IN VITRO CULTURES AND REGENERATION OF *BIENERTIA SINUSPERSICI*

(CHENOPODIACEAE) UNDER INCREASING CONCENTRATIONS OF SODIUM

CHLORIDE AND CARBON DIOXIDE

by

JOSH JEFFREY ROSNOW

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

MASTER OF SCIENCE IN MOLECULAR PLANT SCIENCES

WASHINGTON STATE UNIVERSITY
Department of Molecular Plant Sciences

MAY 2010

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JOSH JEFFREY
ROSNOW find it satisfactory and recommend it be accepted.

_____

Gerald E. Edwards, PhD., Chair

_____

Asaph Cousins, PhD.

_____

Amit Dhingra, PhD.

ACKNOWLEDGMENTS

The members of the Edward's lab, past and present

IN VITRO CULTURES AND REGENERATION OF *BIENERTIA SINUSPERSICI* (CHENOPODIACEAE) UNDER INCREASING CONCENTRATIONS OF SODIUM CHLORIDE AND CARBON DIOXIDE

Abstract

By Josh Jeffrey Rosnow, M.S.
Washington State University
May 2010

Chair: Gerald E. Edwards

The recent discovery of single-cell C_4 photosynthesis in terrestrial plants provided evidence that Kranz anatomy is not required in C_4 plants. Although the majority of C_4 plants have an anatomy that separates carboxylation and decarboxylation activities between two cell types (Kranz anatomy), aquatic macrophytes such as *Hydrilla verticulata*, and *Egeria densa,* and terrestrial plants *Bienertia cycloptera*, *B. sinuspersici* and *Suaeda aralocaspica,* can all conduct C_4 photosynthesis within an individual chlorenchyma cells. The terrestrial single –cell C_4 species provides an interesting opportunity to study the developmental transition of chloroplast from C_3 to C_4 photosynthesis. Investigations into the development of photosynthesis in *B. sinuspersici* in response to environmental and chemical factors were made using callus cells generated from leaf material and using young shoots developed from callus tissue. Cultures under a high level of CO_2 (1.2% versus ambient) increased callus growth, and was essential for development of shoots, roots, and plant regeneration. Green callus appears in a C_3 default mode as they have

minimal levels of C_4 enzymes and lack the structural development of the single-cell C_4 system; whereas, regenerating shoots develop expression of C_4 enzymes and features of photosynthetic cells characteristic of mature leaves in a normal plant. The effect of increasing sodium chloride in the media (0 to 200 mM NaCl) resulted in an increase in tissue osmolality, size of plantlets, and overall growth and regeneration, while having no effect on C_4 protein expression. These results can be used as a foundation for developing a transformation system and provide insight into developmental events that are involved in the formation of single cell C_4 species. As a C_4 system that lacks Kranz anatomy, a better understanding of how *B. sinuspersici* develops may facilitate the design of a transgenic system for incorporating C_4 photosynthesis into major C_3 crop species.

TABLE OF CONTENTS

TABLES: CHAPTER TWO

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

SUPPLEMENTAL FIGURES: CHAPTER TWO

Dedication

This thesis is dedicated to my parents, teachers, and advisors

CHAPTER ONE

GENERAL INTRODUCTION

Modes of Photosynthesis

Photosynthesis in terrestrial plants, aquatic algae, and cyanobacteria involves the

production of carbohydrates and amino acids by utilizing light as an energy source and

CO_2 fixation by the enzyme ribulose-1, 5-bisphosphate carboxylase (Rubisco). The

photosynthetic carbon reduction cycle (alternatively called the Calvin - Benson cycle)

operates in the stroma of the chloroplast, where Rubisco catalyzes the entry of CO_2 into

the cycle, by generating two C_3 compounds (triose phosphates). Plants in which the first

product of atmospheric CO_2 fixation is a C_3 compound, are referred to as C_3 species, and

this metabolic pathway operates in all chlorenchyma cells.

Rubisco is also a bifunctional enzyme acting as an oxygenase, as well as a

carboxylase, utilizing O_2 as a substrate which leads to photorespiration with a loss of

fixed carbon (Edwards et al. 1983). The theoretical minimum energy requirements for

CO_2 fixation, to the level of triose phosphate in C_3 plants, according to the energetics of

the C_3 cycle, in the absence of photorespiration are 3 ATP and 2 NADPH per CO_2. In

warmer climates, CO_2 can limit photosynthesis due to the decreased solubility of CO_2

relative to O_2, decreased specificity factor of Rubisco to utilize CO_2, and under water

limiting conditions reduced stomatal conductance increases CO_2 diffusive resistance from

the atmosphere to the chloroplasts (Ku et al. 1977; Jordan et al. 1984; Bernacchi et al.

2002). When CO_2 is limiting there is an increase in photorespiration which generates

CO_2 and uses more ATP and NADPH to regenerate Calvin cycle intermediates, lowering

the efficiency of carbon assimilation (Edwards *et al.* 1983). Photorespiration can reduce

overall yield in economically important C_3 crops such as wheat, rice, soybean, and potato.

To overcome the effect of limited CO_2 on photosynthesis, some terrestrial plants developed ways to increase the level of CO_2 at the site of Rubisco. Concentrating CO_2 around Rubisco decreases the oxygenase reaction and the loss of carbon through the photorespiratory pathway. These modes of photosynthesis include C_4 and Crassulacean Acid Metabolism (CAM), which differ in terms of spatial (C_4 photosynthesis) verses temporal (CAM photosynthesis) separation of atmospheric CO_2 fixation and reduction of CO_2 to carbohydrate.

Species that concentrate CO_2 through a C_4 dicarboxylic acid cycle during the day are called C_4 plants, and have a leaf anatomy that is different from C_3 plants. In the majority of C_4 plants, the most observable difference seen with a light microscope is the presence of chloroplasts in both mesophyll and distinctive bundle sheath cells, which is called Kranz anatomy due to its wreath-like appearance. Carbonic anhydrase (CA) catalyzes the first enzymatic step in C_4 photosynthesis, where it equilibrates the aerial CO_2 to HCO_3^- in the cytosol of mesophyll cells. The second cytoplasmic step in the C_4 pathway is the formation of the C_4 acid oxaloacetate (OAA) from HCO_3^- and phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxylase (PEPC), which can then be transaminated to aspartate or reduced to malate. The C_4 acids are then shuttled to bundle sheath cells, the compartment containing Rubisco, where they are decarboxylated by C_4-acid-decarboxylating enzymes (Figure 1) (Edwards *et al.* 2004).
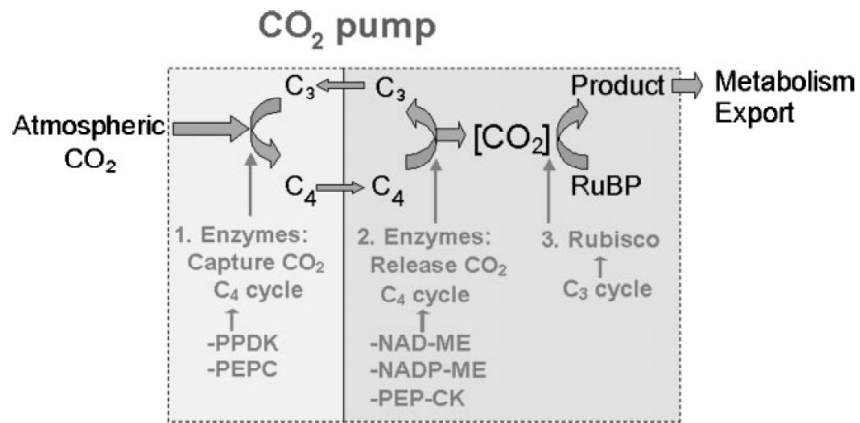
Figure 1 Compartmental depiction of the CO_2 pump for C_4 photosynthesis.

There are three well-defined biochemical subtypes of C_4 plants based on differences in the type of C_4 acid decarboxylases utilized, and their compartmentalization: the chloroplastic NADP-malic enzyme (NADP-ME), the mitochondrial NAD-malic enzyme (NAD-ME), and the cytosolic phosphoenolpyruvate carboxykinase (PEP-CK). The CO_2 released during decarboxylation is assimilated in the Calvin cycle, as in C_3 plants (Edwards *et al.* 1983; Hatch 1987). In the process of decarboxylation of the C_4 acids by malic enzymes, pyruvate is formed in bundle sheath cells, and transported to mesophyll cells for the regeneration of phosphoenolpyruvate (PEP) by pyruvate, Pi dikinase (PPDK), which requires the input of 2 ATP. The extra cost of 2 ATP per CO_2 fixed in C_4 plants, is in addition to the 3 ATP required for the Benson-Calvin cycle. This extra expenditure of energy becomes favorable under conditions when CO_2 availability is limiting, such as drought and salinity. CAM is a metabolic adaptation to arid environments where stomata are closed during much of the day and open at night. During the night, the primary fixation of CO_2 is catalyzed by PEPC, resulting in the formation of malic acid, which is stored in the vacuole. During the day, this acid is decarboxylated to provide CO_2 for fixation by the C_3 cycle. The

3

CAM and C_4 pathway rely on the regulated gene expression, compartmentalization, and activation state of PEPC for proper functioning of the carbon concentrating mechanism.

As noted above, in the mesophyll cells of C_4 plants, primary fixation of CO_2 occurs via PEPC, and the CO_2 is concentrated by the C_4 cycle in the Rubisco containing bundle sheath cells. Thus, C_4 photosynthesis is a highly integrated process characterized by difference in anatomy and biochemistry, with cell specific enzyme expression required for an operational C_4 pathway. There is natural variation in biochemistry of the C_4 pathway and differences in anatomy among C_4 genera. There are also some plant species that have an intermediate C_3-C_4 type of photosynthesis which combine some traits of both types of CO_2 fixation. The evolution of different C_4 systems has occurred at least 50 independent times in 19 families of angiosperms (Sage 2004; Muhaidat et al. 2007). The Chenopodiaceae family has the largest number of C_4 species with the greatest diversity in leaf anatomy among dicot families, having C_4 Kranz anatomy and C_4 single-cell type species, as well as C_3 type species (Voznesenskaya et al. 2008). This diversity suggests that plants under conditions of limiting CO_2 can evolve different mechanisms for survival that include the compartmentation of enzymes and changes in leaf anatomy, which results in the alteration of Rubisco kinetics, and the development of different forms of the C_4 cycle.

C_4 plants evolved from C_3 plants in environments that are conducive to high rates of photorespiration, an adaptation to increase carbon assimilation. Isoforms of the enzymes required for the formation of the C_4 cycle, are already present at low levels in C_3 plants. During evolution of C_4 photosynthesis new isoforms developed with unique promoters and splicing variations, which controls the level of expression and tissue

specificity (Ku et al. 1996; Nomura et al. 2000). Genes encoding C_4 isoforms have been identified in Kranz type plants, but genes which are required to develop Kranz type anatomy have not been identified, and little is known about the transcriptional program regulating C_4 differentiation (Covshoff et al. 2008). Evolution of Kranz anatomy requires close vein spacing, development of bundle sheath cells with chloroplasts having Rubisco and the C_3 cycle, and mesophyll chloroplasts supporting the capture of CO_2 in the C_4 cycle. Understanding leaf development has been limited by lack of identification of genes that effect vein spacing and differentiation of C_4 bundle sheath and mesophyll cells (Mitchell 2007). In the single-cell C_4 system of *B. sinuspersici*, it is known that very early in development the photosynthetic chlorenchyma cells are in a C_3 default mode, with all chloroplasts containing Rubisco; as the leaf matures, there is a transition to a C_4 system with more transcripts and proteins of the C_4 cycle being expressed, and development of dimorphic chloroplasts (Figure 2) (Voznesenskaya et al. 2005; Lara et al. 2008; Park et al. 2009a). How differentiation of chloroplasts is accomplished, and maintained, are major questions in C_4 biology.

Single Cell C_4 photosynthesis

Until a few years ago it was believed that Kranz anatomy was required for the operation of a C_4 cycle in terrestrial plants, with strict compartmentation of C_4 and Calvin cycle enzymes in mesophyll and bundle sheath cell chloroplasts, respectively, to prevent futile CO_2 cycling and leakage (Edwards *et al.* 2004). Kranz anatomy is more or less a fixed property of C_4 leaves, with the expression of Rubisco and C_4 photosynthesis genes confined to leaves and regulated by light and photosynthetic metabolism (Patel et al. 2008). More knowledge about interactions between the chloroplast and nucleus,

mitochondria, and peroxisomes would help to explain the environmental or developmental interactions that regulate the formation of C_4 anatomy for increased acquisition of carbon.

Most C_4 plants and many CAM plants function with obligate C_4 type photosynthesis. There are facultative CAM species which can function in C_3 or CAM mode depending on environment conditions, or stage of development (Winter et al. 1996). A well characterized example is the common ice plant *Mesembryanthemum crystallinum*, where a switch from C_3 to CAM mode is induced by water stress, caused by drought or salinity (Winter et al. 2007). This facultative mode of nighttime CO_2 fixation limits water loss during the day and increases water use efficiency.

With respect to C_4 plants, the aquatic monocots *H. verticillata* (L.f.) Royle and *Edgera densa* are facultative single-cell C_4 NADP-ME species which have developed mechanisms to increase photosynthesis under conditions of low CO_2 availability. These species exhibits C_3 photosynthesis under high levels of dissolved CO_2 during cooler low light conditions in the winter, but when the external CO_2 concentration declines during the summer, and the temperature and light intensity increases the C_4 cycle is induced. The C_4 and Calvin cycles operate in the same cell with PEPC and Rubisco confined to the cytosol and chloroplast, respectively. During C_4 induction, a specific C_4 PEPC isoform is the first enzyme of the C_4 cycle to increase in expression. Once in the C_4 state, inorganic carbon is fixed in the leaf cytosol by PEPC and the C_4 acid is transported into the chloroplast where it is converted to malate, whose subsequent decarboxylation by NADP-ME causes CO_2 accumulation in the vicinity of Rubisco (Bowes et al. 2002; Lara et al. 2002). In this system, ineffective CO_2 cycling is apparently avoided by an intracellular

separation of the initial carboxylation and subsequent decarboxylation events between cytosol and chloroplast, but it is not clear what prevents leakage of CO_2 away from Rubisco and futile cycling. Unlike the terrestrial C_4 plants that have dimorphic chloroplasts, there appears to be only one type of chloroplast present in the NADP-ME *H. verticillata* and *E. densa*. The leaves of these species also show a pH-polarity, similar to mesophyll-cell wall acidification, with the abaxial side pH decreasing to facilitate the uptake of inorganic carbon. At high light intensities and low dissolved carbon concentration, acidification shifts the equilibrium of HCO_3^-/CO_2 towards CO_2, to increase the concentration of CO_2 inside the leaf (Bowes *et al.* 2002; Lara *et al.* 2002). In these examples of facultative aquatic and CAM plants, the photosynthetic carbon metabolism operates within a single cell with no differentiation of two cell types required. This function of C_4 with monomorphic chloroplast is in contrasts with the Kranz type dimorphic chloroplasts where structural differentiation and biochemical specialization of photosynthetic cells is necessary. Another inducible C_4 system in which Kranz type anatomy is formed occurs in the genus *Eleocharis* (Cyperaceae). For example *Eleocharis vivipara* expresses C_4 characteristics under terrestrial conditions and C_3 characteristics under submerged aquatic conditions (Ueno 2001).

The three terrestrial single-cell C_4 species discovered in the family Chenopodiaceae are *Bienertia cycloptera* (Voznesenskaya et al. 2002), *B. sinuspersici* (Akhani et al. 2005), and *Suaeda aralocaspica* (Voznesenskaya et al. 2001). These species grow in semi-desert areas in Central Asia and around the Persian Gulf, under high temperatures, limiting water, and saline soils (Akhani *et al.* 2005). *B. sinuspersici* performs C_4 photosynthesis under high salt concentrations and accumulates large

quantities of salt ions and compatible solutes in their leaves (Park et al. 2009b). The current model of chlorenchyma cell development in *B. sinuspersici* (figure 2), starts with a small heterotrophic cell containing monomorphic chloroplasts, a prominent nucleus, and a few small vacuoles. Chlorenchyma cell maturation leads to more photoautotrophic development and, two cytoplasmic domains, separated by a large central vacuole, interconnected by cytoplasmic channels (Park *et al.* 2009a). The mature chlorenchyma cells once developed have a stable structure, maintaining two cytoplasmic compartments, even when grown under very low light (Lara *et al.* 2008). In mature chlorenchyma cells, peripheral chloroplasts have PPDK and lack Rubisco while the central compartment chloroplasts contain Rubisco and lack PPDK (Voznesenskaya *et al.* 2002). *B. sinuspersici* dimorphic chloroplasts are thought to be specialized for C_4 functions analogous to the dimorphic chloroplasts occurring in Kranz type C_4.

Model for *Bienertia sinuspersici* leaf cell development

Voznesenkaya E. et al., 2005;
Park J. et al., 2009

Green - chloroplast
Red - vesicle or vacuole
Blue - nucleus
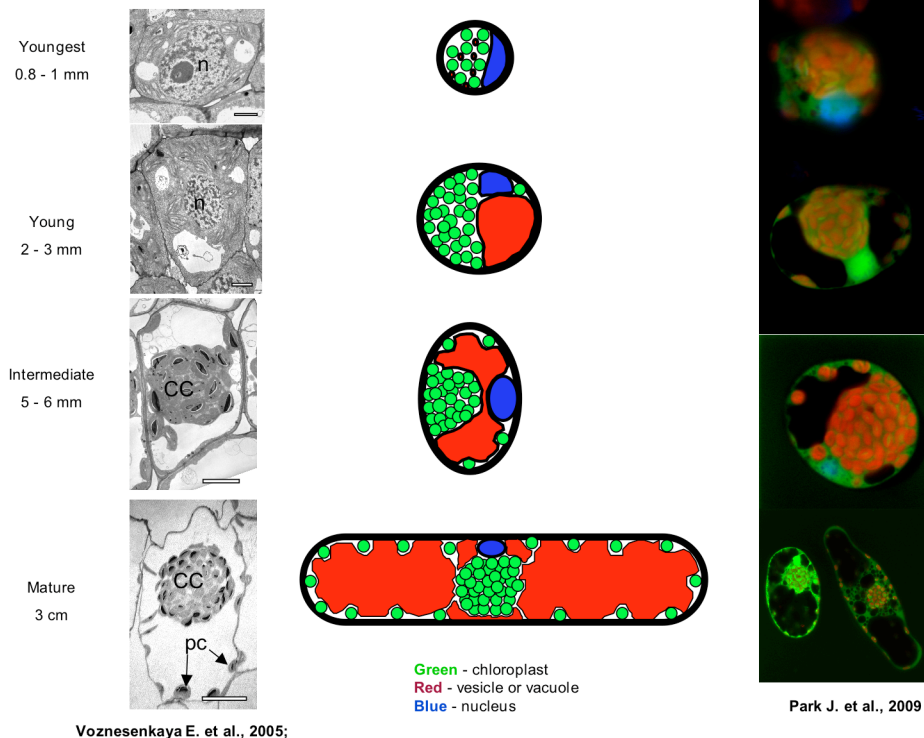
Figure 2. Chlorenchyma cell development model, showing a gradual increase in cell size with the development of a central compartment (CC). Peripheral chloroplasts (PC) are separated from the CC chloroplasts by a large central vacuole, with interconnecting cytoplasmic channels.

