 2002).

### ***Recapture of respired CO<sub>2</sub>***

There is evidence that PEP-Carboxylase recaptures respired CO<sub>2</sub> moving from the roots through the xylem stream (Berveiller and Damesin 2008). Ford et al. (2007) found that rhizospheric carbon contributed to the root anaplerotic pathway, but that the stems of pine seedlings assimilated the carbon as well, suggesting that root carbon can move up through the plant. PEP-C likely recaptures respired CO<sub>2</sub> intracellularly as well (Cramer 2002). The concentration of HCO<sub>3</sub><sup>-</sup> in root cytosol was estimated by Chang and Roberts (1992) as roughly 11mM, with 6.8mM coming from respired CO<sub>2</sub> (mitochondrial) and 4.2mM coming from sources outside of the cell.

### ***Anaplerosis***

The anaplerotic function involves PEP-C replenishing the organic acids of the Krebs Cycle (Figure 17). The various Krebs cycle intermediates most notably including malate, oxaloacetate, citrate, succinate, and  $\alpha$ -ketoglutarate, are important for respiratory production of NADH, but also serve as starting points for other biosynthetic pathways, including amino acids and chlorophyll biosynthesis (Latzko & Kelly 1983). As these metabolites are siphoned off for these other purposes,

a deficit occurs in the Krebs cycle pool, particularly in the part of the cycle that occurs from  $\alpha$ -ketoglutarate to malate (Edwards 1998). Without PEP-C to replenish these organic acids, Krebs cycle activities would eventually stop (Plaxton and Podesta 2006). PEP-C's contribution to the Krebs' cycle has been shown to change, relative to the glycolytic input, depending on which amino acids are being synthesized by the cell (Edwards 1998). Edwards et al (1998) used  $^{13}\text{C}$  labeling, NMR and GC-MS techniques to estimate that PEP-C was the source of  $62\pm 5\%$  of the malate in root tips in their study.

Krebs' cycle replenishment by PEP-C suggests that its activity could have a direct effect on root respiration rates. Increased substrate pool for the Krebs cycle could lead to more cycling, leading to the production of more reducing power (NADH) and more ATP. Johnson et al. (1994) compared respiratory  $\text{O}_2$  consumption between normal and cluster roots (a specialist root type in some plant species which have higher PEP-C content) and found *reduced* consumption in these carbon-fixing roots. In their system, carbon from PEP-C was found preferentially in citrate in the roots, but in even higher amounts in the exudates (also citrate) outside of the roots. It seems likely that the TCA cycle was being 'short-circuited' under these conditions, resulting in less NADH, and decreased respiration rates. I will return to this hypothesis below.

## **FUNCTIONAL SIGNIFICANCE OF ROOT-DERIVED CARBON**

Despite the many potential functions of root PEPC *in vivo*, there is some general agreement among root researchers as to the functional significance of root-derived carbon. Most

studies (and there are relatively few to date) suggest that anaplerosis (as discussed above), root exudation, and to a lesser extent, overall plant biomass are the main contributions that carbon fixed in roots makes to plant metabolism. Each of these will be discussed below.

### ***Whole Plant Effects***

Contributions of root-derived carbon to overall plant biomass seem to vary considerably across species, and with growth conditions. Cramer and Richards (1999) note that these types of whole plant effects seem to be extremely dependent upon growth conditions. They found that they could stimulate plant biomass (in tomato) with root DIC if the plants were grown at high irradiances (1500 $\mu$ E). However at lower irradiance levels, root DIC only stimulated biomass if there was some co-occurring plant stress, such as salinity or high temperature. Notably, Bialczyk et al. (2007) developed a protocol synthesizing various root carbon fixation studies which enabled them to increase tomato yield by 20%. However, they very precisely controlled rhizosphere DIC concentrations (5mM bicarbonate), nitrogen species ratios (4:1  $\text{NO}_3^-:\text{NH}_4^+$ ), and media pH (pH =6.9), conditions which may be very different from edaphic conditions in many natural soils. Interestingly, under their ideal experimental conditions, the tomato fruits had higher concentrations of the Krebs cycle acids malate and citrate than the control fruits.

A few studies have investigated possible impacts of root carbon uptake on above-ground metabolic processes, such as photosynthetic rate, stomatal conductance, transpiration rate and water use efficiency. Effects on these gas exchange characteristics also seem to vary with growth conditions. Tomato plants grown with low light intensities and high RH (80%) had lower stomatal conductance, lower transpiration rates, and higher water use efficiency if grown with rhizosphere

DIC (5000ppm). Plants grown at 40% RH did not exhibit the same differences (Cramer and Richards 1999). These authors conclude that there is a commonality amongst the conditions that allow root DIC to benefit both biomass and water use efficiency: Salinity, high temperature and high irradiance would all likely cause some stomatal closure to control water loss, also causing a reduction in photosynthetic capacity due to lower CO<sub>2</sub> conductance. Plants grown in high DIC were found to have 13-fold higher concentrations of carbon compounds moving up the xylem stream, perhaps allowing these plants to close their stomata more while maintaining the same photosynthetic rate (Cramer and Richard 1999). He et al. (2007, 2010) found similar results in lettuce, such that plants grown aeroponically at elevated root-zone CO<sub>2</sub> and high irradiance (2100 $\mu$ E) had higher photosynthetic rates and lower stomatal conductance than control plants. In the field, abiotic conditions in which soil carbon could have impacts on above ground gas exchange or biomass may be rare and highly unpredictable.

There have been studies utilizing non-crop systems in which the impacts of root-derived carbon are primarily in root metabolic processes, as opposed to growth. A good illustration of this is presented by Vuorninen et al. (1992) who estimated that while root carbon fixation may be only roughly 1-2% of total plant carbon fixation, that amount may represent 25% of the carbon necessary to assimilate nitrogen, highlighting the potential important interaction between root carbon fixation and nitrogen assimilation. Cramer et al. (1999) found that the rates of DIC assimilation were about 30% of the rates of respiration in the root, signifying that DIC uptake is important for the root carbon budget. In this way, contributions to individual metabolic processes appear to be proportionally greater than what a biomass measurement might reveal. The most



common example is the high percentage of root-derived carbon that is allocated to root organic acid exudation, for example, 20-30% in lupine (Johnson 1996).

### ***Root exudation***

Root-derived carbon is commonly found to contribute largely to root organic acid exudation. Organic acid exudation is generally considered to aid in nutrient acquisition by roots, or to protect roots from toxins, such as aluminum, in the soil (Cramer 2002). Although there is clear evidence that root DIC uptake and root organic acid exudation are both elevated in roots exposed to nutrient deficiency (iron or phosphorus), there remains some controversy as to why (Jones 2009). It appears that successful phosphorus acquisition via organic acid exudation may require very high exudation rates, i.e. a burst, of exudation in concert with sudden acidification of the soil and exudation of other anti-microbial compounds, in order to obtain phosphorus before microbes consume the exudates (Lambers 2009, Shane 2004). This suite of behaviors seems to occur mostly in proteoid or cluster roots, a form of specialized root that occurs only in some species (Shane 2004). Organic acid exudation may also contribute to what is known as the rhizosphere “priming effect,” in which plants provide organic carbon to encourage colonization by mycorrhizae or to feed other microbial populations that in turn support plant growth in some way, such as in release of nutrients from decaying organic matter (Lambers 2009).

Many root carbon fixation studies have focused on the relationship of root DIC uptake with nutrient deficiency and/or nutrient acquisition. (Johnson 1994, Cramer et al. 1993, DeNisi 2000, Ollat 2003, Roosta 2008, Plaxton and Podesta 2006). Generally, these studies have found either higher DIC uptake in plants grown under nutrient deficiency or have found higher PEP-C activity in

plants grown under nutrient deficiency. Lupine root carbon fixation was found to occur at higher rates in phosphorus deficient treatments, and PEP-C fixed carbon was found to contribute significantly (66%) to the citrate exuded from the roots (Johnson 1994,1996). In iron-deficient cucumber roots, PEP-C activity was four times greater than control roots, which then returned to control values when iron was re-supplied (DeNisi 2000). Although it is not clear in all of these studies exactly how the DIC uptake is beneficial to the plant, rhizospheric carbon fixation is an important factor for plant nutrient acquisition, since PEP-C and rhizospheric carbon provides carbon skeletons necessary for amino acid formation during nitrogen assimilation (Cramer 2003). There is considerable concrete evidence that the root DIC uptake leads to higher rates of nitrogen assimilation in some systems (Cramer 1999, 2002). Lupine leaves were found to have increasing nitrogen content as rhizosphere CO<sub>2</sub> partial pressures were increased, indicating that rhizosphere CO<sub>2</sub> is also beneficial for N-fixing species (Cramer 2005). However, it is important to note that in these studies the carbon fixation rates and corresponding PEP-C activities for the control roots were non-zero, confirming that there are functions for PEP-C that are independent of nutrient acquisition.

### ***Root Respiration***

In plants, respiration is the release of energy stored in organic molecules, through the three pathways of glycolysis, Krebs cycle, and electron transport chain. In non-photosynthetic tissues such as roots, energy is provided solely by respiration. Plant respiration is very complex, including many alternative enzymes and shunts to biosynthetic pathways at various stages. This

complexity and redundancy may exist because it aids in plant ability to survive stress as sessile organisms (Plaxton and Podesta 2006).

When studying the use of inorganic carbon by roots, there are a few respiratory processes of note. PEP-C, which has been discussed as the root carbon fixation enzyme, is also an important terminal glycolysis enzyme in plant tissues and is considered to be important for the regulation of respiration (Figure 17). Phosphoenolpyruvate (PEP) is one of the final products of glycolysis, and can be a substrate for either Pyruvate kinase (PK) or PEP-C (Plaxton and Podesta 2006). The primary flux of PEP for respiration is through pyruvate kinase. However during various cellular or metabolic conditions, such as phosphate limitation or biosynthesis of amino acids, flux through PEP-C may be equal to or even surpass that of PK (Plaxton and Podesta 2006, Edwards 1998).

The Krebs cycle, important for the biosynthesis of organic acids for exudation and carbon skeletons for amino acid production, and discussed above as a sink for root-derived carbon, is also the pathway for creating reducing power in the form of NADH. NADH is then utilized by the electron transport chain to move protons across the mitochondrial membrane to create the “proton motive force” necessary to create ATP (Figure 17). However, if conditions exist such that there is phosphorus limitation, the production of ATP might not be favorable. In this case, the NADH can be used up by the alternative oxidase (AOX) instead of the traditional, ATP-generating pathway of cytochrome-C oxidase (Cyt C) (Podesta and Plaxton 2006). The utility of AOX in plant respiration is just beginning to be understood, but AOX activity does seem to occur in concert with the biosynthetic function of the Krebs cycle, and has been shown to increase in roots exposed to DIC (Shane 2004, van der Westhuizen 1998).

## EFFECTS OF CO<sub>2</sub> ON ROOT RESPIRATION

### *Short-term or direct effects on root CO<sub>2</sub> production*

There has been substantial controversy amongst researchers as to whether or not there are direct effects of carbon dioxide on respiratory processes. Some authors have reported that as measurement CO<sub>2</sub> concentration is increased, root respiration (CO<sub>2</sub> production) decreases, but others have found no effect (Burton 1997, van der Westhuizen 1998, Bouma 1997). Some authors conclude that uptake of CO<sub>2</sub> by roots is the cause of this apparent decrease, while others have sought out other metabolic causes. CO<sub>2</sub> has been shown to directly inhibit the activities of the respiratory enzymes cytochrome c oxidase and succinate dehydrogenase (Gonzalez-Meler 1996), but the authors of these studies conclude that these enzyme inhibitions do not explain tissue or whole-organ scale impacts on respiration like those cited above (Gonzalez-Meler 2004). They suggest that broader scale direct inhibition of respiration would be unlikely because of two factors: **1.** Plants could simply increase enzyme concentration to make up for the inhibition by CO<sub>2</sub>, and **2.** The activity of AOX, which is not inhibited by CO<sub>2</sub>, would mask the inhibition during tissue or organ-scale measurements because it still consumes O<sub>2</sub> (Gonzalez-Meler 2004). These authors conclude that the many observations of CO<sub>2</sub> inhibition of respiration are due to leaks in the measurement chambers, and that in leaf measurements there were leaks through the leaf tissues themselves (Gonzalez-Meler 2004, Burton 2002, Janke 2001). While a leak could certainly cause this phenomenon in measurements of CO<sub>2</sub> respiration, it does not seem to explain various (direct and indirect) observed effects of CO<sub>2</sub> on oxygen consumption (Cramer 2002, Johnson 1994, Burton 1997, van der Westhuizen 1998).

### ***Short-term effects on O<sub>2</sub> consumption***

Burton et al (1997) found a direct inhibition of O<sub>2</sub> consumption in roots when increasing the measurement CO<sub>2</sub> concentration from 1000 to 5000ppm. They later suggested that these results should be disregarded, although there is not much rationale to do so except that the companion CO<sub>2</sub> production response data is suspected of being confounded by leaks (Burton 2002). Leaks during O<sub>2</sub> measurement would cause the same magnitude of error in O<sub>2</sub> measurement at all CO<sub>2</sub> concentrations, unlike the situation during CO<sub>2</sub> measurement, in which the concentration inside and outside of the chamber are different only for some of the measurements, causing different magnitudes of error. Interestingly, Burton's data shows a non-linear decrease in both CO<sub>2</sub> and O<sub>2</sub> respiration in response to measurement CO<sub>2</sub>. The respiratory decrease would occur at CO<sub>2</sub> concentrations from 0 to 5,000ppm, and then the respiration rate stabilized without further decreases, up to 20,000ppm. This pattern in the data also seems to call into question the idea that the response was caused by leaks. Much stronger evidence that the root respiration was not affected by CO<sub>2</sub> is the actual data from their 2002 study, in which none of the species tested varied their respiration rates from 350ppm to 1000ppm (Burton 2002). They unfortunately did not measure O<sub>2</sub> response in that study.

In a very complicated study, van der Westhuizen and Cramer (1998) measured short-term effects of measurement CO<sub>2</sub> on O<sub>2</sub> consumption in a few different ways. When measurement CO<sub>2</sub> concentration was increased from 0 up to 2000ppm, roots were found to increase their O<sub>2</sub> consumption by 36%, while CO<sub>2</sub> production decreased 54% during the same treatment. When roots grown in NO<sub>3</sub><sup>-</sup> were transferred from 0 into 2000ppm CO<sub>2</sub> and measurements were taken

over the course of 7 hours, O<sub>2</sub> consumption slightly increased over the period of measurement, while CO<sub>2</sub> production increased by 26% after 1.5 hours, then decreased by 70%, stabilizing at the lower rate after 4.5 hours. Interestingly, the same 7-hour experiment was repeated for roots grown in NH<sub>4</sub>, in which the CO<sub>2</sub> production decreased by 59%, and the O<sub>2</sub> consumption also decreased by 7%. They also measured respiration in roots at 0ppm CO<sub>2</sub> and 4000ppm CO<sub>2</sub> after a 1.5-hour incubation, and found no difference in O<sub>2</sub> consumption and a 30% decrease in CO<sub>2</sub> production. The variations in response shown over time, as well as the different O<sub>2</sub> consumption responses to nitrogen nutrition are tantalizing details of this study, because they suggest that changes in root metabolism could be underlying these observations. Similarly, in roots with higher DIC uptake rates, Johnson et al. (1994) reported a 31% decrease in O<sub>2</sub> consumption though the underlying mechanism of this response was uncertain.

### ***Long-term effects on root respiration rates***

Bouma et al. (1997) examined both short-term and long-term effects of CO<sub>2</sub> on CO<sub>2</sub> respiration only (opposed to CO<sub>2</sub> and O<sub>2</sub>), for citrus and bean plants. They used a system in which respiration could be measured continuously for 7 days, during which ambient CO<sub>2</sub> concentration was altered for 2 days at a time. Prior to these respiration measurements, plants were grown in either 600 or 20,000ppm rhizosphere CO<sub>2</sub>. They found no long-term or short-term effects of CO<sub>2</sub> on root CO<sub>2</sub> production in either species. Surprisingly, I have been unable to identify any published studies that attempt to look at both short-term and long-term effects of rhizosphere CO<sub>2</sub> concentration on both CO<sub>2</sub> and O<sub>2</sub> respiration.

## GOAL OF THIS THESIS

Given the many ways in which rhizosphere DIC may impact plant metabolism, it is surprising that more studies have not directly examined the effects of rhizosphere DIC concentration on tree growth and physiology. In this thesis, I present results from a greenhouse study examining the impact of elevated rhizosphere CO<sub>2</sub> concentration on plant growth and physiology of poplar.

