VARIATION FOR WHOLE PLANT WATER USE EFFICIENCY AND LEAF-LEVEL TRAITS AFFECTING DROUGHT TOLERANCE IN SOYBEAN

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

MEHDI FARID

In partial fulfillment of requirements

for the degree of

Master of Science

January, 2011

Your file Votre référence
ISBN: 978-0-494-80006-5
Our file Notre référence
ISBN: 978-0-494-80006-5

ABSTRACT

VARIATION FOR WHOLE PLANT WATER USE EFFICIENCY AND LEAF-LEVEL TRAITS AFFECTING DROUGHT TOLERANCE IN SOYBEAN

Mehdi Farid
University of Guelph, 2010

Advisor:
Dr. Hugh J. Earl

Genotypic variation for water use efficiency and a correlated leaf-level trait, the dark-adapted leaf epidermal conductance (g_{dark}) has been previously identified among soybean cultivars adapted to Ontario, Canada. In the present work, parents of existing soybean mapping populations were screened for variation in these two traits to identify populations that would be suitable for identifying chromosomal regions controlling the traits. Second, a comparison of greenhouse and field data demonstrated that greenhouse screening experiments could predict cultivar differences for g_{dark} in the field, but only when plants in the greenhouse were grown under a cyclic drought treatment. Third, greenhouse experiments were conducted to examine restrictions to photosynthesis in six soybean cultivars during recovery from drought stress. No treatment by cultivar interactions were found. Compared to control plants, drought-stressed plants showed residual limitations to photosynthesis 24 h after rewatering. The lower photosynthetic rates were primarily caused by reduced mesophyll conductance.

ACKNOWLEDGEMENTS

First and foremost, I would like to offer my sincerest gratitude to my supervisor, Dr. Hugh Earl, for his support and guidance of this work. One simply could not wish for a better or friendlier supervisor. I also acknowledge the extensive and critical reviews of this work provided by member of advisory committee, Dr. Istvan Rajcan and Dr. Lewis Lukens.

I am very grateful to friendly and cheerful group of crop physiology laboratory, Godfrey Chu, Laxhman Ramsahoi, Shuang Li and Li Guo whom I might never have finished all my measurements. I also appreciate Jim Hoare and Dietmar Scholz for their support my work. The financial assistance was provided by the Grain Farmers of Ontario, and the Natural Science and Engineering Research Council which made possible this research.

Finally, I thank my wife and daughter for supporting me throughout all my studies at University, for providing a home in which to complete my writing up.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_G - gross rate of leaf photosynthetic CO_2 assimilation

A_N - net rate of leaf photosynthetic CO_2 assimilation

C_a - leaf external CO_2 concentration

C_C - chloroplast CO_2 concentration

C_i - leaf internal CO_2 concentration

CO_2 - carbon dioxide

D_s - leaf stomatal density

DW - dry weight

E_s - water lost by evaporation

ET - evapo-transpiration

F'_M - maximum fluorescence signal from an illuminated leaf sample

F'_S - steady state fluorescence from an illuminated leaf sample

g_c - leaf stomatal conductance to CO_2

g_{dark} - dark-adapted leaf epidermal conductance

g_m- leaf mesophyll conductance

g_s - leaf stomatal conductance

g_w - leaf stomatal conductance to H_2O

J_e - electron transport rate

K_S - CO_2/O_2 specifity of RuBisCo

LA - leaf area

LFW – leaf fresh weight

l_m - mesophyll resistance

LRWC - leaf relative water content

LWC – leaf water content

O_C - partial pressure of oxygen at carboxylation site

PAR - photosynthetically active radiation

PPFD - photosynthetic photon flux density

PS II - photosystem II

QTL - quantitative trait loci

R1 – developmental stage commencing with onset of flowering

R_d - respiration rate of a darkened leaf

RDW - root dry weight

RLWC - rehydrated leaf water content

R / S - root to shoot ratio

SDW - shoot dry weight

SLW – specific leaf weight

r_m - leaf mesophyll resistance

r_s - leaf stomatal resistance

T - plant transpiration rate

TDW – total crop dry weight

TE - plant transpiration efficiency (above ground dry weight / transpiration rate)

T_{max} - daily free water evaporation

V3 – developmental stage with three unfolded trifoliolate mainstem leaves

V_C - rate of carboxylation by RuBisCo

V_O - rate of oxygenation by RuBisCo

W_a - leaf external H_2O vapour concentration

W_i - leaf internal H_2O vapour concentration

WU - water used by the plant

WUE - water use efficiency

WUE_C - water replete water use efficiency

WUE_D - drought water use efficiency

WUE_L - leaf-level water use efficiency

α - leaf total absorptance of photosynthetically active radiation

f_{II} - proportion of absorbed photons absorbed by the light harvesting complex of photosystem II

Γ^* - CO_2 compensation point in the absence of dark respiration

Φ_{II} - quantum efficiency of photosystem II

‰ – part per 1000

CHAPTER 1

General Introduction

1.1 GENERAL INTRODUCTION

Soybean is one of the most important crops for human food, animal feed and industrial uses because of its high concentration of protein (36%), and oil (18%). (Boydak et al., 2002; Dogan et al., 2007 cited from Arıoglu, 1999).

Global warming is increasing the frequency of extreme weather events (Keeling et al., 1995) and especially drought stress. Water shortage is the most significant environmental limitation that reduces crop growth and yield through decreased photosynthesis. In Ontario, soil water deficits likely reduce soybean yields in most years (H.J. Earl, unpublished data). Hence, it is important to seek opportunities to improve water use efficiency (WUE, the amount of crop dry matter produced per unit soil water transpired) and to develop drought-tolerant varieties of soybean. However, a better understanding of the physiological and genetic bases for variation in WUE is the first prerequisite to understand how to improve these traits in soybean.

Briggs et al. (1914) and Shantz et al. (1927) first reported inter-specific variability in WUE of some crops (cited by Zhang et al., 1998). Intra-specific genetic diversity for WUE in soybean has since been reported by several researchers (e.g. Mian et al., 1996; Hufstetler et al., 2007; Walden; 2008). It therefore seems that there is potential to improve WUE in soybean. However, whole-plant WUE is rather difficult to measure in the field.

Recent greenhouse studies with soybean have revealed a strong negative correlation between WUE and another trait, the dark-adapted leaf epidermal conductance (g_{dark}, the physical conductance to water loss through the leaf epidermis and stomata in plants adapted to dark conditions) in some soybean genotypes (Hufstetler et al., 2007; Walden, 2009). Consequently, g_{dark} could possibly serve as a surrogate measurement for WUE, even in field experiments. So far, few researchers have shown genetic diversity for g_{dark} , including Hufstetler et al. (2007) and Walden (2008) in soybean, and Fish and Earl (2009) in cotton.

WUE is a polygenically (quantitatively) controlled trait (Martin et al., 1999; Mian et al., 1996; Bari et al., 2005). Accordingly, finding any molecular marker(s) concurrently associated with WUE and g_{dark} can give an excellent opportunity to soybean breeders to improve WUE more quickly than before.

Water shortage limits plant growth and yield mainly because of plant carbon balance reduction due to reduced photosynthesis (Flexas et al., 2002; Lawlor and Cornic, 2002; Monclus et al., 2006; Galle et al., 2007). To introduce more drought-tolerant plants, it is very important to understand the main physiological factors limiting photosynthesis during recovery of plants after drought stress. This will allow targeting of specific traits that form the actual bottlenecks to carbon assimilation arising from the stress exposure. Additionally, when variation for traits putatively associated with drought tolerance is identified in controlled environment experiments, it is critical to verify that these differences also exist in the field environment.

1.2 RESEARCH OBJECTIVES

1. To screen parents of existing soybean mapping populations for variation in g_{dark} and WUE for potential QTL analysis.
2. To characterize the physiological basis of limitations to photosynthesis in commercial soybean cultivars during recovery from a transient severe water stress event. Specifically, to compare the magnitudes of stomatal and mesophyll resistances to CO_2 diffusion.
3. To determine if genotype differences found in greenhouse studies for g_{dark} or other leaf-level gas exchange parameters potentially related to WUE (stomatal conductance, leaf internal CO_2 concentration) are also observed in the field.

CHAPTER 2

Literature Review

2.1 *Drought stress as a limitation to crop yield*

Macroclimates, the regional climate of a broad area, can change not only seasonally, but also from moment to moment. Therefore, plants may face great climatic variation. Mostly, the environment varies daily or seasonally which is "predictable" for the plant. However, sometimes conditions change in ways to which the plant is not fully adapted. These conditions are not suitable for the plant, and therefore constitute "stress" (Gaspar et al. 2002). Generally, biological stress is defined as change in environmental conditions that might adversely affect a plant's growth or development (Levitt 1980).

Where plants are often subjected to periods of drought, water shortage is the most significant environmental limitation factor which reduces crop growth and yield during the growing season (Begg and Turner, 1976; Evans, 1996; Flexas et al., 2006a; Fuhrer, 2003; Hsiao, 1973; Kramer and Boyer, 1995) including soybean (Araus et al., 2002; Ashley and Ethridge,1978; Batchelor et al., 2002; Cooper et al., 1991; Cox and Jolliff, 1986; De Costa and Shanmugathasan, 2002; Doss et al., 1974; Dogan et al., 2007; Frederick et al., 2001; Frederick et al., 1991; Karam et al., 2005; Korte et al., 1983; Meckel et al., 1984; Mederski and Jeffers, 1973; Sinclair et al.,1992; Sionit and Kramer,1977; Smith and Griffiths, 1993).

2.1.1 *Drought stress in Ontario*

Global warming is increasing the frequency of extreme weather events (Keeling et al., 1995) and will probably make water deficit an even greater restriction for plant productivity in the future (Chaves et al., 2009). According to most climate change

scenarios, the severity of the summer drought may increase as well as the frequency of severe droughts, as Giorgi and Lionello (2008) anticipated for the globe. It is expected that either the total amount of available precipitation will decrease or that precipitation distribution will change because of climate change, often resulting in hot and dry conditions at crops' critical growing stages. Over large areas of the earth, water is already the major factor limiting plant productivity. Even regions that have reasonably wet climates can experience periodic or seasonal droughts, which reduce productivity from that achieved under optimal conditions (Jones 1992; Leuschner et al. 2001). Soil water deficits likely reduce soybean yields in most years in Ontario, at least to some extent; even in a relatively cool, wet year such as 2009 supplemental irrigation was found to significantly enhance soybean yield (H.J. Earl, personal communication).

2.2 *Effects of drought on photosynthesis*

Photosynthesis is sensitive to a number of environmental conditions, including light, temperature, CO_2 concentrations, nutrient supply and water supply. It is now well known that one of the primary physiological targets of water stress is photosynthesis (Chaves, 1991; Cornic, 1994; Lawlor, 1995). Many studies have shown that drought stress primarily limits plant productivity through direct effects on photosynthesis (Chaves 1991; Flexas et al., 2002; Flexas and Medrano, 2002; Galle et al., 2009; Galle et al., 2007; Kramer and Boyer, 1995; Lawlor and Cornic, 2002; Monclus et al., 2006; Ohashi et al., 2000; Quick et al., 1992; Tang et al., 2002). Hence, physiologists have concerned themselves with photosynthesis responses to water shortage for decades (Flexas and Medrano, 2002; Lawlor and Cornic, 2002).

2.2.1 *Importance of the chloroplast CO_2 concentration*

The first step in the Calvin cycle involves fixation of CO_2 by the enzyme RuBisCo in the stroma of the chloroplast. Atmospheric CO_2 is therefore one substrate for photosynthesis, and photosynthesis in C_3 plants can be limited by any factor that causes low CO_2 concentration at the carboxylation site, whether it be reduced atmospheric CO_2 concentration outside the leaf, increased resistance to CO_2 diffusion from the atmosphere to the leaf interior air spaces, or increased resistance to diffusion in the liquid phase from the cell walls to the chloroplast stroma.