Tissue culture

Ironically Gottlieb Haberlandt, the first botanist to hypothesize that the two photosynthetic cell types in Kranz-type plants may have separate functions, was also the first to attempt *in vitro* culture of plants using leaf cells of *Tradescantia reflexa* (Haberlandt 1884; Haberlandt 1902; Yamada 1985). Years later, growth of callus cultures was achieved, as other scientists followed with studies that confirmed the presence of photosynthetic activity in culture (LaRosa et al. 1984; Yamada 1985). Cultured plant cells can be regarded as omnipotent, possessing the complete genetic information necessary for constructing an intact plant. In fact, single cells or protoplasts from different cell types of several plant species have been regenerated into intact plants. Explant material derived from a plant, normally contains a diversity of cell types, and not all are equally capable of embryogenesis or responsive to re-determination (Williams et al. 1986). Some cells are more totipotent than others, having a greater capacity to continue to divide, regenerate a new organ such as the shoot or an entire plant. Induced regeneration from *in vitro* tissue cultures can occur indirectly from callus, or directly from cells of an organized structure such as a stem segment or zygotic embryo (Figure 3). The hypocotyl and cotyledons of germinating seeds are a favorable source of explant

9

material, due to the developmental plasticity and ease of cell proliferation.   Cells of a

maturing tissue such as a leaf are often too specialized or differentiated to be a good

source of cells for *in vitro* growth and plant regeneration.
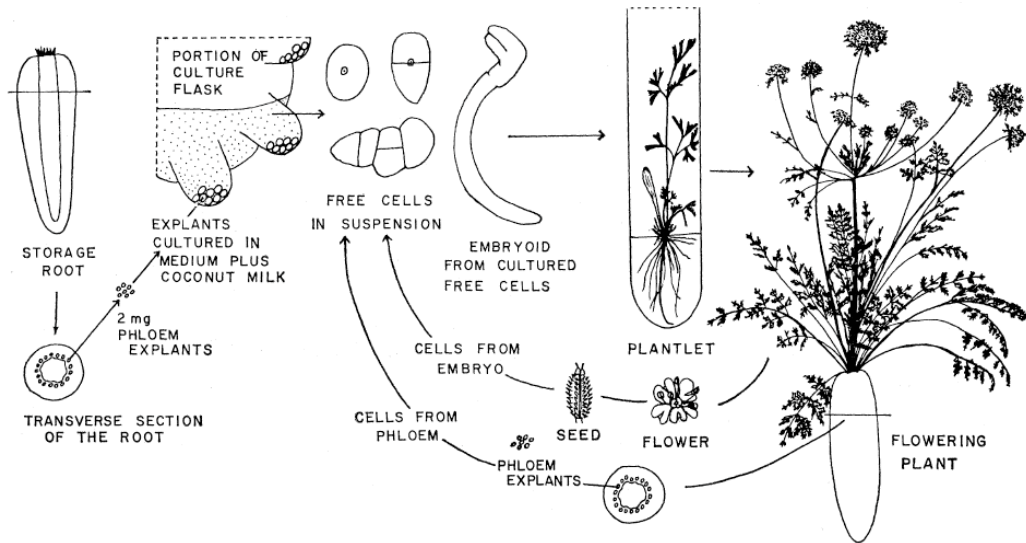
Figure 3. Diagrammatic representation of the cycle of growth of the carrot plant

showing the source of totipotent cells used for *in vitro* cultures is often derived

from the phloem procambium or the embryo (Steward et al. 1964).

Plant regeneration starts with embryogenesis, or the coordinated behavior of cells

in vitro. The explant material may require re-determination to the embryogenic state, by

chemical factors such as hormones that affect coordinated cell growth.   Regeneration of

intact plants from transformed tissues is not always an easy task. In a number of systems

it is easy to transform tissue that is not competent for regeneration. The two primary

modes of introducing new genetic elements into plant cells are micro particle

bombardment and *Agrobacterium* mediated, and both have been used successfully for

many C_3 plants. With respect to C_4 plants, C_4 NADP-ME species *Flaveria bidentis,*

Saccharum spp. L*., Zea mays,* and *Sorghum bicolor* L. *Moench* can be transformed using

Agrobacterium tumefaciens infection of explants followed by regeneration of shoots from

callus, as well as the CAM plant *Kalanchoe blossfeldiana* (Chitty et al. 1994; Aida et al.

1996; Arencibia et al. 1998; Frame et al. 2002; Howe et al. 2006; Sunagawa et al. 2007).

Recently, the inducible CAM plant *Mesembryanthemum crystallinum* and the first NAD-

ME type C_4 species, *Cleome gynandra* L. were successfully transformed using hypocotyl

and cotyledon explant material mediated by *Agrobacterium tumefaciens* (Sunagawa et al.

2007; Newell et al. 2010). The key to these efficient transformation protocols was the

selection for tissue or cells that have the ability to regenerate. However, currently there is

no transformable C_4 plant species, which provides an effective model system similar to

Arabidopsis (easily transformed, fast life cycle, small size, and small genome with

sequence information).

Callus cell cultures can be described as a mixotrophic population of cells, with

most of the cells being heterotrophic when grown on a high exogenous concentration of

sugars, most often sucrose, at much higher concentrations than found in photosynthetic

cells *in vivo*. Heterotrophic metabolism involves the uptake of organic carbon from the

external environment in the form of sugar to support growth via mitochondrial

respiration. When both organic carbon and CO_2 are acquired and utilized in cultures, the

cell metabolism is described as being mixotrophic. Photoautotrophic metabolism is

centered on using light energy to generate chemical energy inside the cell, which is used

to fix CO_2 and assimilate inorganic nitrogen to support growth without any exogenous

sugar or organic carbon. *In vitro* photoautotrophic growth of callus cells is often

achieved by elevating the concentration of the CO_2 in the environment (Berlyn et al.

1975; Husemann et al. 1977; Rogers et al. 1987; Ziegler et al. 1989). Increasing the

available CO_2 concentration surrounding callus cultures helps with chloroplast

development, including thylakoid membrane organization and increases the soluble

protein and chlorophyll content.

The development of callus photoautotrophic metabolism and growth has been

linked with available carbon in the environment and media. Inclusion of 50 mM glucose

in the media of *Chenopodium rubrum* callus cultures was found to suppress Rubisco

small subunit expression, and the expression of C_4 genes in maize mesophyll protoplasts

is lowered when sugars are added (Krapp et al. 1993; Jang et al. 1994). The activity of

PEPC was found to be higher when sugar was replaced with soluble starch in the media

using *M. crystallinum* calli (Yen *et al.* 1995). There have been mixed reports on the

development of C_4 photosynthesis in photomixotrophic callus cultures of Kranz type C_4

plants. *Portulaca oleracea* had up to 45% of the radiolabel CO_2 incorporated in C_4 acids

during a 4 sec. pulse with $^{14}CO_2$ with the label going into to sugars during a chase with

$^{12}CO_2$, suggesting function of a C_4 cycle (Kennedy et al. 1977). *Froelichia gracilis* had

50% of the $^{14}CO_2$ label in malate during the pulse, but whether there was transfer of label

from the C_4 acids was not determined (Laetsch 1974; Kennedy *et al.* 1977). In other C_4

callus, 40% of the total $^{14}CO_2$ in *Gisekia pharnaceoides* L. was found in C_3 compounds

with 30% in malate and 10% in aspartate, while *Zea Mays* was found to perform C_3

photosynthesis with little label in C_4 acids (Kennedy *et al.* 1977; Seeni et al. 1983;

Lavergne et al. 1992). Typical anatomy of green callus has large parenchyma cells

containing chloroplasts making up most of the tissue, with pockets of meristematic cells

and small clusters of lignified cells randomly scattered throughout the callus. Activity of

PEP carboxylase is low in C_3 plants, but can be higher in callus tissue when expressed on a mg of protein basis (Hanson et al. 1972) There have been no reports of callus cultures of single-cell C_4 species, which is the topic of this thesis.

Research Aims

Bienertia sinuspersici is a halophytic species being developed as a model system for studying single-cell C_4 photosynthesis. Mature chlorenchyma cells have dimorphic chloroplasts in two separate cytoplasmic domains that are peripherally and centrally located, analogous to mesophyll and bundle sheath cells of Kranz anatomy. The selective positioning of chloroplasts, peroxisomes and mitochondria allows increased carbon acquisition and decreased photorespiration through C_4 function. This unusual structure of a chlorenchyma cell makes it a good model system for studying organelle interactions. A procedure for plant regeneration needs to be established for future genetic investigations on requirements for development of single-cell C_4 photosynthesis.

Studies on the mechanism of intracellular development of dimorphic chloroplasts, and spatial compartmentalization of C_4 functions, will provide significant information on developmental plant biology. Also, research on halophytic species like *B. sinuspersici* may provide insight into how these plants tolerate stress and the underlying cellular mechanisms that may be necessary to increase future agricultural production, such as high nitrogen use efficiency, salinity acclimation, and drought tolerance. The goal of this research is to develop optimum media composition for regeneration of an entire *B. sinuspersici* plant starting from stem explant.

Works Cited

Aida, R. and Shibata, M. (1996). "Transformation of *Kalanchoe blossfeldiana* mediated by *Agrobacterium tumefaciens* and transgene silencing." <u>Plant Science</u> **121**: 175-185.

Akhani, H., Barroca, J., Koteeva, N., Voznesenskaya, E., Franceschi, V., Edwards, G., Ghaffari, S. M. and Ziegler, H. (2005). "*Bienertia sinuspersici* (Chenopodiaceae): A new species from southwest asia and discovery of a third terrestrial C_4 plant without Kranz anatomy." <u>Systematic Botany</u> **30**: 290-301.

Arencibia, A. D., Carmona, E. R., Tellez, P., Chan, M.-T., Yu, S.-M., Trujillo, L. E. and Oramas, P. (1998). "An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens* " <u>Transgenic Research</u> **7**: 213-222.

Berlyn, M. B. and Zelitch, I. (1975). "Photoautotrophic growth and photosynthesis in tobacco callus cells." <u>Plant Physiology</u> **56**: 752-756.

Bernacchi, C. J., Portis, A. R., Nakano, H., von Caemmerer, S. and Long, S. P. (2002). "Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo." <u>Plant Physiology</u> **130**: 1992-1998.

Bowes, G., Rao, S. K., Estavillo, G. M. and Reiskind, J. B. (2002). "C_4 mechanisms in aquatic angiosperms: comparisons with terrestrial C_4 systems." Functional Plant Biology **29**: 379-392.

Chitty, J. A., Furbank, R. T., Marshall, J. S., Chen, Z. and Taylor, W. C. (1994). "Genetic transformation of the C_4 plant, *Flaveria bidentis*." The Plant Journal **6**(6): 949-956.

Covshoff, S., Majeran, W., Liu, P., Kolkman, J. M., van Wijk, K. J. and Brutnell, T. P. (2008). "Deregulation of Maize C_4 Photosynthetic Development in a Mesophyll Cell-Defective Mutant." Plant Physiology **146**: 1469-1481.

Edwards, G. and Walker, D. (1983). C_3, C_4: Mechanism, and Cellular and Environmental Regulation, of Photosynthesis. London, Blackwell Scientific Publications.

Edwards, G. E., Dai, Z., Cheng, S. H. and Ku, M. S. B. (1996). Factors affecting the induction of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. Crassulacean acid metabolism: biochemistry, ecophysiology and evolution. K. Winter and J. A. C. Smith. New York, Springer. **114:** 449.

Edwards, G. E., Franceschi, V. R. and Voznesenskaya, E. V. (2004). "Single-Cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm." Annual Review Plant Biology **55**: 173-96.

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Pegg, S. E. K., Li, B., Nettleton, D. S., Pei, D. and Wang, K. (2002). "Agrobacterium tumefaciens-Mediated transformation of maize embryos using a standard binary vector system." Plant Physiology **129**: 13-22.

Haberlandt, G. (1884). "Physiological Plant Anatomy (Transl. M. Drummond)." Macmillan, London.

Haberlandt, G. (1902). Sitz.-Ber **111**: 69.

Hatch, M. D. (1987). "C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure." Biochimica et Biophysica Acta **895**: 81-106.

Hanson, A. D. and Edelman, J. (1972). "Photosynthesis by carrot tissue cultures." Planta **102**: 11-25.

Howe, A., Sato, S., Dweikat, I., Fromm, M. and Clemente, T. (2006). "Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum." Plant Cell Reports **25**: 784-791.

Husemann, W. and Barz, W. (1977). "Photoautotrophic growth and photosynthesis in cell suspension cultures of *Chenopodium rubrum*." Physiol. Plant **40**: 77 - 81.

Jang, J.-C. and Sheen, J. (1994). "Sugar sensing in higher plants." <u>The Plant Cell</u> **6**: 1665-1679.

Jordan, D. B. and Ogren, W. L. (1984). "The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase." <u>Planta</u> **161**: 308-313.

Kennedy, R. A., Barnes, J. E. and Laetsch, W. M. (1977). "Photosynthesis in C_4 plant tissue cultures: Significance of Kranz anatomy to C_4 acid metabolism in C_4 plants." <u>Plant Physiology</u> **59**: 600-603.

Krapp, A., Hofmann, B., Schafer, C. and Stitt, M. (1993). "Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis?" <u>The Plant Journal</u> **3**: 817-828.

Ku, M. S. B., Kano-Murakami, Y. and Matsuoka, M. (1996). "Evolution and expression of C_4 photosynthesis genes." <u>Plant Physiology</u> **111**: 949-957.

Ku, S.-B. and Edwards, G. E. (1977). "Oxygen inhibition of photosynthesis I. Temperature dependence and relation to O_2/CO_2 solubility ratio." <u>Plant Physiology</u> **59**: 986-990.

Lara, M. V., Casati, P. and Andreo, C. S. (2002). "CO_2-concentrating mechanism in *Egeria densa*, a submersed aquatic plant." <u>Physiologia Plantarum</u> **115**: 487-495.

Lara, M. V., Offermann, S., Smith, M., Okita, T. W., Andreo, C. S. and Edwards, G. E. (2008). "Leaf development in the single-cell C_4 system in *Bienertia sinuspersici*: Expression of genes and peptide levels for C_4 metabolism in relation to chlorenchyma structure under different light conditions." Plant Physiology **148**: 593-610.

Laetsch, W. M. (1974). "The C_4 syndrome: a structural analysis." Annual Review Plant Physiology **25**: 27-52.

LaRosa, P. C., Hasegawa, P. M. and Bressan, R. A. (1984). "Photoautotrophic potato cells: Transition from heterotrophic to autotrophic growth." Physiol. Plant **61**: 279 - 286.

Lavergne, D., Nato, A., Dupuis, J.-M., Pean, M. and Chagvardieff, P. (1992). "Evidence for the expression of morphological and biochemical characteristics of C_3-photosynthesis in chlorophyllous callus cultures of *Zea mays*." Physiologia Plantarum **84**: 292-300.

Mitchell, P. L. (2007). Catching up with the literature for C_4 rice: what we know and didn't then. Charting new pathways to C_4 rice. J. E. Sheehy, P. L. Mitchell and B. Hardy. Los Banos, International rice research institute**: 55-78.

Muhaidat, R., Sage, R. F. and Dengler, N. G. (2007). "Diversity of Kranz anatomy and biochemistry in C_4 Eudictos." American Journal of Botany **94**: 362-381.

Newell, C. A., Brown, N. J., Liu, Z., Pflug, A., Gowik, U., Westhoff, P. and Hibberd, J. M. (2010). "*Agrobacterium tumefaciens* - mediated transformation of *Cleome gynandra* L., a C_4 dicotyledon that is closely related to *Arabidopsis thaliana* " Journal of Experimental Botany **61**: 1311-1319.

Nomura, M., Sentoku, N., Nishimura, A., Lin, J.-H., Hondar, C., Taniguchi, M., Ishida, Y., Ohta, S., Komari, T., Miyao-Tokutomi, M., Kano-Murakami, Y., Tajima, S., Ku, M. S. B. and Matsuoka, M. (2000). "The evolution of C_4 plants: Acquisition of cis-rgulatory sequences in the promoter of C_4 type pyruvate, orthophosphate dikinase gene." The Plant Journal **22**: 211-221.

Park, J., Knoblauch, M., Okita, T. W. and Edwards, G. E. (2009a). "Structural changes in the vacuole and cytoskeleton are key to development of the two cytoplasmic domains supporting single-cell C_4 photosynthesis in *Bienertia sinuspersici*." Planta **229**: 369-382.

Park, J., Okita, T. W. and Edwards, G. E. (2009b). "Salt tolerant mechanisms in single-cell C_4 species *Bienertia sinuspersici* and *Suaeda aralocaspica* (Chenopodiaceae)." Plant Science.

Patel, M. and Berry, J. O. (2008). "Rubisco gene expression in C_4 plants." Journal of Experimental Botany **59**: 1625-1634.

Rogers, S. M. D., Ogren, W. L. and Widholm, J. M. (1987). "Photosynthetic characteristics of a photoautotrophic cell suspension culture of soybean." Plant Physiology **84**: 1451-1456.

Sage, R. F. (2004). "The evolution of C_4 photosynthesis." New Phytologist **161**: 341-370.

Seeni, S. and Gnanam, A. (1983). "Photosynthesis in cell suspension culture of a C_4 plant, *Gisekia pharnaceoides* L." Plant and Cell Physiology **24**: 1033-1041.

Steward, F. C., Mapes, M. O., Kent, A. E. and Holsten, R. D. (1964). "Biochemical and morphogenetic studies with cells yield new evidence on their metabolism and totipotency." Science **143**: 20-27.

Sunagawa, H., Agarie, S., Umemoto, M., Makishi, Y. and Nose, A. (2007). "Effect of urea-type cytokinins on the adventitious shoots regeneration from cotyledonary node explant in the common ice plant, *Mesembryanthemum crystallinum*." Plant Production Science **10**: 47-56.

Voznesenskaya, E. V., Akhani, H., Koteyeva, N. K., Chuong, S. D. X., Roalson, E. H., Kiirats, O., Franceschi, V. R. and Edwards, G. E. (2008). "Structural, biochemical, and physiological characterization of photosynthesis in two C_4 subspecies of *Tecticornia indica* and the C_3 species *Tecticornia pergranulata* (Chenopodiaceae)." Journal of Experimental Botany **59**: 1715-1734.

Voznesenskaya, E. V., Franceschi, V. R., Kiirats, O., Artyusheva, E. G., Freitag, H. and Edwards, G. E. (2002). "Proof of C_4 photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae)." <u>The Plant Journal</u> **31**: 649-662.

Voznesenskaya, E. V., Franceschi, V. R., Kiirats, O., Freitag, H. and Edwards, G. E. (2001). "Kranz anatomy is not essential for terrestrial C_4 plant photosynthesis." <u>Nature</u> **414**: 543-546.

Voznesenskaya, E. V., Koteyeva, N. K., Chuong, S. D. X., Akhani, H., Edwards, G. E. and Franceschi, V. R. (2005). "Differentiation of cellular and biochemical features of the single-cell C_4 syndrome during leaf development in *Bienertia cycloptera* (Chenopodiaceae)." <u>American Journal of Botany</u> **91**: 1784-1795.