In this study I use clonal cuttings of both *Populus deltoides* (Eastern Cottonwood) and *Populus balsamifera trichocarpa* to test a series of hypotheses regarding the effects of rhizosphere DIC on plant metabolism and physiology. At the whole-plant level, I test the hypotheses that elevated root DIC will enhance biomass accumulation, decrease the root-shoot ratio, stimulate photosynthetic assimilation and enhance whole plant water use efficiency in a model tree species. At the root-level, I explore the involvement of PEP-C in the root fixation of carbon, and present some of the first enzymology measures of PEP-C activity from tree roots. Although previous studies have failed to show a direct regulation of PEP-C activity by rhizosphere DIC (Cramer 1999, 2002), here I test the hypothesis that poplar roots grown in elevated rhizosphere DIC will have higher levels of PEP-C activity, consistent with a role for this enzyme in mediating root carbon metabolism in rapidly growing tree roots. Finally, a primary goal of this study is to measure both the short-term and long-term effects of root DIC exposure on root CO<sub>2</sub> production and O<sub>2</sub> consumption. Given the proposed relationship between root DIC concentration and rhizodeposition, I specifically test the hypothesis that variation in root O<sub>2</sub> consumption in response to elevated DIC is due to reduced flux through the bulk of the Krebs cycle, leading to enhanced

exudation of organic acids, and resulting in less NADH available to support mitochondrial respiration. By focusing my efforts on examining the complex interactions occurring at the intersection between respiration and biosynthesis, the results of this thesis provide a mechanistic foundation for resolving the contradictory effects of rhizosphere DIC concentration on root respiration, oxygen consumption, and rhizodeposition, in root cells.



## Chapter II Methods

### EXPERIMENTAL PLANT MATERIAL

All experimental material used in this thesis was harvested from either clonal cuttings of *Populus deltoides* (Brassos, TX) or *Populus balsamifera* ssp. *trichocarpa* (collected locally as stem cuttings from Kelley Point Park, Portland, OR). Stem cuttings (approximately 30-40cm) from both species were regularly clonally propagated and maintained in potted culture in greenhouses on the campus of Portland State University. For the elevated DIC experiments, the entire experimental protocol was repeated three independent times. The first experiment was performed with stem cuttings from our greenhouse collection of *P. deltoides*, and subsequent experiments were performed with stem cuttings of *P. balsamifera* ssp. *Trichocarpa*, as a pest outbreak significantly impacted the growth of our *P. deltoides* collections within the PSU research greenhouse. In every case, multiple cuttings were taken from each individual and then pairs of cuttings from each individual, chosen to be a similar mass, were divided out into the two treatments. This was done in order to help control for genetic and size effects. Total sample size after all three repetitions was 25.

### ROOT CHAMBER DESIGN

I designed and constructed root chambers (i.e. “bubblers”) from the basic description provided by Ford et al. (2007) (Figure 18). The chambers were built to be 4-liter hydroponic tanks

made of 8" diameter PVC pipe with a square plastic (PET-G) base and circular Plexiglas top. Two hose barbs and a plug were threaded into the PVC, about 2" from the lid. One hose barb functioned as the gas inlet, and had 3/8" tubing running (guided and secured by narrow PVC pipe) to the bottom of the chamber. This configuration ensured that gas was released at the bottom of the nutrient solution and helped maintain steady flow rates as well. The other hose barb functioned as the outlet, so that atmospheric pressure was maintained inside the tanks. The plug was used to replenish water and nutrient solution and monitor pH without opening the lid. Silicon aquarium adhesive was used to assemble parts.

The Plexiglas lid had a 1" hole drilled into the center with a 1.5" slip joint nut glued around it (Gorilla glue "Plastic Welder" adhesive), to create threads. Poplar cuttings were fitted, prior to rooting, with 2 hand-cut rubber gaskets designed to form a tight seal when inserted into a 1" : 1.5" galvanized adapter that could then be threaded onto the lid. All points of contact were coated in grease (Vaseline) to prevent leaks, and then the irregular circumference around tree stem was wrapped with self-adhering silicon tape. These were tight seals yet able to flex if the lid was lifted or the tree moved. The lids were closed onto the PVC tanks with 3 bungee cords crossing the lid, and compressing a gasket made of caulk backer rod. Bungee cords ran through aluminum "guides" (adhered to the lids with Gorilla glue "Plastic Welder") and fastened to eye bolts in the base.

The seals were tested by turning on air flow into the inlets, and connecting the outlet to a flow meter to check for positive flow coming out. In addition, air around the lids, as well as the poplar canopy was periodically monitored for  $P_{CO_2}$  using a LI-COR LI-840 CO<sub>2</sub>/H<sub>2</sub>O gas analyzer. Finally, exhaust tubing was connected from the outlet barbs to a soda lime trap. The exhaust

tubing was of greater diameter than the inlet, to provide a path of least resistance, and to reduce buildup of water in the tubing. Any water droplets were drained from the exhaust tubes every other day when the trees were watered.

Because each treatment lasted for 10 weeks at a time, much longer than a pre-mixed gas tank would last, I built a system that could actively mix 100% CO<sub>2</sub> into room air and maintain rhizosphere CO<sub>2</sub> concentrations within the root chambers. The flow from three standard aquarium pumps was merged into one line (with a flow meter in line) to a 1L Nalgene bottle. The CO<sub>2</sub> tank line ran to the Nalgene bottle with 2 flow meters in line—one with a dial and the other with finer scale markings for more precise control. Inside the Nalgene bottle was a length of stiff tubing running from the bottom of the bottle (to optimize mixing) and out to a valve manifold constructed of Swagelok fittings and valves to split the flow to the four bubblers. The gas mixer was calibrated using the LI-COR LI-840 CO<sub>2</sub>/H<sub>2</sub>O gas analyzer to determine the appropriate settings to attain rhizosphere-like CO<sub>2</sub> levels (approximately 15,000ppm CO<sub>2</sub>). The control bubblers also received air flow merged from 3 aquarium pumps split four ways. Flow from the CO<sub>2</sub> tank was <2% that of air flow, so that difference in flow rates between control and treatment were quite small.

## **RHIZOSPHERE MANIPULATIONS**

### ***Preliminary sodium bicarbonate treatments***

Poplar stem cuttings (approximately 30cm) (*P. deltoides*) were rooted in a modified Hoagland's hydroponic solution (1mM KNO<sub>3</sub>, 1mM Ca(NO<sub>3</sub>), 1mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1mM MgSO<sub>4</sub>, 10μM

H<sub>3</sub>BO<sub>3</sub>, 2μM MnCl<sub>2</sub>, 0.5μM ZnSO<sub>4</sub>, 0.2μM CuSO<sub>4</sub>, 0.02μM Na<sub>2</sub>MoO<sub>4</sub>, 5μM FeEDTA) buffered to between pH 6.0 and 6.8 with 2mM potassium-phosphate buffer, and bubbled with room air. After rooting (approximately 5 weeks), the rooted cuttings were transferred into 7-liter containers, two to a container with a screen placed between the root balls so that they would not grow into each other. A small air stone was installed on each side of the screen to provide aeration of the solution with room air. Two pots, containing two trees each, were given a control treatment of the buffered hydroponic solution described above. The other two pots, also with two trees each, received increasing (from 2 up to 6mM) additions of sodium bicarbonate over the course of the treatment (11 weeks). Solutions were corrected for pH every day with either HCl or KOH. However, the buffer capacity chosen was not able to maintain pH despite this daily effort, which is why in later experiments I changed to actual carbon dioxide gas bubbling, and MES buffer.

### ***Root carbon dioxide treatments***

Poplar stem cuttings were transferred to the experimental greenhouse, and rooted (4 weeks) in a modified Hoagland's hydroponic solution (1mM KNO<sub>3</sub>, 1mM Ca(NO<sub>3</sub>), 1mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 1mM MgSO<sub>4</sub>, 10μM H<sub>3</sub>BO<sub>3</sub>, 2μM MnCl<sub>2</sub>, 0.5μM ZnSO<sub>4</sub>, 0.2μM CuSO<sub>4</sub>, 0.02μM Na<sub>2</sub>MoO<sub>4</sub>, 15μM FeEDTA) buffered to pH=5.8 with 3mM MES, and bubbled with room air. Plants were given supplemental nutrients every 3 days, and solution was changed completely every 10 days throughout the experiment (10 weeks and 12 weeks). During the final replicate of the experiment, the volume of all water and nutrient solution added was recorded and standardized to total dry weight biomass to determine total water use over the course of the experiment (analyzed by one-way ANOVA for root treatment effect).

After 4 weeks of culture, tree stems were sealed into the lids of custom-fabricated closed hydroponic tanks bubbled with either 15,000ppm or 400ppm (room air) carbon dioxide gas (CO<sub>2</sub>). Sealing the tanks ensured that the tree canopy was not enriched with CO<sub>2</sub>. The carbon dioxide partial pressure of the gas entering the hydroponic tanks, and the partial pressure in the room around the leaves was regularly measured using a LI-COR LI-840 CO<sub>2</sub>/H<sub>2</sub>O gas analyzer. Exhaust vents on the tanks allowed atmospheric pressure to be maintained, and were scrubbed of CO<sub>2</sub> using drierite and soda lime. The CO<sub>2</sub> was bubbled constantly through the containers, but was turned off whenever the containers had to be opened, so as to not flood the tree canopy with CO<sub>2</sub>. The trees were maintained with a 14-hour day length and temperatures ranging from 15-30°C within the greenhouse.

#### ***Quantification of Dissolved Inorganic Carbon (DIC)***

Water samples were taken from tanks at the time of root sampling (for enzyme activity), and sealed in vials with septa until inorganic carbon analysis using a Shimadzu TOC-V CSH/CSN analyzer. Root treatments had significantly different concentrations of inorganic carbon (two-way ANOVA  $p=0.003^*$ ). Mean DIC concentration in media bubbled with 15,000ppm CO<sub>2</sub> was 30mg/L, and 5.5mg/L in media bubbled with 400ppm CO<sub>2</sub>.

#### **ROOT PEP-CARBOXYLASE ACTIVITY**

Roots were sampled for PEP-C Activity by pooling 5 root tips (25mm total lengths from each individual plant), and homogenizing in extraction buffer (100mM Tricine-KOH pH 8, 5mM MgCl<sub>2</sub>,

5% Glycerol, 0.25mM EDTA, 14mM  $\beta$ ME, 2% PVP-40). Root PEP-C activity was quantified using a NADH-coupled enzyme assay (50mM Tricine-KOH pH 8, 5mM DTT, 10mM  $\text{MgSO}_4$ , 2.5mM  $\text{NaHCO}_3$ , 15% Glycerol, 2mM PEP, 0.2mM NADH, 0.5 U/mL Malate Dehydrogenase) and measured spectrophotometrically at 25°C in a Shimadzu UV-1700 UV-VIS Spectrophotometer, following the methods of Rosenstiel et al (2004).

### ***Malate inhibition of PEP-C Activity***

Root extracts from 4 plants grown under ambient conditions were pooled, and the same enzyme activity assay was performed as above. Malate was added to assays at 0, 0.1, 0.5, 1.0 and 1.4mM concentration.

### ***Effects of pH on PEP-C Activity***

The same activity assay as above was performed, using 7 different Tris-HCl buffers from pH 6 to pH 9. After each assay was performed, the cuvette pH was determined using pH papers, to confirm the pH.

### ***Statistical Analyses***

All statistical analyses were performed using JMP software (SAS Institute, Inc., 2007). Effect of treatment and day of assay on PEP-C activity was analyzed with a two-way ANOVA. Data from both the bicarbonate and gas-bubbling root treatments were pooled together, as no significant difference was found between the types of inorganic carbon treatment given. Malate inhibition data were analyzed with a one-way ANOVA and a Tukey post-hoc test. The effect of pH on PEP-C

activity was analyzed with a single-factor ANOVA and Tukey post-hoc test.

## ROOT EXUDATES

Approximately 0.1g of intact roots were placed in a 20mL syringe filled with 15mL buffer (2.5mM  $\text{CaSO}_4$ , 2.5mM potassium phosphate pH=5.8) and bubbled with either 400ppm or 15,000ppm  $\text{CO}_2$ . The buffer was chosen based on recommendations by Aulakh (2001) that  $\text{CaSO}_4$  solution is a better choice than deionized water or nutrient solution for collecting root exudates. DI water could over-inflate exudation rates by upsetting the ion balance, but nutrient solution may introduce variation in root metabolism due to nutrient uptake. The syringes had an exhaust opening drilled into the area of the head space, and attached with a hose barb to exhaust tubing out to a soda lime trap. The top of the syringe, where the roots entered, was plugged with a Vaseline-coated cotton ball. Total exudates were collected for 1.5 hours, (with a few exceptions which were recorded), flash frozen in liquid nitrogen and lyophilized. Exudate samples, resuspended in 0.25mL 0.005 N  $\text{H}_2\text{SO}_4$  and centrifuged (1000 x g, 2min) to pellet out particles and excess  $\text{CaSO}_4$ , were then analyzed for organic acid composition using high performance liquid chromatography (HPLC).

The Krebs Cycle organic acids were separated using an Aldrich OA-1000 Column (300mm x 65mm with a guard column before the pump), using a mobile phase of 0.005 N  $\text{H}_2\text{SO}_4$  (70 °C FIAtron TC-50 thermal controller, Rheodyne injector), and UV detection at 254nm (ESA Model 520 UV/Vis absorbance detector). The pump (Rainin Dynamax) was programmed with a variable flow

rate designed to separate the Krebs cycle acids into easily quantifiable peaks, while reducing the overall run time of other compounds eluting after the peaks of interest. Two different programs were used due to a change in backpressure in the system that caused the program to have to be corrected for the final 10 samples. The primary program ran 3 minutes at a flow rate of 2.5mL/min, a 2 minute ramp to 0.75mL/min, then a 26 min run at 0.75mL/min during which the Krebs cycle acids would elute. Then, the pump would undergo another ramp up to 3.5mL/min and a 20 min run at 3.5mL/min to more rapidly elute the remaining organic acids in the samples, and ensure that all of the sample had passed the detector. Finally, there was a 2-minute ramp back to 2.5mL/min in preparation for the next run. The other program was designed to obtain the same results as the first, and PSI generated by the pump under the new conditions was used to match new flow rates to the old ones. This program used 3.5mL/min instead of 2.5mL/min, 1.25mL/min instead of 0.75 and 4.25 mL/min instead of 3.5. The length of the 1.25mL/min portion was lengthened to 37 minutes, and the final run time was lengthened to 75 minutes to ensure that all of the sample had passed the detector.

### ***Quantification***

Peaks were recorded and analyzed using PeakSimple software (SRI Instruments, with SRI Model 202 data module). Retention times and peak area quantification of samples were verified by injecting authentic standards of: oxalic acid, citrate, alpha-ketoglutarate, pyruvate, succinate, malate, and fumarate. In this method, citrate and alpha-ketoglutarate co-elute, as do malate and fumarate. For all samples, these peaks were determined as means of the standard equations for



the co-eluting peaks. Peak area-concentration relationships were found to be linear for all standards.

### ***Statistical Analyses***

All Krebs cycle exudates were analyzed for effect of root treatment with two-way ANOVA, with treatment and sampling day as factors. All Krebs cycle exudation rate data (except total malate and malate secondary peak) were log transformed to satisfy the assumptions of the ANOVA.

### **ROOT RESPIRATION**

Following exudate collection (see above) root samples (approx 20cm) were harvested and placed into either a liquid phase or gas phase O<sub>2</sub> electrode (Hansatech Instruments). Liquid phase measurements were conducted in a buffer (2.5mM CaSO<sub>4</sub>, 2.5mM Potassium phosphate buffer pH=5.8, Aulakh 2001) equilibrated with either room air (400ppm CO<sub>2</sub>) or 15,000ppm CO<sub>2</sub> using the manufacturer's recommended procedures. The roots were kept in place (not clogging the stir bar or lid) by fitting them into a tiny basket (9mmx20mm) made of plastic screening and nylon thread. The rate of O<sub>2</sub> consumption was measured at 25°C for 10 minutes after a 5 min equilibration period. Gas phase measurements were conducted with excised roots placed on a moistened felt inside the chamber, and exposed to either room air or approximately 1000ppm CO<sub>2</sub>. Desired CO<sub>2</sub> concentrations were introduced into the chamber by removing half of the chamber air and then replenishing it with the measurement gas. (For example, by injecting 2000ppm to achieve

1000ppm in the chamber). Samples were then transferred into the Li-COR insect respiration chamber and CO<sub>2</sub> release measured at 400ppm and 1000ppm with a Li-COR 6400 portable photosynthesis system using the on-board dynamic CO<sub>2</sub> mixing system (Li-COR Inc., Lincoln, NE USA). Root respiration CO<sub>2</sub> response curve was measured with the Li-COR 6400 using 3 root samples grown at control conditions and exposed to 5 measurement CO<sub>2</sub> concentrations ranging from 400 - 2000ppm. For all respiration measurements, following respiration measures, samples were dried at 60°C for 48hrs and root respiration rates were standardized and expressed per unit dry mass.