Flexas et al. (2008) indicated that CO_2 concentration in the chloroplast (Cc) is roughly 20-30% less than that of the ambient CO_2 concentration (C_a). In addition, there are many studies illustrating that in woody plants, CO_2 concentration in the chloroplast is significantly less than that in the substomatal cavities (C_i) (Evans et al., 1986; Epron et al., 1995; Warren et al., 2003). Roupsard et al. (1996) also showed that under well watered conditions, the concentration of CO_2 in the chloroplasts was much lower than the calculated substomatal CO_2 concentration in oak. Moreover, the works mentioned above generally found that in the species with the lowest assimilation rates, the lowest values of chloroplast CO_2 concentration were recorded. Therefore, C_C is one of the major limiting factors determining the CO_2 assimilation rate. The value of C_C depends on the diffusion of CO_2 from the substomatal cavity to the interior of the chloroplasts. In other words, C_C is a function of two obstacles against CO_2 diffusion from the atmosphere towards chloroplast cells, namely stomatal and mesophyll resistances.

2.2.2 *Stomatal resistance*

Stomatal conductance (g_s) (inverse of stomatal resistance) plays a fundamental role in the plant-atmosphere water relationship (Chen et al., 1999, cited by Zhang, 2007). CO_2 diffusion into the leaf mesophyll and water vapour diffusion from the leaf to the atmosphere are mainly regulated by the stomatal opening, controlled by a complex system of physiological processes.

There are two historical beliefs about the predominant signals causing stomatal closure: hydraulic signals (leaf water potential, cell turgor) and chemical signals (abscisic acid). The early idea regarding stomatal closure in reaction to stresses like soil water deficit was that, as a result of soil water content reduction, leaf water potential and cell turgor pressure would decline, and that was the main signal which could induce stomata to close. In contrast, it has been observed that stomata may actually start to close in response to low soil water content even when there is no decrease in leaf water potential. Therefore, it seems stomatal closure is a function of soil water potentials more than leaf water potentials. In this regard, Zhang and Davies (1989; 1990) demonstrated that leaf stomatal closure correlated with concentration of abscisic acid (ABA) in plant leaves, and also with ABA concentrations in the xylem stream between roots and leaves.

Currently, the majority of physiologists accept that a combination of both kinds of signalling mentioned above cause stomata to close at different times (Comstock 2002; Assama et al. 2002).

2.2.3 *Mesophyll diffusive resistance*

The resistance to CO_2 influx from the atmosphere to the chloroplast is one of the important limiting factors for leaf photosynthesis either under optimal conditions or under stress conditions. While the stomatal component of this resistance has been broadly appreciated, the diffusion path from the substomatal cavities to the sites of carboxylation in the chloroplast has been neglected as an important resistance to CO_2 flux, especially in the 1970s and early 1980s (Gaastra, 1959; Farquhar and Sharkey, 1982). It was only during the 1990s that diffusion limitations other than stomatal closure or leaf boundary layer effects were considered as a major subject of research (Parkhurst, 1994).

There is now some agreement that it is the diffusive limitation to CO_2 influx, rather than only biochemical factors, that are restricting photosynthesis. These restrictions include not only stomatal resistance, but also decreased non-stomatal, internal or mesophyll conductance to CO_2 (g_m)\ (Roupsard et al., 1996; Flexas et al., 2002; Ennahli & Earl, 2005). Thus, it is now generally accepted that a high conductance to CO_2 diffusion in the mesophyll (g_m) is required to support high rates of photosynthesis.

2.2.3.1 *Gas phase vs. liquid phase components of g_m*

The physical resistance to diffusion of CO_2 in the mesophyll includes both gas phase (diffusion in intercellular air spaces) and liquid phase components. However, most available evidence suggests that the majority of the leaf internal resistance to CO_2 movement is in the liquid phase. The gas phase conductance can be estimated by

contrasting gas exchange in normal air with air in which the nitrogen has been replaced by helium ('helox') where CO_2 diffuses 2.3 times more quickly. For instance, the rates of photosynthesis of six amphistomatous species were, on average, 2% faster in helox than air; and photosynthesis of five hypostomatous species were 12% faster in helox than air (Parkhurst and Mott, 1990). What this means is that gas phase resistance to mesophyll diffusion is likely a negligible limitation in leaves of most plants.

2.2.3.2 *Physical vs. biochemical components of g_m*

Physiologists used to believe that g_m was constant over a short periods of time (i.e., one day or less) (Evans and von Caemmerer 1996), because they believed that leaf anatomy and morphology were the two main determinants of g_m (Evans et al., 1994; Syvertsen et al., 1995). Currently, it is recognized that g_m can change much more quickly than can leaf anatomy and/or morphology, implicating non-structural factors in determination of g_m. It is now clear that in the liquid phase, resistance to CO_2 diffusion is a function of structural features such as cell wall thickness (Miyazawa and Terashima, 2001) and the surface area of mesophyll cells or chloroplasts exposed to the intercellular air spaces (Evans et al., 1994), but also that it has a biochemical component (Terashima et al. 2005; Fabre et al., 2007, cited by Warren, 2008). Evidence has suggested two promising candidates to play this biochemical role: carbonic anhydrase, and aquaporins.

Carbonic anhydrase in plants exists in three different classes including a, b, and c; and the b class is the most abundant one (Majeau et al., 1994; Price et al., 1994). For the first time a role for carbonic anhydrase in CO_2 movement through the mesophyll was

shown in tobacco using antisense technology to reduce the amounts of b-carbonic anhydrase to 1–10% of wild-type plants (Majeau et al., 1994; Price et al., 1994), which caused the concentration of CO_2 in the chloroplast to decline. In addition, a parallel reduction in g_m and the carbonic anhydrase amount was reported in leaves of rice suffering from zinc deficiency (Sasaki et al., 1998).

Aquaporins, which are the most abundant proteins in plant plasma membranes, have recently been considered with respect to internal leaf CO_2 flux in a number of studies (Terashima and Ono, 2002; Uehlein et al., 2003; Hanba et al., 2004; Flexas et al., 2006b). At least some aquaporins are playing a role in CO_2 flux in the leaves. For instance, Terashima and Ono (2002) indirectly showed this role for aquaporins using a non-specific inhibitor of aquaporins, $HgCl_2$, which reduced g_m in *Vicia faba*. In addition Hanba et al. (2004) showed that overexpression of barley aquaporin (HvPip2; 1) in transgenic rice increased the leaf internal conductance by 40% compared with control leaves. Recently Flexas et al. (2006c) also found that changes in aquaporin content were related to changes in g_m.

2.2.3.3 *Variation of g_m in time*

There is a little knowledge about the rate of change in g_m over time, in comparison with the ample evidence about how quickly g_s changes (Valladares et al., 1997; Tausz et al., 2005). Mesophyl conductance (g_m) used to be considered constant over the period of one day (Evans and von Caemmerer, 1996), because it was thought that leaf anatomy and morphology were the principal determinants of internal conductance (Evans et al., 1994;

Syvertsen et al., 1995). However, Flexas et al. (2007) reported that in response to water shortage or limitation in ambient CO_2 (C_a), g_m can significantly change within 5 or 10 minutes. This supports the idea of a vital role of g_m in the photosynthetic response of plants to drought stress, since it responds similarly to g_s both in the short term (minutes to hours) and over longer periods (days to weeks) (Centritto et al., 2003; Flexas et al., 2007). This dynamic nature may make g_m an important trait for improving plant photosynthesis responses to environmental stresses (Flexas et al., 2008; Warren, 2006).

2.2.3.4 *Inter- and intra-specific variation for g_m*

Mesophyl conductance (g_m) could potentially be an important trait for improving plant photosynthesis responses to environmental stresses if it shows high genetic diversity. Genetic variability in g_m has previously been reported in a number of species, including wheat, where g_m varied between 0.20 and 0.43 mol m^{-2} s^{-1} among five cultivars (Evans &Vellen 1996, cited by Barbour et al., 2010), European chestnut provenances (Lauteri et al. 1997), among *Populus* populations from different latitudes (Soolanayakanahally et al. 2009), and among *Phaseolus vulgaris* genotypes (Flowers et al. 2007) after exposure to high ozone concentration. Evans et al. (1994) found that tobacco transgenic plants with antisense genes to components of the RuBisCo small subunit, had lower g_m than wild-type plants when grown under the same light conditions. Inter- and intra-specific diversity for g_m has been reported by a number of other authors (e.g., von Caemmerer and Evans, 1991; Harley et al., 1992; Loreto et al., 1992; Epron et al., 1995; Warren et al., 2003).

Very recently, Barbour et al. (2010) found significantly lower g_m in four *Hordeum vulgare* genotypes than in *H. bulbosum* genotypes. They also showed significant differences between genotypes within each species, with the tetraploid *H. bulbosum* (HB4) having higher g_m than the diploid (HB2), and the variety 'Dash' having the highest g_m among the *H. vulgare* genotypes.

2.2.3.5 *Relative magnitudes of g_s and g_m*

There has been a long debate about the relative importance and physiological nature of two main classes of drought stress-induced limitations, g_s and g_m, the two main conductances that dominate the diffusive pathway from the atmosphere to the carboxylation site in the chloroplast. Currently, g_s is seen as just the first obstacle in the CO_2 diffusion pathway, with internal conductance (g_m) being the second quantitatively important barrier, significantly restricting CO_2 diffusion from the substomatal cavity towards site of carboxylation in the chloroplast stroma (Warren, 2008).

It is well established that in all studied species (e.g. by Evans et al., 1986; Lloyd et al., 1992; Epron et al., 1995; Warren et al., 2003) g_m is finite and C_C is noticeably less than C_i, indicating that g_m results in a significant decrease in CO_2 concentration. Quantitatively, g_m is sometimes found to be larger that g_s. In most cases g_s and g_m caused similar relative limitations for photosynthesis in well-watered plants (Epron et al., 1995; Warren et al., 2003; Yamori et al., 2006).

2.2.3.6 *Measuring g_m*

Estimates of the CO_2 mole fraction in the chloroplast stroma (C_C), which make it possible to quantify g_m, have not been available until recently. A number of different methods have been developed to estimate the resistance to CO_2 diffusion from the intercellular airspaces within the leaf through the mesophyll to the sites of carboxylation during photosynthesis. However, two methods are the most popular ones: simultaneous measurement of gas exchange with instantaneous carbon isotope discrimination (Evans et al., 1986); and a combination of gas exchange and chlorophyll fluorescence measurements (Bongi and Loreto, 1989). There are also other methods, including one based on the difference in the chloroplastic (C_C) and intercellular (C_i) photocompensation points (Caemmerer and Evans, 1981), and another based on the reduction in initial slope of an A_N/C_i curve from its theoretical maximum (Evans and Terashima, 1988).