Williams, E. G. and Maheswaran, G. (1986). "Somatic Embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group." <u>Annals of Botany</u> **57**: 443-462.

Winter, K. and Holtum, J. A. M. (2007). "Environment or development? Lifetime net CO_2 exchange and control of the expression of Crassulacean Acid Metabolism in *Mesembryanthemum crystallinum*." <u>Plant Physiology</u> **143**: 98-107.

Winter, K. and Smith, A. J. (1996). <u>Crassulacean acid metabolism: Biochemistry, ecophysiology, and evolution</u>. Berlin, Springer.

Yamada, Y., Ed. (1985). <u>Photosynthetic Potential of Plant Cell Cultures</u>. Advances in Biochemical Engineering/Biotechnology. Berlin, Springer-Verlag.

Yen, H. E., Grimes, H. D. and Edwards, G. E. (1995). "The effects of high salinity, water-deficit, and absicisic acid on phosphoenolpyruvate carboxylase activity and proline accumulation in *Mesembryanthemum crystallinum* cell - cultures." <u>Journal of Plant Physiology</u> **145**: 557-564.

Ziegler, P., Scheibe, R. and Universitat Bayreuth, L. P. (1989). "Greening and growth of suspension-cultured cells of Chenopodium rubrum under conditions of heterotrophic and autotrophic nutrition." <u>Plant, Cell and Environment</u> **12**: 725 - 735.

CHAPTER TWO

DEVELOPMENT OF *IN VITRO* REGENERATION OF *BIENERTIA SINUSPERSICI*

FROM MATURE EXPLANTS UNDER PHOTOAUTOTROPHIC CULTURE

CONDITIONS

Abstract

Bienertia sinuspersici has an unusual form of C_4 photosynthesis that occurs in individual photosynthetic cells, by development of two cytoplasmic domains with dimorphic chloroplasts. The objective of the study was to find appropriate conditions for the *in vitro* culture of stem explants in order to develop an efficient plant regeneration system. Stem explants developed either callus with or without red nodular structures (RNS). Increasing the CO_2 concentration during growth of callus without RNS caused an increase in overall size, protein content, and photosystem II yield, while structural and biochemical analysis indicated the cells had C_3 type photosynthesis similar to the youngest chlorenchyma cells which develop during leaf initiation in *B. sinuspersici*. Plants were successfully regenerated *in vitro* from callus having RNS. The same concentration of MS salts and vitamins, with 5 mM phosphate, was used throughout the three stages of regeneration. The hormones used were 1 mg l^{-1} dichloropenoxy-acetic acid (2,4-D) for the first three weeks (Stage 1), 2 mg l^{-1} 6-benzylamino purine (6-BAP) for the next three weeks (Stage 2), with no hormones for the last three weeks (Stage 3). The source of organic carbon in the media was 87 mM sucrose for Stage 1, 43 mM sucrose plus 1.5% soluble starch for Stage 2, and no organic carbon source for Stage 3. During Stage 3 the effect of 1.2% CO_2 versus current ambient CO_2 on photoautotrophic

growth was studied. Raising the concentration of CO_2 to 1.2% compared to current ambient CO_2, had a large effect on regeneration success, increasing efficiency of shoot and root development, and increasing size of plantlets, leaf soluble protein and chlorophyll concentration. Anatomical analysis of plantlets under 1.2% CO_2 showed leaves developed C_4 type chlorenchyma cells, including expression of key C_4 biochemical enzymes. Increasing salinity in the media from 0 to 200 mM NaCl, increased tissue osmolality, average plantlet area and regeneration success, but did not affect protein or chlorophyll content. In summary, rooted plantlets of the single-cell C_4 halophyte *B. sinuspersici* can be generated by culture of stem explants.

Introduction

The recent discovery of single-cell C_4 photosynthesis in terrestrial land plants raises many important questions about the mechanism of development and function of this form of carbon acquisition.   Although the majority of C_4 plants have an anatomy that separates C_4 carboxylation and decarboxylation activities between two cell types (Kranz anatomy), three terrestrial plants in the family Chenopodiaceae *Bienertia cycloptera*, *B. sinuspersici* and *Suaeda aralocaspica,* all can conduct C_4 photosynthesis within individual cells (for review on single-cell C_4 photosynthesis see Edwards *et al.* 2004). These species are unique in their development of two cytoplasmic domains with dimorphic chloroplasts for spatial separation of C_4 function. These are NAD-ME type C_4 species that are proposed to function analogous to this type with Kranz anatomy. In the scheme for NAD-ME subtype having Kranz anatomy, alanine plus atmospheric CO_2 is converted to aspartate in the mesophyll cells (through carbonic anhydrase, alanine

aminotransferase, pyruvate Pi dikinase, PEP carboxylase and aspartate aminotransferase). In the bundle sheath cells, aspartate is metabolized to pyruvate plus CO_2 in the mitochondria (through aspartate aminotransferase, malate dehydrogenase, and NAD-malic enzyme) the pyruvate converted to alanine in the cytosol, and the CO_2 assimilated by Rubisco in the C_3 cycle. This results in a intercellular shuttle of aspartate and alanine in the C_4 cycle (Kanai et al. 1999; Sage et al. 1999). In this C_4 system one can monitor the levels of PEPC, PPDK, NAD-ME and Rubisco as indicators of expression of C_4 and C_3 pathway. *Bienertia* spp. have a peripheral compartment with one chloroplast type which is proposed to function analogous to mesophyll cells, and a central compartment having another chloroplast type and mitochondria which are proposed to function analogous to bundle sheath cells in C_4 species having Kranz anatomy.

The current model for chlorenchyma cell development in *Bienertia* spp. begins with a small heterotrophic cell containing monomorphic chloroplasts, a prominent nucleus, and a few small vacuoles. During photoautotrophic development a large central vacuole separates two cytoplasmic domains, with one type of chloroplast in the periphery of the cell interconnected by channels to the central compartment containing another type of chloroplast, along with mitochondria and peroxisomes (Voznesenskaya et al. 2005; Park et al. 2009a). The metabolic switch from small dividing heterotrophic cells that are sinks for sucrose into mature photoautotrophic cells that are net exporters of sucrose, is an important developmental transition for carbon fixation.

In the current study, *in vitro* development of green callus and regeneration of plantlets from stem explants of *B. sinuspersici* was evaluated. Exploring the effects of different chemical and environmental factors on callus and regenerating shoots will help

establish what chemical or developmental signals are required for optimal growth. Analyzing expression of Rubisco and C_4 cycle enzymes, and structural development of chlorenchyma cells was used to evaluate the degree of development of C_3 versus C_4 photosynthesis during regeneration in callus versus plantlets. Since *B. sinuspersici* is a halophyte growing in the salty coastal areas surrounding the Persian Gulf (Akhani et al. 2005), increasing concentrations of NaCl (0 to 200 mM) in the media was evaluated. Additionally, growth under high CO_2 was evaluated to determine if it effects autotrophic growth and development of the C_4 system.

Materials and Methods

Plant Material

B. sinuspersici was grown in growth chambers with a day/night temperatures of 35º/18ºC, and a 14/10 h photoperiod, with a photosynthetic flux density of 1100 μmol m^{-2} s^{-1}. Since *B. sinuspersici* is a halophyte, for optimum growth 150 mM sodium chloride was added to the nutrient medium of Peter Professional fertilizer (20:20:20) given once a week; otherwise, plants were watered on alternative days as needed.

Tissue Growth

Explant material of *B. sinuspersici* was washed in a 20% bleach solution with 0.2% (w/v) Trition X-100, rinsed once in 70% ethanol for 30 sec, and then subsequently rinsed three times in sterilized water for five min each. Stem segments were cut into 1-5 cm pieces and placed in Petri dishes (100 mm). Initial comparisons of culture media was made with Chu N_6 (Chu et al. 1975) and MS (Murashige et al. 1962) media. The major difference between the two media types is the concentration of available nitrogen, with

26

MS media having 41.2 mM NH_4 and 60 mM NO_3 while, N_6 has 3.5 mM NH_4 and 28 mM NO_3, see supplemental table S1 for full composition of the media used. Using regenerated shoots of *B. sinuspersici* initiated from mature leaf explants it was found that MS medium (pH 5.8) containing 0.3% gelrite, was the best media to use for *in vitro* cultures (Fig. S1), as it produced less hyperhydric shoots. For Stage 1, explant material was placed on MS media with 5 mM phosphate, buffered with MES (pH 5.8) containing 0.3% gelrite, 1mg l^{-1} 2,4-D, supplemented with 87 mM sucrose. Different concentrations of salt (0, 25, 50, 100, and 200 mM NaCl) were added to the plates and continued at these levels for each subsequent re-plating. The plates were placed in a tissue cabinet at 25ºC with a photosynthetic photon flux density of 70.5 µmol quanta $m^{-2}sec^{-1}$. After 3 weeks of growth on 2,4-D media, red nodular structures (RNS) were replated to Stage 2 media, which was same as above, except 43 mM sucrose and 1.5% starch was used with 2 mg l^{-1} BAP, and plates were placed back in the tissue cabinet (25ºC, 70.5 PPFD). After another 3 weeks of growth, regenerating plantlets or callus was transferred to Stage 3 MS media with 5 mM phosphate and no organic carbon, in full-gas micro boxes (Green Arc Inc.) These boxes were placed inside a larger 76 X 61 X 61 cm hinged Plexiglas box, inside a growth chamber set at 30ºC with a PPFD of 273 µmol quanta m^{-2} sec^{-1} (measured inside the full-gas microbox inside the Plexiglas box).   The Plexiglas boxes contained either CO_2 at current ambient or 1.2%. The latter concentration of CO_2 was obtained by controlled feeding of CO_2 into the chamber from a CO_2 gas cylinder (measured using an infrared CO_2 analyzer).   The composition of the media for the 3 Stages is shown in Figure 1.
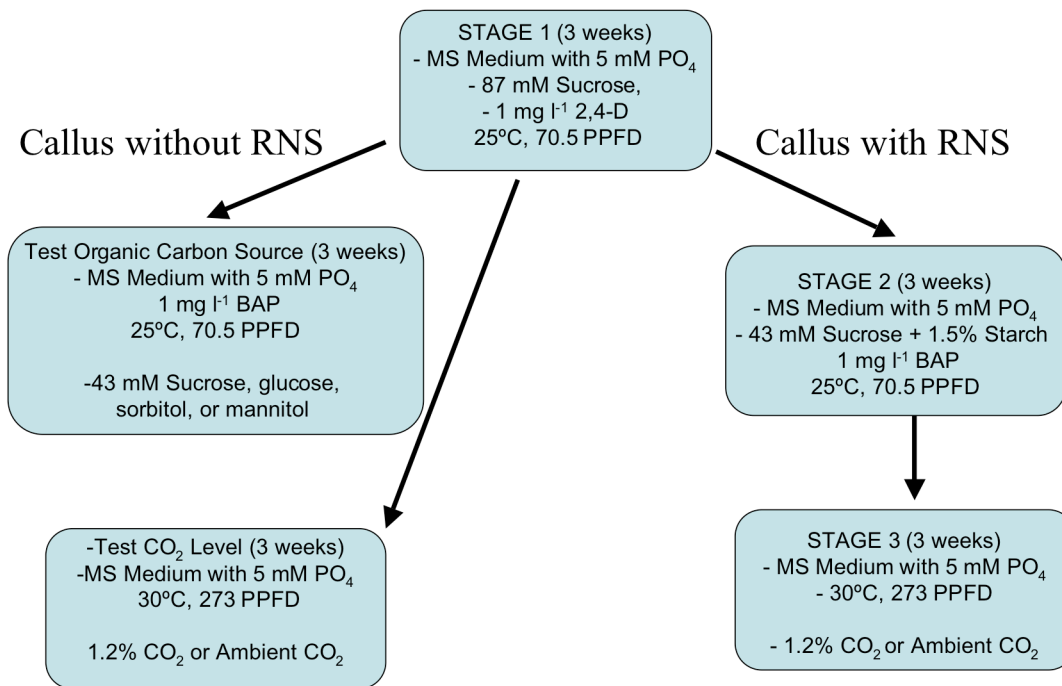
```
                    STAGE 1 (3 weeks)
                 - MS Medium with 5 mM PO_4
                      - 87 mM Sucrose,
                      - 1 mg l^-1 2,4-D
Callus without RNS    25ºC, 70.5 PPFD        Callus with RNS


   Test Organic Carbon Source (3 weeks)
        - MS Medium with 5 mM PO_4               STAGE 2 (3 weeks)
              1 mg l^-1 BAP                  - MS Medium with 5 mM PO_4
            25ºC, 70.5 PPFD              - 43 mM Sucrose + 1.5% Starch
                                                  1 mg l^-1 BAP
       -43 mM Sucrose, glucose,                 25ºC, 70.5 PPFD
          sorbitol, or mannitol


      -Test CO_2 Level (3 weeks)                 STAGE 3 (3 weeks)
     -MS Medium with 5 mM PO_4               - MS Medium with 5 mM PO_4
           30ºC, 273 PPFD                        - 30ºC, 273 PPFD

       1.2% CO_2 or Ambient CO_2             - 1.2% CO_2 or Ambient CO_2
```

Figure 1. Media composition used for each Stage of growth.

PPFD = photosynthetic photon flux density (μmol m^{-2}s^{-1})

RNS = red nodular structures

Chlorophyll fluorescence measurements

Fluorescence measurements were made on calluses using an OS-500 modulated

fluorometer (Opti-Sciences, Inc., Tyngsboro, MA, USA), at ambient CO_2 concentrations.

The OS-500 probe was held at an angle of 60º from the exposed surface of the callus so it

would not interfere with the incident illumination. Initially the maximum intrinsic yield

of PS II was determined by measuring the F_v/F_m ratio on callus, 30 min to 60 min before

chamber lights came on in the morning. $F_v/F_m = (F_m - F_o)/F_m$, where F_o is determined in

the absence of light, and F_m is determined after a saturating pulse of light (about 9000

μmol quanta m^{-2} s^{-1}). The quantum yield of PS II was determined during photochemistry

(ϕ_{II}) with increasing light intensity up to 561 μmol quanta m^{-2} sec^{-1}. Three measurements

were made each day, with three replicate days being analyzed.   At steady-state

photosynthesis under a given light level, F_s was monitored continuously, and, for periodic

determination of F'_m saturating pulses (900-ms duration) of white light (about 9000 μmol

quanta m^{-2} s^{-1}) were applied automatically at 96-s intervals. Opti-Science software was

used to measure F'_m and F_s, after each saturating pulse of light, and to calculate values of

Φ_{II}. Φ_{II} was calculated as $(F'_m - F_s)/F'_m$, for each steady-state condition, an average of

three values of Φ_{II} was taken. Measurements were made on three samples from each box,

measuring 5 boxes in each treatment.

Chlorophyll and Soluble Protein determination

Total chlorophyll and soluble protein concentration was determined from the

same crude extract of tissue culture that was homogenized with a mortar and pestle in

liquid nitrogen.   200 mg of completely ground tissue was placed in 500 μL of 100 mM

Tris-HCl, pH 7.5 buffer containing 10 mM $MgCl_2$, 15 mM β-mercaptoethanol and 1 mM

phenylmethylsulfonyl fluoride.   Chlorophyll concentration was determined using the

procedure described by (Knudson et al. 1977). A 200 μl aliquot from the 200 mg extract

of leaf material was mixed in a 1.5 mL eppendorf tube with 200 μL of 100% ethanol.

Material was kept in the dark until assayed to prevent chlorophyll degradation. The

ethanol in the leaf extract was replaced by centrifugation at 16,000 g for 5 minutes,

removing the supernatant, and re-suspending the pellet in 200 μL of fresh 100% ethanol.

The eppendorf tube was washed once with 200 μL of 100% ethanol resulting in a total of

1 mL of supernatant that was used in determining chlorophyll content spectroscopically.

Measurement at wavelengths of 649, 665, and 700 nm was made to determine chlorophyll b, chlorophyll a, and background, respectively.

For protein determination the samples were centrifuged at 16,000g for 5 min at 4ºC, and the resulting supernatant was used for protein concentration determination and western blot analysis. Protein concentration was determined using the assay (Bradford 1976) with the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard.

Immunoblotting

The supernatant fractions from crude extracts of callus or plantlets was mixed with 0.25 M Tris-HCl, pH 7.5, 2% SDS, 0.5% (v/v) ß-mercaptoethanol, and 0.1% (v/v) bromphenol blue and boiled for 5 min. for SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). A total of 15 µg of soluble protein was loaded on to a 12.5% (w/v) polyacrylamide gel according to (Laemmli 1970), and separated by gel electrophoresis (10 V/cm for 1 h in a Bio-Rad Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, the gel was transblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) that was presoaked in 1X transblotting buffer (20 mM Tris base pH 8.3, 150 mM glycine, 0.025% SDS, and 20 % methanol (v/v) for 5 min. The membranes were transblotted for 1 h (400 mA constant current) at 4 °C. Proteins were initially visualized by reversible staining with 0.1% Ponceau S in 5% acetic acid (v/v) for 2 min, followed by three, 5 min washes with 5% acetic acid to removed background staining. The membrane was then rinsed 3 times with distilled water and blocked with blocking buffer (3% skim dry milk in

Tris-buffered Saline Tween buffer (TBST) (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Tween-20 detergent) for 30 min.

The primary antibody (in blocking buffer) was applied to the membrane which was placed on an orbital shaker to incubate at 4 °C overnight. The primary antibody was removed and the membrane was rinsed with TBST three times, 10 min each rinse. Bound primary antibodies were located using secondary, alkaline-phosphatase conjugated goat anti-rabbit IgG. The secondary antibody (in blocking buffer) was applied to the membrane and incubated for 2 h at room temperature on an orbital shaker. The secondary antibody was removed and the membrane was again rinsed with TBST buffer three times, 10 min each rinse. The membrane was then incubated in activation buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM MgCl) for 5 min. Chromogenic detection was then performed by the addition of the color substrate solution (50 µl 5-bromo-4-chloro-3-indolyl phosphate dipotassium salt (BCIP) (35 mg/ml) per 10 ml activation buffer, and 50 µl nitroblue tetrazolium salt (NBT) (70 mg/ml) per 10 ml activation buffer). Color development was stopped with washes of distilled water. Primary antibodies for PEPC, NAD-ME, PPDK and Rubisco LSU were used to determine the relative expression levels across different treatments. Bound antibodies were located using an alkaline phosphate-conjugated goat anti-rabbit antibody, according to the manufacturer's instructions (Bio-Rad). The antibodies used for detection, with the dilutions in parentheses were as follows; anti-*Amaranthus viridis* PEPC (1:100,000; (Colombo et al. 1998), serum against the α-subunit of NAD-ME from *Amaranthus hypochondriacus* (1:2,000; (Long et al. 1994), anti-*Zea mays* PPDK (1:50,000; courtesy of Dr. T. Sugiyama), anti-spinach (*Spinacia oleracea*) Rubisco LSU (1:10,000; courtesy

of Dr. B McFadden). The molecular mass of the polypeptides was estimated from a marker standard. The intensities of bands in western blots was quantified with Image J software and expressed relative to levels in the mature leaves.