### ***Statistical Analyses***

Root CO<sub>2</sub> respiration data was analyzed for effects of root treatment, measurement CO<sub>2</sub> and sampling date, using a two-way ANOVA, as well as a Tukey post-hoc test for the interaction of measurement CO<sub>2</sub> and root treatment. Respiration rate was log transformed to satisfy the assumptions of the ANOVA. Root respiration CO<sub>2</sub> response curve was analyzed with a one-way ANOVA and post-hoc Tukey analysis. Root O<sub>2</sub> respiration data collected in the gas phase and liquid phase was analyzed separately due to the large variation in the results. Both sets of data were analyzed with ANOVA to determine how root treatment, measurement CO<sub>2</sub> concentration, date, and measurement CO<sub>2</sub> and treatment cross-interaction affected root respiration. Gas phase root O<sub>2</sub> respiration data was square root transformed to satisfy the equal variances assumption of the ANOVA. Liquid phase data was transformed (squared) to satisfy the equal variances assumption of the ANOVA.

## LEAF GAS EXCHANGE

Maximum rates of leaf photosynthesis (leaf net assimilation), leaf stomatal conductance, leaf transpiration rate (all at a light intensity of  $1500\mu\text{E}$ ), and leaf dark respiration were measured using a flow-through leaf cuvette using a Li-COR 6400, following standard protocols. Leaves were chosen according to similar stem position and chlorophyll content. Leaf temperature and relative humidity (RH) were controlled within the cuvette using the Li-COR instrument. Temperature and relative humidity during measurements are presented in Table 1. Leaf temperature was regulated near to  $25^{\circ}\text{C}$  by controlling the block temperature. Relative humidity was maintained near to 65% primarily by changing the amount of airflow through drierite, and when necessary, by changing flow rate.  $\text{CO}_2$  concentration was maintained at 400ppm. Chlorophyll content was determined using a Konica Minolta Chlorophyll meter. Three measurements (relative absorbance units) were taken and averaged for each intact leaf, each of which was also for gas exchange measurements.

### ***Statistical Analyses***

Maximum leaf net assimilation, leaf stomatal conductance, leaf transpiration rate, and leaf dark respiration were all analyzed with two-way ANOVA to determine the effect of root treatment and sampling date on gas exchange characteristics. Leaf chlorophyll content, RH and leaf temperature had also been recorded and were included as covariates in the ANOVA. The effect of root treatment on chlorophyll content was analyzed with a two-way ANOVA with treatment and sampling date as factors.

## **WHOLE PLANT BIOMASS**

At the end of each experiment, all tree tissue (roots, stems, leaves) were harvested and dried for 10 days at 60°C for total dry weight (DW) biomass and root to shoot ratio determination. In the first replicate, stems were cut above the uppermost root to divide roots from shoots, but subsequently, all roots were cut off of the stem, and the entire stem was counted as shoot.

### ***Statistical Analyses***

Shoot DW, root DW, total DW biomass and root to shoot ratio were all analyzed for the effects root treatment and sampling date, using a two-way ANOVA. Initial stem cutting mass was included as a covariate. Shoot, root and total DW biomass were all sine-transformed to satisfy the equal variance assumptions of the ANOVA.

## Chapter III Results

### LEAF GAS EXCHANGE

Root carbon dioxide treatment did not have any significant effect on any of the physiological leaf responses we measured (Table 1). Maximum photosynthetic rate, measured at 1500  $\mu\text{mol}/\text{m}^2/\text{s}$  ( $\mu\text{E}$ ) was  $15.0 \pm 0.8 \mu\text{mol CO}_2/\text{m}^2/\text{s}$  in the 15,000 group and  $15.9 \pm 1.0 \mu\text{mol CO}_2/\text{m}^2/\text{s}$  in the 400 group ( $n=17$  each  $\pm$  s.e.). Stomatal conductance was  $0.30 \pm 0.02 \text{ mmol H}_2\text{O}/\text{m}^2/\text{s}$  in the 15,000 group, and  $0.31 \pm 0.03 \text{ mmol H}_2\text{O}/\text{m}^2/\text{s}$  in the 400 group ( $n=17$  each  $\pm$  s.e.). Transpiration rate in the 15,000 group was  $3.4 \pm 0.3$  and  $3.5 \pm 0.4$  in the 400 group ( $n=17$  each  $\pm$  s.e.) Mean dark respiration was identical in both treatment groups,  $1.7 \mu\text{mol CO}_2/\text{m}^2/\text{s}$  ( $n=17,16$  respectively  $\pm 0.14$  and  $0.18$  s.e. respectively). In the case of photosynthesis and dark respiration, measurements on some days were significantly different than other days. Additionally, RH was significantly correlated with stomatal conductance ( $r^2=0.15$   $p=0.03^*$ ) and transpiration rate.

### BIOMASS AND TOTAL WATER USE

Root carbon dioxide treatment did not have any significant effect on biomass accumulation or root to shoot ratio in the gas-bubbling experiments (Table 2). In the earlier sodium bicarbonate experiment, we found a significantly smaller root to shoot ratio in bicarbonate-treated plants. However, I believe the method for dividing root and shoot used in that experiment was not

sufficiently accurate. Initial biomass was significantly correlated with shoot biomass and total biomass, but not root biomass (data not shown). Root carbon dioxide treatment did not have any significant effect on total water use over the course of the experiment (Table 2). The 15,000 group (n=3) used, on average,  $0.399 \pm 0.019$  L/gDW and the 400 group (n=4) used  $0.375 \pm 0.024$  L/gDW ( $\pm$  s.e.).

## ROOT PEP CARBOXYLASE ACTIVITY

In both types of inorganic carbon treatments, i.e. sodium bicarbonate additions as well as carbon dioxide gas aeration, roots grown in higher inorganic carbon concentrations were found to have higher PEP-C activity (18% higher, Figure 1). The inorganic carbon type was not found to have a significant effect, so I pooled data from both experiment types. Malate concentration inhibited PEP-C activity (Figure 2). The 100 $\mu$ M malate concentration was found to slightly inhibit maximum PEP-C activity, and 500 $\mu$ M reduced activity, significantly, by 33%. The highest malate concentration I tested was 1.4mM, which inhibited PEP-C activity by 69%. Root PEP-C activity was found to be insensitive to pH between 7 and 8.3, but was slightly inhibited at pH 6 and pH 9 (Figure 3).

## RESPIRATION RATE

CO<sub>2</sub> Production

Long-term root treatment did not have an effect on root CO<sub>2</sub> production when the treatment groups were measured at the same CO<sub>2</sub> concentration (Figure 4A). However, roots grown at 15,000ppm and measured at 1,000ppm had a lower respiration rate than roots grown at 400ppm and measured at 400ppm (Figure 4B). Measurement CO<sub>2</sub> concentration did influence root CO<sub>2</sub> production (Figure 4B, Figure 5, Figure 9). Roots consistently reduced CO<sub>2</sub> respiration in response to a change from 400ppm to 1000ppm CO<sub>2</sub>, although the magnitude of response varied (Figure 8). Some roots were found to have negative respiration rates when exposed to 1000ppm CO<sub>2</sub>, suggesting a net uptake of CO<sub>2</sub>. When measurement  $P_{CO_2}$  was decreased back to 400ppm, roots increased respiration back to a similar rate as was previously measured at 400ppm (Figure 8). When individual root responses to measurement CO<sub>2</sub> were analyzed for root treatment effect (i.e. Resp @ 400 – Resp at 1000), there was a marginal difference ( $p=0.08$  two-factor anova, Figure 9). This trend is masked if all root samples are pooled as in Figure 4B.

### O<sub>2</sub> Consumption

Growth at 15,000ppm CO<sub>2</sub> lead to a decrease in rate of O<sub>2</sub> consumption (Figure 6A,  $p=0.02^*$ ), when measurements were in the liquid phase. DIC concentration in the chamber did not have a significant effect on root O<sub>2</sub> consumption, even when determined from individual roots (Figure 6B, Figure 9).

Analysis of the root O<sub>2</sub> consumption data revealed some issues with our calibration of the O<sub>2</sub> electrode. Unlike root CO<sub>2</sub> production, in which the overall respiration rates at 400ppm and 1000ppm were fairly predictable, O<sub>2</sub> consumption rates were quite different if they were measured in the liquid vs. the gas phase (Figures 6 & 7). Roots measured in gas phase had higher respiration

rates than those measured in liquid phase, and the treatment effect was reversed in root respiration measured at the gas phase (Figure 7). Due to the small size of the chamber (approx 6mL) and the process of calibrating the electrode mV signal using air with 400ppm CO<sub>2</sub>, we conjecture that the subsequent measurements conducted at 1000ppm may be inflated due to reduced  $P_{O_2}$  in the chamber. Additionally, any build up of CO<sub>2</sub> produced by the root sample would cause the same effect, possibly resulting in the higher O<sub>2</sub> consumption values in the 15,000 group. I have decided to exclude all gas phase O<sub>2</sub> measurements from the analysis. It is important to note that there may also be a calibration issue within the liquid phase data as well, in that the zero O<sub>2</sub> signal achieved by adding sodium sulfite was generally 15-20% of the saturation signal, which is considerably higher than the 1% recommended by the manufacturers. This calls into question the absolute values we report, but not the relationships between the treatments, and is also why I've chosen not to present or discuss the respiratory quotients ( $RQ=CO_2/O_2$ ).

## ROOT EXUDATES

Krebs cycle exudation rates varied considerably amongst the root samples, and even within the same plant, when sampled on different days. Despite this variation, a recognizable exudation pattern exists amongst all samples. Succinate was, by far, the primary Krebs cycle exudate, forming on average  $90\pm 7\%$  of the exuded Krebs acids (Figure 10). Pyruvate had the smallest percentages in the exudate profile, generally forming less than 0.5% of the exuded Krebs acids (Figure 11). Malate was found to elute in two separate peaks, the first included about 23% of the peak area, and the second, which elutes with fumarate, contained the rest. I attempted to



include this peak in the analysis, because the ratio of the two peaks was very consistent across all standards that were injected. However, in actual samples, the smaller malate peak frequently came out with larger area than the main peak, which suggests that some unknown compound that I did not quantify may have been present in the samples, eluting with that peak. I therefore decided to exclude that peak from further analysis at this time.

When individual organic acid exudation rates were analyzed as percentages (data not shown), no significant difference existed between the two treatments in any of the individual exudates. The 15,000 group exuded a higher percentage of succinate, and therefore the 400 group exuded higher percentages of all of the other Krebs organic acids. However, this trend was not statistically significant.

When considered as a rate (nmol/gDW/s) there was an overall trend of higher exudation amongst the 400ppm CO<sub>2</sub> treatment group, with significantly higher exudation found in the averaged citrate and  $\alpha$ -ketoglutarate peak as well as the averaged malate and fumarate peak (Figure 11). Pyruvate exudation was marginally higher in the 400ppm group, as well. Unfortunately, we cannot differentiate between citrate and  $\alpha$ -ketoglutarate, nor malate and fumarate in our analysis, and so the contribution of these individual components is obscured.

## **RELATIONSHIPS BETWEEN ROOT EXUDATION AND RESPIRATION**

These data were analyzed for correlations between root exudation and respiration. Because root respiration and root exudation were not measured simultaneously, it is difficult to

draw strong conclusions from these data. However the relationships are interesting, nonetheless. Only results from CO<sub>2</sub> production measurements are presented, because the liquid phase O<sub>2</sub> electrode sample size (n=9) was too small for analyzing the root treatment groups separately.

### ***CO<sub>2</sub> production and Krebs cycle exudation***

Overall, there is not a relationship between CO<sub>2</sub> production and Krebs cycle organic acid exudation. However, I found correlations between CO<sub>2</sub> production and succinate, pyruvate and citrate exudation rates, but for each, it is only in a single treatment group, and only at one of the measurement CO<sub>2</sub> concentrations.

I found negative correlations between root CO<sub>2</sub> production in roots grown at 400ppm CO<sub>2</sub> and both succinate and pyruvate exudation rates (Figure 12B, Figure 14B). Exudates were collected before the respiration measurement. This indicates that root CO<sub>2</sub> production, when measured at 1000ppm, was lower in roots that had exuded more succinate and pyruvate ( $r^2=0.42$ , and  $0.44$ , respectively,  $p=0.009^*$  and  $0.007^*$ ). Interestingly, both of these trends are driven by root samples that exhibited negative respiration rates when exposed to 1000ppm CO<sub>2</sub> (Also see Figure 8), and I will discuss later why these individual samples are of more interest than the overall relationships presented here. These same roots also had the greatest difference in respiration rates at 400ppm vs. 1000ppm, leading to the correlations presented in Figures 13 and 15. Roots that exuded more pyruvate also more dramatically reduced their respiration in response to exposure to 1000ppm CO<sub>2</sub> ( $r^2=0.6$ ,  $p=0.0008^*$  Figure 15). This same relationship was marginally significant with respect to succinate exudation ( $r^2=0.26$ ,  $p=0.05$ , Figure 13).

Citrate exudation was found to be negatively correlated with root CO<sub>2</sub> production in roots grown at 15,000ppm (Figure 16A). The more citrate these roots had produced, the lower their CO<sub>2</sub> production ( $r^2=0.42$ ,  $p=0.04^*$ ). This trend does not hold for the 400 treatment group, or for the CO<sub>2</sub> production measured at 1000ppm.

## Chapter IV Discussion

Atmospheric CO<sub>2</sub> has been rising steadily, since the industrial period, with no clear immediate sign of global stabilization (Tans, 2009). Although substantial research effort has focused on the impacts of elevated atmospheric CO<sub>2</sub> on plant metabolism and physiology, relatively few studies have examined the specific effects of elevated rhizospheric CO<sub>2</sub> on plant processes, despite the fact that high soil  $P_{\text{CO}_2}$  is almost certainly the condition under which roots evolved. Fewer studies still have examined the impacts of elevated rhizospheric CO<sub>2</sub> on plant processes in forest trees, a key biotic driver influencing terrestrial primary productivity and the atmospheric carbon budget. In this thesis, I present results from a study aimed at examining the impacts of rhizospheric CO<sub>2</sub> on fundamental aspects of root physiology and metabolism in poplar, an ecologically and economically relevant, emerging model system.

As rhizosphere dissolved inorganic carbon (DIC) concentration has been shown to influence biomass accumulation in some plant systems, namely crops (Bialczyk 2007, Cramer and Richards 1999), I hypothesized that increased rhizospheric DIC would lead to an increase in biomass in poplar as well. I hypothesized that root carbon uptake, as observed in other plant systems, would impact root to shoot ratios in poplar, assuming that roots grown in higher DIC environments would fix more carbon, and that because of the relationship with respiration via the Krebs cycle that has been discussed (see introduction), root growth would be impacted in these plants, potentially promoting a decreased biomass accumulation. However, in poplar, I found no effect of root-zone DIC on root to shoot ratio, above-ground gas exchange parameters (particularly net assimilation and stomatal conductance) or whole-plant biomass accumulation. However, these

plants were grown hydroponically within greenhouses on the campus of Portland State University, under shade cloth, with ideal nutrient conditions and, as a result, these trees were not exposed to environmental conditions (high irradiance, high humidity, salinity stress) that have been shown to be necessary in order for root DIC to impact biomass accumulation and whole-plant processes (Bialczyk 2007, Cramer and Richards 1999, He et al. 2007, 2010). Previous studies have also shown that nitrogen form, as well as exposure to aluminum (a stressor) can influence the effect of rhizospheric DIC on biomass accumulation and plant growth (Cramer and Richards 1999, Viktor and Cramer 2003). In tomato seedlings grown at high irradiance, nitrogen form had no effect on biomass at 360ppm rhizospheric CO<sub>2</sub>, but at 5,000ppm rhizospheric CO<sub>2</sub>, plants fed with 2mM NO<sub>3</sub><sup>-</sup> had significantly higher biomass than plants grown with 2mM NH<sub>4</sub><sup>+</sup>. Both nutrient treatments (at 5000ppm) had more biomass than the 360ppm plants (Cramer and Richards 1999). In an effort to focus the current study on the direct effects of elevated CO<sub>2</sub> on root metabolism in Poplar, independent of other interactive effects, nutrient supply and plant water status were maintained at optimal conditions in my hydroponic system. The trees exhibited no signs of differential nutrient status with DIC treatment as they exhibited both similar leaf chlorophyll contents and rates of net assimilation (total leaf C/N analyses are currently underway), two well-established indicators of whole plant nutrient status (Chang and Robinson 2003).