2.2.3.6.1 *Carbon isotope discrimination method*

This method requires carbon isotope fractionation to be measured simultaneously with gas exchange, and is based on different diffusion and carboxylation rates of $^{12}CO_2$ and $^{13}CO_2$ (reviewed by Pons et al., 2009). $^{13}CO_2$ diffuses more slowly through the boundary layer (2.9‰) and stomata (4.4‰), slower through the liquid phase (1.8‰), and is carboxylated much more slowly than $^{12}CO_2$ (27–30‰) (Farquhar et al. 1982, 1989; Evans et al., 1986). Measurements of g_m using ^{13}C discrimination were first used by Evans et al. (1986). Stable isotopic fractionation occurs during photosynthetic CO_2 fixation. Specifically, the heavier isotope of carbon, ^{13}C, is discriminated against during diffusion in the gaseous and the liquid phases and during biochemical carboxylation

(Farquhar et al., 1982). These effects are mainly due to the lower diffusivity of $^{13}CO_2$ in both the air and liquid phases relative to $^{12}CO_2$, and to discrimination by carboxylating enzymes such as RuBisCo, which preferentially bind molecular species containing the lighter isotopes ($^{12}CO_2$). Hence, the photosynthetic products are generally enriched in the lighter isotope ^{12}C compared with the substrate atmospheric CO_2. In C_3 species, the isotopic discrimination is related to the relative contribution of diffusion and carboxylation, which is reflected in the ratio of CO_2 concentration at the sites of carboxylation (C_C) to that in the surrounding atmosphere (C_a). Carbon isotope discrimination is proportional to the concentration of CO_2 in chloroplasts (C_C), while gas-exchange measurements of transpiration estimate the substomatal concentration of CO_2 (C_i) and net CO_2 assimilation (A_N). Then, the internal conductance or mesophyll conductance may be calculated as $g_m = A_N/(C_i - C_c)$. In C_3 plants, the average of these discriminations against $^{13}CO_2$ are between - 20/1000 and - 30/1000, with the predominant effect of discrimination due to carboxylation, which is why discrimination is mostly proportional to C_C (reviewed by Warren, 2006).

2.2.3.6.2 *Combined chlorophyll fluorescence / gas exchange method*

Kautsky et al. (1960) first found changes in the yield of chlorophyll fluorescence. They found that after transferring plants from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a time period of around 1 s. This rise has subsequently been explained as a consequence of reduction of electron acceptors in the photosynthetic pathway, downstream of photosystem II (PSII). Once PSII absorbs light and excites an electron, it is not able to accept another photon until it has passed the first

excited electron onto a subsequent electron carrier. During this period, the reaction centre is said to be 'closed'. When the reaction centre is closed, the efficiency of photochemistry will decrease which causes compensating increases in the fluorescence yield and heat dissipation.

In the intervening years, fluorescence theory has been further developed. With the advent of modulated chlorophyll fluorometers, it has become possible to estimate the quantum efficiency of photosytem II (Φ_{II}) in illuminated leaves based only on fluorescence signals. First, the fluorescence yield of the leaf sample under ambient light is measured. This measurement is usually called steady state fluorescence yield (F_S). Second, a fully saturating pulse from the chlorophyll fluorometer is required, effecting complete closure of all PSII reaction centers, and the fluorescence yield rises to a maximum, F'_M. At this point, as was demonstrated by Genty et al. (1989, cited by Earl and Ennahli, 2004), Φ_{II} can be calculated as:

$$\Phi_{II} = (F'_M - F_S) / F'_M \qquad (1),$$

where Φ_{II} is the fraction of photons absorbed by the light harvesting complex of photosystem II that is used for photochemistry.

Next, the linear flux of electrons in PSII (J_e), or the electron transport rate, is easily calculated as:

$$J_e = \alpha \times f_{II} \times PPFD \times \Phi_{II} \qquad (2),$$

where α is leaf absorptance of incident PPFD, and f_{II} is the proportion of absorbed photons absorbed by the light harvesting complex of PSII (Loreto et al., 1994).

Accepting that four electrons are needed per carboxylation or oxygenation of RuBP by RuBisCO in C_3 plants, and assuming that other sinks for electrons are negligible, then:

$$J_e = 4V_C + 4V_O \qquad (3),$$

where V_C is the rate of carboxylation by RuBisCO, and V_O is the rate of oxygenation by RuBisCO. For each oxygenation event, 0.5 CO_2 are expected to be released due to photorespiration, so gross photosynthesis (A_G) can be calculated as:

$$A_G = V_C - 0.5V_O \qquad (4),$$

Then, it is possible to calculate the V_C/V_O ratio by combining equations (3) and (4) as:

$$V_C/V_O = (J_e + 8A_G) / (2\ J_e - 8A_G) \qquad (5),$$

In practice, net leaf exchange of CO_2 is measured using a non-dispersive infrared gas analyser, and A_G is estimated as $A_G = A_N + R_D$, where A_N is the net CO_2 assimilation rate measured in the illuminated leaf at the same time that Φ_{II} was measured, and R_D is the respiratory CO_2 release measured for the same leaf in the dark.

As Ennahli and Earl (2005) cited from Lal et al. (1996), CO_2 concentration in the chloroplast (C_C) may be calculated as follows:

$$C_C = (V_C/V_O) \times (O_C/Ks) \qquad (6),$$

where O_C is the partial pressure of oxygen at the carboxylation site and Ks is the CO_2/O_2 specificity of RuBisCo at a particular leaf temperature.

Finally, g_m can be estimated as:

$$g_m = A_N / (C_i - C_C) \qquad (7),$$

where C_i is internal (substomatal) CO_2 concentration derived from leaf gas exchange measurements.

2.2.4 *Leaf-level responses of photosynthesis to water stress*

Leaf photosynthesis is reduced at mild leaf water deficits or even before any change in leaf water status has occurred in response to a decrease in soil water potential (Gollan et al., 1986, cited by Chaves, 1991) or in air humidity (Lange et al., 1971; Bunce, 1981).

Water deficit is known to alter a variety of biochemical and physiological processes either at the stomatal level or at the level of the leaf mesophyll.

2.2.4.1 *Stomatal vs. non-stomatal limitations to photosynthesis under water stress*

Grassi and Magnani (2005) divided photosynthetic limitation processes associated with water stress into three categories: stomatal diffusive limitations, non–stomatal diffusive limitations, and biochemical limitations (i.e. carboxylation activity). They indicated that restrictions to CO_2 diffusion within the mesophyll caused the highest non-stomatal limitation under water stress.

However, there is a long controversy over the mechanisms by which water stress decreases photosynthetic assimilation of CO_2. Water deficit-induced reduction of the net photosynthetic CO_2 assimilation rate in some cases was attributed primarily to stomatal

closure (Chaves, 1991; Cornic, 1994; Flexas et al., 2004; Flexas et al., 2006a; Sharkey, 1990). Stomatal closure is often considered to be a short term response of plants to drought stress, whilst the non-stomatal limitations are usually thought to come into play only during longer and more severe water stresses. More recently, increased diffusive resistance of the mesophyll cells has been suggested as one of the main reasons for photosynthesis suppression induced by water shortage in tobacco (Galle et al., 2009) and cotton (Enahhli and Earl, 2005).

There are many reports showing that even under mild water shortage, photosynthesis reduction cannot be attributed entirely to the observed stomatal limitations (Ni and Pallardy 1992; Ramanujulu et al. 1998; Yordanov et al. 2000). This suggests that under drought stress, resistance to CO_2 diffusion from the substomatal cavity to the chloroplast (g_m) may be as important a limiting factor as g_s. Indeed, there is now some agreement that CO_2 influx limitations on photosynthesis are overriding under the majority of situations of drought stress, and include not only stomatal closure, but also decreased internal conductance to CO_2 (g_m) (Roupsard et al., 1996; Flexas et al., 2002; Ennahli & Earl, 2005).

2.2.4.2 *Effects of water stress on g_m*

To date, it has been shown that g_m is sensitive to water shortage and is decreased under water deficit conditions in several species, including grapevines (Flexas et al., 2002), oak trees (Grassi and Magnani 2005; Roupsard et al., 1996), soybean and tobacco (Flexas et al., 2006c), and ten Mediterranean species occurring naturally in the Balearic

Islands including two evergreen sclerophyll shrubs (*Pistacia lentiscus* and *Hypericum balearicum*), two evergreen sclerophyll semishrubs (*Limonium gibertii* and *Limonium magallufianum*), three summer semideciduous shrubs (*Lavatera maritima*, *Phlomis italica* and *Cistus albidus*), two perennial herbs (*Beta maritima* ssp. *maritima* and *B. maritima* ssp. *marcosii*), and an annual herb (*Diplotaxis ibicensis*) (Galmes et al. 2007). Finding any clear effects of drought stress on g_m would be interesting because they may, at least partially, explain non-stomatal limitations of photosynthesis.

2.2.4.3 *Recovery of photosynthesis following relief of stress*

The extent to which photosynthetic capability has the ability to recover rapidly following a transient exposure to water stress may play an important role in plant adaptation to drought environments. Countless researchers have reported effects of drought stress on plant growth (Delgado et al., 1992), photosynthesis (Boyer, 1970; Ogren and Oquist, 1985), plant cell metabolism (Bohnert et al., 1995; Nonami et al., 1997), etc. However, the vast majority of these studies were conducted by exposing the plants to water shortage stresses, then studying plants' responses under the stress conditions. Perhaps more important from an agricultural perspective is not the photosynthetic activity during the stress (which is usually minimal), but rather the ability of the crop to recover full photosynthetic competence once the stress is relieved.

Recognizing that the capability for photosynthetic recovery from an extreme water stress condition determines future growth and survival of plants in their habitat, some experiments have been conducted on the recovery of the photosynthesis rate from

drought stress (Boyer,1971; Subramanian and Maheswari, 1990; Djekoun and Planchon, 1991; Heckathorn et al., 1997; Widodo et al., 2003). However, factors affecting the degree of recovery of photosynthesis after relief of stress have not been fully understood.

Recently this topic has gained greatly attention (Ennahli and Earl, 2005; Miyashita et al., 2005; Flexas et al., 2006a; Galle et al., 2007; Galmes et al., 2007) and Galmes et al. (2007) have interestingly shown a residual reduction of g_m following re-watering. Moreover, for the first time Galmes et al. (2007) applied the photosynthesis limitation analysis proposed by Grassi and Magnani (2005) to survey ten different Mediterranean species, and showed that on the day after re-watering, limited recovery of g_m was the main limiting factor for photosynthesis recovery in many of these plants. On the other hand, in some species including the *Vitis* hybrid R-110 (*Vitis berlandieri* x *V. rupestris*), g_s reduction after re-watering showed a considerable limitation to photosynthesis recovery, while it increased the intrinsic water-use efficiency (Bogeat-Triboulot et al., 2007; Galle and Feller, 2007; Pou et al., 2008). More recently, Gomes et al. (2008) conducted a photosynthesis limitation analysis and indicated that mesophyll limitations were generally more important than stomatal limitations during recovery, but in this study, the effects of biochemistry and mesophyll diffusion conductance on mesophyll limitations were not separated.

When drought stress is relieved, photosynthesis may not immediately return to pre-drought levels. The recovery period depends on species, and after severe water shortage can sometimes take from weeks to even months in tree species, while stomata

are slow in regaining their pre-drought conductance, or damaged photosynthetic machinery is repaired (Kozlowski and Pallardy, 1997). Conversely, in field-grown maize, Earl and Davis (2003) found rapid and essentially complete recovery of leaf photosynthetic activity following rewatering, even under stress conditions that sharply reduced final crop yields.

2.2.5 *Canopy-scale effects of water stress on photosynthesis*

Soil water shortage causes reductions in whole canopy photosynthesis through two main mechanisms: i) decrease in interception of photosynthetically active radiation (PAR) due to reduced leaf area expansion, wilting, and early senescence of leaves and ii) decreased radiation use efficiency (RUE). Earl and Davis (2003) reported that reduced RUE was the dominant effect and that a decrease in PAR absorptance was of negligible importance except under very severe drought stress in maize. In contrast, Stone et al. (2001) indicated that sweet corn yield was significantly affected not only by reduced RUE, but also because of reduced total radiation interception, particularly for water deficit treatments applied during early growth stages.