Light Microscopy

Samples were fixed at 4ºC in 4% (v/v) paraformaldehyde and 2% (v/v) gluteraldhyde in 0.1 M Cacodylate buffer, pH 7.2. The samples were dehydrated with a graded ethanol series and embedded in Spurs Resin. 700 nm cross sections were made on a Reicher Ultracut R ultra microtome and placed onto gelatin coded slides (Surgipath Medical Ind., Inc., Richmond, IL) for light microscopy. Sections were stained using 1% toluidine blue in 1% borax and images were captured using an Olympus BH-2 Light Microscope (Olympus Optical Co., Ltd., Tokyo, Japan) and a Jenoptik ProgRes C12plus digital camera (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) with ProgRes Capture Pro software (Jenoptik, Jena, Germany). Pictures of RNS were captured using a stereomicroscope and a Jenoptik ProgRes C12 plus digital camera (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) with ProgRes Capture Pro software (Jenoptik, Jena, Germany).

Osmolality determination

The osmolarity of the samples was measured using a vapor pressure osmometer model 5500 (Wescor Inc., Logan, UT, USA). Plantlet samples were removed using tweezers after three weeks of growth in full-gas microboxes (9 weeks total). Plantlet material was placed in a microfuge tube and gently macerated using a small plastic pestle. Samples

were centrifuged for 5 min at 16,000 g. The supernatant was loaded onto the reference disc in the sample holder of the osmometer using 10 µl size samples. The osmometer was calibrated using 290 and 1000 milliosmol standards before each use.

Statistical analysis

The entire procedure, with culture through the three stages over 9 weeks was repeated three times. For each replicate the treatments at each stage consisted of 0, 25, 50, 100, 200 mm NaCl and for Stage 3 the concentration of CO_2 was either 1.2% or current ambient. For each treatment in Stage 3 (final 3 weeks of growth) tissue was grown in a separate microboxes box, with each box containing 5-10 individual shoots. Measurements were made within each box by pooling leaves randomly sampled from individual shoots. Three independent replicates were measured for each experiment (unless otherwise noted). One-way or two-way statistical analysis of variance (ANOVA) was performed with Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). Tukey's HSD (honest significant difference) testes were used to analyze differences between salt levels. All analyses were performed at the $P < 0.05$ level.

Results

Stage 1

For Stage 1, explant material was placed on MS media with 5 mM phosphate, buffered with MES (pH 5.8) containing 0.3% gelrite, 1 mg l^{-1} 2,4-D, supplemented with 87 mM sucrose. For each treatment in Stage 1, there were 3 Petri dishes with an average of 20 – 30 explants per dish. After 3 weeks of growth on auxin plates, most stem explants of *B. sinuspersici* developed callus with or without red nodular structures (RNS)

approximately 1 mm in diameter (Fig. 2). Cross section analysis of RNS showed the development of a central vascular system, with many smaller cells located near the vascular system, and a few larger cells on the periphery of the RNS (Fig. 3). There was no observed difference in the size of callus across the salt treatments, with larger explants tended to have larger calli.

Experiments on callus without RNS

Following growth in Stage 1, *B. sinuspersici* callus without RNS were used to optimize, the organic carbon source that produced the most vigorous growth, by supplementing the media with 43 mM sucrose, glucose, mannitol, or sorbitol. Visually the callus grown on sucrose or glucose had a greener color than did callus grown on mannitol or sorbitol (Fig. S2). Also, the increase in fresh weight and final surface area of the callus was greater when grown on sugars compared to polyols (Fig 4). Sucrose was selected as the best organic carbon source for growth.

Following Stage 1, callus without RNS were selected and grown for 3 weeks in microboxes on MS medium with 5 mM phosphate, buffered with MES (pH 5.8) containing 0.3% gelrite, at 30°C with a PPFD of 273 μmol quanta m^{-2} sec^{-1}, under ambient or elevated CO_2 levels. The average surface area of callus when grown on carbon free media was greater when grown at 1.2% CO_2 compared to current ambient CO_2 (Fig. 5). The average soluble protein content in callus grown at 1.2% CO_2 was 3.2 μg mg^{-1} fresh weight compared to1 μg mg^{-1} fresh weight for callus grown at current ambient CO_2. Callus chlorophyll fluorescence showed that the average F_v/F_m ratio for callus grown at 1.2% CO_2 was .74 compared to an average F_v/F_m ratio for callus grown at current ambient CO_2 of .61 (Fig. 5). The steady-state yield of photosystem II was greater at all measured

light intensity for callus grown at 1.2% CO_2 compared to callus grown at current ambient CO_2 (Fig. 5). Anatomical analysis of callus cell structure shows that there are numerous small chloroplasts present in callus when grown at 1.2% CO_2, with a few being associated to the nucleus, light micrographs shows that cells from callus grown at 1.2% CO_2 had thicker cell walls than cells from callus grown at current ambient CO_2 (Fig. 6) Western blot analysis using 15μg of *B. sinuspersici* callus protein grown under photoautotrophic conditions 1.2% CO_2, showed expression of Rubisco, but no detectable expression of C_4 biochemical proteins (Fig. 7).

Regeneration from callus having RNS

From stem explants, the efficiency of callus formation having RNS at the end of Stage 1 was 26 - 30 %, with no observed difference across the salt treatments. All RNS callus from each Petri dish were combined on to one Petri dish and continued to grow on Stage 2 media. Stage 2 media was MS medium with 5 mM phosphate buffered with MES (pH 5.8) containing 0.3% gelrite, supplemented with 43 mM sucrose, 1.5% starch and 2 mg l^{-1} BAP, grown in a tissue cabinet at 25ºC with a 70.5 PPFD. After 6 weeks of growth the size of the RNS structure increased to approximately 5 mm in diameter, with a loss of the red/pink color and a slight greening (Fig. 8), with no significant difference in the size of RNS under different NaCl levels (Fig 9).

Following Stage 2, RNS were grown in microboxes on MS medium with 5 mM phosphate, buffered with MES (pH 5.8) containing 0.3% gelrite, at 30ºC with a PPFD of 273 μmol quanta m^{-2} sec^{-1}, under different CO_2 levels. At the end of stage 3, the percent efficiency of regenerating shoots from callus having RNS (after Stage 1) across all salt treatments was 57% under 1.2% CO_2 and only 17% under ambient CO_2 (Table 1). The

highest shoot regeneration efficiency was 96%, when grown at 1.2% CO_2 with 200 mM NaCl. The rooting efficiency from Stage 3 shoots across all salt treatments was 9% under 1.2% CO_2 and 3% under ambient CO_2 (Table 1). The highest rooting efficiency was 11%, when grown at 1.2% CO_2 with 200 mM NaCl. After 9 weeks of growth, shoots of *B. sinuspersici* grown in microboxes at 1.2% CO_2 were larger and had a darker green color compared to shoots grown at current ambient CO_2 (Fig 10). The average area of each plantlet grown at 1.2% CO_2 was greater (2 to 3 fold) than that grown at current ambient CO_2 (Fig. 9). Cross-section analysis of leaves grown at 1.2% CO_2, showed the association of chloroplasts and mitochondria to form a central compartment indicative of single-cell C_4 development (Fig. 11).

A large increase in the plantlet osmolality, from approximately 250 to 1000 mosmolal, was seen in the leaves as the NaCl concentration of the media increased (Fig. 12). There was on average a 25% greater increase in leaf osmolarity observed in plantlets that were grown at 1.2% CO_2 compared to plantlets grown at current ambient CO_2.

The amount of protein and chlorophyll per mg fresh weight was higher in regenerating plantlets grown at 1.2% CO_2 than at current ambient CO_2. On average there was a four fold increase in chlorophyll and soluble protein content when shoots were grown at 1.2% CO_2 compared to current ambient CO_2 (Table 2). There was no significant affect of NaCl on the chlorophyll and protein content.

Result from the western blots, loading 15 µg of protein per lane, showed that regenerated plantlets had expression of Rubisco and key C_4 enzymes under 1.2% CO_2. There was no significant effect of salinity on expression of Rubisco LSU, PEPC, PPDK, and NAD-ME (Fig. 13). The resulting bands were quantified using Image J and the

relative intensity was compared to mature leaves of a plant growing in soil as a control. Compared to *B. sinuspersici* growing in soil, NAD-ME in the plantlets had the same relative intensity, while PEPC, PPDK and Rubisco had only 40-60% expression in plantlets compared to leaves from a mature plant grown in soil (Fig 14). Western blots of ambient CO_2 grown shoots had very low or no detectable immunolabeling for these photosynthetic proteins (not shown). Root induction was approximately 9% under growth at 1.2% CO_2 with no carbon source or hormones in the media compared to 2% at ambient CO_2 with no carbon source or hormones in the media (Table 1). There was more root formation observed on shoots when NaCl was included in the media, with 200 mM NaCl producing the most roots. Only rooted plantlets grown under 1.2% CO_2 were successfully transferred to a sand/soil mix and continued to grow in the CO_2 enriched chamber (see example in Fig. 15). Rooted plants were also able to grow at ambient CO_2.

Discussion

The purpose of this study was to develop a regeneration system for *B. sinuspersici* as the first step towards a transformation protocol to gain knowledge about the development of single-cell C_4 photosynthesis. The specific aims of this study was to determine the effect that CO_2 enrichment and salinity had on regeneration success, and to determine the forms of photosynthesis in green callus and regenerating shoots. During shoot regeneration, it was observed that MS media produced less hyperhydric leaves compared to Chu N_6 media, a result often associated with water availability, microelements and/or hormonal imbalance (Wu et al. 2009). Developing shoots, with no roots to regulate the intake of nutrients from the media, are subject to limited nutrient

37

uptake and an imbalance of nutrients absorbed.

Culture of callus lacking RNS

During callus culture which lacked RNS, it was observed that the sugars, sucrose and glucose, were much better sources of carbon than the polyol, sorbitol or manitol for *in vitro* growth. Species *Fraxinus pennsylvanica* and celery synthesize mannitol, and are capable of utilizing it as the sole carbon source for growth in callus cultures (Wolter et al. 1966; Stoop et al. 1993), while 11 Rosaceae species such as *Malus* and *Prunus persica* which synthesize sorbitol, are able to use it as an organic carbon source for growth (Coffin et al. 1976). Species that do not synthesize polyols as compatible solutes or export as major photosynthetic products may have a limited ability to utilize polyols as a carbon source. Chenopodiaceae species including *B. sinuspersici,* are noted for synthesizing glycine betaine as a major compatible solute (Park *et al.* 2009b). Sucrose was selected as the optimal organic carbon source for *in vitro* cultures of *B. sinuspersici.*

When callus cultures which lacked RNS following Stage 1 were cultured in 1.2 % versus current ambient CO_2, there was photoautotrophic growth under 1.2% CO_2 increasing the overall surface area and producing thicker cell walls. Raising the CO_2 level in the air surrounding callus cultures of *Nicotiana tabacum* L. increased the growth rate (Berlyn *et al.* 1975). The maximum intrinsic yield of PS II (F_v/F_m) of dark adapted tissue represents an estimate of the maximum photochemical efficiency of PS II and is a valid indicator of photoinhibitory damages (Krause et al. 1991). The lower F_v/F_m ratio in culture under ambient CO_2 indicates some photoinhibiton. PS II yield measured in the light was also greater when *B. sinuspersici* callus was grown in 1.2% CO_2 versus current ambient CO_2, suggesting an increased capacity for carbon assimilation. A different result

from the photoautrophically grown bryophyte callus of *Marchantia polymorpha*, where the concentration of CO_2 had no effect on the F_v/F_m ratio, or Rubisco content (Bockers et al. 1997).

There have been mixed reports on the development of C_4 photosynthesis in photomixotrophic callus cultures of Kranz type C_4 plants. Based on $^{14}CO_2$ labeling in C_4 acids it was concluded that *Amaranthus retroflexus* L., *Portulaca oleracea* and *Froelichia gracilis* perform C_4 photosynthesis (Usuda et al. 1971; Laetsch et al. 1972; Kennedy *et al.* 1977). Photomixotrophic C_4 callus of *Gisekia pharnaceoides* L. and *Zea Mays* were found to perform C_3 photosynthesis based on little label in C_4 acids using $^{14}CO_2$ and low PEPC activity in callus compared to mature leaves respectively(Seeni *et al.* 1983; Lavergne *et al.* 1992). Autotrophic callus cultures of Kranz C_4 species *Amaranthus cruentus* and *A. powellii* were found to perform C_3 photosynthesis, having a lower PEPC activity than autotrophic cultures of C_3 species *Datura innoxia* and *Nicotiana tabacum (*Xu *et al.* 1988*).* All reports of C_4 chlorophyllous callus are devoid of any morphological organization or tissue-like organization typical of Kranz anatomy in C_4 leaves. Analysis of *B. sinuspersici* callus cell structure, grown autotrophically at 1.2% CO_2 showed no evidence for the expression of single-cell C_4 photosynthesis. Western blot analysis of *B. sinuspersici* callus grown at 1.2% CO_2 showed expression of Rubisco and very low PEPC and NAD-ME with no PPDK being detected. This biochemical gene expression is similar to the youngest cell age during leaf development *in vivo* (Voznesenskaya *et al.* 2005), indicating callus have C_3 type chlorenchyma and photosynthesis.

Cultures of callus having RNS

Shoot organogenesis was observed from callus of *B. sinuspersici* which developed RNS after Stage 1 of growth on auxin containing media, whereas callus which were green but did not produce RNS were not able to generate shoots. The development of pink colored tissue has been observed in field grown *B. sinuspersici* in the stem and leaf segments (Akhani *et al.* 2005), and also in plant material from our growth chambers. The production of red/pink pigment in the RNS, which is presumably anthocyanin, may be associated with environmental radiation, and is often observed in young seedlings (Mol et al. 1996). The excision of explant material and washing in combination with changes in light quantity and the hormone 2,4-D may be involved in the production of this pigment.

Transferring *B. sinuspersici* callus having RNS from Stage 1 to plates with 2 mg l^{-1} 6-BAP containing 43 mM sucrose with 1.5% starch produced a more green nodular structure. BAP has been found to stimulate the formation of chlorophyll and chloroplasts in leaves (Kinoshita et al. 1984; Arnold et al. 1986). Higher concentrations of 6-BAP, up to 7 mg l^{-1}, were observed to disrupted shoot organogenesis in *B. sinuspersici* by the unnatural development of multiple leaves from the same location on the stem.

A successful regeneration protocol from undifferentiated tissue is expected to gradually increase the expression of photoautotrophic genes. Having an available source of sugars (sucrose or glucose) in the media has been found to suppress Rubisco small subunit expression in autotrophic *Chenopodium* callus cultures, decrease expression of genes involved in the C_4 cycle in maize mesophyll protoplasts, and produce a rapid drop in photosynthetic rates in photomixotrophic cell-suspension cultures of *Alternanthera philoxeroides* (Krapp et al. 1993; Jang et al. 1994; Mudalige et al. 2006). In

Mesembryanthemum crystallinum and *Glycine max* calli, replacement of sucrose media with soluble starch as the external carbon supply was found to increase chlorophyll and soluble protein content, and PEPC activity (Yen et al. 1995; Yen et al. 1999). Having starch present in the media may help with chloroplast development and photoautrophic growth by providing a steady, low level of glucose, through excretion of amylase from the culture that hydrolyses starch and avoids high-sugar suppression of photosynthetic genes.

Photoautotrophic growth *in vitro* is generally associated with higher light intensities compared to mixotrophic conditions, and accompanied with CO_2 enrichment, which has been shown to promote photosynthesis (reviewed in (Nguyen et al. 1998). Placing developing shoots of *B. sinuspersici* on carbon free media after 6 weeks of growth, with no hormones resulted in photoautotrophic growth during Stage 3 under 1.2% CO_2, compared to very limited growth at current ambient CO_2. It has also been shown that photosynthetic rates of banana (*Musa* spp.) were the highest under increased light and CO_2 concentrations (Nguyen et al. 2001). The apparent health of the shoot material and development of photosynthetic machinery is indicative of photoautotrophic growth. The average chlorophyll and soluble protein content per mg of fresh weight was 3 to 4 fold higher when shoots of *B. sinuspersici* were grown at 1.2% CO_2 compared to growth at current ambient CO_2, indicating increase photoautotrophic growth. Western blot analysis of shoots after Stage 3 showed that under 1.2% CO_2 major C_4 enzymes are expressed. This biochemical gene expression is similar to the intermediate cell age during leaf development *in vivo* (Voznesenskaya *et al.* 2005), indicating that regenerating shoots have C_4 type chlorenchyma and photosynthesis.

Low doses of NaCl (50 mM) for tissue cultures of halophytic species such as *M. crystallinum* (CAM), *Atriplex halimus* (C4), and C3 species *Thellungiella holophila, Salicornia europaea* and *Suaeda maritima* promoted growth (Yen *et al.* 1995; Bajji et al. 1998; Moghaieb et al. 2004; Zhao et al. 2009). In comparison, NaCl treatment in cultures of glycophytic species such as *Jatropha curcas, Arabidopsis thaliana,* and *Saccharum officinarum* decreased growth in a concentration dependent manner (Kumar et al. 2008; Patade et al. 2008; Zhao *et al.* 2009). To maintain an osmotic gradient for the uptake of water, many halophytic plants accumulate inorganic ions and compatible solutes to a concentration equal to or greater than that of the surrounding solution (Flowers et al. 2008). Osmotic adjustment has been observed with halophytic Chenopodiaceae C3 species *Salicornia europaea* and *Suaeda maritima*, and in the single-cell C4 species *B. sinuspersici* and *Suaeda aralocaspica* as the concentration of NaCl increased (Moghaieb *et al.* 2004; Park et al. 2009b). There was a greater increase in internal osmolality of *B. sinuspersici* plantlets growing under 1.2% CO_2 in comparison to plantlets grown at current ambient CO_2. The greater osmolality under 1.2% CO_2 could be due to the increased capacity for photosynthesis and in turn the production of compatible solutes.

The survivorship of developed plantlets is dependent on initiation of taproots to provide nutrients to the vascular system and developing leaves. The amount of roots that formed from developing plantlets is a product of the environment in which they are growing. Increasing the concentration of CO_2 during *in vitro* culture of *Paulownia fortunei* increased the shoot multiplication and rooting rates (Vallikhan et al. 2003). The initiation of roots from *B. sinuspersici* shoot was much greater when grown at 1.2% CO_2 than when grown at current ambient CO_2. *In vitro* micro propagation, including leaf and

root development, of Plantain (*Musa*) was best under photomixotrophic (30 g l^{-1} sucrose) conditions with elevated CO_2 (1.2%) (Aragon et al. 2010). This was not the case for *Eucalyptus tereticornis* shoots, where the CO_2 concentration had no effect on root initiation (Khan et al. 2002). Following development of plantlets in microboxes, rooted plants can be transferred to a sand/soil mixture with continued growth under 1.2% CO_2 for 3 more weeks, which are then able to acclimate to ambient CO_2 conditions. In this high CO_2 concentration there was normal development of single-cell C_4 photosynthesis in plantlets. At this level of CO_2, photorespiration will be very suppressed, and the C_4 cycle not needed. This indicates growth under very high CO_2 does not induce C_3 type photosynthesis, and there is obligate formation of the C_4 system during leaf development. The coordinated growth of plant organs, in this study the leaf, seems to be a requirement for the development of single-cell C_4 photosynthesis. The apparent formation of "C_3 type" photosynthetic tissue in callus and C_4 in regenerating shoots provides a means for stable separation of *Bienertia sinuspersici* in a C_3 versus C_4 mode.