Although I did not observe any direct effect of root DIC on Poplar growth, it is possible for carbon acquired by the roots to be allocated to biosynthetic pathways that do not impact respiratory processes, most notably asparagine synthesis from oxaloacetate (Edwards 1998, Plaxton and Podesta 2006). In that pathway, the oxaloacetate need not enter the mitochondria at all.

Additionally, activity of the alternative oxidase pathway during electron transport allows for respiration to occur uncoupled to energy production (Plaxton and Podesta 2006). If AOX activity was high during root DIC uptake, then carbon gains would not directly result in gains of energy or growth.

Similar to the effects of DIC on biomass accumulation and leaf-level physiology, I found no differential effect of rhizosphere DIC on whole-plant water use. These whole-plant results are consistent with leaf level estimates of water use efficiency and stomatal conductance measured with the portable photosynthesis system. This lack of effect of elevated rhizospheric DIC on plant water use efficiency is in stark contrast to the well-established impact of atmospheric CO<sub>2</sub> concentration on plant water use efficiency in C<sub>3</sub> plants. Taken together, these results suggest that there were minimal impacts of rhizospheric DIC under the environmental conditions examined, suggesting that poplar plants, at the whole plant level, are capable of growing normally under a wide range of rhizospheric CO<sub>2</sub> partial pressures. Further, results from these whole-plant analyses indicate that there is no evidence of any residual CO<sub>2</sub> fertilization (i.e. root-chamber leakage) within the canopy as a result of my experimental manipulations.

Although root DIC did not lead to dramatic changes in whole-plant growth, biomass allocation, leaf chlorophyll content, or leaf gas exchange characteristics, I did find several treatment-specific effects of DIC on Poplar root physiology. In particular, I found that Poplar roots grown at a higher rhizosphere DIC concentration (15,000ppm CO<sub>2</sub>) had significantly greater extractable root PEP-C activity, when measured with an *in vitro* assay. Surprisingly, this direct effect of rhizosphere DIC on PEP-C activity has not been found in other studies (Cramer and

Richards 1999, Cramer 2002). Of course, the *in vitro* PEP-C assay does not give information about actual *in vivo* rates of activity or regulation of the enzyme. Poplar root PEP-C activities are within the reported range of values for other root PEP-Cs. Results from poplar are roughly double (~100 nmol/min/mg protein vs. 54nmol/min/mg protein) of that reported in cucumber roots (also *in vitro*, DeNisi and Zochi 2000). However, *in-vitro* PEP-C activity in Lupine roots (300 (normal) and 480 (cluster roots) nmol/min/mg protein) were triple that of poplar (Johnson 1994). In this study I did not attempt to compare root malate content between our treatments, or cytosolic pH, nor any other conditions which could have impacted the actual *in vivo* daily rates of PEP-C activity. Nonetheless, rhizosphere DIC affected the roots' PEP-C activity when measured under the same conditions *in vitro*. The higher PEP-C activity in the 15,000ppm DIC treatment group likely represents a larger pool of the PEP-C enzyme in those roots. A larger pool of PEP-C might be necessary to compensate for higher, inhibitory malate concentrations that could result in roots grown in higher DIC. These results are the first hint suggesting that root DIC may perturb root function without necessarily impacting whole plant growth or physiology under nutrient rich conditions.

The effect of pH on poplar root PEP-C activity is similar to that found in corn roots and in cucumber roots (Dong et al. 1998, De Nisi and Zochi 2000). Dong et al compared root PEP-C with leaf PEP-C in corn and found that at pH 7.3, root PEP-C activity was 10% less than at pH 8, whereas leaf PEP-C activity decreased by 60%. We found no decrease in root PEP-C activity between pH 7 and pH 8.3. They conclude, as we do, that root PEP-C maintains activity at a broader pH range than leaf PEP-C (Dong et al. 1998). In cucumber roots, PEP-C was also found to maintain similar rates of activity throughout a pH range of 8 to 9.5 (De Nisi and Zochi 2000). This

difference between root and leaf PEP-C is unsurprising given that root cells are exposed to relatively dramatic changes in extracellular pH due to contact with the soil environment.

Our malate inhibition data is consistent with that of Chang and Roberts (1992) who measured *in vivo* rates of PEP-C activity in corn root tips. We did not measure PEP-C activity at the malate concentration that likely exists *in vivo*, around 3.5-7.5 mM, as was found in corn root tips (Chang 1992). Chang and Roberts (1992) found that *in vivo* PEP-C activities in the corn root tips were in the range of 0.08-0.18  $\mu\text{mol}/\text{min}/\text{g}$ , more than 50% less than the rates we found at 1.4mM malate. We can conclude that the *in vivo* rates of PEP-C activity for our poplar roots could be much lower than the rates we measured with our *in vitro* assay, because we don't know the cytosolic pH, nor do we know how much endogenous malate remained at the end of the enzyme extraction process.

Root PEPC has been suggested to have several functions, including impacts on rhizodeposition (Johnson 1994, 1996), anaplerotic replenishment of the respiratory Krebs cycle (Edwards 1998, Plaxton and Podesta 2006), and nitrogen assimilation (Cramer 2005). Since I found that PEP-C activity increased in response to root DIC concentration in poplar, I further explored the effects of root DIC on both characteristics of rhizodeposition and respiration in my experimental system. I predicted that the enhanced PEP-C activity I observed would mean increased rhizospheric DIC uptake, and I formed a general prediction about the how that additional carbon would impact root respiration and rhizodeposition. Specifically, I predicted that there is an inverse relationship between root respiration and Krebs cycle exudation, due to both  $\text{CO}_2$



production and O<sub>2</sub> consumption being tied to cycling of organic acids, the relative pool of which would decrease with higher exudation rates.

Despite the fact that roots grown in 15,000ppm rhizosphere CO<sub>2</sub> had higher PEP-C activity, they generally exhibited lower total rates of root exudation as well as significantly lower root exudation rates of citrate and  $\alpha$ -ketoglutarate as well as malate and fumarate. I highlight these compounds as they are generally found to be exuded at higher rates from roots with increased PEP-C activity (Johnson et al. 1996, Shane et al. 2004, Dechassa and Schenk 2004). Therefore, it is surprising that elevated root DIC led to a decrease in exudation rate despite the greater PEP-C activities. An increase in exudation of these organic acids is generally linked to roots expressing nutrient deficiency and nutrient deficient roots are shown to have higher PEP-C activity (Johnson 1994, De Nisi 2000). However, in the absence of nutrient deficiency (my system), it is likely that PEP-C activity may be less predictive of root exudation of organic acids and may reflect other roles of PEP-C in this system.

Despite the importance of root exudation of organic acids in mediating plant-soil interactions, relatively few studies have directly examined the impacts of elevated rhizospheric CO<sub>2</sub> on exudation in non-cluster root forming species (cluster root formers represent a specific adaption for coping with low nutrient environments). Shane et al (2004) report exudation rates for cluster roots and non-cluster roots grown under phosphorus deficiency, in *Hakea prostrata*. The range of exudation rates for citrate and malate for poplar roots is quite similar. The *H. prostrata* non-cluster roots exuded approximately 0.05 nmol/gFW/s of both malate and citrate. The cluster roots exuded 0.5 nmol/gFW/s of each compound. The range of each of these exuded by my poplar roots, when

reported on a fresh weight basis is 0.19-0.5 nmol/gFW/s, well within the range of cluster root exudation rates. Similarly, even the lowest exudation rates of my poplar roots are higher than those reported by Dechassa and Schenk (2004) in phosphorus deficient cabbage, carrot and potato. Citrate exudation was 0.12nmol/cm/hr in P-deficient cabbage compared with 3-7nmol/cm/hr as the range of citrate exudation in poplar roots (calculated using a conversion factor of 0.00025gDW/cm, common in our root samples). Of course, centimeter of root is not a consistent method for standardizing exudation rates, in that the cabbage roots are likely completely different in diameter or mass from poplar roots. Nonetheless, according to these estimates it appears that nutrient deficient cabbage roots are not exuding more organic acids than the poplar roots in our study. It should be noted that our citrate and malate exudation rates are pooled with other compounds (a-ketoglutarate and fumarate), but that the comparison is still informative. Taken together the results from poplar suggest that exudation rate of this species is relatively high compared to other systems investigated to date. The total Krebs cycle organic acid rate (in the range 8-22 nmol/gFW/s) was considerably higher than the reports ( 3.5nmol/gFW/s) of Shane et al (2004) for cluster root exudation of similar compounds (malate, citrate, succinate, lactate, and aconitate). Poplar exudation of citrate, malate, and succinate is several hundred times higher than rates exuded from cabbage, carrot and potato, when compared per cm root (Dechassa and Schenk 2004). There is another report of poplar Krebs cycle exudation rates, although differences in collection methods make the rates difficult to compare. Naik et al. (2009) report exudation rates in two poplar species with roots exposed to varying aluminum concentration. They did not report values of exudation in the roots without aluminum exposure, but interestingly, the species appear to differ in which organic acids they preferentially exude in response to aluminum. These authors

also note that their poplar exudation rates are much higher than those of other forest species published thus far.

The most significant (on average 90% of total) of the exudates quantified in my system was succinate. Succinate exudation is also reported in other poplar species *P. tremuloides* and *P. trichocarpa*, but not in the same dominant role I observed (Naik et al. 2009). Succinate exudation was about 70% of organic acid exudation reported in cabbage and potato, and about 30% in carrot. However, when exposed to phosphorus deficiency, the percentage of succinate exudation dropped off in cabbage, because rates of citrate exudation increased 6-fold. Potato increased its succinate exudation 30-fold, but not citrate or malate, in response to P deficiency (Dechassa and Schenk 2004). Some succinate exudation is also reported in cluster root exudation by Shane et al (2004), but not in the same dominant role seen in my poplar. In fact, they did not find it exuded by the non-cluster roots at all, and don't appear to have detected it in root tissues. Johnson et al (1996) did not detect succinate in root exudates, but did find pools of succinate root tissue, along with citrate and malate. Cramer, Shane, and Lambers (2005) measured organic acid concentrations in phloem and xylem of lupine, and detected succinate in the phloem only, suggesting that succinate may be traveling from the shoot. It is unclear whether the succinate exuded by poplar roots originates in the shoot or root, as we did not measure tissue concentrations. Despite the high variability in succinate exudation observed in my experimental roots, there was nearly a 40% reduction in succinate exudation rate in roots grown under elevated DIC.

Although I hypothesized that greater root DIC would lead to enhanced exudation of organic acids, results from this study show that increased DIC actually reduced exudation rates of organic

acids. As growth at 400ppm root CO<sub>2</sub> is likely “sub-ambient” for roots growing in a natural soil environment, it is tempting to speculate that roots under these ‘non-physiological’ conditions may be suffering from slight nutrient deficiency, without the inorganic carbon supply which may be utilized for nitrogen assimilation, and are enhancing the release of organic acids as is reported in nutrient deficient species (Johnson 1994,1996, Shane 2004, Dechassa and Schenk 2004). This is the first report I am aware of showing that increased root DIC concentration alters the magnitude of compounds released to the rhizosphere in trees. Though additional experiments are warranted, these results suggest that root DIC concentration may play a key role in mediating the capacity for root nutrient assimilation – with higher DIC concentrations leading to greater root PEP-C activity and greater capacity to quickly assimilate N belowground. Future studies clearly need to continue to examine the impacts of elevated DIC on root exudation. In particular, it would be interesting to explore whether organic acid exudation is related to cellular ionic balance.

Despite the increase in root PEP-C activity at greater DIC, I did not find strong support for my hypothesis that there is a simple relationship between root carbon fixation via PEP-C activity, respiration rate and exudation of Krebs cycle organic acids. I predicted two suites of responses consistent with our model of the functions of root-fixed carbon. Specifically, I predicted that roots would assimilate more DIC via PEP-C, exude organic acids such as malate and citrate, bypassing the full Krebs cycle and NADH production, resulting in reduced CO<sub>2</sub> production and O<sub>2</sub> consumption. However, my hypothesis would lead to a different prediction in the case that PEP-C is replenishing the Krebs Cycle during biosynthesis of compounds not destined for exudation, such as amino acids: If roots with higher PEP-C activity are not engaged in organic acid exudation, the

DIC would contribute to more complete cycling through the Krebs Cycle and higher CO<sub>2</sub> production and O<sub>2</sub> consumption. This set of hypotheses is consistent with the variation that has been observed in root respiratory responses to DIC/CO<sub>2</sub>.

Ideally, to test this hypothesis, measurements of exudation rates, CO<sub>2</sub> production and O<sub>2</sub> consumption would be taken simultaneously. However, I was only able to measure them consecutively on the same roots. This experimental design includes the assumption that respiration rates measured in the hour(s) following exudation collection and excision will continue to reflect those processes that were occurring in the intact roots. This experimental design also introduces some uncertainty into the interpretation of the results. Specifically, it is difficult to point to direct causes for the correlations we see in our data because they did not coincide exactly in time.

A key aspect of this study was to examine both the long-term and short term effects of DIC on root respiration. To do so, I measured root respiration in poplar using a variety of techniques. I examined the influence of DIC on Poplar root respiration in a liquid phase oxygen electrode at their growth DIC concentrations. Root respiration rate was reduced 27% on a dry-mass basis when measured at 15,000ppm CO<sub>2</sub>. These results are remarkably similar to Johnson et al., (1994) who also showed roughly a 31% reduction in O<sub>2</sub> consumption with elevated DIC despite an increase in extractable PEP-C activity. We found no short-term impact of DIC concentration on O<sub>2</sub> consumption values. This is also confirmed by the linearity of O<sub>2</sub> consumption rates over the course of the measurement (15 minutes), during which the root's own respiration was contributing to a build-up in chamber CO<sub>2</sub> concentration. The magnitude of O<sub>2</sub> consumption rates in my poplar were considerably lower than those reported in lupine or in tomato, but much closer to values

reported in harsh hakea, barley, and pine (Johnson 1994, van der Westhuizen and Cramer 1998, Shane 2004, Rigano 1996, Drake 2008). Again, I am unsure about the accuracy of my values due to possible inadequate removal of O<sub>2</sub> during calibration of the instrument. In addition, I attempted to measure root respiration with an oxygen electrode in the gas phase, but issues with calibration invalidate those attempts, so that data will not be discussed further here.

I also measured the response of root respiration to DIC using an infrared gas analyzer to quantify CO<sub>2</sub> exchange (opposed to O<sub>2</sub>). Consistent with the results from the oxygen electrode, results from the IRGA analyses show suppression of respiration at elevated DIC in both long-term and short-term. It is most reasonable to compare respiration of roots grown at 15,000 to those of roots grown at 400ppm when respiration is measured at a CO<sub>2</sub> concentration values as close as possible to the growth condition (1000 and 400 respectively). Unlike the case of O<sub>2</sub> respiration, CO<sub>2</sub> production does appear to experience a short term, instantaneous suppression of respiration due to measurement CO<sub>2</sub> concentration. Rates of Poplar root CO<sub>2</sub> production are within the range of values reported in other studies (Shane 2004, van der Westhuizen and Cramer 1998, Bouma 1997, Drake 2008).

Despite the variability in measured respiration rate in response to growth DIC environment, there was a consistent reduction of root respiration rate with increasing measurement CO<sub>2</sub> concentration (with respiration rates decreasing consistently from 400ppm to 2000ppm CO<sub>2</sub>). There are 3 possible explanations for observed inhibition of CO<sub>2</sub> production at higher measurement CO<sub>2</sub> concentrations. 1) The difference in CO<sub>2</sub> production could be explained by the change in the difference in partial pressure of CO<sub>2</sub> ( $\Delta P_{CO_2}$ ) between the two measurement conditions. Given the

same mitochondrial respiration rate in the roots, the driving force for diffusion of CO<sub>2</sub> out of the root is less at 1000ppm than at 400ppm. However, we found considerable variation in the magnitude of response to measurement CO<sub>2</sub>, which seems inconsistent with changes related to diffusional rates. This variation does not correlate with root mass (a stand-in for diffusional distance), suggesting that diffusion does not explain all of the response to measurement CO<sub>2</sub> that we found. **2)** It is also possible, and has been suggested by other researchers as well, that reduction in apparent CO<sub>2</sub> production at higher CO<sub>2</sub> is due to a change in the uptake of CO<sub>2</sub> by the root, resulting in a net decrease in production (van der Westhuizen and Cramer 1998, Cramer 2002). If this is the case, it would be very difficult to use CO<sub>2</sub> production measurements as an indicator of metabolic activity, because the same CO<sub>2</sub> production value could encompass quite a degree of variation in mitochondrial respiration, depending on how much is being obscured by uptake. If responses to measurement CO<sub>2</sub> concentration are due to changes in uptake of CO<sub>2</sub>, then our results suggest that the 400 group took up more CO<sub>2</sub> in response to measurement CO<sub>2</sub>, assuming that mitochondrial respiration is staying constant (data not significant ( $p=0.08$ )).