2.3 *Water use efficiency*

2.3.1 *Definitions of water use efficiency*

From the farmer's perspective, water use efficiency means getting more crop yield per drop of irrigation water or rain, but for human society it means getting more value in terms of economic benefit and human nutrition per unit of water resource used. From the crop physiologist's perspective, water use efficiency is the ratio of total dry

matter production (phytomass) or economic yield to total crop evapo-transpiration. Thus, it can be expressed in the following equation (Hatfield et al. 2001):

$$WUE = Y / ET \qquad (8)$$

where WUE is water use efficiency, Y is total harvestable biomass and / or marketed yield, and ET, evapo-transpiration, is the total water lost via evaporation, both from the soil surface, and transpiration from plants.

As explained above, evaporation and transpiration are often considered as one parameter, namely evapo-transpiration, because it is obviously rather difficult to separate them especially in field measurements. In pots, these two factors can be easily separated and WUE is precisely determined by the gravimetric method, such as used by Earl (2003) .

Hatfield et al. (2001) recalled that de Wit (1958) showed a linear relationship between accumulated dry matter (Y) and cumulative transpiration (T) with high solar radiation. Subsequently, Hatfield et al. (2001) described this relationship by:

$$Y / T = m / T_{max} \qquad (9)$$

where Y is the total dry matter production, T is the transpiration, m is a coefficient, and T_{max} is the daily free water evaporation.

In addition, Richards et al. in 1991 expressed this term in another way:

$$WUE\ (biomass) = \frac{TE}{1 + E_s / T} \qquad (10)$$

where TE is the transpiration efficiency (above ground dry weight / transpiration), E_s is the water lost by evaporation from the soil surface, and T is water lost through transpiration by the crop.

As can be seen, there are many ways to define the term water use efficiency. However, it is generally defined in three different ways (Stanhill, 1986 cited by Guo et al. 2006):

1. Input-based WUE is the ratio of the yield or total biomass produced to total water inputs, i.e., precipitation plus rainfall.

2. At the whole plant or crop level, WUE may also be defined as the ratio of yield or total biomass produced to total water actually used (i.e., total evapotranspiration).

3. Instantaneous leaf-level WUE (WUE_L) is defined as the ratio of photosynthetic carbon assimilation rate (A_N), responsible for dry matter production, to the transpiration rate (T) (Udayakumar et al. 1998). Leaf level water use efficiency is also called *intrinsic water use efficiency* (Condon et al. 2002).

2.3.2 *Genetic variation for WUE*

Briggs (1914) and Shantz and Piemeisel (1927) first reported inter-specific variability in WUE of some crops including maize, sorghum, millet, wheat, oats, barley, potato, alfalfa and soybean (cited by Zhang et al. 1989). Since then, intraspecific genetic diversity for WUE has been reported by numerous researchers (e.g. , Farquhar and

Richards (1984) in wheat, Mian et al. (1996), Hufstetler et al. (2007), Earl (2002) and Walden (2008) in soybean, Ehdai and Waines (1993) and Farquhar and Richards (1984) in bread wheat, Hubick and Farquhar (1989) in barley, Martin et al. (1999) in tomato, Stiller (2005) and Fish and Earl (2009) in cotton, and Anyia et al. (2007) in barley).

2.3.3 *Improvement of water use efficiency*

There are many strategies to increase the water use efficiency. It is instructive to consider these in the context of the three different definitions of WUE discussed above:

1. Farm level improvement: many modern strategies for increasing whole plant level water use efficiency have been recommended and have already been incorporated into irrigation practices; for instance water-saving irrigation techniques and increasing canal network density to reduce runoff, seepage and unproductive evaporation, and modern agronomic practices such as better nutrient and weed management (Toung and Bhuiyan, 1999), suitable density for planting (Payne et al., 2001), and better distribution of planting (stand uniformity) (Ritchie and Basso, 2008).

Furthermore, breeding for plant characteristics that maximize water extraction capabilities, such as deep roots, high hydraulic capacities for water transport, and high stomatal conductance can sometimes increase farm level WUE simply by increasing total productive water use and consequently by decreasing the evaporation from the surface of the soil (Shan and Xu, 1991, cited by Guo et al. 2006; Richards et al. 2002; Ritchie and

Basso, 2008). To achieve further improvement in water use efficiency, researchers should focus on plant and leaf level factors.

2 and 3. Plant level and intrinsic level improvement: In spite of ongoing attempts to increase the plant level water use efficiency, progress in this area has been minor (Udayakumar et al. 1998; Johnson and Yangyang 1999).

Typically, a healthy plant transpires 700-1300 mol H_2O for the fixation of 1 mol CO_2 (Heldt, 1997). However, plants are different in terms of their capacity to minimize the amount of water lost per unit carbon fixed. One of the main reasons for these differences can be differences in intrinsic water use efficiency (WUE_L). As mentioned in equation (11) this parameter is the ratio of the net photosynthetic rate (A_N) to the transpiration rate (T). A_N is determined by stomatal conductance to CO_2 (g_c) and a concentration gradient of CO_2 between outside the leaf and inside the leaf (C_a-C_i) (equation 14) ; and T is controlled by stomatal conductance to H_2O (g_w) and the H_2O concentration gradient between inside and outside the leaf (W_i-W_a) (equation 12).

$$A_N = g_c (C_a - C_i) \qquad (11)$$

$$T = g_w (W_i - W_a) \qquad (12)$$

Because of a similar diffusion pathway for CO_2 and H_2O between the leaf intercellular air spaces and the atmosphere, and in harmony with equations 11 and 12, the intrinsic water use efficiency can be calculated by:

$$WUE_L = A_N / T = g_c (C_a - C_i) / g_w (W_i - W_a) \qquad (13)$$

Noting that the ratio of the diffusivities of CO_2 and H_2O in air is approximately 0.6, equation (13) can also be written as (e.g. Condon et al. 2002, 2004):

$$WUE_L = 0.6\,(1- C_i / C_a\,) / (\,W_i - W_a\,) \qquad (14)$$

According to equation (14), there are two possible ways to improve the leaf level water use efficiency. The first one is a decrease in the C_i / C_a value (or, increasing the value of $[1- (C_i / C_a\,)]$. The second is a reduction in the value of $(W_i - W_a)$. For non-stressed C_3 plants, the C_i/C_a ratio is typically about 0.7(Farquhar et al. 1989, cited by Guo et al., 2006; Condon et al. 2002), and controlled by the balance between the leaf internal "demand" for CO_2 or ''photosynthetic capacity'' (Condon et al. 2002) and the CO_2 diffusive process associated with the stomata, the stomatal conductance (g_s) (Udayakumar et al. 1998 ; Condon et al. 2002), although there is not any explicit mention of g_s in equation (14). In other words, the supply of leaf interior CO_2 is determined by stomatal conductance (g_s), while photosynthetic capacity or intrinsic mesophyll efficiency determines the demand for CO_2 WUE_L improvement is possible through a lower value of C_i / C_a, due to lower g_s and / or higher mesophyll efficiency (Udayakumar et al. 1998; Earl 2002). Plants with high WUE_L have been designated as either ''conductance types'' or ''capacity types'', depending on whether their advantage arises from differences in stomatal (g_s) or non-stomatal (mesophyll) factors, respectively, but both types of strategies may occur together in the same genotypes (Farquhar et al. 1989).

The WUE_L can theoretically be greatly improved by a relatively small change in the C_i/C_a ratio (equation 14). A decrease of 0.1 from 0.7 to 0.6 in the ratio of C_i/C_a theoretically causes a 33% growth in WUE_L which is proportional to $(1- C_i/C_a)$.

Lower T and hence biomass is often the result of increasing WUE. If C_i/C_a goes down as a result of increased photosynthetic capacity then CO_2 assimilation rate (A_N) per unit T will climb. In contrast, if C_i/C_a sinks as a result of lower stomatal conductance (g_s) then there will be a decline in A_N. Udayakumar et al. (1998) believed that this last issue is the main obstacle against any progress in WUE_L improvement.

It is notable that Farquhar et al. (1984) in wheat, Martin and Thorstenton (1993) in tomato, Acevedo (1993) in barley, Meinzer et al. (1990) in coffee and Lu et al. (1996) in cotton (all cited by Udayakumar et al. in 1998), and Earl (2002) in soybean showed that the genetic variability in WUE_L was caused by stomatal factors. Conversely, Condon et al. in wheat, Hubick et al.(1988) in groundnut, Hall et al. (1993) in cowpea, White (1993) in beans and Matus et al. (1995) in canola (all cited in Condon et al. 1998) reported that the variation in C_i and hence WUE_L was dependent on mesophyll factors.

As mentioned above, in ''conductance types'' lower C_i/C_a to improve WUE_L brings about a reduction in A (and consequently, biomass). There is also probably another disadvantage associated with a reduction in stomatal conductance (g_s). Any reduction in g_s will be followed by increased leaf temperature and W_i unless the boundary layer conductance of the leaf is very large. Then, because of this increase in W_i, the water

vapour gradient between the air inside and outside the leaf ($W_i - W_a$) will increase and hence, transpiration (T) per unit g_s will rise (Condon et al. 2002, and 2004). Even so, the proven existence of differences in WUE among genotypes that differ in WUE_L because of differences in g_s (i.e. conductance type WUE differences) indicates that this trait may still be a legitimate target for genetic improvement.

2.3.4 *Genetic markers for WUE*

Lin et al. (1998, cited by Bari et al., 2005) reported that in tomato 22 genomic regions distributed on 11 chromosomes were controlling WUE, where each trait had its own unique set of "Quantitative Trait Loci" (QTLs). These data demonstrated that there is a number of linked markers for WUE parameters and WUE is a polygenically (quantitatively) controlled trait in various environments (Martin et al. 1999). Also, in soybean, Mian et al. (1996) found molecular markers associated with WUE; and Bari et al. (2005) found significant genotypic heterogeneity in a soybean population of recombinant inbred lines, with the responsiveness to water abundance being a key contributor to higher mean yield.

Traits such as yield and WUE are controlled by more than one pair of genes. These kinds of traits are called quantitative, polygenic, multifactorial or complex (Collard et al. 2005). "The regions within genomes that contain genes associated with a particular quantitative trait are known as quantitative trait loci (QTLs)" (Collard et al. 2005).

Because there is genetic diversity in WUE, using molecular markers linked to QTL conditioning WUE has been suggested as an indirect criterion to improve water use efficiency (Mian et al., 1998). There have subsequently been a number of studies the have mapped QTL for water-use efficiency (WUE) in crops (Xu et al., 2004 cited by Bari et al., 2005).

2.3.5 *Leaf dark conductance as an indicator of WUE*

Dark-adapted epidermal conductance (g_{dark}) is water lost through the leaf epidermis and stomata in plants adapted to dark conditions, when stomatal conductance is minimal or zero.

2.3.5.1 *Measurement of g_{dark}*

To estimate g_{dark}, leaf gas exchange can be measured on an attached leaf using an open flow system or on a freshly detached leaf using a closed re-circulating system. Walden (2009) using both methods mentioned above examined the correlation between g_{dark} and WUE (see below), and found the best correlation when g_{dark} was measured using the closed recirculating system and freshly detached leaves.

2.3.5.2 *Variation for g_{dark}*

So far, a few researchers have reported g_{dark} variation, such as Fish and Earl (2009) in cotton and Hufstetler et al. (2007) and Walden (2009) in soybean. For instance, Walden (2009) showed significant variation for g_{dark} among 12 Ontario-adapted soybean

varieties. She identified two existing mapping populations whose parents differed for the g_{dark} trait: "OAC Salem x Nattosan", and "AC Colibri x OT91-3".