Works Cited

Aida, R. and Shibata, M. (1996). "Transformation of *Kalanchoe blossfeldiana* mediated by *Agrobacterium tumefaciens* and transgene silencing." <u>Plant Science</u> **121**: 175-185.

Akhani, H., Barroca, J., Koteeva, N., Voznesenskaya, E., Franceschi, V., Edwards, G., Ghaffari, S. M. and Ziegler, H. (2005). "*Bienertia sinuspersici* (Chenopodiaceae): A new species from southwest Asia and discovery of a third terrestrial C_4 plant without Kranz anatomy." <u>Systematic Botany</u> **30**: 290-301.

Aragon, C. E., Escalona, M., Rodrigues, R., Canal, M. J., Capote, I., Pina, D. and Gonzalez-Olmedo, J. (2010). "Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors." <u>In Vitro Cell. Dev. Biol. - Plant</u> **46**: 89-94.

Arencibia, A. D., Carmona, E. R., Tellez, P., Chan, M.-T., Yu, S.-M., Trujillo, L. E. and Oramas, P. (1998). "An efficient protocol for sugarcane (*Saccharum* spp. L.) transformation mediated by *Agrobacterium tumefaciens* " <u>Transgenic Research</u> **7**: 213-222.

Arnold, V. and Fletcher, R. A. (1986). "Stimulation of chlorophyll synthesis by benzyladenine and potassium in excised and intact cucumber cotyledons." <u>Physiologia Plantarum</u> **68**: 169-174.

Bajji, M., Kinet, J.-M. and Lutts, S. (1998). "Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures." Plant Science **137**: 131-142.

Berlyn, M. B. and Zelitch, I. (1975). "Photoautotrophic growth and photosynthesis in tobacco callus cells." Plant Physiology **56**: 752-756.

Bernacchi, C. J., Portis, A. R., Nakano, H., von Caemmerer, S. and Long, S. P. (2002). "Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo." Plant Physiology **130**: 1992-1998.

Bockers, M., Capkova, V., Ticha, I. and Schafer, C. (1997). "Growth at high CO_2 affects the chloroplast number but not the photosynthetic efficiency of photoautotrophic *Marchantia polymorpha* culture cells." Plant Cell, Tissue and Organ Culture **48**: 103-110.

Bowes, G., Rao, S. K., Estavillo, G. M. and Reiskind, J. B. (2002). "C_4 mechanisms in aquatic angiosperms: comparisons with terrestrial C_4 systems." Functional Plant Biology **29**: 379-392.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Analytical Biochemistry **72**: 248-254.

Chitty, J. A., Furbank, R. T., Marshall, J. S., Chen, Z. and Taylor, W. C. (1994). "Genetic transformation of the C_4 plant, *Flaveria bidentis*." The Plant Journal **6**: 949-956.

Chu, C. C., Wang, C. S., Sun, C. S., Hus, C., Ying, K. C., Chu, C. Y. and Bi, F. Y. (1975). "Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources." Sci. Sin. **18**: 659-668.

Coffin, R., Taper, C. D. and Chong, C. (1976). "Sorbitol and sucrose as carbon source for callus culture of some species of the Rosaceae." Canadian journal of botany **54**: 547-551.

Colombo, S. L., Andreo, C. S. and Chollet, R. (1998). "The interaction of shikimic acid and protein phosphorylation with PEP carboxylase from the C_4 dicot *Amaranthus viridis*." Phytochemistry **48**: 55-59.

Covshoff, S., Majeran, W., Liu, P., Kolkman, J. M., van Wijk, K. J. and Brutnell, T. P. (2008). "Deregulation of Maize C_4 Photosynthetic Development in a Mesophyll Cell-Defective Mutant." Plant Physiology **146**: 1469-1481.

Doulis, A. G., Debian, N., Kingston-Smith, A. H. and Foyer, C. H. (1997). "Differential localization of antioxidants in maize leaves." Plant Physiology **114**: 1031-1037.

Edwards, G. and Walker, D. (1983). C_3, C_4: Mechanism, and Cellular and Environmental Regulation, of Photosynthesis. London, Blackwell Scientific Publications.

Edwards, G. E., Franceschi, V. R. and Voznesenskaya, E. V. (2004). "Single-Cell C_4 photosynthesis versus the dual-cell (Kranz) paradigm." Annual Review Plant Biology **55**: 173-96.

Flowers, T. J. and Colmer, T. D. (2008). "Tansley Review: Salinity tolerance in halophytes." New Phytologist **179**: 945-963.

Frame, B. R., Shou, H., Chikwamba, R. K., Zhang, Z., Xiang, C., Fonger, T. M., Pegg, S. E. K., Li, B., Nettleton, D. S., Pei, D. and Wang, K. (2002). "Agrobacterium tumefaciens-Mediated transformation of maize embryos using a standard binary vector system." Plant Physiology **129**: 13-22.

Haberlandt, G. (1884). "Physiological Plant Anatomy (Transl. M. Drummond)." Macmillan, London.

Haberlandt, G. (1902). Sitz.-Ber **111**: 69.

Hatch, M. D. (1987). "C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure." Biochimica et Biophysica Acta **895**: 81-106.

Howe, A., Sato, S., Dweikat, I., Fromm, M. and Clemente, T. (2006). "Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum." Plant Cell Reports **25**: 784-791.

Husemann, W. and Barz, W. (1977). "Photoautotrophic growth and photosynthesis in cell suspension cultures of *Chenopodium rubrum*." Physiol. Plant **40**: 77 - 81.

Jang, J.-C. and Sheen, J. (1994). "Sugar sensing in higher plants." The Plant Cell **6**: 1665-1679.

Jiang, K., Schwarzer, E., Zhang, S., Ruzin, S., Machen, T., Remington, S. J. and Feldman, L. (2006). "Expression and characterization of a redox-sensing green fluorescent protein (reduction-oxidation-sensitive green fluorescent protein) in Arabidopsis." Plant Physiology **141**: 397-403.

Jordan, D. B. and Ogren, W. L. (1984). "The CO_2/O_2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase." Planta **161**: 308-313.

Kanai, R. and Edwards, G. E. (1999). The biochemistry of C_4 photosynthesis. San Diego, Academic Press.

Kennedy, R. A., Barnes, J. E. and Laetsch, W. M. (1977). "Photosynthesis in C_4 plant tissue cultures: Significance of Kranz anatomy to C_4 acid metabolism in C_4 plants." Plant Physiology **59**: 600-603.

Khan, P. S. S. V., Kozai, T., Nguyen, Q. T., Kubota, C. and Dhawan, V. (2002). "Growth and net photosynthetic rates of *Eucalyptus tereticornis* Smith under photomixotrophic and various photoautotrophic micropropagation conditions." Plant Cell, Tissue and Organ Culture **71**: 141-146.

Kinoshita, I. and Tsuji, H. (1984). "Benzyladenin-induced increase in DNA content per chloroplast in intact bean leaves." Plant Physiology **76**: 575-578.

Knudson, L. L., Tibbitts, T. W. and Edwards, G. E. (1977). "Measurement of ozone injury by determination of leaf chlorophyll concentration." Plant Physiology **60**: 606-608.

Krapp, A., Hofmann, B., Schafer, C. and Stitt, M. (1993). "Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis?" The Plant Journal **3**: 817-828.

Krause, G. H. and Weis, E. (1991). "Chlorophyll fluorescence and photosynthesis: The basics." Annual Review Plant Physiology Plant Molecular Biology **42**: 313-49.

Ku, M. S. B., Kano-Murakami, Y. and Matsuoka, M. (1996). "Evolution and expression of C_4 photosynthesis genes." Plant Physiology **111**: 949-957.

Ku, S.-B. and Edwards, G. E. (1977). "Oxygen inhibition of photosynthesis I. Temperature dependence and relation to O_2/CO_2 solubility ratio." Plant Physiology **59**: 986-990.

Kumar, N., Pamidimarri, S. D. V. N., Kaur, M., Boricha, G. and Reddy, M. P. (2008). "Effects of NaCl on growth, ion accumulation, protein, proline contents and antioxidant enzymes activity in callus cultures of Jatropha curcas." Biologia **63**: 378-382.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of Bacteriophage T4." Nature **227**: 680-686.

Laetsch, W. M. (1974). "The C_4 syndrome: a structural analysis." Annual Review Plant Physiology **25**: 27-52.

Laetsch, W. M. and Kortschak, H. P. (1972). "Chloroplast structure and function in tissue cultures of a C_4 plant." Plant Physiology **49**: 1021-1023.

Lara, M. V., Casati, P. and Andreo, C. S. (2002). "CO_2-concentrating mechanism in *Egeria densa*, a submersed aquatic plant." Physiologia Plantarum **115**: 487-495.

Lara, M. V., Offermann, S., Smith, M., Okita, T. W., Andreo, C. S. and Edwards, G. E. (2008). "Leaf development in the single-cell C_4 system in *Bienertia sinuspersici*: Expression of genes and peptide levels for C_4 metabolism in relation to chlorenchyma structure under different light conditions." Plant Physiology **148**: 593-610.

LaRosa, P. C., Hasegawa, P. M. and Bressan, R. A. (1984). "Photoautotrophic potato cells: Transition from heterotrophic to autotrophic growth." Physiol. Plant **61**: 279 - 286.

Lavergne, D., Nato, A., Dupuis, J.-M., Pean, M. and Chagvardieff, P. (1992). "Evidence for the expression of morphological and biochemical characteristics of C_3-photosynthesis in chlorophyllous callus cultures of *Zea mays*." Physiologia Plantarum **84**: 292-300.

Long, J. J., Wang, J.-L. and Berry, J. O. (1994). "Cloning and analysis of the C_4 photosynthetic NAD-dependent malic enzyme of Amaranth mitochondria." The Journal of Biological Chemistry **269**: 2827-2833.

Mitchell, P. L. (2007). Catching up with the literature for C_4 rice: what we know and didn't then. Charting new pathways to C_4 rice. J. E. Sheehy, P. L. Mitchell and B. Hardy. Los Banos, International rice research institute**:** 55-78.

Moghaieb, R. E. A., Saneoka, H. and Fujita, K. (2004). "Effect of salinity on osmotic adjustment, glycinebetaine accumulation and the betaine aldehyde dehydrogenase gene

expression in two halophytic plants, *Salicornia europaea* and *Suaeda maritima*." Plant Science **166**: 1345-1349.

Mol, J., Jenkins, G., Schafer, E. and Weiss, D. (1996). "Signal perception, transduction, and gene expression involved in anthocyanin biosynthesis." Critical Reviews in Plant Sciences, **15**: 525-557.

Mudalige, R. G. and Longstreth, D. J. (2006). "Effects of salinity on photosynthetic characteristics in photomixotrophic cell-suspension cultures from *Alternanthera philoxeroides*." Plant Cell, Tissue and Organ Culture **84**: 301-308.

Muhaidat, R., Sage, R. F. and Dengler, N. G. (2007). "Diversity of Kranz anatomy and biochemistry in C$_4$ Eudictos." American Journal of Botany **94**: 362-381.

Murashige, T. and Shoog, F. (1962). "A revised medium for rapid growth and bio assays with tobacco tissue cultures." Physiologia Plantarum **15**: 473-497.

Newell, C. A., Brown, N. J., Liu, Z., Pflug, A., Gowik, U., Westhoff, P. and Hibberd, J. M. (2010). "*Agrobacterium tumefaciens* - mediated transformation of *Cleome gynandra* L., a C$_4$ dicotyledon that is closely related to *Arabidopsis thaliana* " Journal of Experimental Botany **61**: 1311-1319.

Nguyen, Q. T. and Kozai, T. (1998). "Environmental effects of the growth of plantlets in micropropagation." <u>Environ. Control in Biol.</u> **36**: 59-75.

Nguyen, Q. T. and Kozai, T. (2001). "Growth of *in vitro* banana (*Musa* spp.) shoots under photomixotrophic and photoautotrophic conditions." <u>In Vitro Cell. Dev. Biol. - Plant</u> **37**: 824-829.

Nomura, M., Sentoku, N., Nishimura, A., Lin, J.-H., Hondar, C., Taniguchi, M., Ishida, Y., Ohta, S., Komari, T., Miyao-Tokutomi, M., Kano-Murakami, Y., Tajima, S., Ku, M. S. B. and Matsuoka, M. (2000). "The evolution of C_4 plants: Acquisition of cis-rgulatory sequences in the promoter of C_4 type pyruvate, orthophosphate dikinase gene." <u>The Plant Journal</u> **22**: 211-221.

Park, J., Knoblauch, M., Okita, T. W. and Edwards, G. E. (2009a). "Structural changes in the vacuole and cytoskeleton are key to development of the two cytoplasmic domains supporting single-cell C_4 photosynthesis in *Bienertia sinuspersici*." <u>Planta</u> **229**: 369-382.

Park, J., Okita, T. W. and Edwards, G. E. (2009b). "Salt tolerant mechanisms in single-cell C_4 species *Bienertia sinuspersici* and *Suaeda aralocaspica* (Chenopodiaceae)." <u>Plant Science</u>.

Patade, V. Y., Suprasanna, P. and Bapat, V. A. (2008). "Effects of salt stress in relation to osmotic adjustment on sugarcane (*Saccharum officinarum* L.) callus cultures." Plant Growth Regulation **55**: 169-173.

Patel, M. and Berry, J. O. (2008). "Rubisco gene expression in C_4 plants." Journal of Experimental Botany **59**: 1625-1634.

Rogers, S. M. D., Ogren, W. L. and Widholm, J. M. (1987). "Photosynthetic characteristics of a photoautotrophic cell suspension culture of soybean." Plant Physiology **84**: 1451-1456.

Sage, R. F. (2004). "The evolution of C_4 photosynthesis." New Phytologist **161**: 341-370.

Sage, R. F. and Monson, R. K., Eds. (1999). C4 Plant Biology. Physiological Ecology. San Diego, Academic Press.

Seeni, S. and Gnanam, A. (1983). "Photosynthesis in cell suspension culture of a C_4 plant, *Gisekia pharnaceoides* L." Plant and Cell Physiology **24**: 1033-1041.

Steward, F. C., Mapes, M. O., Kent, A. E. and Holsten, R. D. (1964). "Biochemical and morphogenetic studies with cells yield new evidence on their metabolism and totipotency." Science **143**: 20-27.

Stoop, J. M. H. and Pharr, D. M. (1993). "Effect of different carbon sources on relative growth rate, internal carbohydrates, and mannitol 1-oxidoreductase activity in celery suspension cultures." Plant Physiology **103**: 1001-1008.

Sunagawa, H., Agarie, S., Umemoto, M., Makishi, Y. and Nose, A. (2007). "Effect of urea-type cytokinins on the adventitious shoots regeneration from cotyledonary node explant in the common ice plant, *Mesembryanthemum crystallinum*." Plant Production Science **10**: 47-56.

Ueno, O. (2001). "Environmental regulation of C3 and C4 differentiation in the amphibious sedge Eleocharis vivipara." Plant Physiology **127**: 1524-1532.

Usuda, H., Kanai, R. and Takeuchi, M. (1971). "Comparison of carbon dioxide fixation and the fine structure in various assimilatory tissues of *Amaranthus retroflexus* L." Plant and Cell Physiology **12**: 917-930.

Vallikhan, P. S. S., Kozai, T., Nguyen, Q. T., Kubota, C. and Dhawan, V. (2003). "Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions." Biologia Plantarium **46**: 161-166.

Voznesenskaya, E. V., Akhani, H., Koteyeva, N. K., Chuong, S. D. X., Roalson, E. H., Kiirats, O., Franceschi, V. R. and Edwards, G. E. (2008). "Structural, biochemical, and physiological characterization of photosynthesis in two C_4 subspecies of *Tecticornia*

indica and the C$_3$ species *Tecticornia pergranulata* (Chenopodiaceae)." Journal of Experimental Botany **59**: 1715-1734.

Voznesenskaya, E. V., Franceschi, V. R., Kiirats, O., Artyusheva, E. G., Freitag, H. and Edwards, G. E. (2002). "Proof of C$_4$ photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae)." The Plant Journal **31**: 649-662.

Voznesenskaya, E. V., Franceschi, V. R., Kiirats, O., Freitag, H. and Edwards, G. E. (2001). "Kranz anatomy is not essential for terrestrial C$_4$ plant photosynthesis." Nature **414**: 543-546.

Voznesenskaya, E. V., Koteyeva, N. K., Chuong, S. D. X., Akhani, H., Edwards, G. E. and Franceschi, V. R. (2005). "Differentiation of cellular and biochemical features of the single-cell C$_4$ syndrome during leaf development in *Bienertia cycloptera* (Chenopodiaceae)." American Journal of Botany **91**: 1784-1795.

Wang, J., Seliskar, D. M. and Gallagher, J. L. (2003). "Tissue culture and plant regeneration of *Spartina Alterniflora*: implications for wetland restoration." Wetlands **23**: 386-393.

Williams, E. G. and Maheswaran, G. (1986). "Somatic Embryogenesis: Factors influencing coordinated behavior of cells as an embryogenic group." Annals of Botany **57**: 443-462.

Winter, K. and Holtum, J. A. M. (2007). "Environment or development? Lifetime net CO_2 exchange and control of the expression of Crassulacean Acid Metabolism in *Mesembryanthemum crystallinum*." Plant Physiology **143**: 98-107.

Winter, K. and Smith, A. J. (1996). Crassulacean acid metabolism. Berlin, Springer.

Wolter, K. E. and Skoog, F. (1966). "Nutritional requirements of *Fraxinus* callus cultures." American Journal of Botany **53**: 263-269.

Wu, Z., Chen, L. J. and Long, Y. J. (2009). "Analysis of ultrastructure and reactive oxygen species of hyperhydric garlic (*Allium sativum* L.) shoots." In Vitro Cell. Dev. Biol. - Plant **45**: 483-490.

Xu, C., Blair, L. C., Rogers, S. M. D., Govindjee and Widholm, J. M. (1988). "Characteristics of five new photoautotrophic suspension cultures including two *Amaranthus* species and a cotton strain growing on ambient CO_2 levels." Plant Physiology **88**: 1297-1302.

Yamada, Y., Ed. (1985). Photosynthetic Potential of Plant Cell Cultures. Advances in Biochemical Engineering/Biotechnology. Berlin, Springer-Verlag.

Yen, H. E., Chen, Y.-C., Yen, S.-K. and Lin, J.-H. (1999). "Sugar uptake by photomixotrophic soybean cultures." Botanical Bulletin of Academia Sinica **40**: 147-152.

Yen, H. E., Grimes, H. D. and Edwards, G. E. (1995). "The effects of high salinity, water-deficit, and absicisic acid on phosphoenolpyruvate carboxylase activity and proline accumulation in *Mesembryanthemum crystallinum* cell - cultures." Journal of Plant Physiology **145**: 557-564.

Zhao, X., Tan, H. J., Liu, Y. B., Li, X. R. and Chen, G. X. (2009). "Effect of salt stress on growth and osmotic regulation in *Thellungiella* and Arabidopsis callus." Plant Cell Tissue and Organ culture.

Ziegler, P., Scheibe, R. and Universitat Bayreuth, L. P. (1989). "Greening and growth of suspension-cultured cells of Chenopodium rubrum under conditions of heterotrophic and autotrophic nutrition." Plant, Cell and Environment **12**: 725 - 735.