**3)** The third possibility is that there is an actual inhibition of some metabolic process, such as in the way CO<sub>2</sub> has been found to inhibit the respiratory enzymes cytochrome c oxidase (COX) and succinate dehydrogenase (Gonzalez-Meler 1996). If at higher external CO<sub>2</sub> concentrations, more CO<sub>2</sub> stays in the root, then inhibitions of these enzymes would increase. The marginal difference we found in CO<sub>2</sub> sensitivity between root treatment types could be due to acclimation in the roots already grown at higher external CO<sub>2</sub> concentrations. However, the O<sub>2</sub> consumption data appears to contradict this interpretation, as the roots grown at higher DIC seemed to be more

sensitive to CO<sub>2</sub>, when O<sub>2</sub> consumption is analyzed instead of CO<sub>2</sub> production. Also, Gonzalez-Meler et al (1996) found that sensitivity of respiratory enzymes to CO<sub>2</sub> was stronger when the mitochondria had been previously exposed to CO<sub>2</sub>.

As both root respiration and organic acid exudation rates were reduced with increased root DIC in Poplar, I was interested in exploring the relationship between root respiration and Krebs cycle organic acid exudation in my experimental system. Some of the Krebs cycle organic acid exudation rates were found to be negatively correlated with respiration rate (when quantified as CO<sub>2</sub> exchange with an IRGA) but often, only for one treatment group at one measurement CO<sub>2</sub> concentration. This result does not strongly support the broad relationships between organic acid exudation and root respiration that I had hypothesized. The correlations I found between exudation and respiration do suggest that more detailed exploration of their relationship would be worthwhile. Most interesting is the observation that roots that exuded the highest quantities of succinate and pyruvate actually seem to take up CO<sub>2</sub> when placed in the Li-Cor 6400 chamber at 1000ppm CO<sub>2</sub>. It is possible that this response is indicative that the high exudation rates immediately prior to excision caused a deficit in organic acids, and that the CO<sub>2</sub> uptake is evidence of the roots capitalizing on available rhizospheric CO<sub>2</sub>. These results, however small, do provide more support that instantaneous responses to measurement CO<sub>2</sub> have some basis in metabolism, regardless of issues with chamber leaks in other studies.

In light of my results, and data presented in the studies of root respiration to date, and notwithstanding my hypotheses when I began this thesis, it appears that measurements of root respiration are in many ways uncoupled from actual energy usage by the organism. Both CO<sub>2</sub>



production (due to consumption of mitochondrial CO<sub>2</sub> by PEP-C, or by uptake of external CO<sub>2</sub> into the root) and O<sub>2</sub> consumption (due to the function of the alternative oxidase) can be measured without assurance that any predictable quantity of ATP is produced in association. Additionally, the current push to estimate root respiration is for inclusion in models of the carbon cycle under global change scenarios. However, it appears that due to high sensitivity of roots to CO<sub>2</sub> concentration under some conditions, estimating what occurs in intact soil conditions may be difficult.

### ***Conclusions***

Results from this thesis suggest that rhizospheric DIC concentration does not impact leaf gas exchange, whole plant biomass or water use efficiency in poplar under the growth conditions used. However, elevated DIC (similar to field conditions) did increase root PEP-C activity, and was found to alter relationships with exudation of some Krebs cycle organic acids. Consistent with some previous studies (van der Westuizen and Cramer 1998), measurement CO<sub>2</sub> concentration was observed to have an effect on root CO<sub>2</sub> production, most likely due to a combination of changes to diffusional rates as well as root uptake of CO<sub>2</sub>. Though I was unable to find support for my hypothesis linking root exudation and organic acid exudation across treatments, results from this study do provide an important new perspective on organic acid exudation in the roots of rapidly growing forest trees. Exudation rates observed in poplar roots are sensitive to growth root DIC concentration. Exudation rates for roots grown at 400ppm CO<sub>2</sub> are remarkably similar to the rates observed in cluster roots of lupine (Shane 2004), suggesting that we may be grossly underestimating the magnitude and diversity of compounds released by tree roots to the rhizosphere. Although root DIC did not directly impact poplar growth in the conditions examined,

the substantial increase in organic acid exudation at low DIC, suggests a functional relationship between root carbon accumulation (mediated by PEP-C) and organic acid exudation. If these results can be extrapolated to field conditions they would have important implications for the understanding the relationship between plant roots and the rhizosphere in an environment with increasing CO<sub>2</sub>. The increased activity of root PEP-C and decreased rates of exudation of organic acids, collectively suggests that growth of plant roots in a high DIC environment may facilitate root nitrogen assimilation by providing additional carbon skeletons for N-assimilation, and thereby reduce the release of organic acids to the soil environment to stimulate nutrient mobilization. Clearly more work is need on the integration of these processes under field conditions.

### ***Future Directions***

I think that the impacts of rhizosphere DIC on roots should be further explored, and there are some additional measurements that I would make to clarify relationships between DIC and root metabolic processes. Hydroponics are excellent for allowing the relatively unhindered study of roots, and the growth conditions could be manipulated to more closely replicate conditions in the rhizosphere. I chose to only manipulate the CO<sub>2</sub> conditions surrounding the roots, but O<sub>2</sub> could be manipulated as well to more closely match natural soil conditions. Additionally, measurement of the relative activities of pyruvate kinase (PK) and PEP-C in roots would give more information about flux of carbon through the Krebs cycle. PK and PEP-C Enzyme content, as well as organic acid content of roots would help elucidate the sources of exudates. Simultaneous collection of exudates during respiration measurements (liquid phase O<sub>2</sub> electrode) would also improve understanding of the relationship between these processes, although it might require a custom built chamber. More

finely-controlled nutrient conditions would also be important, and it might be useful to perform root experiments on agar plates in an attempt to observe quantitative responses to different nutrient conditions or pH.

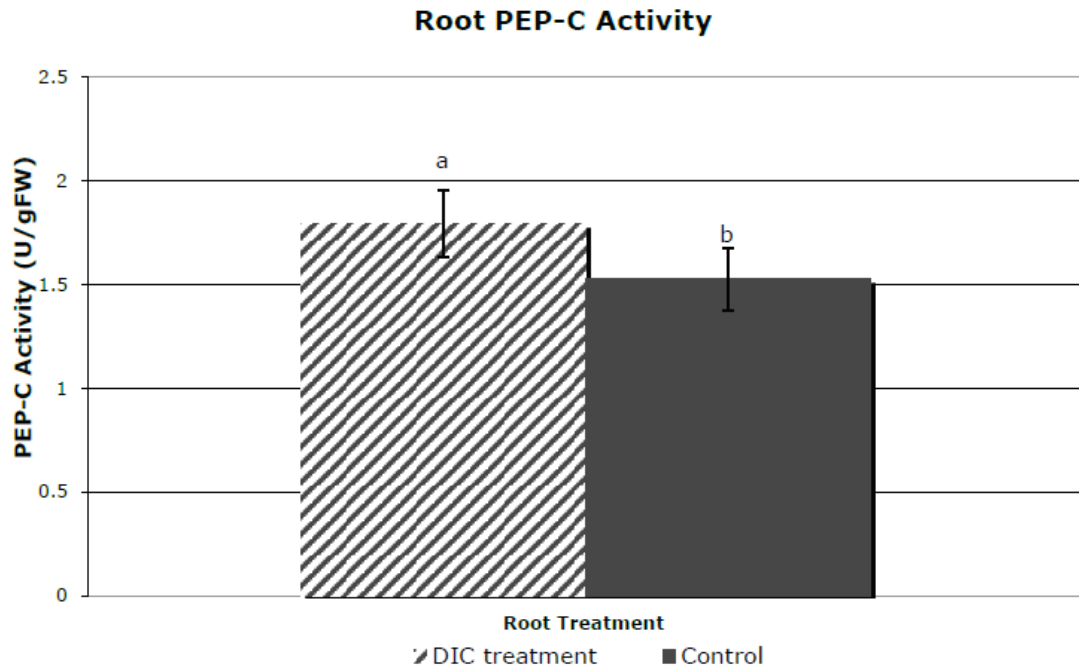
## Tables and Figures

**Table 1: Leaf gas exchange characteristics** of poplar trees grown hydroponically with either 15,000 or 400ppm root-zone CO<sub>2</sub>. Measurements were simultaneous for photosynthesis, stomatal conductance and transpiration rate, so RH and leaf temp reported for photosynthesis applies to all. Root treatment was found to cause no significant difference for any of these gas exchange characteristics (two-way ANOVA to determine effect of treatment and day on each characteristic and with RH, temp, and chlorophyll content as covariates. Treatment did not affect chlorophyll content. There were some significant day effects, and RH did influence stomatal conductance and transpiration despite the relatively high degree of RH control.

n=34	Treatment Mean ± s.e.	Root treatment ppm CO <sub>2</sub>	Mean measurement RH (%) ± s.d.	Mean leaf temp (°C) ± s.d.
Dark Respiration μmol CO <sub>2</sub> /m <sup>2</sup> /s	1.73±0.14	15,000	62.7±2.7	23.5±0.7
	1.73±0.18	400	63.6±1.9	23.6±0.5
Max Photosynthesis μmol CO <sub>2</sub> /m <sup>2</sup> /s	15.0±0.8	15,000	64.8±0.8	25.0±0.7
	15.9±1.0	400	64.9±0.9	25.1±0.5
Stomatal conductance mol H <sub>2</sub> O/m <sup>2</sup> /s	0.30±0.02	15,000		
	0.31±0.03	400		
Transpiration rate mmol H <sub>2</sub> O/m <sup>2</sup> /s	3.4±0.26	15,000	Leaf Chlorophyll Content ± s.e.	39.9±1.2
	3.5±0.37	400		41.2±1.2

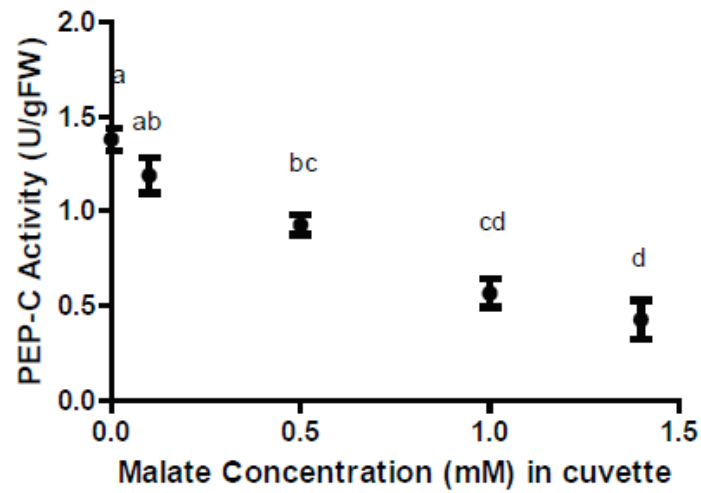
**Table 2: Whole plant biomass and water use** in poplar trees grown hydroponically with either 15,000 or 400ppm root zone CO<sub>2</sub>. Biomass data is pooled from two experiments (n=13). Water use was only recorded for the second experiment (n=7). Root treatment did not have a significant effect on plant biomass. (Two-factor ANOVAs with root treatment and day as factors and initial mass as a covariate. Shoot, root and total dry weights were sine-transformed to satisfy equal variances assumption of the ANOVA.) Water use was not significantly influenced by root treatment (One-way ANOVA).

Root CO <sub>2</sub> treatment	Shoot dry weight (g) ± s.e.	Root dry weight (g) ± s.e.	Total dry weight (g) ± s.e.	Root to shoot ratio ± s.e.	Total water use (L/gDW) ± s.e.
15,000 ppm	92.4 ± 9.6	8.8 ± 2.3	101.1 ± 11.8	0.09 ± 0.01	0.40±0.01
400 ppm	80.4 ± 8.9	9.6 ± 3.3	90.0 ± 11.9	0.10 ± 0.02	0.38±0.02

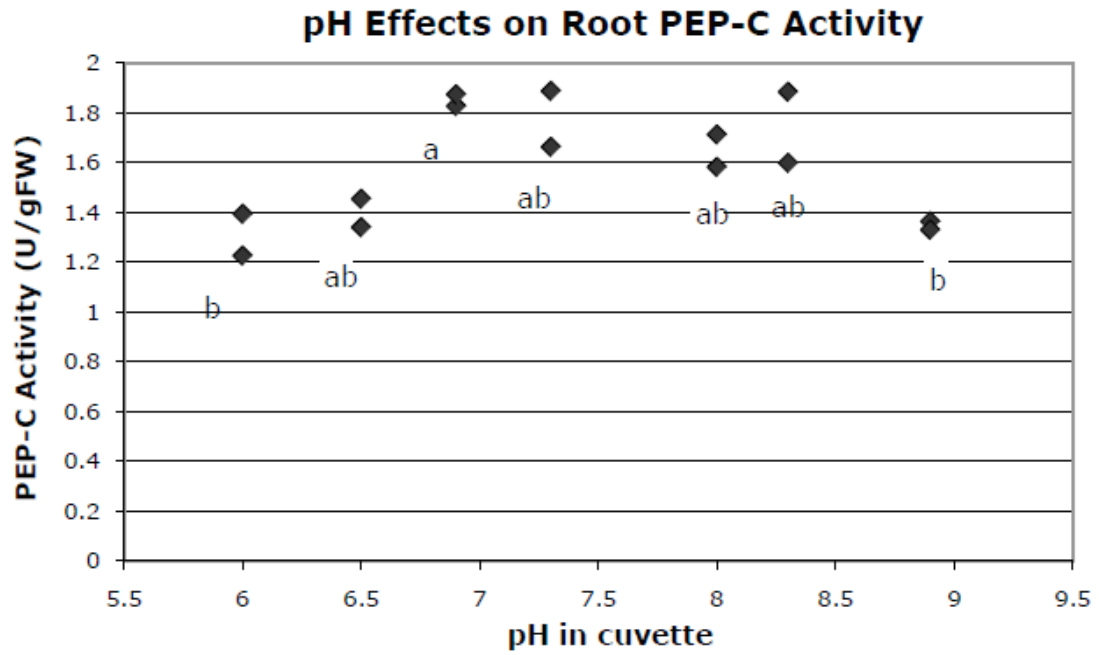


**Figure 1: Root PEP-C activities** for poplar grown hydroponically in either elevated or ambient dissolved inorganic carbon (DIC). Data from sodium bicarbonate and CO<sub>2</sub> gas-bubbling treatments were pooled as DIC treatment type was not found to be significantly different. Effects of root treatment and sampling day on PEP-C activity were analyzed with a two-way ANOVA ( $p=0.008^*$ ). PEP-C activity was log-transformed to satisfy equal variances assumption of the ANOVA. Levels not connected by same letter are significantly different (Student's t-test). Values are means ( $n=26$ )  $\pm$  s.e.