2.3.5.3 *Correlation between WUE and g_{dark}*

In 2007, Hufstetler et al. reported a range of about 20% in dry matter-based WUE among 23 soybean varieties, breeding lines and plant introductions and demonstrated the unique finding that another physiological parameter, namely g_{dark}, was strongly negatively correlated with whole plant WUE ($r = -0.74$, $P < 0.0001$). A similar correlation between WUE and g_{dark} was subsequently found in cotton ($r = -0.75$, $P<0.0001$), in a comparison involving 22 commercial cultivars, primitive race stocks and converted lines (Fish and Earl, 2009). Walden (2009) also found a stronger correlation between WUE and g_{dark} under water replete conditions ($r = -0.70$, $p = 0.01$) than across two different watering treatments ($r = -0.64$, $p = 0.03$) in twelve Ontario-adapted soybean genotypes, including six conventional and six RR (glyphosate-tolerant) varieties.

2.3.5.4 *Physiological basis for the correlation between WUE and g_{dark}*

Earl (2002) confirmed that WUE in soybean may be related to differences in leaf C_i, consistent with established theory (Farquhar et al., 1989) about the physiological basis of genotypic differences in WUE. However, it is not clear why WUE should be strongly related (negatively correlated) to g_{dark}. Muchow and Sinclair (1989) found g_{min} (minimum epidermal conductance in a wilted leaf) to be strongly positively correlated with stomatal density (number of stomata per unit leaf area) in sorghum. They reasoned that since the stomatal complex itself is not as well cuticularized as the rest of the

epidermis, even when stomata are closed, leaves with high stomatal density would also have high evaporation through the epidermis. Following from this, one possible explanation for the correlation between WUE and g_{dark} in soybean is that genotypes with high stomatal density also tend to have high stomatal conductance, and therefore high C_i and therefore low WUE_L, leading ultimately to low whole-plant dry matter-based WUE.

Walden (2009) mentioned that water stress reduced stomatal density significantly and significant genotype differences in stomatal density were found, but there was no "genotype x treatment" interaction for stomatal density. Most importantly, stomatal density was not found to be correlated with either g_{dark} or WUE (Walden, 2009). Hence, g_{dark} does not seem to be related to stomatal density in soybean. Instead, g_{dark} (again, measured on dark-adapted leaves) appears to accurately predict the stomatal conductance and C_i of those same leaves during steady-state photosynthesis (Walden, 2009). She also reported a strong correlation between g_{dark} and either g_s or C_i, which is a very surprising result, since there are no previous reports of dark-adapted leaf conductance to water vapour serving as an accurate predictor of leaf gas exchange activity in the light. Because Walden (2009) illustrated that g_{dark} was an accurate predictor of C_i, it could be an accurate predictor of WUE as well.

2.3.6 *Effects of water stress on WUE*

There are contradictory observations regarding the effect of drought stress on WUE in the literature. Many researchers have worked to figure out the reaction of dry matter-based WUE to water shortage to find a way to increase WUE under drought

stress as a drought tolerance criterion. However, plant reaction to water deficit for WUE (dry matter-based) did not follow a particular trend. For example, Zhao et al. (2004) reported that dry matter-based WUE increased with water stress up to tillering, but decreased with water stress after tillering. Liu et al. (2005) indicated that WUE was improved at mild soil water deficits. Walden (2009) reported a decrease in WUE in soybean under cyclic drought conditions compared to a water replete treatment. In contrast, Earl (2002), also in soybean, found that WUE was higher in a drought treatment, while Hufstetler et al. (2007) reported no significant difference in WUE of soybean between a drought treatment and a water replete treatment.

It seems that plant reaction to drought stress in terms of dry matter-based WUE is a function of plant species, phenological stage, genetic background, the timing of the water shortage treatment, and its severity.

2.3.6.1 *Inverse relationship between WUE_L and C_i*

Short-term measurement of CO_2 and H_2O vapour exchange can be used to instantaneously measure WUE_L, (Ehleringer et al., 1986; Garten and Taylor, 1992). Measurements of gas exchange are then used to estimate substomatal (internal) CO_2 concentration (C_i) (Farquhar et al., 1982; Ehleringer et al., 1986; Ehleringer et al., 1987). Earl (2002) confirmed that WUE in soybean may be related to differences in leaf C_i; plants that had lower C_is showed higher WUE, as predicted by equation (14).

2.3.6.2 *Effects of g_m on C_c and WUE*

By the theory presented above, WUE varies inversely with the C_i / C_a ratio. Since the plant has essentially no control over C_a, in practice this means that plants that maintain low C_i should have high WUE, or at least high WUE_L. With this in mind, it is clear that g_m can directly affect WUE_L. At a given stomatal conductance, a high g_m results in a higher C_C, and therefore supports a higher A_N. A higher A_N at the same stomatal conductance, by mathematical definition (Eqn. 14) results in a lower C_i, and therefore a higher WUE_L (Warren at al., 2008; Flexas et al., 2008). However, to date there are no examples in the literature where genotypic differences in either WUE_L or WUE could be attributed to differences in g_m *per se*.

CHAPTER 3

Screening of Parents of Soybean Mapping populations for Variation in Water Use Efficiency and Dark-Adapted Leaf Epidermal Conductance

3.1 ABSTRACT

To improve crop yields under water stress, increasing crop water use efficiency (WUE, the amount of crop dry matter produced per unit water transpired) is a major demand and challenge at present. One problem is that WUE is very difficult to measure in the field, which makes improvement difficult. However, a recent finding showed that dark-adapted leaf epidermal conductance (g_{dark}) is strongly correlated to WUE. This is potentially important, because of the relative ease of g_{dark} measurement in the field. The discovery of any shared quantitative trait loci (QTLs) conditioning both WUE and g_{dark} would further support the use of g_{dark} as a surrogate measurement for WUE in the field, and could facilitate future breeding activity for WUE improvement. Identifying QTL controlling both WUE and g_{dark} first requires the identification of mapping populations with parents that differ for the traits. A greenhouse study was conducted to compare parents of three existing mapping populations for both WUE and g_{dark}. Among three sets of recombinant inbred line (RIL) population parents, one set was found with significant ($P < 0.01$) parental differences for both WUE and g_{dark}. However, the difference in WUE was only 6% of the biparental mean value, which was not considered sufficient to pursue phenotyping of the entire RIL population for QTL identification. Consequently, it is suggested to screen parents of additional RIL populations in the future, to identify populations with more extreme values for both traits.

3.2 INTRODUCTION

To meet the world population forecasted food demand (Wallace, 2000), global food production must be increased. However, with current changes in the global environment, namely global warming, more frequent severe drought stresses are expected to be experienced by crops (Keeling et al., 1995). Even under current climatic conditions, water stress is one of the most important factors restricting soybean yields under dryland conditions in North America (Specht et al., 1986), including in Ontario (H.J. Earl, unpublished data). Hence, it is important to seek opportunities to improve soybean productivity under conditions of limited soil water availability. One approach is to increase water use efficiency (WUE, amount of crop dry matter produced per unit soil water transpired) of commercial soybean varieties. Hence, WUE improvement should be a target of breeders.

Briggs and Shantz (1914) and Shantz and Piemeisel (1927) (both cited by Zhang et al., 1998) first reported inter-specific variability for WUE in some crops. Since then, intra-specific genetic diversity for WUE has been reported by numerous researchers. For instance, Mian et al. (1996), Hufstetler et al. (2007) and Walden (2009) found variation for WUE in soybean. Thus, it seems that there is potentially good opportunity to improve WUE. However, the difficulty of measuring WUE in the field is a major obstacle preventing improvement. On the other hand, Fish and Earl (2009), Hufstetler et al. (2007), and Walden (2009) reported the surprising finding that a leaf trait, dark- adapted leaf epidermal conductance (g_{dark}), was closely related to whole-plant WUE. This finding is significant because g_{dark} is much simpler to measure than WUE, so it could possibly

serve as a surrogate measurement for WUE even in field experiments. Furthermore, the unexpected strong correlation between WUE and g_{dark} may provide further insight into the genetic basis of the naturally-occurring variation for WUE in these species.

Evidence suggests that WUE is a polygenic trait. Brendel et al. (2006) detected ten quantitative trait loci (QTL) for WUE in *Quercus robur* based on carbon isotope discrimination and Julier et al. (2010) found nine markers/alleles with a significant effect on WUE variation in alfalfa. Based on these and another study done by Mian et al. (1996) who showed four quantitative trait loci (QTL) conditioning WUE, it can be concluded that WUE is a quantitatively controlled trait in soybean, and so it is expected to be conditioned by multiple QTL. Using molecular markers linked to QTL, conditioning WUE as an indirect criterion to improve water use efficiency has been suggested by Mian et al. (1996). There have subsequently been a number of studies that have mapped QTL for WUE in crops (e.g. Bari et al., 2005; Xu et al. 2004).

By contrast, few researchers have investigated the genetic diversity for g_{dark}, (e.g. Hufstetler et al. (2007) and Walden (2009) in soybean and Fish and Earl (2009) in cotton). Moreover, no QTL have ever been reported for g_{dark} in any species. Discovery of common QTL conditioning both WUE and g_{dark} would further solidify the genotypic relationship between these two traits. This is important because:

1. If g_{dark} really is highly predictive of WUE, one could measure g_{dark} instead of WUE to more efficiently identify genetic diversity for WUE in

the available soybean germplasm. This diversity could then be introduced into elite lines to increase WUE.

2. g_{dark} could also be used in lieu of WUE in RIL phenotyping efforts to identify additional QTL controlling WUE.

3.3 RESEARCH OBJECTIVE

To better understand the genetic relationship between WUE and g_{dark}, this study was designed to identify parents of existing recombinant inbred line (RIL) populations that show differences for WUE and g_{dark} , these would allowing for QTL mapping of these traits.

3.4 MATERIALS AND METHODS

3.4.1. *Plant materials*

To find appropriate populations for phenotyping and subsequent mapping of QTL controlling WUE and g_{dark}, parents of several existing soybean mapping population were chosen for screening, including "OAC Salem x Nattosan", "AC Colibri x OT91-3", and "AC 756 x RCAT Angora", which were reported by Walden (2009) to have parents with extreme value of g_{dark}, and also "Heinong-38 x OAC Millennium", with different origins (Figure 3.1). However, one line (OAC Salem) had poor seed viability; in six replications with 10 seeds planted per pot, no seedling emergence was seen. Hence, the experiment was only conducted with the remaining three pairs of mapping population parents mentioned above.

3.4.2 *Greenhouse culture*

A greenhouse study was conducted at the University of Guelph (Guelph, Ontario) between December and February 2009. The culture system used in this experiment was developed over one year of preliminary studies. All parents were grown in 2.5-L white plastic containers without drainage holes, filled with 2400 g of a 2:1 by volume mixture of granitic (non-calcareous) sand (B-sand, Hutcheson Sand Mixes, Huntsville ON) and top soil (a triple mix of one part top soil: one part peat moss: one part composted manure, Meadowville Gardens, Guelph) with a pH of 7.5 after fertilizing and watering.

For each replication, two additional pots were prepared, with drainage holes. These were saturated with water, capped with lids with a small hole, and then allowed to

drain overnight to determine the saturated weight. Next, the soil from those pots was dried in a forced air oven at 80°C for about 48 h to constant weight to determine the soil dry weight. These data were used to calculate the water holding capacity of the medium.

A commercial fertilizer (20-20-20 plus micronutrients, Plant Products Co. Inc., Brampton ON) was added as a 1% solution (w/v) at the rate of 100 mL per pot before planting. Additional water was added to bring the soil water content to 75% of pot capacity and then 10 seeds were planted per pot, each inoculated with 1 mL of commercial liquid inoculant (*Bradyrhizobium japonicum* and a patented strain of *Bacillus subtilis*, Becker Underwood, Saskatoon, SK, Canada). Approximately one week after planting, at the VC growth stage, seedlings were thinned to one per pot and an additional 120 mL of fertilizer solution was applied per pot. Then, pots were capped with fitted white lids, each with two 5-mm diameter holes, one for the seedling to grow through and another one to water the plants using a funnel (Figure 3.2).