Figure 2. Development of red nodular structure (RNS), after Stage 1 from stem explant of *B. sinuspersici,* grown in 100 mm Petri dishes on MS media with 5 mM phosphate supplemented with 87 mM sucrose, and 1 mg l^{-1} 2,4-D, under current ambient CO_2, 25ºC and 70.5 µmol m^{-2} sec^{-1} (PPFD). Scale bar = 1 mm.
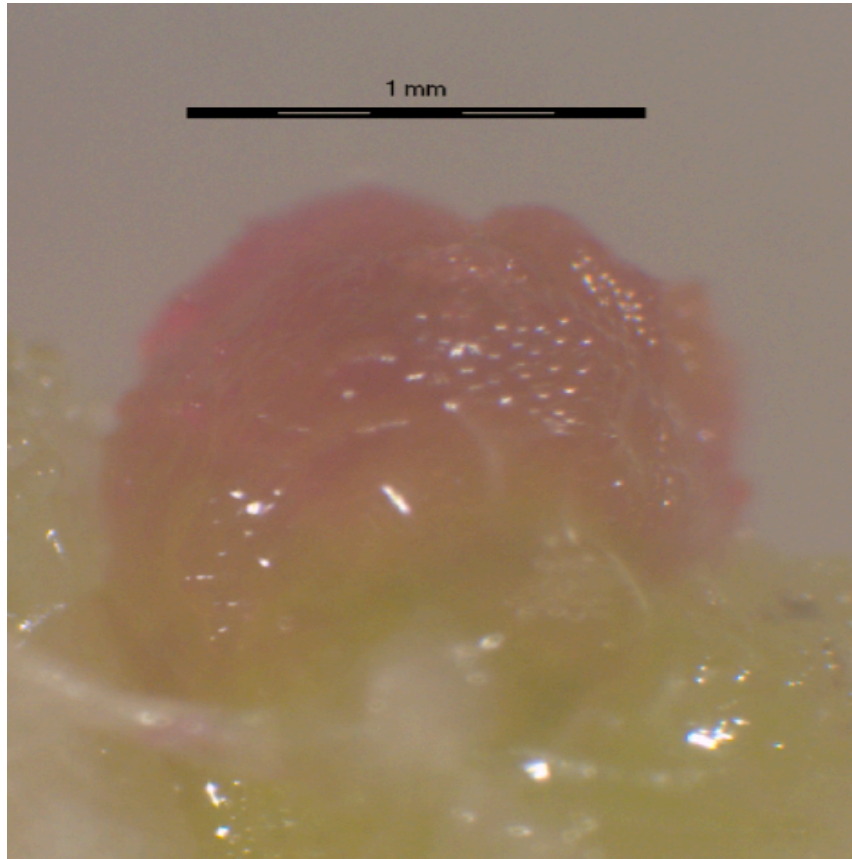
Figure 3. Light micrograph of Red Nodular Structure (RNS) from *Bienertia sinuspersici* after Stage 1, from stem explant of *B. sinuspersici,* grown in 100 mm Petri dishes on MS media with 5 mM phosphate supplemented with 87 mM sucrose, and 1 mg 2,4-D l^{-1}, under current ambient CO_2, 25ºC and 70.5 μmol m^{-2} sec^{-1} (PPFD). Longitudinal section through center of developing shoot, showing different cell morphologies and development of a central vascular system. Scale bar = 1 mm.
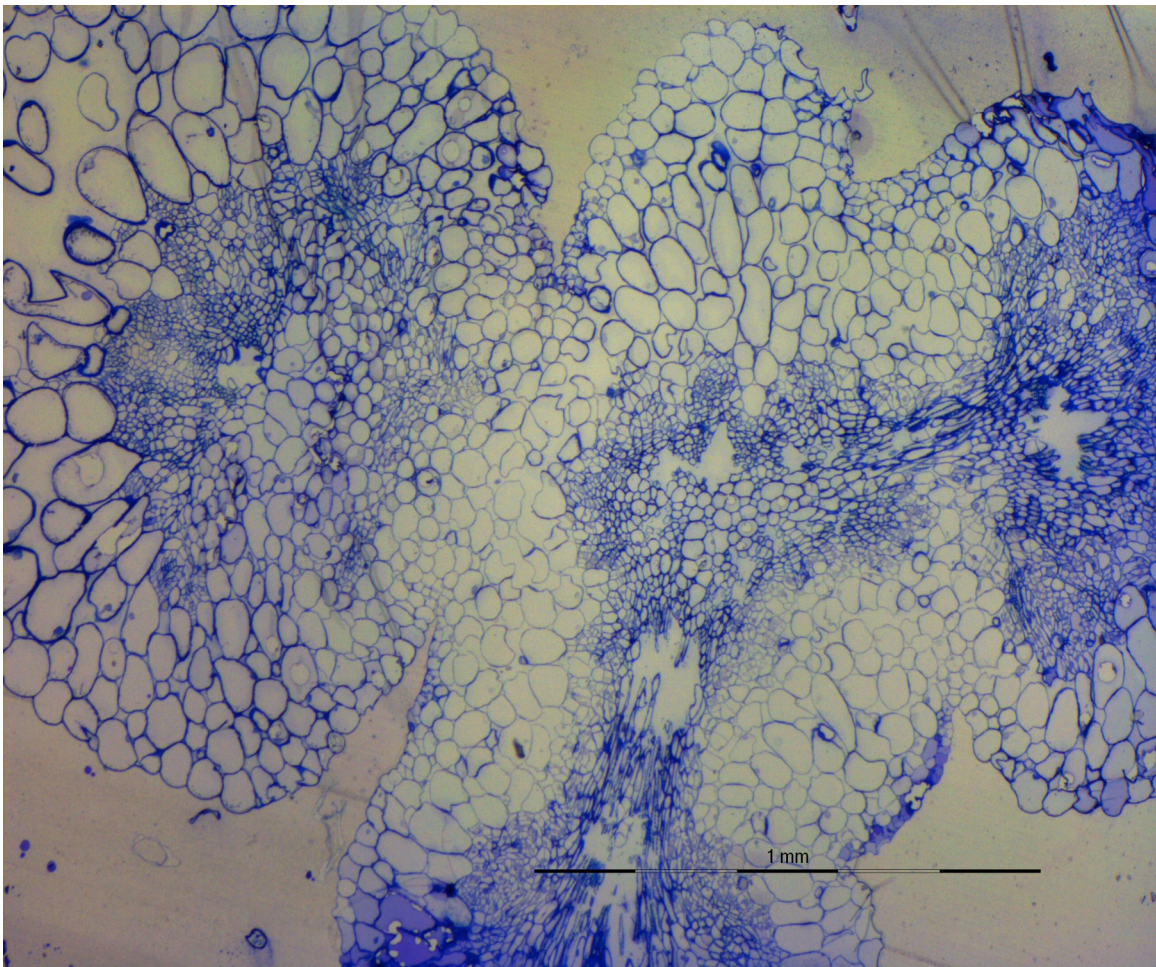
Figure 4. *B. sinuspersici* callus growth during Stage 2, grown on MS media with 5 mM

phosphate supplemented with 10 mM NaCl, and either 43 mM sucrose, glucose, sorbitol,

or mannitol, at 25ºC with a photosynthetic photon flux density of 70.5 μmol quanta m^{-2}sec^{-1}. A) The increase in fresh weight B) The change in surface area. Error bars

represent the standard error of three replicates. Different letters represent statistically

significant differences, as determined by ANOVA.

A

B
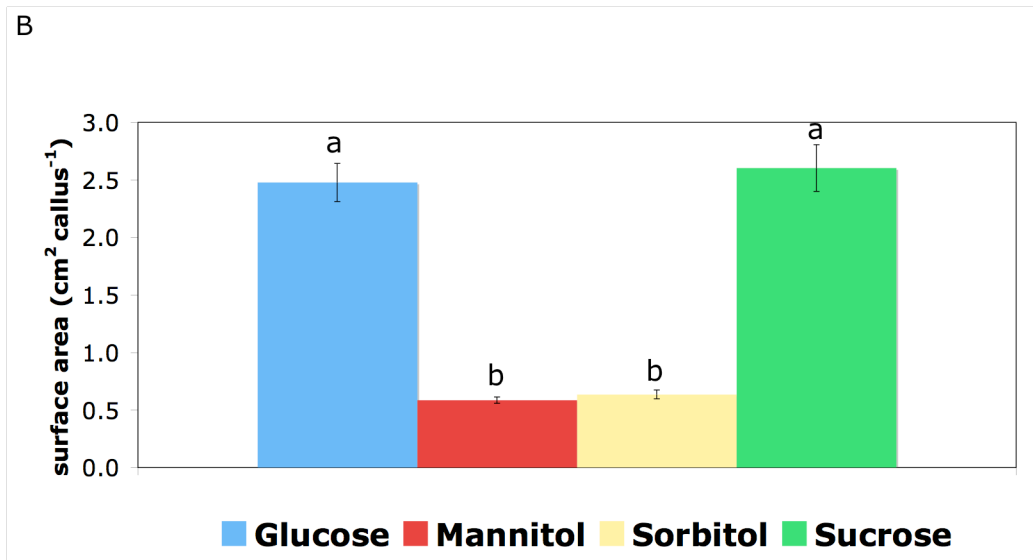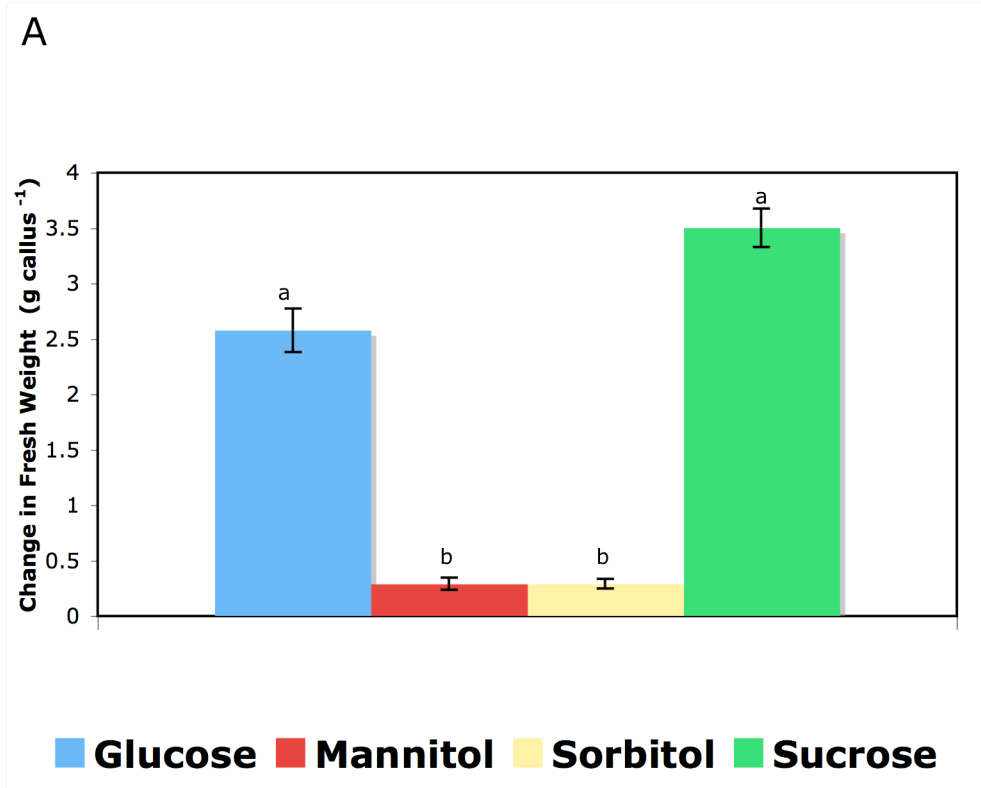
Figure 5. Change in *B. sinuspersici* callus (without shoots) after Stage 3, grown on carbon free MS media with 5 mM phosphate supplemented with 10 mM NaCl, at 30ºC with a photon flux density of 273 μmol m^{-2} sec^{-1}, at either 1.2% CO_2 or current ambient CO_2. A) Measured surface area B) Fv/Fm for *B. sinuspersici* callus, C) PS II yield for *B. sinuspersici* callus. Error bars represent the standard error of three replicates. Different letters represent statistically significant differences, as determined by ANOVA.