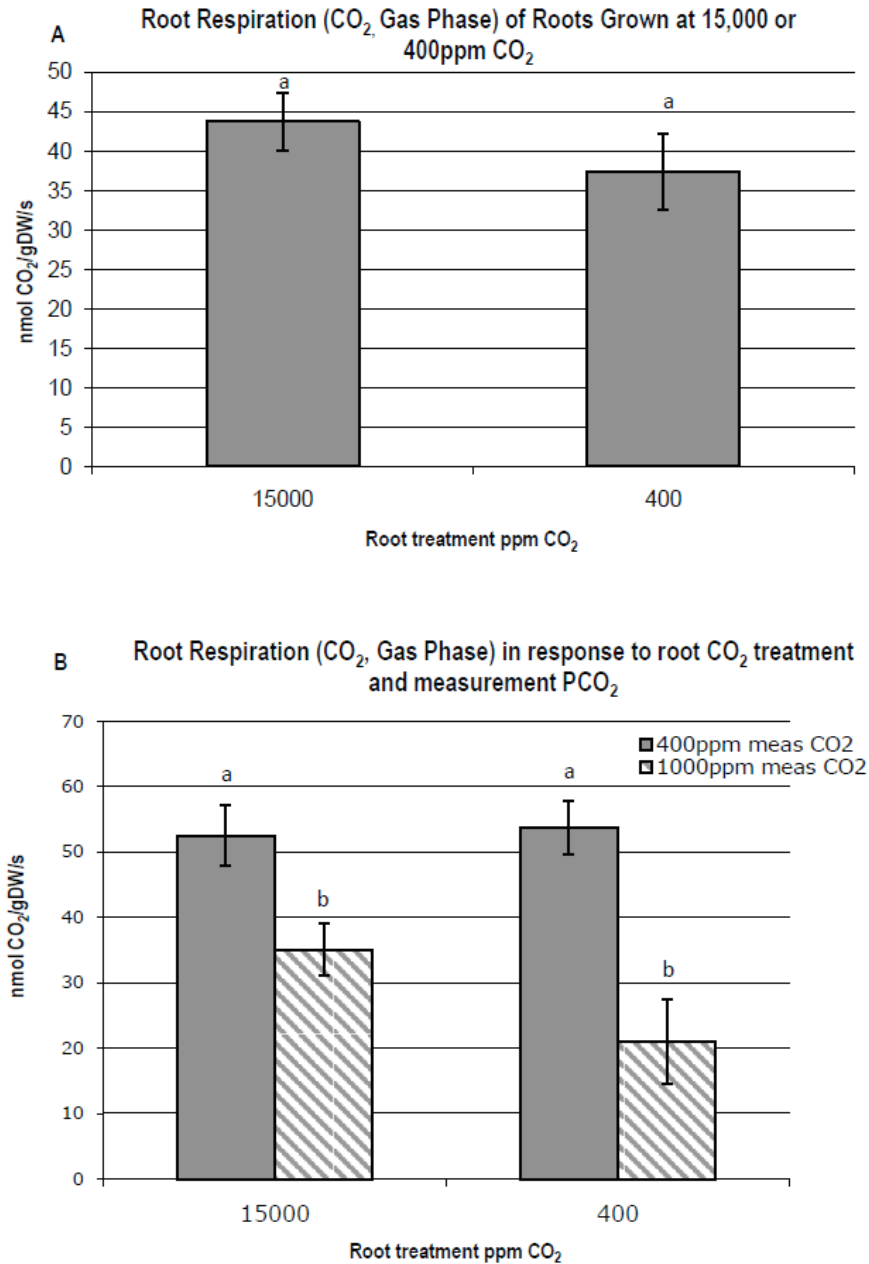
### Malate Inhibition of Root PEP-C Activity



**Figure 2: Malate inhibition of root PEP-C activity** of hydroponically grown poplar roots. Extract of 4 plants' root tips pooled and exposed to different malate concentrations in the cuvette (at pH=8). Effect of different concentrations of malate on PEP-C activity was analyzed with a one-way ANOVA ( $p \leq 0.0001^*$ ) and Tukey post-hoc test. Levels not connected by same letter are significantly different. Values are means ( $n=3$ )  $\pm$  s.d.

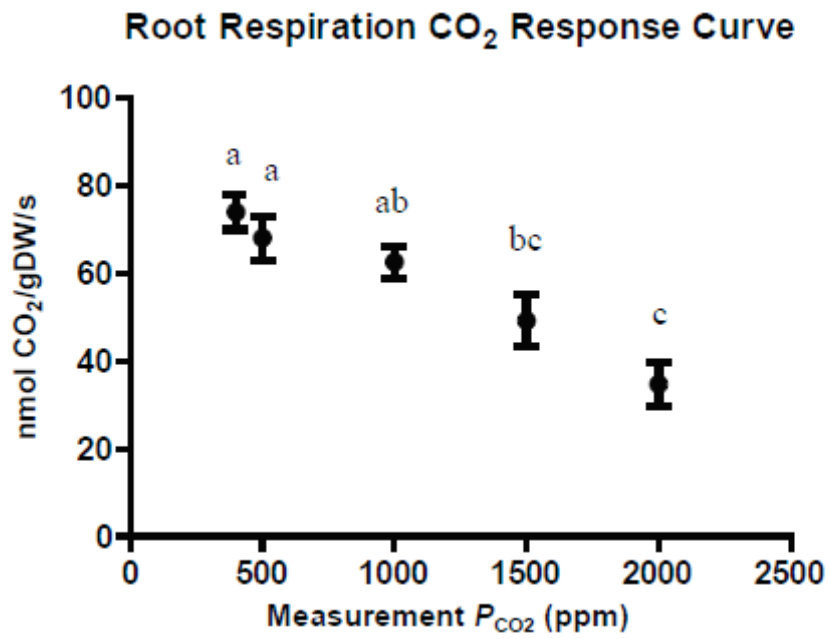


**Figure 3: pH effects on root PEP-C activity** of hydroponically grown poplar roots. Pooled extracts (n=4) of root tips exposed to different pH in cuvette. Effect of pH on PEP-C activity was analyzed with a one way ANOVA ( $p=0.01^*$ ) and Tukey post-hoc test: Levels not connected by same letter are significantly different.

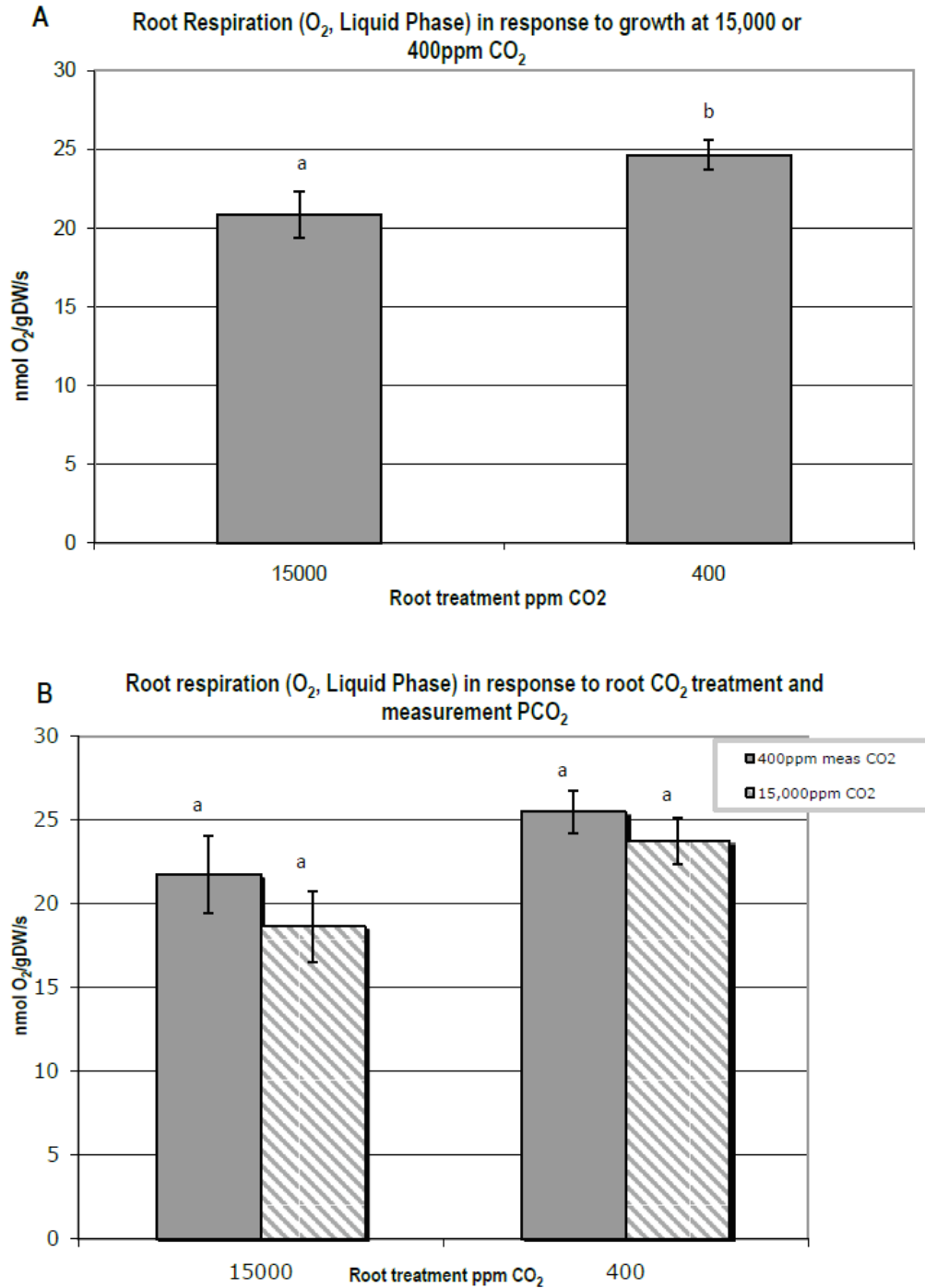


**Figure 4: Respiration Rates** of poplar roots grown hydroponically in either 15,000 or 400ppm  $\text{CO}_2$  (root treatment n.s.) . Values are means ( $n=18,30$ )  $\pm$  s.e. **(A)**. Respiration was measured at either 400 or 1000ppm  $\text{CO}_2$ . Values are means ( $n=9-15$ )  $\pm$  s.e. **(B)**. Effects of root treatment, measurement  $\text{CO}_2$ , sampling day, and meas  $\text{CO}_2$  x trtmt cross interaction on root  $\text{CO}_2$  production were analyzed with two-way ANOVA (measurement  $[\text{CO}_2]$   $p < 0.0001^*$ ). Respiration rate was log transformed to satisfy assumptions of ANOVA. Some days were also significantly different. The interaction between root treatment and measurement  $\text{CO}_2$  was not significant. Levels not connected by same letter are significantly different.

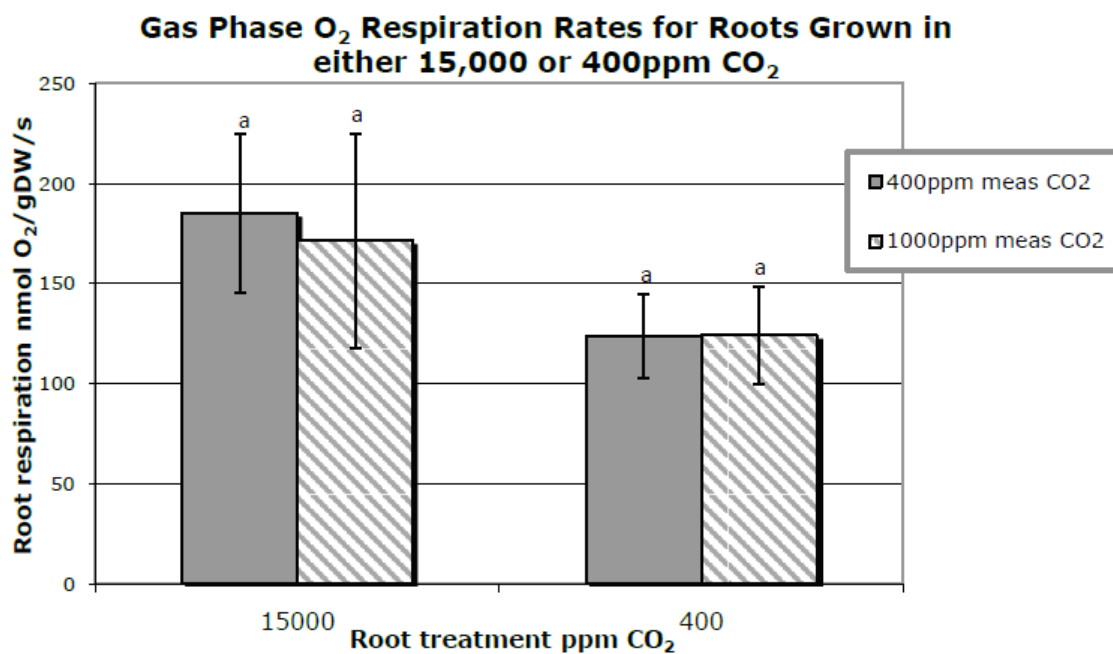




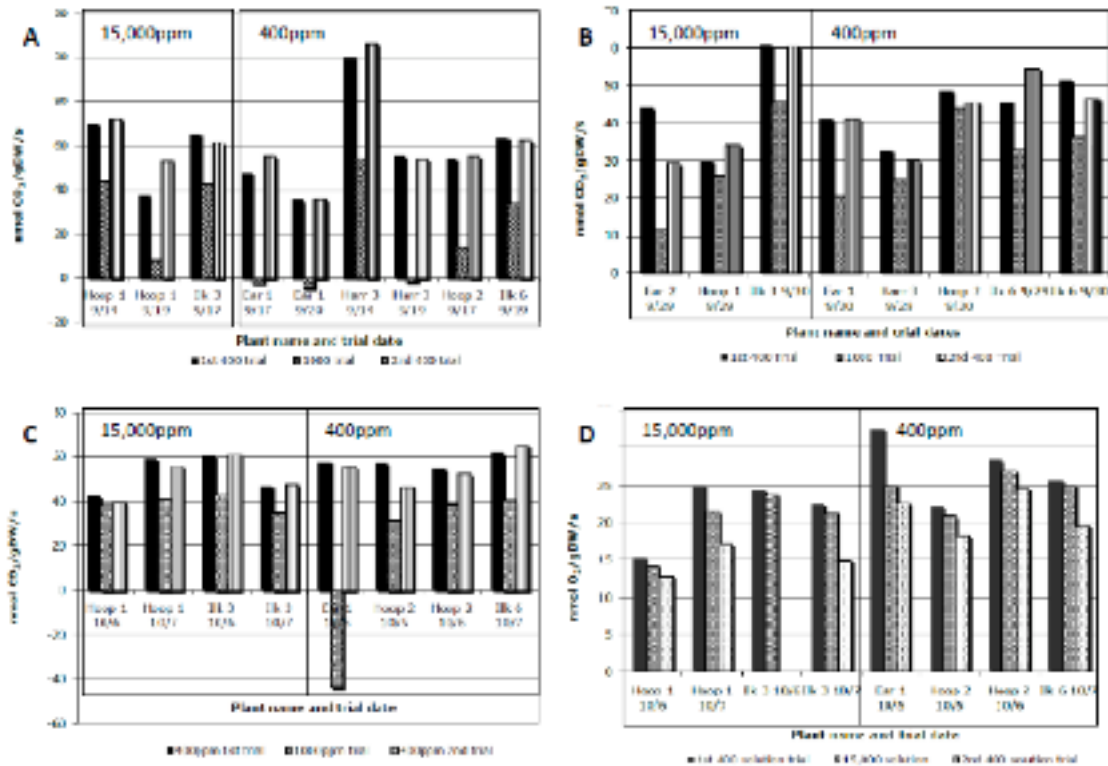
**Figure 5: Respiration rate CO<sub>2</sub>-response curve** of roots grown at 400ppm. Effect of different measurement [CO<sub>2</sub>] on root CO<sub>2</sub> production was analyzed with a one-way ANOVA ( $p=0.0001^*$ ) and tukey post-hoc test. Levels not connected by same letter are significantly different. Values are means ( $n=3$ )  $\pm$  s.e.



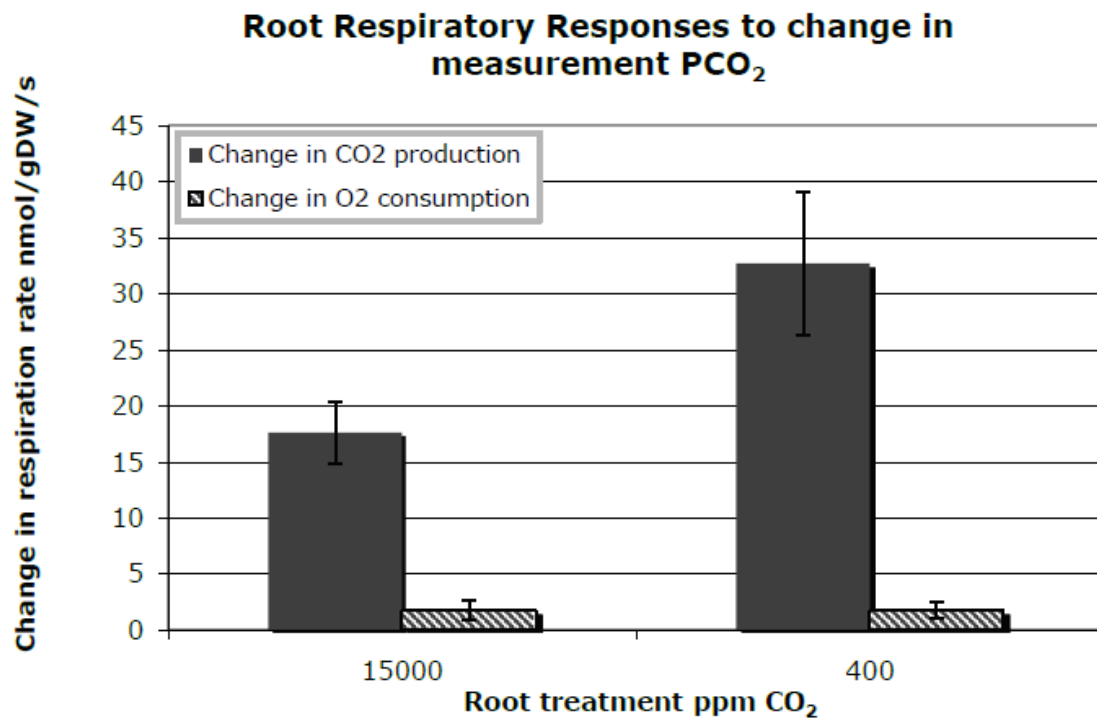
**Figure 6: Liquid phase  $O_2$  electrode respiration rates** of roots grown at either 15,000 or 400ppm  $CO_2$ . Values are means ( $n=8-10$ )  $\pm$  s.e. (A). Measurements were conducted in a closed chamber filled with buffer solution equilibrated with 400 or 15,000ppm  $CO_2$ . Values are means ( $n=4-5$ )  $\pm$  s.e. (B). Effects of root treatment, measurement  $CO_2$ , day, and trtmt x meas  $CO_2$  cross-interaction were analyzed with a two-way ANOVA. (Root treatment  $p=0.02^*$  In-chamber [DIC] not significant.) Respiration rates squared in order to satisfy equal variance assumptions of ANOVA.