Plants were arranged in a Randomized Complete Block Design (RCBD) with eight replications, planted sequentially with a 5-d interval between replications. Greenhouse conditions were day / night temperature settings of 25 / 18°C and a 16-h photoperiod. Day length was extended with overhead high pressure sodium and metal halide lamps delivering an additional flux of photosynthetically active radiation (PAR) of approximately 600 $\mu mol\ m^{-2}\ s^{-1}$ at the tops of the pots during the experiment.

Plants in the greenhouse were kept well watered at all the times by frequent watering with a semi-automated computer controlled watering system (Figure 3.3). Each

pot was placed on an electronic balance, and then identified by scanning a bar code label on the pot. Its current weight was recorded by the computer software, and then the pot received water through 3-mm diameter tubing connected to a solenoid valve until it reached the target weight, after which the final pot weight was also recorded. Pots were watered frequently enough to maintain the relative soil water content above 50% of pot capacity; in this culture system this is adequate to avoid even mild water stress (Earl, 2003). The pots were weighed every other day until the V3 stage, and then every day thereafter.

3.4.3 *Measurement of g_{dark}*

Forty days after planting, when plants were at approximately the R1 growth stage (first flower), all pots were watered to their target weight at the end of the day (8:00 pm), then moved to a dark room at 20°C. After 40 h of dark adaptation, gas exchange measurements were made on two leaf positions per plant: one leaflet from the second youngest fully expanded leaf, and another from the third youngest fully expanded leaf. Measurements were made in a custom-made closed (recirculating) gas exchange system, consisting of a 0.7-L PVC plastic chamber with removable lid, connected to an LI-840 gas analyser (Licor Inc., Lincoln NE) (Figure 3.4). Measurements were made on freshly detached leaflets. Care was taken not to expose the leaflets to light levels that would induce stomatal opening; all procedures were carried out in dim light (< 3 μmol m^{-2} s^{-1}) from a green light source. Following the gas exchange measurement, the leaflet area was determined using an LI-3100C leaf area meter (Licor) (Figure 3.5).

After putting the leaflet in the chamber and sealing the chamber, data were recorded for a period of approximately 180 s at 5-s intervals. Water vapour concentration [H_2O] within the sealed chamber gradually increased at a declining rate during the course of the measurement (Figure 3.6). In all cases a second order polynomial regression with an R^2 higher than 0.98 could be fit to the [H_2O] versus time data; the slope of the tangent to this curve was calculated as the first derivative of the fitted curve at the midpoint of the measurement. Then, the transpiration rate (E, in mol H_2O m^{-2} s^{-1}) was calculated as:

$$E = \alpha \times n / A,$$

where α is the slope of the [H_2O] / time curve at the midpoint time (mol H_2O mol^{-1} air s^{-1}), A is the leaf area (m^2), and n is the number of moles of gas within the measurement system, calculated as:

$$n = pV / RT,$$

where p is the absolute pressure (kPa) of air in the chamber, V is the volume of gas in the chamber (L), T is the Kelvin temperature of the air inside the chamber at the midpoint time of the measurement, and R is the gas constant (8.3144 kPa L mol^{-1} K^{-1}). Then, leaf dark-adapted epidermal conductance (g_{dark}), in mol m^{-2} s^{-1}, was calculated as:

$$g_{dark} = E / (W_i - W_a)$$

where W_a is the water vapour concentration inside the chamber at the midpoint time of the measurement (mol H_2O / mol water), and W_i was the leaf internal water vapour concentration. Presuming leaf internal air was at the saturation vapour pressure (e_S) estimated as:

$$e_S = 0.61121e^{(17.502 \times t) / (240.97 + t)} \text{ (Buck, 1981)}$$

where t is the leaf temperature (°C), then, W_i was calculated as:

$$W_i = e_S / p.$$

3.4.4 *WUE measurement*

Following gas exchange measurements, shoots were harvested, roots thoroughly washed and patted dry with paper towel, then root and shoot fresh weights (including the leaves used for g_{dark} measurement) determined. After that, roots and shoots were dried to constant weight in a forced air oven at 80°C, and the dry weights were measured. Then, WUE was calculated as:

$$WUE = \frac{DW}{[Water - (W_{final} - W_{initial})] + (FW - DW)} \times 1000$$

where WUE is whole plant water use efficiency, DW is total plant dry weight, FW is the total plant fresh weight, water is total water added to the pot between the capping and harvest dates, W_{final} is the final pot weight after watering the pot on the harvesting date, and $W_{initial}$ is the initial pot weight (final weight of pot after watering on the first day of capping).

3.4.5 *Data analysis*

The results were analysed using Proc Mixed in SAS (version 9.2 SAS Institute, Cary, NC, USA, 2007). An analysis of residuals was used to identify observations that were outliers, and then outliers were removed; to perform this test, the internal

studentized residual was computed for each observation. Then the observations with internal studentized residuals having an absolute value higher than the critical value at a Type I error rate of 0.05 for Lund's test of studentized residuals were declared outliers (Bowley, 2008). Next, a Type III error (rate of 0.05) was used for analysis of variance because of some missing data caused by removing the outliers. The two-sided $LSD_{0.05}$ was calculated for each pair of population parents from the standard error of LSMEANS to determine any significant difference between two parents in terms of WUE and/or g_{dark}.

3.5 RESULTS

As can be seen in Tables 3.1 and 3.2, there were significant differences between Heinong-38 and OAC Millennium for g_{dark} and WUE ($p = 0.0004$ and $p = 0.009$, respectively). In contrast, AC756 and RCAT Angora , and AC Colibri and OT91-3 showed significant differences for WUE but no significant difference for g_{dark}.

Comparing Figure 3(A) and Figure 3(B), the parents with higher g_{dark} showed lower WUE, except for "AC Colibri × OT91-3", two parents with the lowest no significant difference for WUE.

3.6 DISCUSSION

No common markers for WUE and g_{dark} have been identified to date. To undertake a QTL analysis to find common QTL concurrently controlling these two traits, it was necessary to have at least one pair of parents with extreme values for both traits.

Heinong-38 and OAC Millennium differed significantly for both traits. Although the difference in g_{dark} was 42% (Table 3.1), it was only around 6% for WUE (Table 3.2), which was not considered sufficient to pursue phenotyping of the entire RIL population for QTL identification. AC Colibri and OT91-3 also differed significantly for both traits, but the differences were even smaller than for Heinong-38 and OAC Millennium. So, this study was not successful in identifying an existing RIL population with sufficiently large differences in both WUE and g_{dark} to justify undertaking the phenotyping effort required to complete a QTL analysis. Parents of additional soybean RIL populations

should be screened as they become available. Alternatively, the possibility exists that one of the populations considered in the present work might still be suitable, if the RILs showed transgressive segregation, i.e. some RILs with significantly higher or lower WUE than either parent because of genetic segregation. Potentially, the RIL progeny of these parents could be suitable for subsequent phenotyping.

In the present work, in both cases where significant differences in both WUE and g_{dark} were found, the parent with higher g_{dark} had lower WUE. This is in agreement with the previous findings of Fish and Earl (2009; cotton), Hufstetler et al. (2007; soybean) and Walden (2009; soybean) who found a negative correlation between WUE and g_{dark}.

Table 3.1 Comparison of g_{dark} of parents of soybean mapping populations (LSMEAN±SE).

Genotype	n	g_{dark} (mmol $m^{-2} s^{-1}$)	p-value
AC Colibri	8	34.1±3.0	
OT91-3	8	26.7±3.0	0.06
Heinong-38	8	37.5±3.0	
OAC Millennium	7	22.0±3.2	0.0004
AC 756	6	22.4±3.4	
RCAT Angora	7	21.2±3.2	0.77

Table 3.2: Comparison of WUE of parents of soybean mapping populations(LSMEAN±SE).

Genotype	n	WUE (g L^{-1})	p-value
AC Colibri	8	4.00±0.06	
OT91-3	8	4.20±0.06	0.03
Heinong-38	8	4.16±0.06	
OAC Millennium	7	4.41±0.07	0.009
AC 756	7	4.20±0.07	
RCAT Angora	7	3.89±0.07	0.002

Figure 3.1: Mapping population parents.

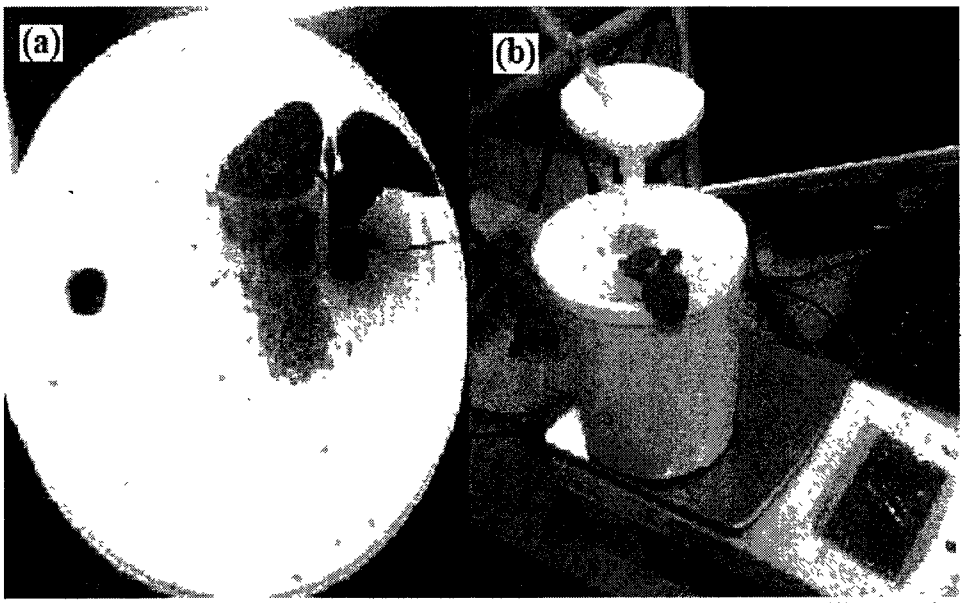

Figure 3.2: Fitted lids with two holes - one to accommodate the seedling, and another to water the plants using a funnel.

Figure. 3.3. Computer-automated weighing and watering system.

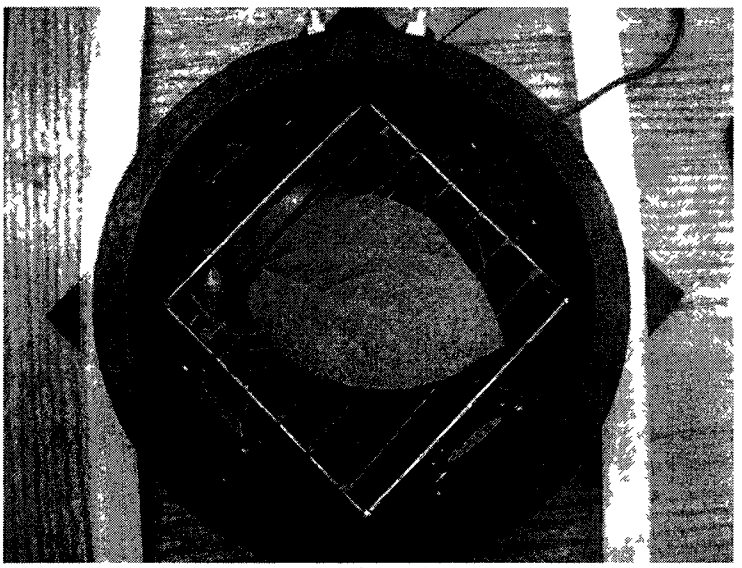

Figure 3.4: Dark-adapted leaf conductance (g_{dark}) was measured on freshly detached leaves in a closed, re-circulating gas exchange system (chamber shown with lid removed).