A

B

C
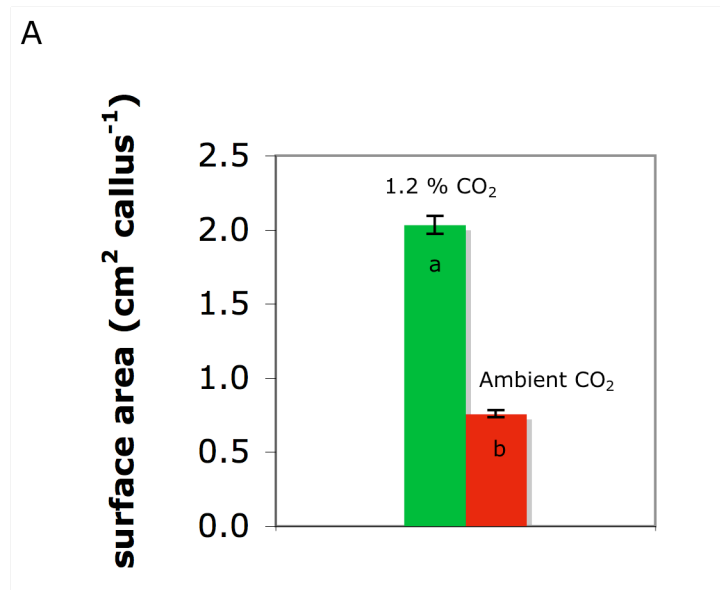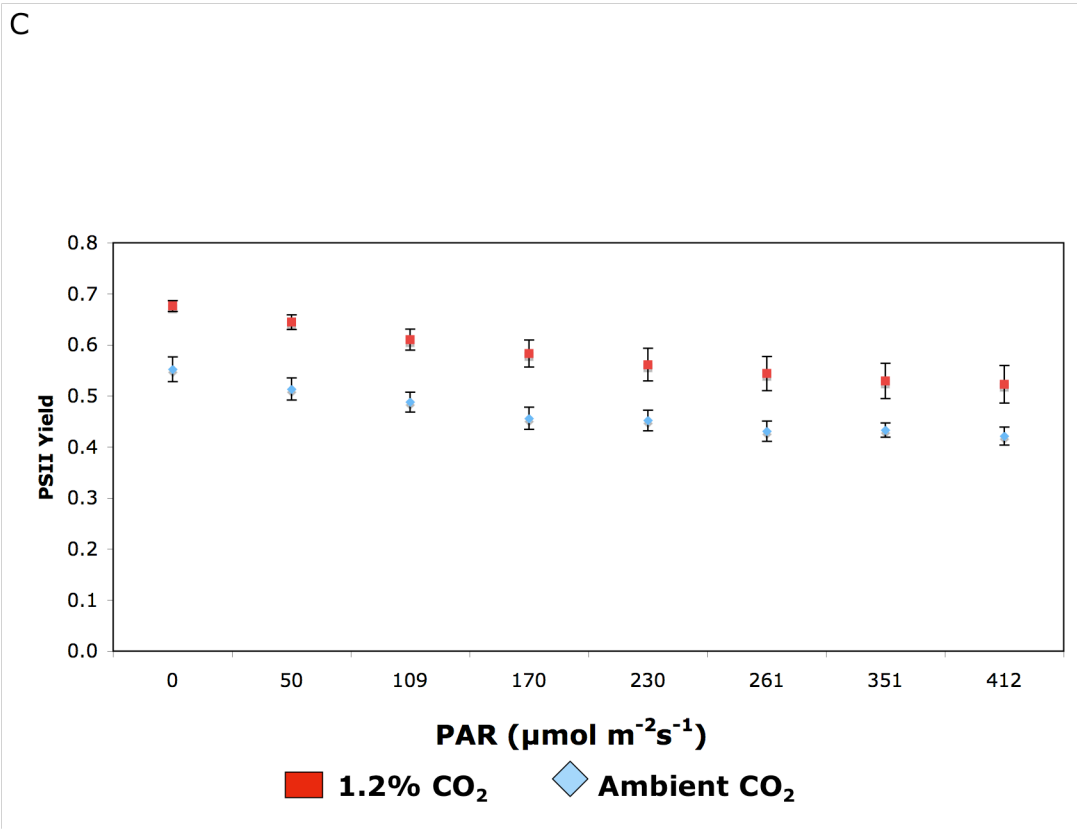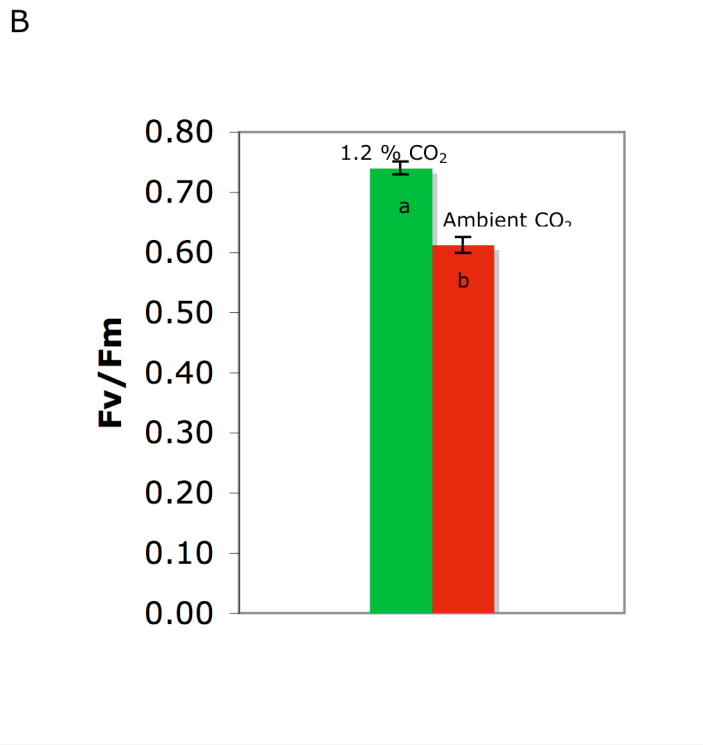
Figure 6. Effect of CO_2 concentration on *B. sinuspersici* callus structure from Stage 3, grown on carbon free MS media with 5 mM phosphate supplemented with 10 mM NaCl, at 30ºC with a photon flux density of 273 µmol m^{-2} sec^{-1}, at either 1.2% CO_2 or current ambient CO_2, A) Light micrograph of *B. sinuspersici* callus grown at 1.2%, section through callus showing large cells with thick cell walls. Scale bar = 50 µm. B) Light micrograph of *B. sinuspersici* callus grown at current ambient CO_2, section through callus showing different cell morphologies. Scale bar = 50 µm. C) Light microscope image of *B. sinuspersici* callus cell grown at 1.2% CO_2, showing association of organelles with nucleus. Scale bar = 50 µm. D) Transmission electron microscopy (TEM) image of callus grown at 1.2% CO_2, showing association of organelles with nucleus. Scale bar = 0.5 µm.
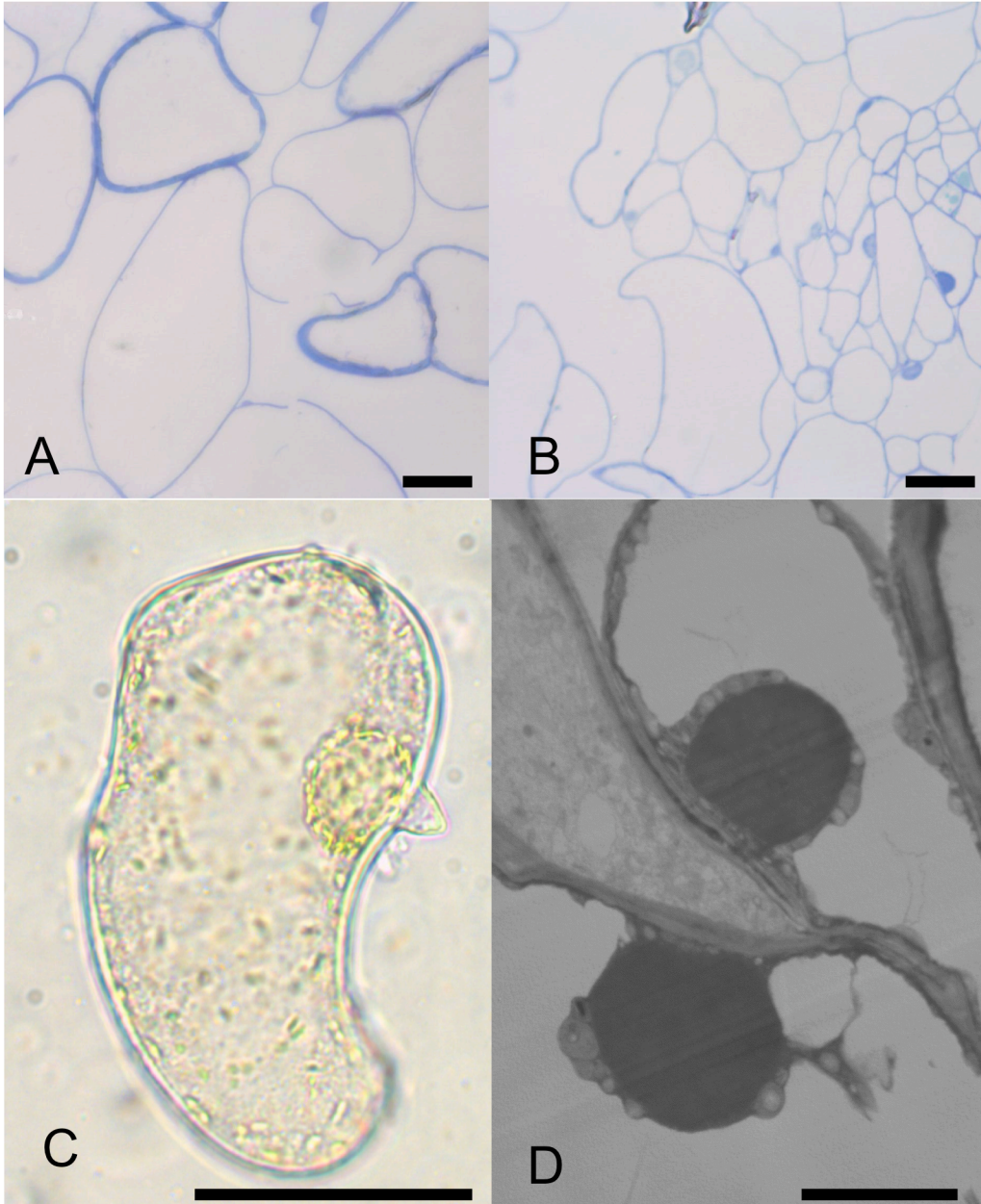
Figure 7. Western blots of photosynthetic portion *B. sinuspersici* callus at end of Stage 3,
grown on carbon free MS media with 5 mM phosphate supplemented with 10 mM NaCl,
at 30ºC with a photon flux density of 273 μmol m^{-2} sec^{-1}, under photoautotrophic
conditions (1.2% CO_2). Callus is compared to a mature plant grown in soil under
ambient CO_2. Under photoautotrophic conditions there is expression of Rubisco Large
subunit in callus tissue, and a detectable PEPC band. 15μg of protein was applied per
lane.
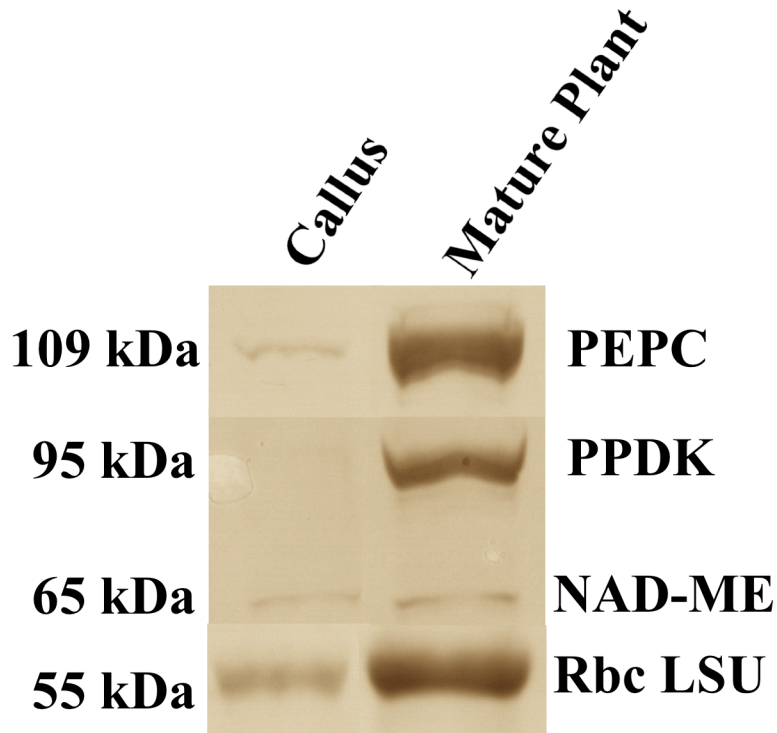
Figure 8. Growth of red nodular structure (RNS) after Stage 2, from stem explant of *B. sinuspersici,* grown in 100 mm Petri dishes on MS media with 5 mM phosphate supplemented with 43 mM sucrose plus 1.5% starch, and 2 mg 6-BAP l^{-1}, under current ambient CO_2, 25°C and 70.5 μmol m^{-2} sec^{-1} (PPFD).
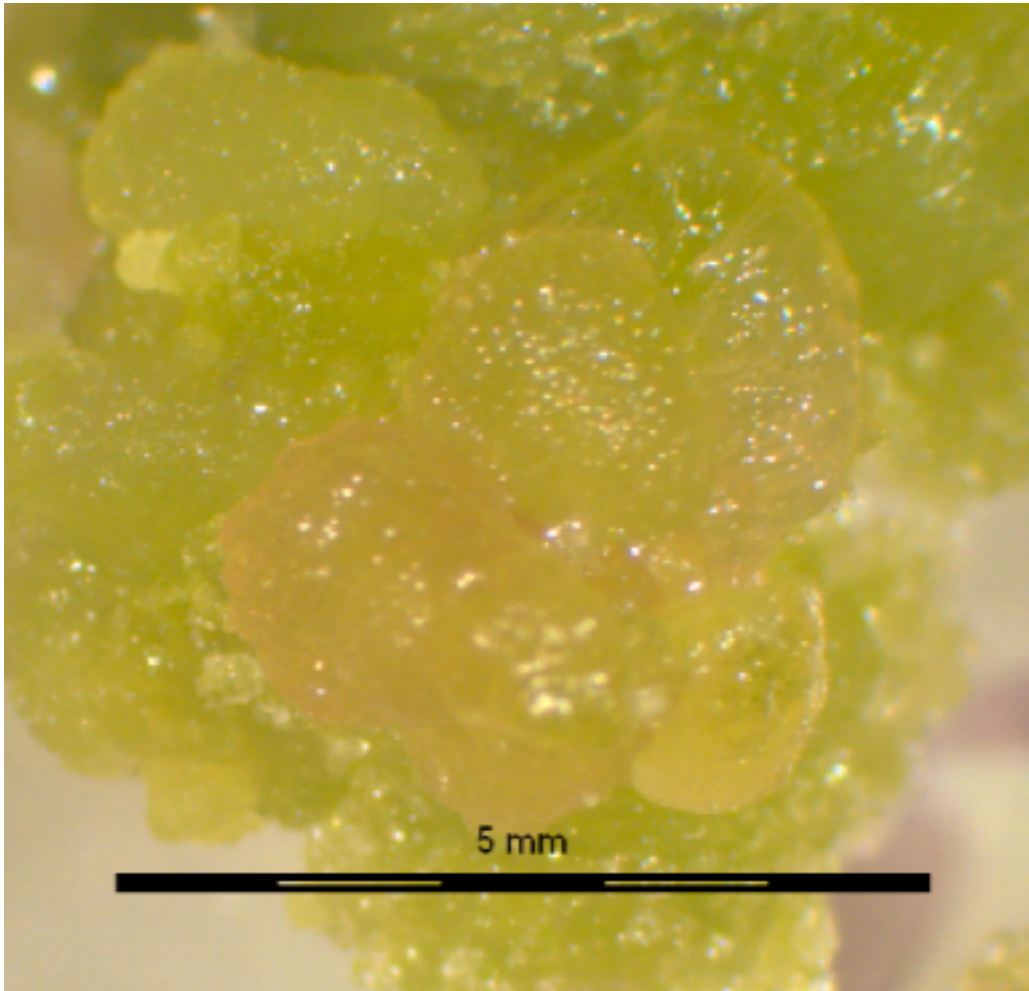
Figure 9. Average area of regenerating plantlet under different NaCl concentrations and 1.2% CO_2 compared to current ambient CO_2 concentrations. The relative size of each plantlet was quantified using ImageJ analysis program. Error bars represent the standard error of three replicates. Different capital letters represent statistically significant differences across salt levels, as determined by Tukey's HSD tests. Different lowercase letters represent statistically significant differences between the two CO_2 levels, as determined by ANOVA tests. P = 0.05
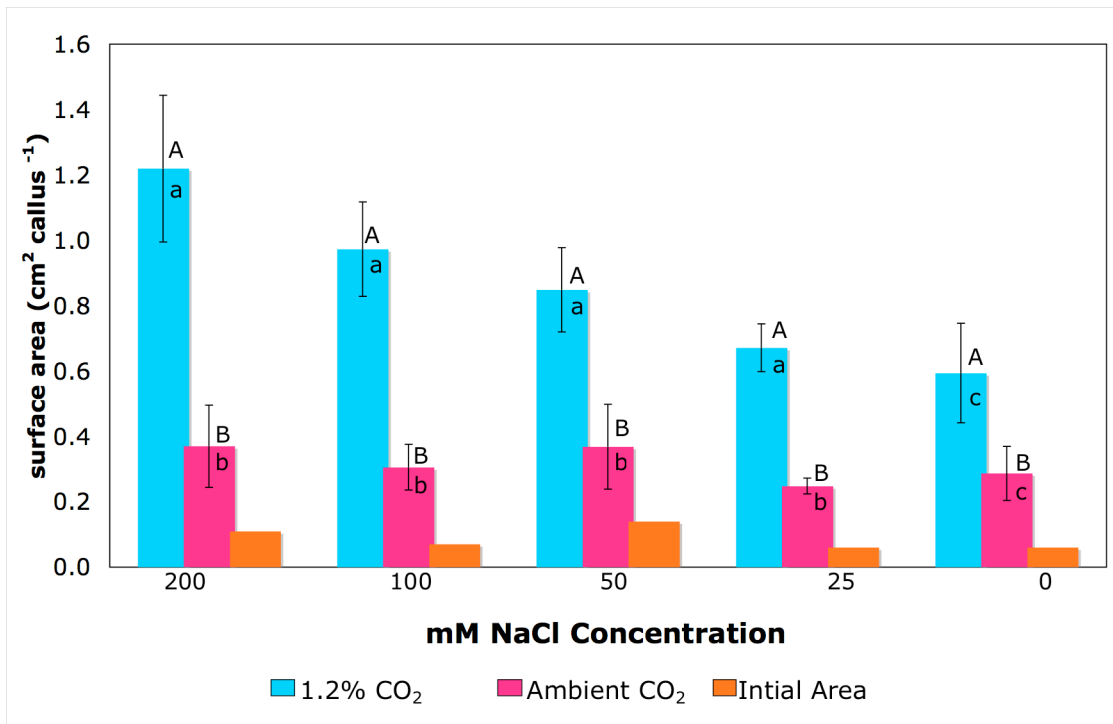
Table 1. Frequency of rooting and survival of *B. sinuspersici* rooted plants after transplanting to soil during Stage 3, grown on carbon free MS media with 5 mM phosphate, at 30ºC with a photon flux density of 273 $\mu mol\ m^{-2}\ sec^{-1}$, under varying NaCl levels. Success rate grown at 1.2% CO_2 versus current ambient CO_2. N = 3

	NaCl Media Concentration					
	0 mM	25 mM	50 mM	100 mM	200 mM	Total all Salt Levels
1.2% CO_2						
Starting callus with RNS	109	118	132	122	117	598
Shoots developed from Explant	39	58	61	69	112	339
Shoots with roots	1	5	8	3	12	29
Plants that grew in soil						8
Ambient CO_2						
Starting callus with RNS	109	118	132	122	117	598
Shoots developed from Explant	12	18	26	17	31	104
Shoots with roots	0	0	2	1	0	3
Plants that grew in soil						0

Figure 10. Shoots of *B. sinuspersici* after Stage 3, grown in microboxes on carbon free MS media with 5 mM phosphate, at 30ºC with a photon flux density of 273 μmol m^{-2} sec^{-1}, at either 1.2% CO_2 or current ambient CO_2. Images are of plantlets grown with 100 mM NaCl.   A) 1.2% CO_2. Scale bar = 0.5 mm. B) current ambient CO_2. Scale bar = 0.5 mm.
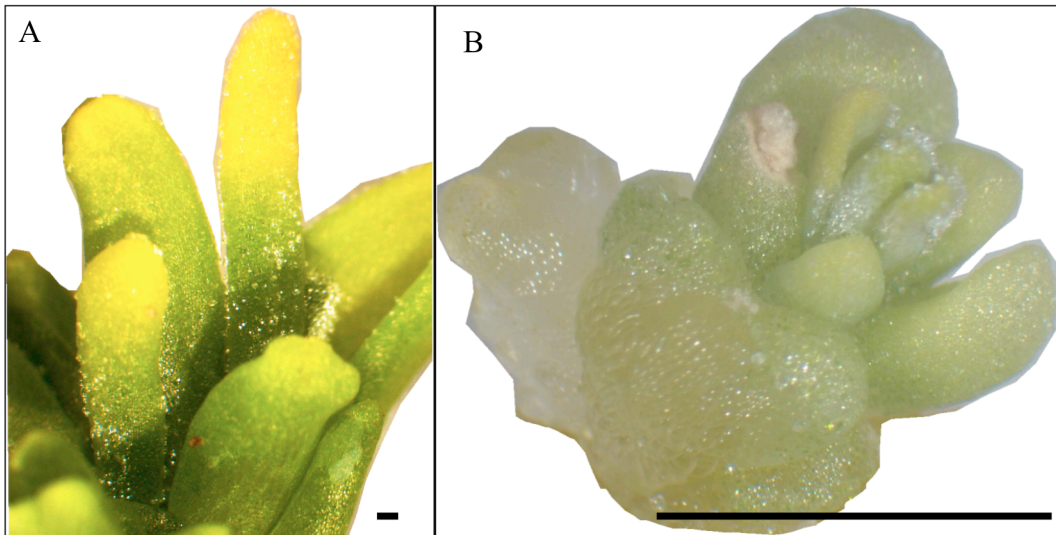
Figure 11. Light micrograph of leaf cross-section of *B. sinuspersici* from regenerating

shoots after Stage 3, at 1.2% CO_2, with 50 mM NaCl. Cross-section through center of

leaf, showing mature chlorenchyma cell, water storage cells and a central vascular
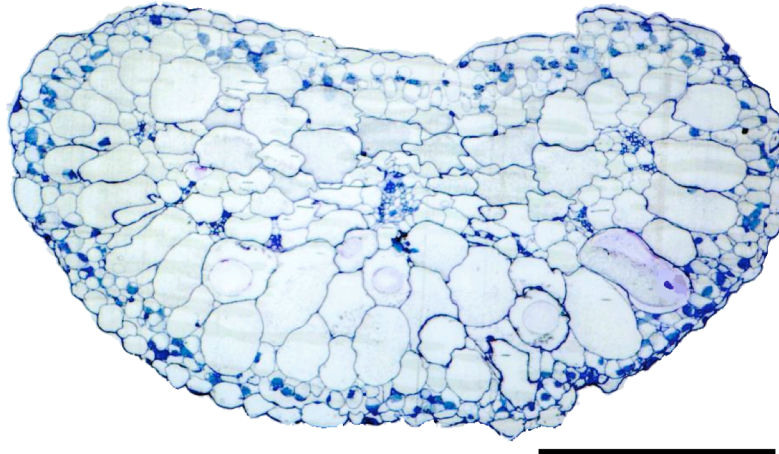
system. Scale bar = 500 µm.