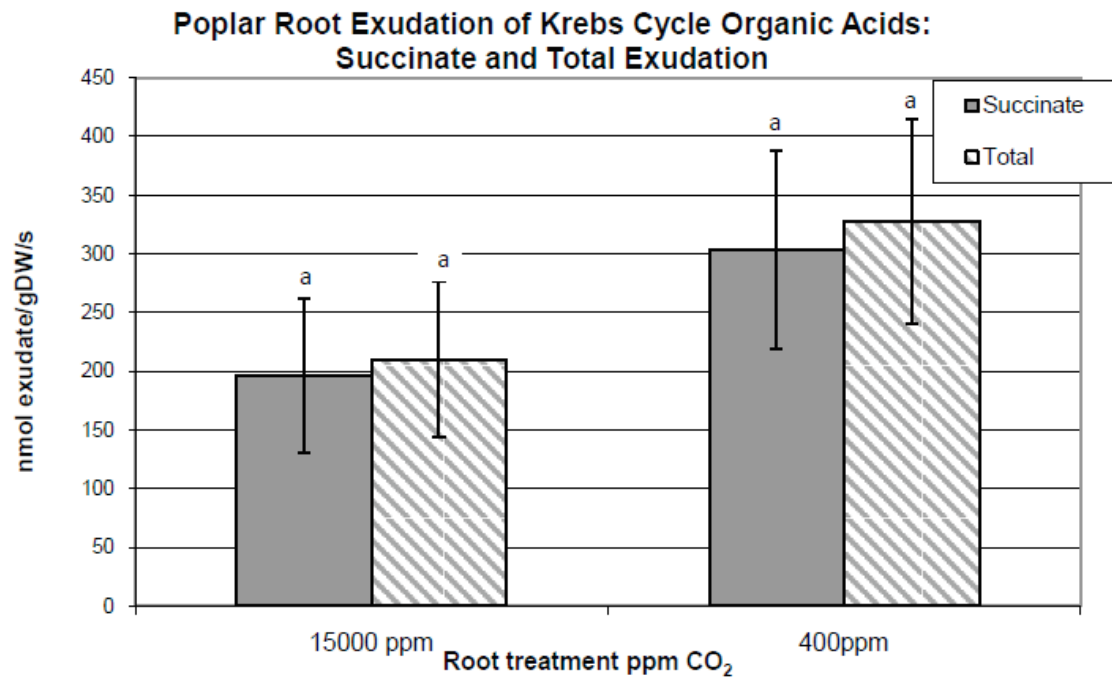
**Figure 7: Gas phase O<sub>2</sub> electrode respiration rates** of roots grown hydroponically at either 15,000 or 400ppm CO<sub>2</sub>. Measurements were conducted in a closed chamber injected with either room air (~400ppm) or 1000ppm CO<sub>2</sub>. Root treatment p=0.04\*). Effects of root treatment, measurement CO<sub>2</sub>, day, and trtmt x meas CO<sub>2</sub> cross-interaction were analyzed with a two-way ANOVA. Respiration rate square root-transformed to satisfy assumptions of ANOVA. Values are means (n=5-11) ± s.e. Note: Issues with electrode calibration led me to exclude data collected in this method.



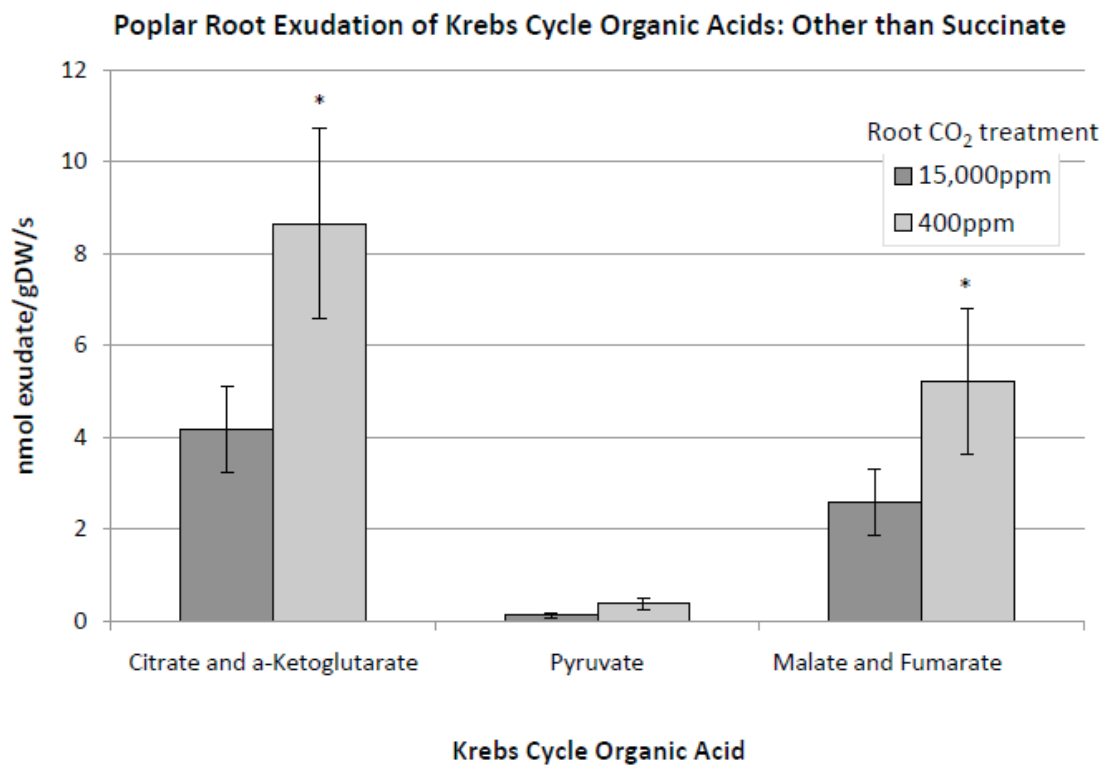
**Figure 8: Variation in individual root respiration rates, showing each different measurement [CO<sub>2</sub>] trial. Root CO<sub>2</sub> production measured in Li-Cor 6400 on measurement dates 9/14/10-9/19/10 (A), 9/29/10-9/30/10 (B), and 10/5/10-10/7/10 (C). Root O<sub>2</sub> consumption measured in the liquid phase 10/5/10-10/7/10 (D).**



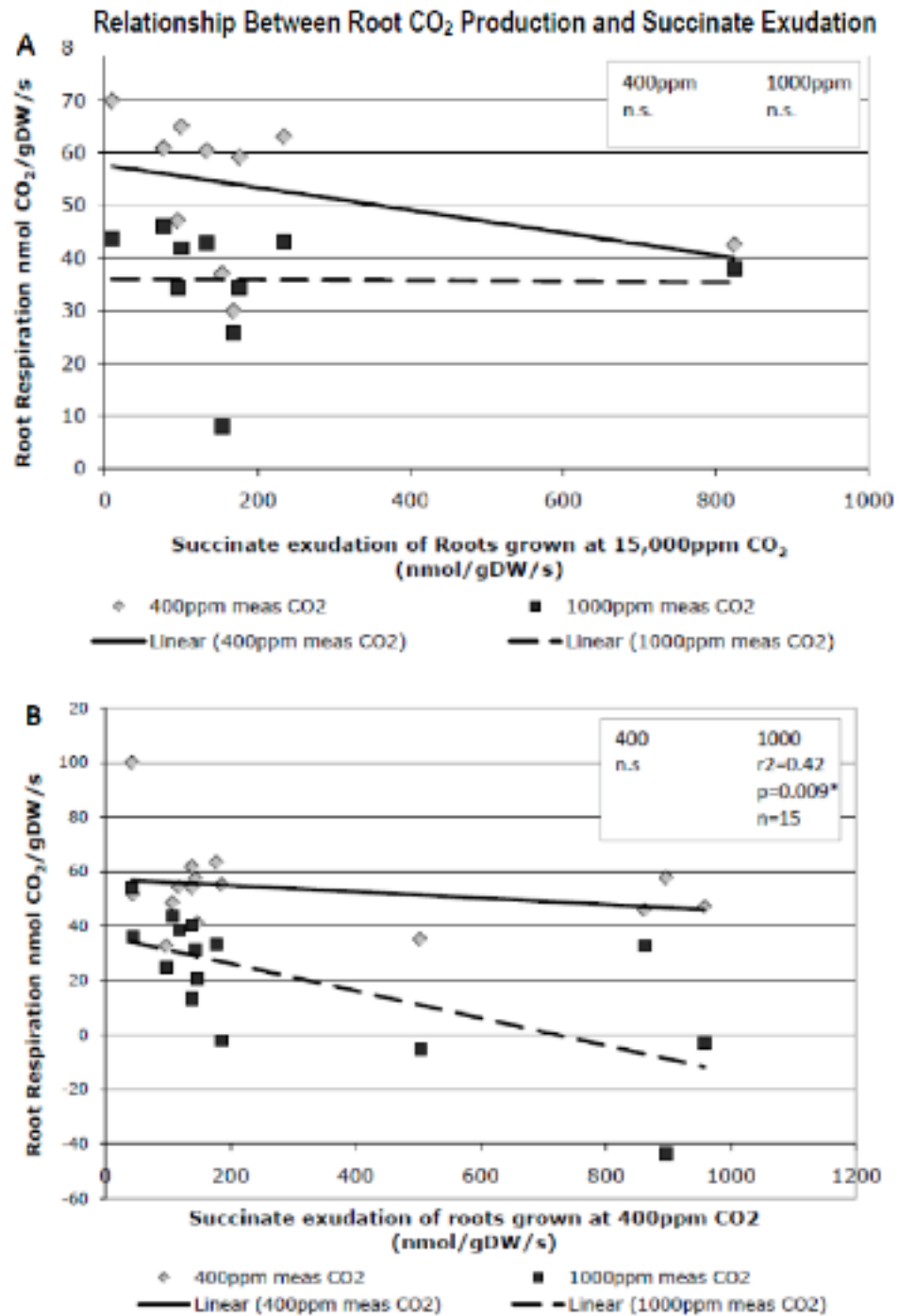
**Figure 9: Root respiratory responses to change in measurement CO<sub>2</sub> concentration** (change from 400ppm to 1000ppm), in poplar roots grown at either 15,000 or 400ppm CO<sub>2</sub>. Effects of root treatment and sampling day on respiratory response to measurement CO<sub>2</sub> were analyzed with two-way ANOVAs. Roots grown at 400ppm had a marginally greater decrease in CO<sub>2</sub> production ( $p=0.08$ ) in response to change in measurement CO<sub>2</sub>. Root O<sub>2</sub> consumption did not change in response to measurement CO<sub>2</sub>. Data was log-transformed to satisfy equal variance assumptions of the ANOVA. Values are means ( $n=9-15$  CO<sub>2</sub>, and  $n=4-5$  O<sub>2</sub>)  $\pm$  s.e.



**Figure 10: Root Krebs cycle acid exudation: Succinate and total exudation.** Effect of root treatment and sampling day on exudation rates was analyzed two-way ANOVAs and Student's t-tests. Both succinate and total exudation rates were log transformed to satisfy the assumptions of the ANOVA. Levels connected by same letter are not significantly different. Values are means ( $n=11-15$ )  $\pm$  s.e.

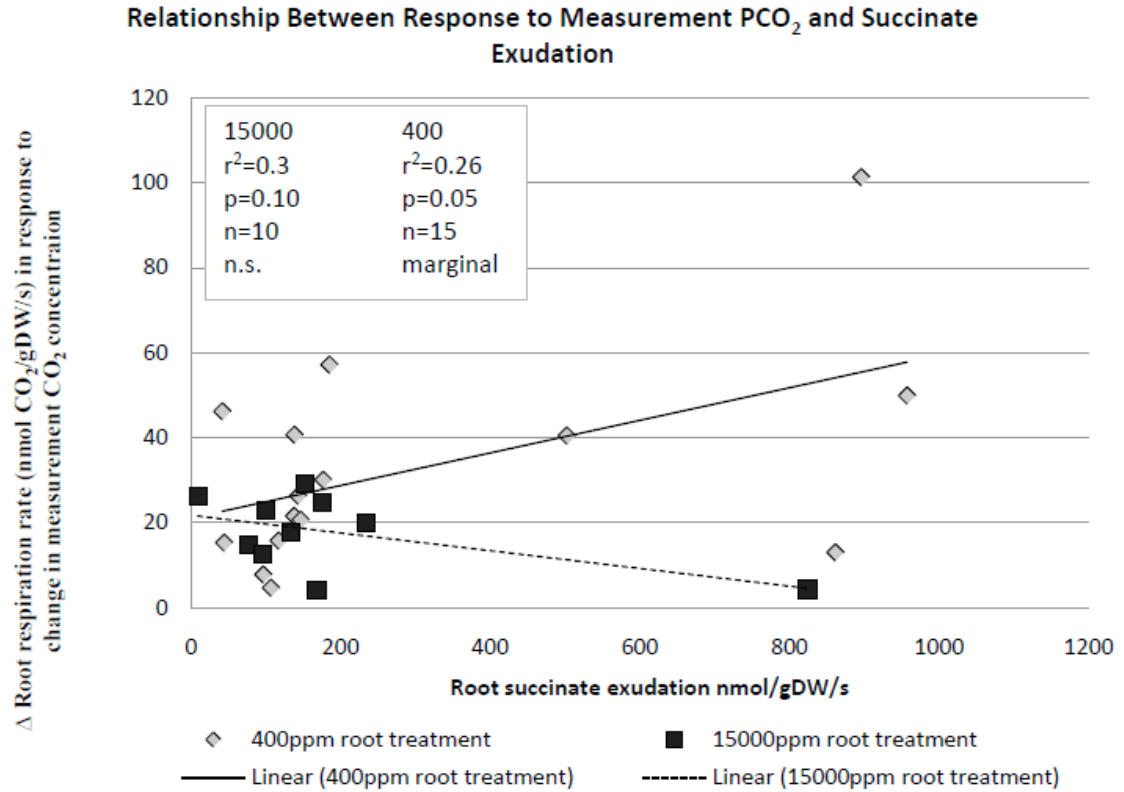


**Figure 11: Krebs cycle organic acid exudation** (excluding succinate) in poplar roots grown hydroponically at either 15,000 or 400ppm CO<sub>2</sub>. Effect of root treatment and sampling day were analyzed with two-way ANOVAs and Student's t-tests. All exudation rates were log transformed to satisfy the assumptions of the ANOVAs. Levels connected by same letter are not significantly different. Values are means (n=11-15) ± s.e.