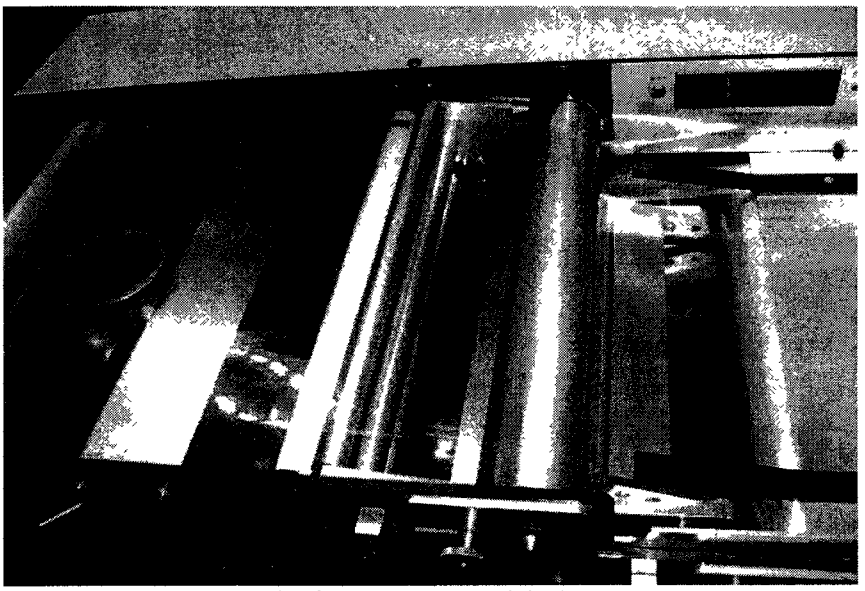

Figure 3.5: LI-3100C leaf area meter (LICOR) to measure leaflet area

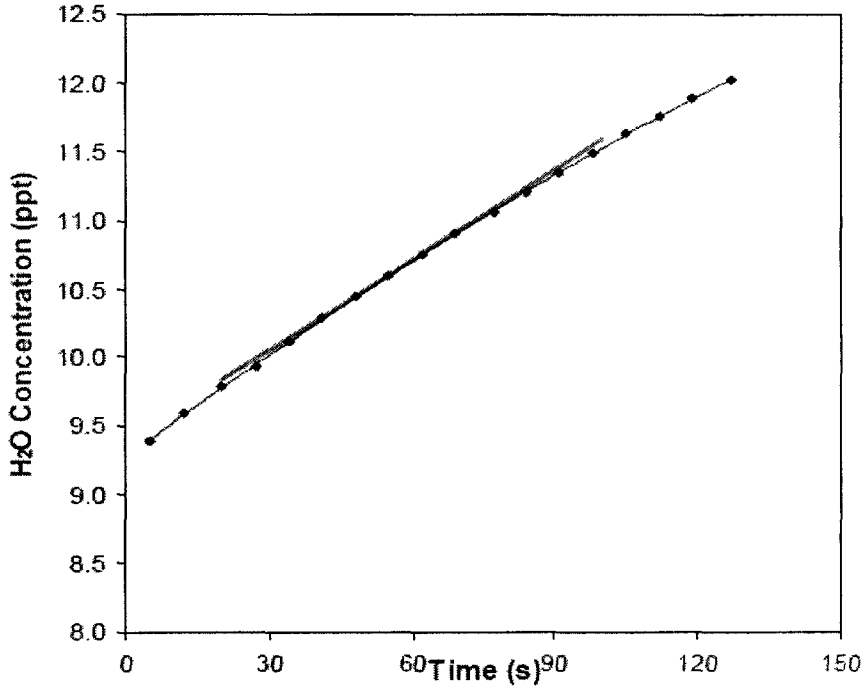

Figure 3.6: Increase in chamber [H_2O] over time due to transpiration from a dark-adapted freshly detached leaf in the chamber of the closed gas exchange system. The transpiration rate was calculated from the slope of the tangent line at the mid point of measurement.

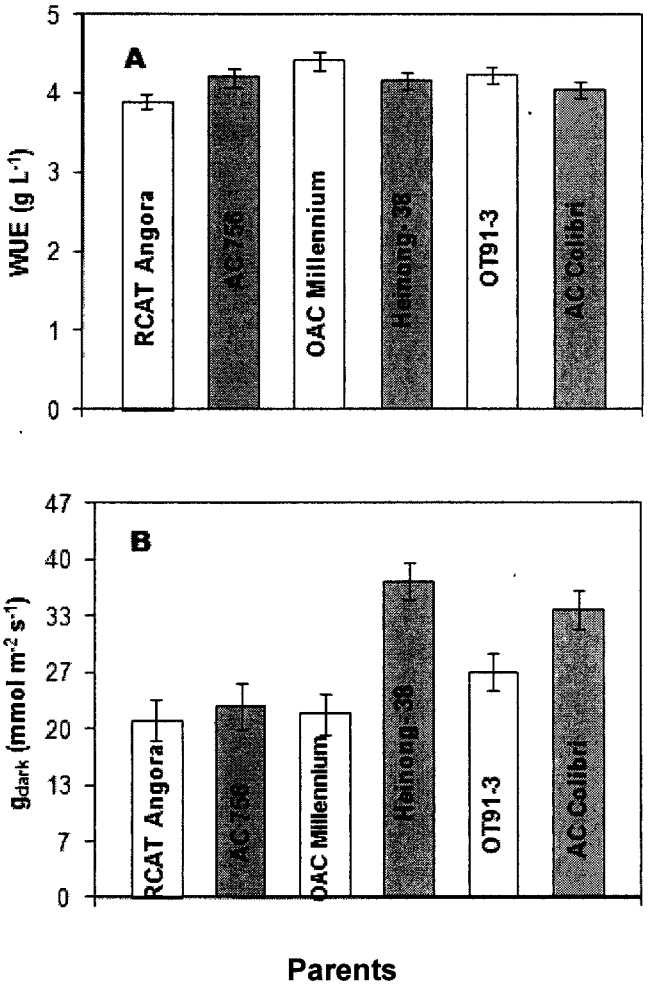

Figure 3.7: (A) water use efficiency (WUE) and (B) dark- adapted epidermal conductance (g_{dark}) for three different pairs of parents of soybean mapping populations. Error bars represent the 95% confidence interval of each LSMEAN.

CHAPTER 4

Component Limitations to Photosynthesis in Soybean During Recovery from Drought Stress

4.1 ABSTRACT

It is generally agreed that water stress reduces photosynthesis in soybean and other species by inducing stomatal closure and thus reducing leaf internal CO_2 concentrations. However, less is known about the magnitude and physiological basis of residual limitations to photosynthesis that may persist following relief of water stress. A controlled environment experiment was conducted to compare six Ontario-adapted commercial soybean cultivars for their ability to recover photosynthetic capacity following a simulated water stress. Plants were exposed to two cycles of controlled soil dry down over a period of two weeks in a greenhouse. The water stress treatment reduced both shoot dry matter and total plant water use by approximately 50%, with no effect on whole plant water use efficiency. Combined leaf gas exchange and chlorophyll fluorescence analysis was used to quantify component limitations to leaf photosynthesis of fully expanded leaves 24 h after re-watering. No treatment x cultivar interactions were found for leaf-level measurements, so analysis was combined across cultivars. Compared to control plants, plants that had been exposed to water stress had reduced stomatal conductance and also lower leaf net CO_2 assimilation rates. However, gas phase leaf internal CO_2 concentrations were only slightly reduced. In contrast, chloroplast CO_2 concentrations were strongly reduced, as was mesophyll conductance to CO_2. It is concluded that increased resistance to CO_2 diffusion between the substomatal cavity and chloroplasts constitutes a major component of the persistent limitation to photosynthesis in soybean following recovery from water stress.

4.2 INTRODUCTION

A soybean crop requires roughly 450–700 mm of water during its 90- to 120-day growing season (Doorenbos and Kassam, 1979 cited by Dogan et al. 2007), so soybean growth and yield can be impacted by water deficit when in-season rainfall and stored soil moisture are insufficient to meet this demand. In many soybean production regions, water shortage is the most significant environmental factor limiting growth and yield (Araus et al., 2002; Ashley and Ethridge, 1978; Batchelor et al., 2002; Chaves et al., 2003; Cooper et al., 1991; Cox and Jolliff, 1986; De Costa and Shanmugathasan, 2002; Doss et al., 1974; Dogan et al., 2007; Frederick et al., 2001; Frederick and Hesketh, 1991; Karam et al., 2005; Korte, 1983; Meckel et al., 1984; Mederski and Jeffers, 1973; Pandey et al., 1984; Sinclair et al.,1992; Sionit and Kramer, 1977; Smith and Griffiths, 1993) mainly because of photosynthesis depression (Quick et al., 1992; Flexas et al., 2002; Lawlor and Cornic, 2002; Monclus et al., 2006; Galle et al., 2007).

Although photosynthesis responses of plants to water deficit have been the subject of study by plant physiologists for decades, in the majority of such studies the focus has been on physiological responses during the actual drought stress treatments themselves. In a crop production context, of equal or perhaps greater importance is the ability of the crop to regain full photosynthetic capacity once the soil water deficit stress is relieved, since crops are very often exposed to short term, cyclic drought stress episodes. Only recently has this aspect of drought stress physiology received appropriate attention (e.g. Ennahli and Earl, 2005; Miyashita et al., 2005; Flexas et al., 2006a; Galle et al., 2007; Galmes et al., 2007).

In the classical view, photosynthetic limitations induced by water stress were broadly divided into two categories: stomatal vs non-stomatal. Stomatal limitations arise from stomatal closure during water deficits, leading to an increased resistance to diffusion of CO_2 from the atmosphere into the leaf interior, and therefore a reduced leaf internal CO_2 concentration (C_i). The value of C_i is calculated from leaf gas exchange measurements; C_i specifically estimates the CO_2 concentration in the gas phase in the sub-stomatal cavity. In some cases it was observed that photosynthetic rates were suppressed more than could be accounted for by stomatal effects (that is, by the reduction in C_i); such residual effects were considered to be "non-stomatal", and were generally assumed to be biochemical in nature (e.g. Ephrath, et al., 1993, cited by Ennahli and Earl, 2005; Faver et al., 1996; Medrano et al., 2002). However, in recent years this view has changed, since it has become apparent that non-stomatal limitations also have a significant diffusive component. Specifically, the resistance to CO_2 diffusion from the sub-stomatal cavity to the carboxylation site in the interior of the chloroplast is similar in magnitude to the stomatal resistance (see reviews by Warren, 2008 and Flexas et al., 2008). That is, in addition to reductions in stomatal conductance (g_s), water stress may also reduce the mesophyll conductance to CO_2 (g_m). This shift in understanding has important implications for the search for specific traits or even specific genes that could enhance crop productivity under water stress.

4.3 RESEARCH OBJECTIVES

1. To compare six commercial soybean cultivars for their ability to recover photosynthetic capacity following relief of a severe, cyclic drought stress treatment.

2. To determine the relative importance of stomatal vs mesophyll resistance to CO_2 diffusion, as component limitations to photosynthesis following recovery from drought stress in soybean.