Figure 12. Leaf osmolality of plantlets under varying NaCl levels in *B. sinuspersici*. The leaf osmolality was measured using a vapor pressure osmometer at each NaCl level at the end of Stage 3. Error bars represent the standard error of three replicates. Different capital letters represent statistically significant differences across salt levels at 1.2% CO_2, and different lowercase letters represent statistically significant differences at current ambient CO_2, as determined by Tukey's HSD tests. Difference between capital letters and lowercase letters represent statistically significant differences between the two CO_2 levels, as determined by ANOVA tests. P = 0.05
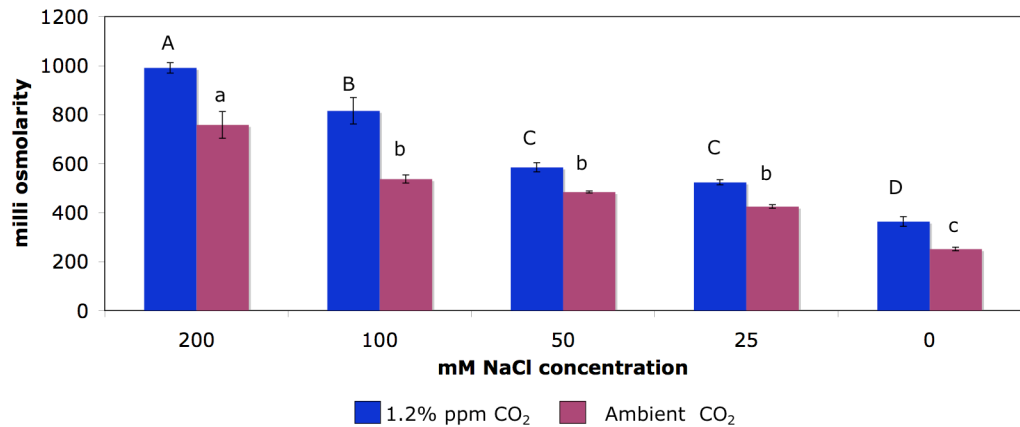
Table 2. Effects of salt treatment and CO_2 concentration on soluble protein and chlorophyll content in *B. sinuspersici* plantlets at the end of Stage 3. The fresh weights were measured after removal from the microboxes. Difference between upper and lowercase letter denotes a statistically significant difference between high and low CO_2 as determined by ANOVA analysis. Difference in upper or lower case letters denotes a statistically significant difference across salt treatments as determined by Tukey's HSD tests. P = 0.05

A) chlorophyll a+b concentration μg/mg FW

NaCl	1.2% ppm CO_2	Ambient CO_2
0 mM	0.143 +/- .03 [A]	0.02 +/- .02 [a]
25 mM	0.141 +/- .02 [A]	0.022 +/- .01 [a]
50 mM	0.159 +/- .03 [A]	0.057 +/- .03 [a]
100 mM	0.121 +/- .02 [A]	0.044 +/- .03 [a]
200 mM	0.150 +/- .02 [A]	0.05 +/- .03 [a]
all groups	0.142 +/- .01	.041 +/- .01

B) soluble protein μg/mg FW

NaCl	1.2% ppm CO_2	Ambient CO_2
0 mM	4.71 +/- .56 [A]	.83 +/- 1.4 [a]
25 mM	5.51 +/- .56 [A]	1.23 +/- 1.2 [a]
50 mM	4.84 +/- .65 [A]	1.32 +/- 1.2 [a]
100 mM	3.82 +/- .24 [A]	0.68 +/- .6 [a]
200 mM	3.85 +/- .44 [A]	1.81 +/- .6 [a]
all groups	4.55 +/- .23	1.19 +/- 1.0

Figure 13. Western blot analysis for photosynthetic portions of *B. sinuspersici* plantlets grown on carbon free MS media with 5 mM phosphate, at 30ºC with a photon flux density of 273 μmol m^{-2} sec^{-1}, in 1.2% CO_2 and varying NaCl levels. The relative intensity was compared to a mature plant growing in soil under normal CO_2 and treated monthly with 150 mM NaCl. 15μg of protein was applied per lane.
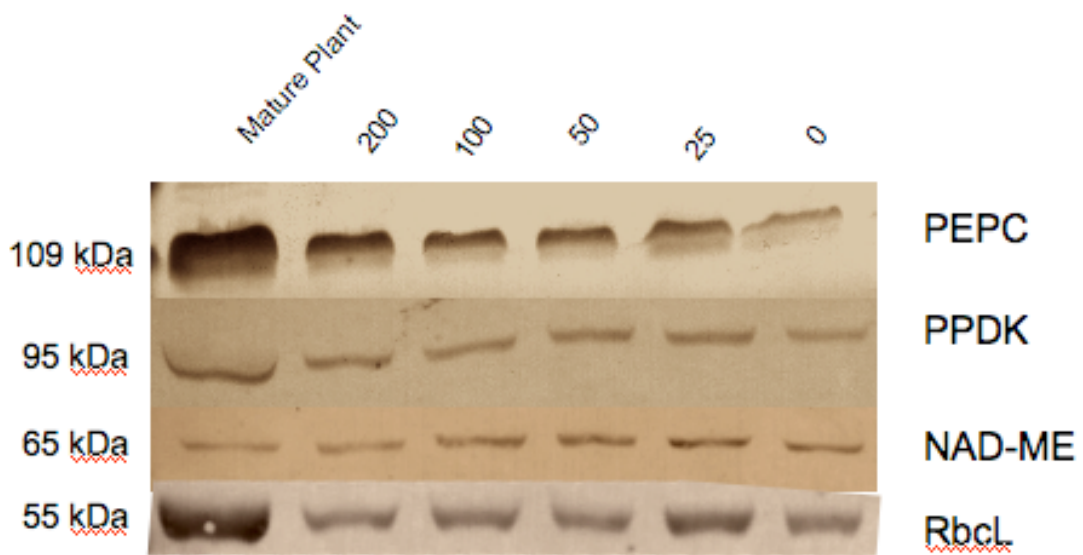
Figure 14. A comparison of the average intensity of each band from Western blot analysis of plantlets grown at 1.2% CO_2 during Stage 3, under varying NaCl levels in *B. sinuspersici* plantlets (quantified using ImageJ analysis program). The relative intensity was compared to a mature plant growing in soul under normal CO_2 treated monthly with 150 mM NaCl. N=3
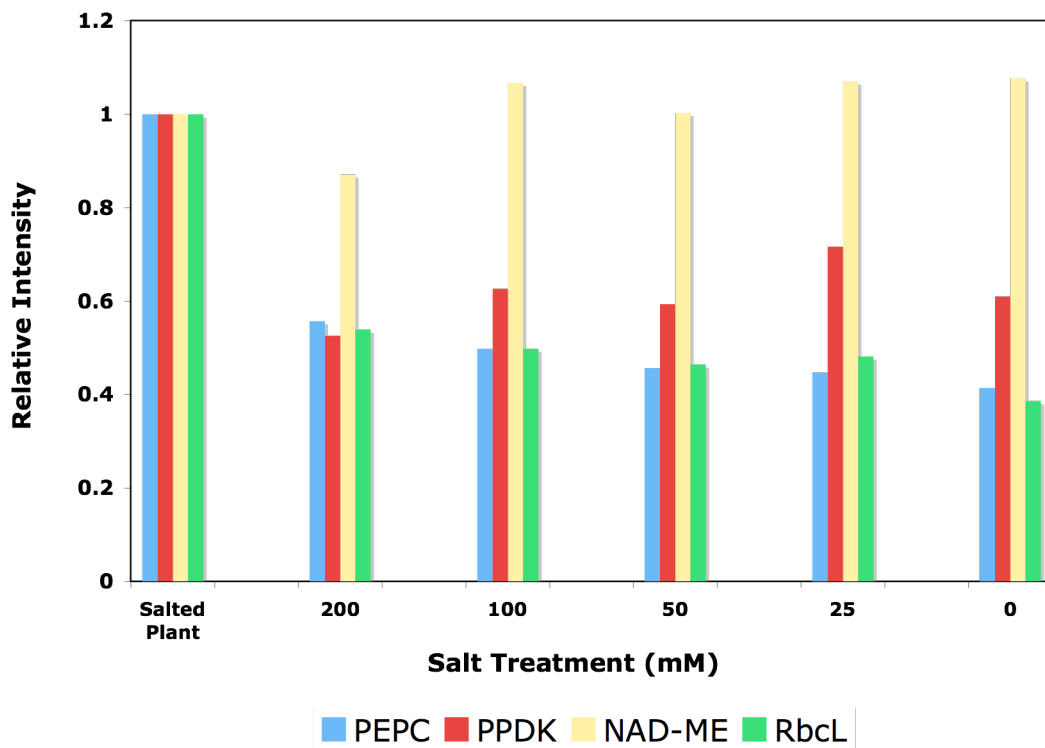
Figure 15. Rooted *B. sinuspersici* plant growing in a microbox containing a sand and soil

mixture under 1.2% CO_2.   Picture was taken 12 weeks after initiation of culture explants.

Supplemental Figure 1. Media comparison of Chu N_6 and MS.

MS Major Elements	mM	N6 Macronutrients	mM
NH_4NO_3	41.2	NH_4SO_4	3.481
KNO_3	18.8	KNO_3	27.992
$CaCl_2$	3	$CaCl_2$	1.088
$MgSO_4$	1.5	$MgSO_4$	0.751
KH_2PO_4	1.25	KH_2PO_4	2.296
Na_2-EDTA	Na 0.20	Na2-EDTA	0.100
$FeSO_4$	Fe 0.10	$FeSO_4$	0.100
		Fe - EDTA	13.620
MS Minor Elements	µM	MS Micro Nutrients	µM
$MnSO_4$	131.9	$MnSO_4$	100.0
H_3BO_3	100.3	H_3BO_3	100.3
$ZnSO_4$	29.9	$ZnSO_4$	29.9
KI	5.0	KI	5.0
Na_2MoO_4	1.0	Na_2MoO_4	1.0
$CuSO_4$	0.1	$CuSO_4$	0.1
$CoCl_2$	0.1	$CoCl_2$	0.1
Vitamins	mg/l^{-1}	MS Vitamins	mg/l^{-1}
Nicotinic acid	0.5	Nicotinic acid	0.5
Pyridoxine	0.5	Pyridoxine	0.5
Thiamine	0.1	Thiamine	0.1
Glycine	2	Glycine	2
myo-inositol	100	myo-inositol	100

Supplemental Figure 2. Comparison of MS media versus Chu N_6 media. Following culture conditions for Stage 1 and Stage 2 with either MS or Chu N_6, pictured is regenerating shoots, grown at 25ºC with a photosynthetic photon flux density of 70.5 µmol quanta $m^{-2}sec^{-1}$. A) Shoots grown on Chu N_6 media supplemented with 43 mM sucrose, are hyperhydric and have more anthocyanin (pink color) compared to B) shoots grown on Murashigue and Skoog media supplemented with 43 mM sucrose. Scale bars = 600 mm.
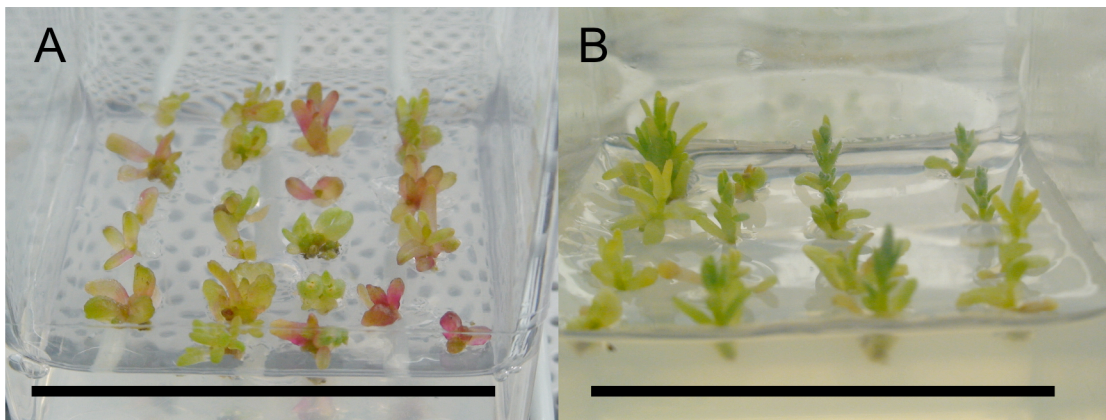
Supplemental Figure 3. The appearance of *B. sinuspersici* callus after Stage 2, grown on MS media with 5 mM phosphate supplemented with 10 mM NaCl, and either A) 43 mM sucrose, B) 43 mM glucose, C) 43 mM sorbitol, or D) 43 mM mannitol. Grown at 25ºC with a photosynthetic photon flux density of 70.5 µmol quanta m^{-2}sec^{-1}. Callus growth on either sucrose or glucose produced larger greener callus than callus grown on sorbitol or mannitol.
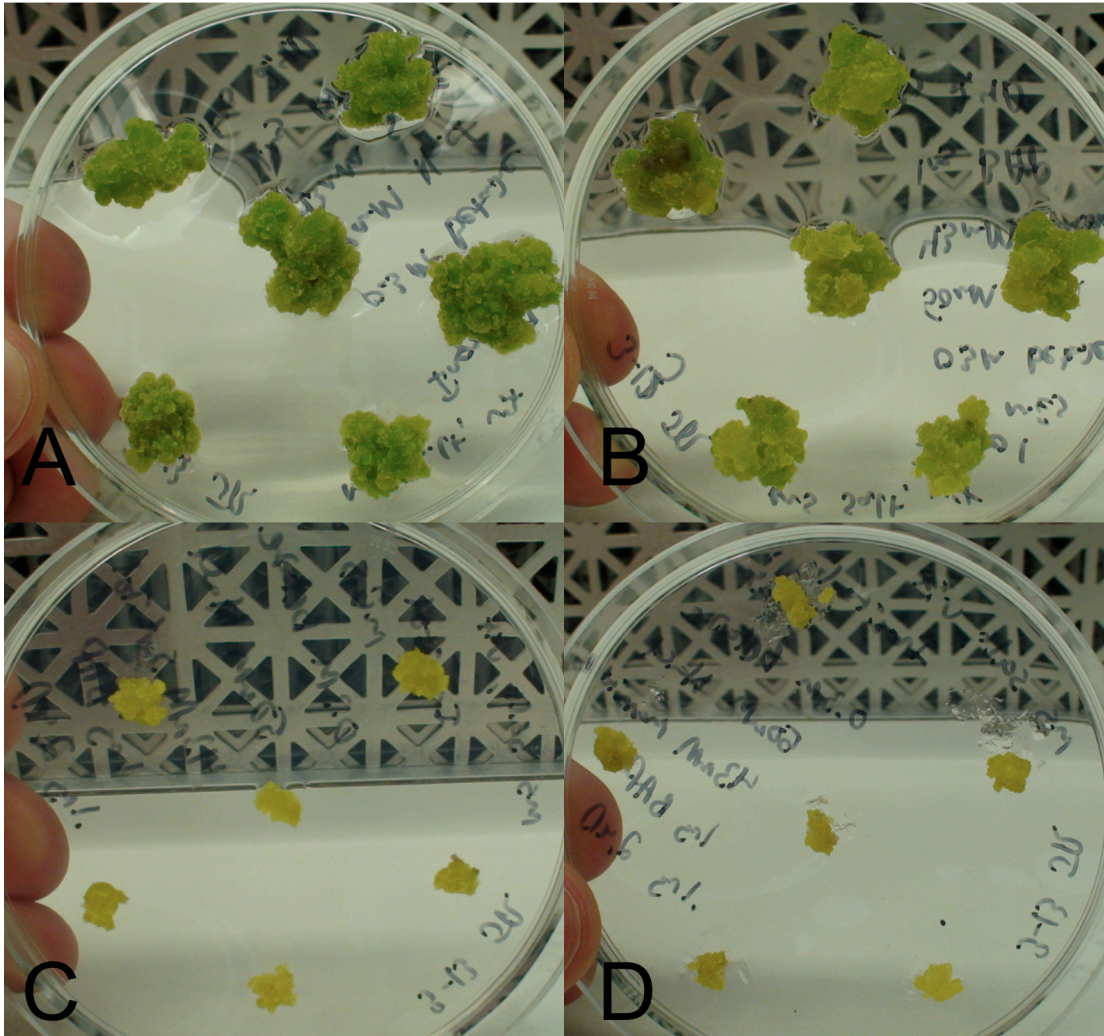
CHAPTER 3

FUTURE RESEARCH PERSPECTIVES FOR *BIENERTIA SINUSPERSICI*

The specialized anatomy seen in *Bienertia sinuspersici* chlorenchyma cells make it a unique system to study developmental plant biology, specifically the mechanism of formation of cytoplasmic domains and the interaction and compartmentation of organelles in a photosynthetic cell. The use of this species as a model system will depend on the ease of applying techniques and genomic resources used with other plant species. The research of this thesis focused on developing optimal *in vitro* culture conditions for the regeneration of *B. sinuspersici*. The results show that elevated CO_2 and 200 mM NaCl produced a shoot regeneration success from callus having RNS of 96% compared to a shoot regeneration success rate of 26% at the same salt level but with ambient CO_2. The lower rooting efficiency of 10% from callus having RNS at elevated CO_2 and 200 mM NaCl might be enhanced by further modification of culture conditions (in Stage 3, or a Stage 4). One test for increasing root regeneration is addition of 1% glucose and an auxin hormone such as indole-3-butyric acid (IBA) to half strength MS media (in a Stage 4), which has been done successfully with *Spartina alterniflora* (Wang *et al.* 2003). With the identification of appropriate culture conditions for shoot development, optimizing rooting conditions is now possible, and can be combined with transformation efforts.

If transformation was routinely possible there are many constructs that could be used to test the requirements for function of C_4 photosynthesis in *B. sinuspersici*. For example if a PEPC knockdown mutant was generated, such that the C_4 biochemical cycle

was not functioning, one could test whether this causes a structural reversion to C_3 type chlorenchyma having chloroplast with the C_3 cycle located at the periphery of the cell. Obtaining evidence that the anatomy is not dependent on the biochemistry would be useful for future engineering strategies. Manipulation of C_4 biochemistry has been performed in *Flaveria bidentis,* and NADP-ME type C_4 species*,* where anti-sense lines against carbonic anhydrase, PEPC, NADP-MDH, PPDK, and Rubisco have been generated, which provides insight into C_4 function (Furbank et al. 1997).

In *Bienertia* anti-sense constructs for the enzymes of the NAD – ME C_4 cycle, could be generated to determine the effect of knocking out individual enzymes in the biochemical cycle, and provide direct proof of requirements for cycle function. Other anti-sense lines against highly expressed transcription factors identified in *B. sinuspersici* by 454 sequencing compared to related species, could be tested to determine if they have a role in development of single cell C_4 photosynthesis. Lastly, anti-sense lines against components of the cytoskeleton such as actin or tubulin could be tested to see if they disrupt the positioning of organelles.

An ideal transformation construct should provide information about the physiological difference between the dimorphic chloroplasts. One physiological difference that has been found in the Kranz species maize is the concentration of H_2O_2 present in the chloroplast of mesophyll and bundle sheath cells. Studies on the antioxidant capacity of the two chloroplast types, showed that bundle sheath chloroplast have an accumulation of ascorbate peroxidase activity similar to accumulation of Rubisco (Doulis et al. 1997). The differential distribution of antioxidant capacity between the two-chloroplast types could play a role in C_4 biochemical protein accumulation.

Redox sensitive roGFP has been successfully used in Arabidopsis to measure the redox potential of mitochondria and the cytoplasm *in vivo*, where a greater buffering capacity was found inside the mitochondria compared to the cytoplasm (Jiang *et al.* 2006). A preliminary test to determine if there is a difference in redox capacity between the two-chloroplast types in *B. sinuspersici* using a roGFP is possible with constructs that have already been generated. Testing of these constructs will be done on protoplasts of *B. sinuspersici* where transformation is all ready possible, and then attempted on RNS to generate stable transformants to study the developmental change. With the identification of effective *in vitro* culture and regeneration conditions for *B. sinuspersici* it is now possible to test transformation of RNS, applying selection for transformed tissue during Stage I or II of regeneration.

Works Cited

Doulis, A. G., Debian, N., Kingston-Smith, A. H. and Foyer, C. H. (1997). "Differential localization of antioxidants in maize leaves." Plant Physiology **114**: 1031-1037.

Furbank, R. T., Chitty, J. A., Jenkins, C. L. D., Taylor, W. C., Trevanion, S. J., von Caemmerer, S. and Ashton, A. R. (1997). "Genetic manipulation of key photosynthetic enzymes in the C_4 plant *Flaveria bidentis*." Australian Journal of Plant Physiology **24**: 477-485.

Jiang, K., Schwarzer, E., Zhang, S., Ruzin, S., Machen, T., Remington, S. J. and Feldman, L. (2006). "Expression and characterization of a redox-sensing green fluorescent protein (reduction-oxidation-sensitive green fluorescent protein) in Arabidopsis." Plant Physiology **141**: 397-403.

Wang, J., Seliskar, D. M. and Gallagher, J. L. (2003). "Tissue culture and plant regeneration of *Spartina Alterniflora*: implications for wetland restoration." Wetlands **23**: 386-393.