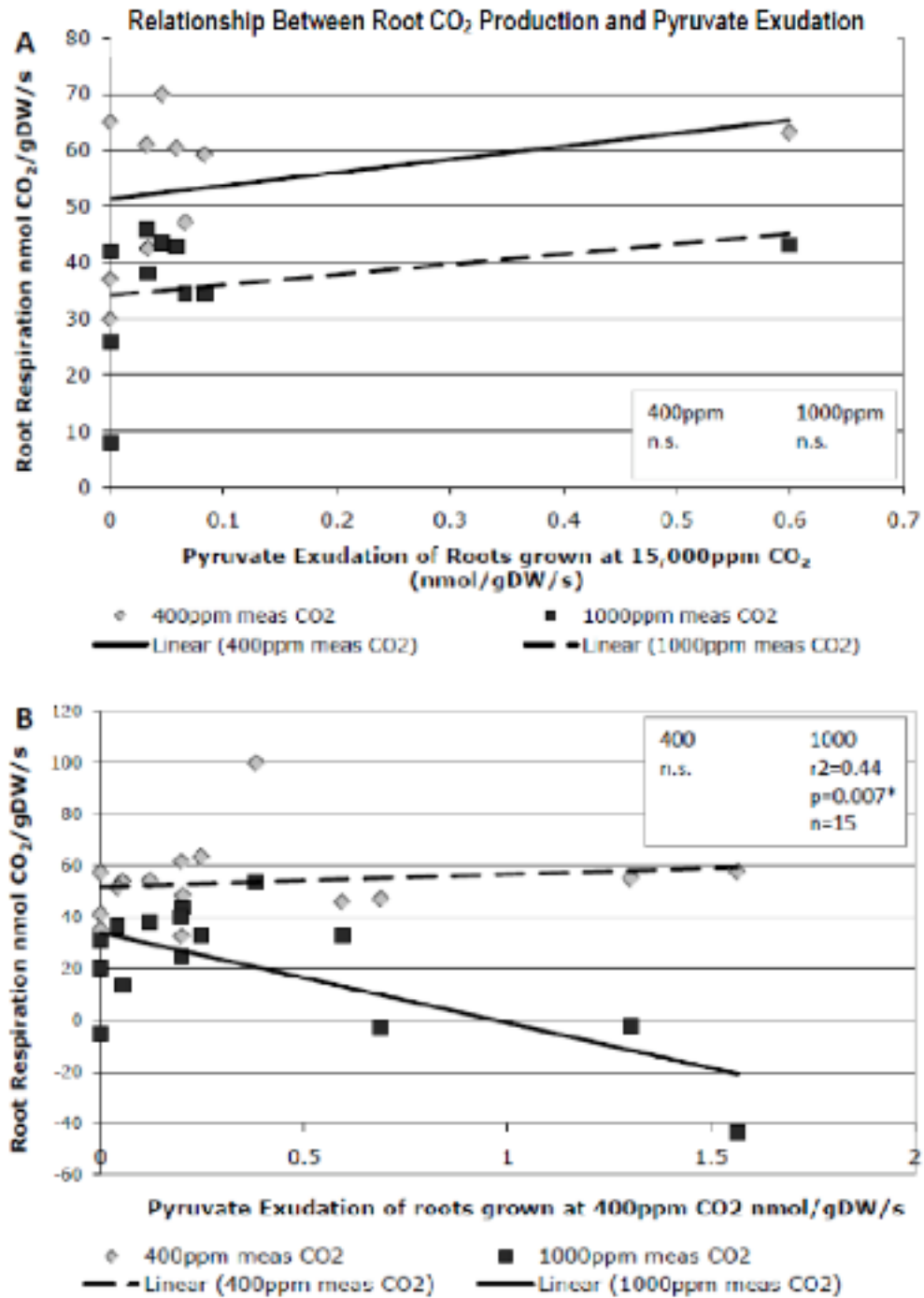


**Figure 12: Succinate exudation and root respiration (@ 400 and 1000ppm measurement CO<sub>2</sub>) of poplar roots grown hydroponically at either 15,000 (A) or 400ppm (B) CO<sub>2</sub> (n=25). Respiration measured at 400ppm was log transformed to normalize.**

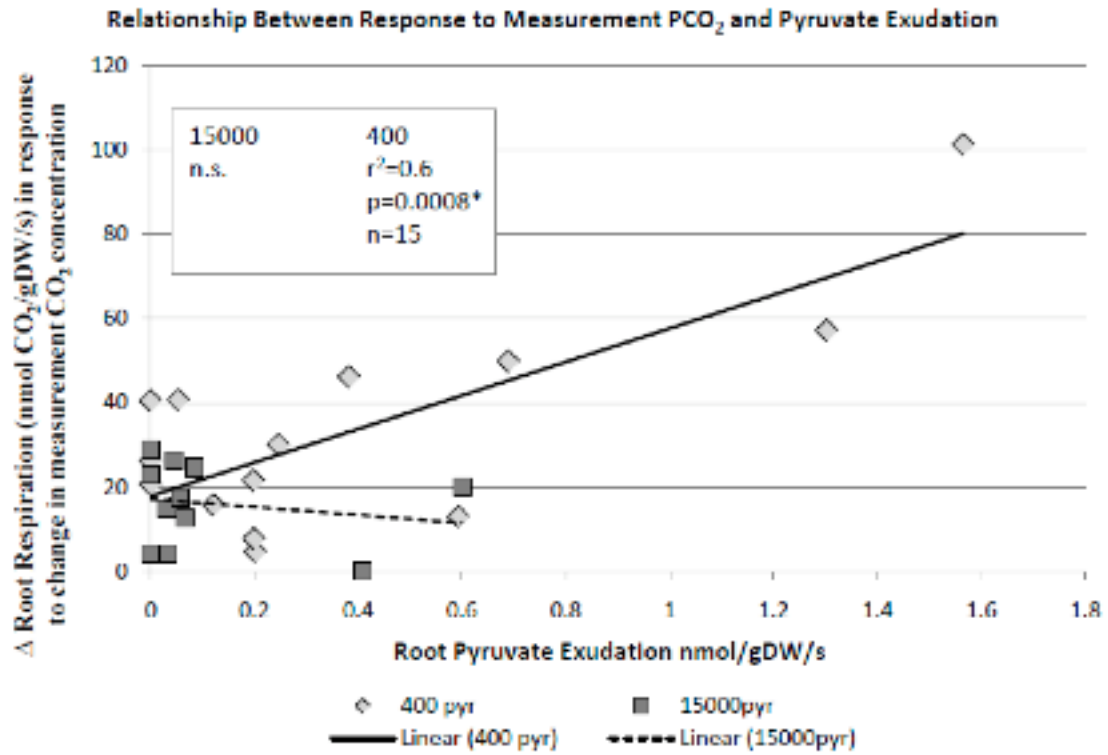




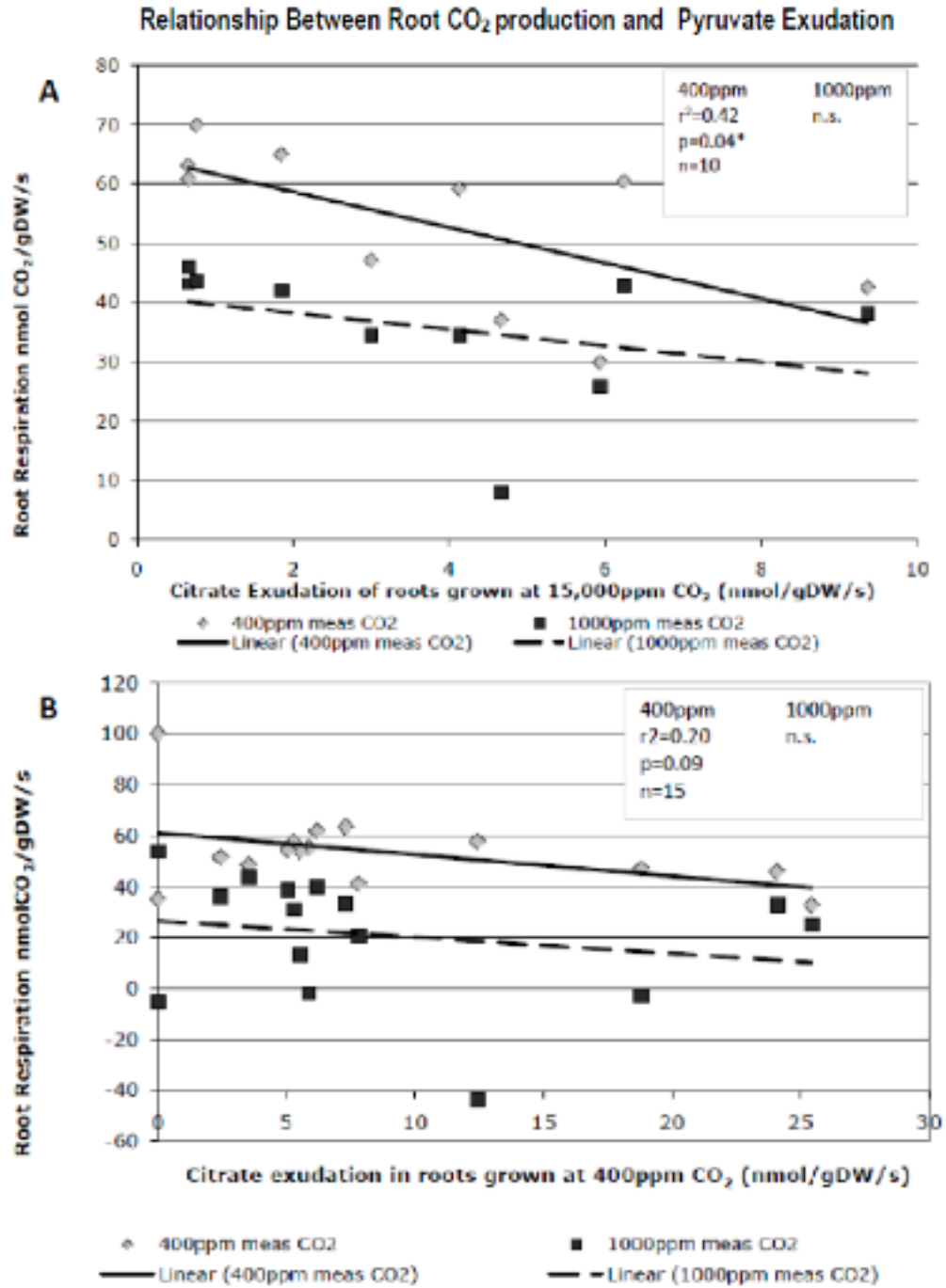
**Figure 13: Change in root respiration in response to change in measurement CO<sub>2</sub> concentration.** Roots grown at either 15,000 or 400ppm CO<sub>2</sub>. Relationship with succinate exudation in same roots (n=25). Data fulfills assumption of normality.



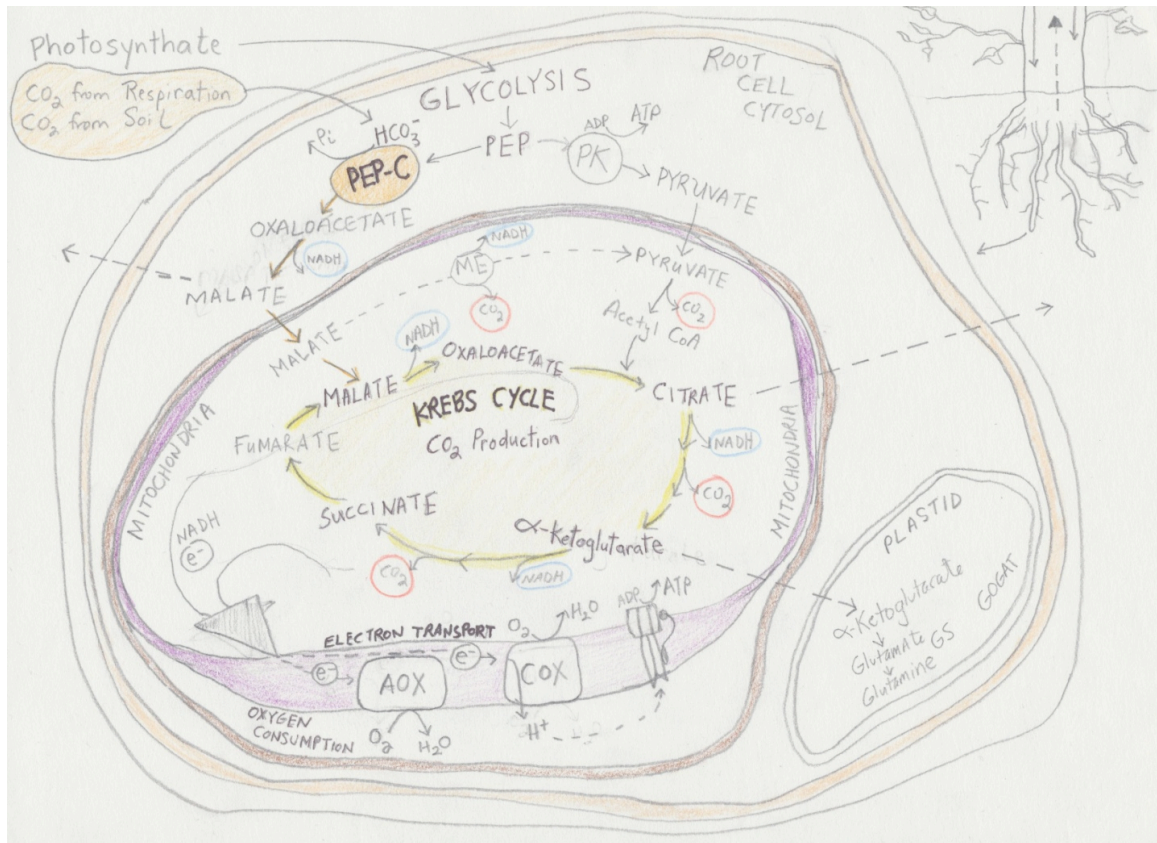
**Figure 14: Pyruvate exudation and root respiration (@ 400ppm and 1000ppm CO<sub>2</sub>) of roots grown hydroponically at 15,000 (A) or 400ppm (B) CO<sub>2</sub> (n=25). Respiration measured at 400ppm was log transformed to normalize.**



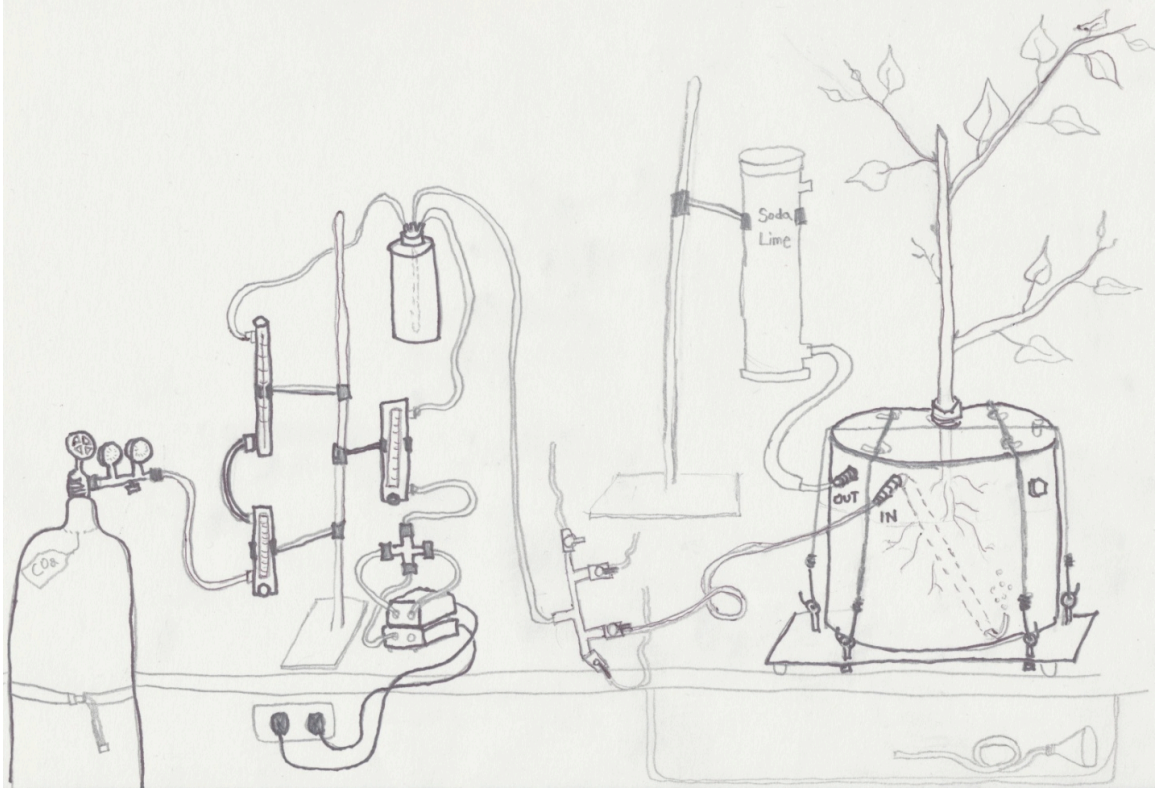
**Figure 15: Change in root respiration in response to change in measurement CO<sub>2</sub> concentration** in roots grown at 15,000 and 400ppm CO<sub>2</sub>. Relationship with pyruvate exudation rates of same roots (n=25). Data fulfills assumption of normality.



**Figure 16: Citrate exudation and root respiration (@ 400 and 1000ppm measurement CO<sub>2</sub>) in poplar roots grown hydroponically at either 15,000 (A) or 400 (B) ppm CO<sub>2</sub> (n=25). Data fulfills assumption of normality.**



**Figure 17: Representation of metabolic activities in a root cell associated with root carbon fixation.** Matarese 2010, after Plaxton and Podesta 2006.



**Figure 18:** Illustration of gas-mixer and hydroponic bubbler system utilized during the experiment (Matarese 2010).

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