4. 4 MATERIALS AND METHODS

4.4.1 *Plant materials*

Six Ontario-adapted, commercial soybean varieties, including OAC Bayfield, OAC Lakeview, RCAT Pinehurst, RCAT Corbett, RCAT Matrix, and 26-02R already known to differ for dark-adapted leaf epidermal conductance (g_{dark}; Walden, 2009) were grown in a University of Guelph greenhouse from March 24th, 2010 to April 30th, 2010.

4.4.2 *Growth medium*

A 2:1 v/v mixture of granitic sand (B-sand, Hutcheson Sand Mixes, Huntsville ON) and a peat-based potting mix (Premier pro-mix PGX, Premier Horticulture Inc., Quakertown, PA) were used as a medium for this experiment. The second fraction of the medium was different from the previous experiment (Chapter 3) because nutrient deficiency symptoms were observed with that previous system, perhaps attributable to inconsistencies in the composition of the commercial topsoil fraction. Then, 2.5-L white plastic containers without drainage holes were filled with 3400 g of the soil mixture.

For each replication, two additional 2.5-L pots were prepared, with four drainage holes covered by nylon screen. These were saturated with water, capped with lids with a small hole, and then allowed to drain overnight to determine the saturated weight. Next, the soil from those pots was dried in a forced air oven at 80°C to constant weight to determine the soil dry weight. These data were used to calculate the water holding capacity of the medium.

4.4.3 *Fertilizing and planting*

After adding water to bring the soil water content to 50% of pot capacity, a commercial fertilizer (20-20-20 plus micronutrients, Plant Products Co. Inc., Brampton Ontario, Canada) was added as a 1% solution (w/v) at the rate of 100 mL per pot. Ten seeds were planted per pot in holes 1 cm in depth, in two separate pots for each of the six varieties in each replication, and inoculated by 1 ml per seed of a commercial liquid soybean inoculant (*Bradyrhizobium japonicum* and a patented strain of *Bacillus subtilis*; Becker Underwood, Saskatoon, SK). Additional water was added to bring the soil water content to 75% of pot capacity.

After thinning to one seedling per pot at the V1 growth stage, another 100 ml of fertilizer solution was added to each pot, and pots were capped with fitted white plastic lids, each with two holes 1 cm in diameter, one for the seedling to grow through and another one to water the plants using a funnel. Pots were arranged in a Randomized Complete Block Design (RCBD) with 6 replications planted sequentially with a 1-d interval between replications, and grown in a greenhouse with day / night temperature settings of 25 / 18°C and a 16-h photoperiod. Overhead high pressure sodium and metal halide lamps delivered an additional flux of photosynthetically active radiation of approximately 600 μmol m^{-2} s^{-1} at the top of the pots (Figure 4.1).

4.4.4 *Treatments*

There were two watering treatments: (1) Water replete) plants were kept well-watered at all times by daily computer-automated weighing and watering of pots to

prevent water stress, and pot water content was maintained between 55 and 75% relative soil water content (RSWC), which is sufficient to prevent water stress in this culture system (Hufstetler et al., 2007); (2) Cyclic drought stress) beginning at the V2 plant growth stage the RSWC of every each pot was allowed to decline by 10% per day over one week (from 75% to 15%) and then maintained at 15% for one more day. Then pots were watered to 75% of RSWC for one day, and then the 1-week drought cycle was initiated again. At the end of the second cycle the RSWC was returned to 75%, and the plants were allowed to recover for 24 h prior to making gas exchange and chlorophyll fluorescence measurements.

4.4.5 *Fluorescence and gas exchange measurement*

At 24 h after re-watering, gas exchange and fluorescence measurements were made using two Portable Photosynthesis Systems (LI-6400, LI-COR Inc., Lincoln NE) each equipped with an LED-based fluorescence / light source attachment called the Leaf Chamber Fluorometer (Model 6400-40) (Figure 4.2) . For each measurement, one attached leaflet of the second youngest fully expanded leaf was put in the chamber such that it completely covered the 2-cm^2 circular chamber area. Leaf temperature was maintained at 25°C using the chamber's Peltier coolers, and chamber CO_2 concentration was controlled at 360 $\mu L\ L^{-1}$ using the system's CO_2 injector (Model 6400-01, LI-COR). The sample side (chamber) flow rate was 250 $\mu mol\ s^{-1}$. Measurements were made at two PPFD levels - 250 and 1200 $\mu mol\ m^{-2}\ s^{-1}$ - provided by a mixture of red (90%) and blue (10%) LEDs. The leaf was allowed to reach steady state stomatal conductance (g_s) and net CO_2 assimilation rate (A_N), and then leaf gas exchange data were recorded and the steady

state chlorophyll fluorescence signal (F_S) and maximum (light saturated) chlorophyll fluorescence signal (F'_M) were determined. The modulation rate of the fluorometer measuring light was 0.5 kHz for the determination of F_S, and then increased to 20 kHz during the saturation pulse protocol for determining F'_M. The "ramp pulse" protocol of the LI-6400 was used to estimate the true F'_M at infinite pulse intensity, similar to the method proposed by Earl and Ennahli (2004).

The quantum efficiency of Photosytem II (Φ_{II}) was calculated according to Genty et al. (1989):

$$\Phi_{II} = (F'_M - F_S) / F'_M,$$

Then, the Photosytem II linear electron flux (J_e) was calculated as:

$$J_e = \alpha \times f_{II} \times PPFD \times \Phi_{II},$$

(Loreto et al., 1994) where α is leaf absorptance of incident PPFD, and f_{II} is the proportion of α x PPFD absorbed by the light harvesting complex of PSII, assumed to be 0.5 for C_3 plants (Earl and Tollenaar, 1998).

The CO_2 concentration at the carboxylation site in the chloroplast (C_C) was calculated according to Lal et al. (1996) as:

$$C_C = (V_C/V_0) \times (O_C/K_S),$$

where V_C/V_0 is the ratio of carboxylation rate to oxygenation rate, which was calculated as:

$$V_C/V_0 = (J_e + 8A_G) / (2J_e - 8A_G), \quad \text{(Ennahli and Earl, 2005)}$$

where A_G is the gross CO_2 assimilation rate, estimated as:

$$A_G = A_N + R_d,$$

where A_N and R_d were net CO_2 assimilation rate and dark respiration rate, respectively. O_C is the partial pressure of oxygen at the carboxylation site of chloroplast, assuming that the atmospheric to chloroplastic O_2 concentration gradient is negligible (Gerbaud and André, 1987). K_S is the temperature-adjusted CO_2 / O_2 specificity of RuBisCO calculated as:

$$K_S = O_C /(2\Gamma^*)$$

where Γ^* is the CO_2 compensation point in the absence of dark respiration, calculated as per Bernacchi et al. (2001):

$$\Gamma^* = e^{(19.02-37.83/T/0.0083144)}/1000000$$

where T is leaf temperature (K).

Finally, the mesophyll conductance (g_m) was calculated according to Epron et al. (1995) as:

$$g_m = A_N / (C_i - C_C) ,$$

where C_i is the substomatal CO_2 concentration determined from gas exchange measurements.

4.4.6 *Leaf absorptance of actinic light (α)*

Leaf absorptance of photosynthetically active radiation (α) within the LI-6400 chamber was estimated for another leaflet of the same leaf used for the gas exchange / fluorescence measurements using an LI-1800-12B external integrating sphere (LI-COR, Figure 4.3), in combination with a diode array reflectance spectrometer (Unispec DC, PP Systems, Harverhill, MA, Figure 4.4). Only one channel of the spectrometer was used for these measurements, and the fibre optic of this channel was fitted to the appropriate port

of the integrating sphere using a custom made adapter. Each data scan consisted of 256 readings at 3.2-nm intervals between 300 and 1100 nm. The leaf sample was installed in the sample port of the sphere, adaxial surface facing inward, and a reference scan was made with the columnated halogen light source aimed at the internal reflective (barium sulphate) standard. Then, a reflectance scan was made with the light source aimed at the leaf sample inside the sphere, and finally the leaf was turned adaxial side outward and a transmittance scan was made with the light source aimed at the leaf surface from the outside of the sphere. For each leaf, fractional absorptance at the centre of each 3.2-nm waveband (A_λ) was calculated as:

$$A_\lambda = 1 - \text{reflectance}_\lambda \ / \ \text{reference}_\lambda - \text{transmittance}_\lambda \ / \ \text{reference}_\lambda$$

Finally, the total absorptance of photosynthetically active radiation (PAR) in the chamber (α) was calculated by multiplying A_λ in each 3.2-nm waveband by the fraction of total PAR from the light source in that waveband, and then summing the products.

4.4.7 *Leaf water status*

After taking three 2-cm diameter leaf disk samples from the same leaflet used for the gas exchange measurements, their fresh weight was recorded and then they were submersed in distilled water for 24 h at room temperature (25°C) to determine the leaf disk turgid weight. Next, the disks were dried in a forced air oven at 80°C for about 24 h to determine their dry weight. The leaf relative water content (LRWC) was calculated as:

$$\text{LRWC} = (\text{fresh weight} - \text{dry weight}) \ / \ (\text{turgid weight} - \text{dry weight})$$

4.4.8 *Dark-adapted epidermal conductance and dark respiration*

After doing all measurements mentioned above, plants were moved to a dark room at 20°C at the end of the day. After 36 h of dark adaptation, leaf water vapour and CO_2 exchange measurements were made on the third leaflet of the same leaf used for previous measurements, the second youngest fully expanded leaf, using the closed gas exchange system described in Section 3.4.2.1. All measurements were carried out in dim light (< 3 μmol m^{-2} s^{-1}) from a green light source. Dark-adapted leaf conductance to water vapour (g_{dark}) was calculated as described previously, and measured dark respiration was adjusted to a temperature of 25°C, assuming a Q_{10} of 2.0. Following the gas exchange measurement, the leaflet area (LA) was determined using an LI-3100C leaf area meter (Licor Inc., Lincoln NE).

4.4.9 *Relative mesophyll diffusive resistance*

To calculate the relative mesophyll resistance (l_m), first stomatal resistance (r_s) and mesophyll resistance (r_m) were calculated as follows:

$$r_s = 1/g_s \ , \ r_m = 1/g_m$$

Then, l_m according to Jones (1985) was calculated as:

$$l_m = r_m / (r_s + r_m)$$

4.4.10 *Data analysis*

An analysis of residuals was used to identify observations that were outliers; the internal studentized residuals were computed for each observation. Then the observations

with internal studentized residuals having an absolute value higher than the critical value at a Type I error rate of 0.05 for Lund's test of studentized residuals were declared as outliers and removed (Bowley, 2008). Next, the results were analysed using PROC GLM in SAS in order to determine whether there was significant variation between the two treatments for any measured parameters, and if there were any interactions between cultivar and treatment using a Type III error rate of 0.05.

4.5 RESULTS

4.5.1 *Effects of water stress on plant growth and leaf water status*

As can be seen in Table 4.1, there was a significant genotype main effect for g_{dark} ($p < 0.0001$), leaf area (LA) ($p = 0.0004$), and R_d ($p = 0.02$). Furthermore, g_s and C_C showed a significant difference among cultivars at the light level of 250 μmol m^{-2} s^{-1} ($p = 0.02$; $p = 0.004$, respectively). In addition to g_s and C_C, g_m at the light level of 1200 μmol m^{-2} s^{-1} showed significant differences among cultivars ($p = 0.02$; $p = 0.0002$; $p = 0.03$, respectively) (Table 4.1).

Treatment x cultivar interactions were found for none of the traits measured in the experiment, so subsequent analyses concentrated on treatment main effects. Compared to control plants, plants exposed to water stress showed significant decreases of around 50% for total plant dry weight (TDW), shoot dry weight (SDW), and root dry weight (RDW), while the root : shoot ratio (R/S) did not show significant change (Table 4.2).

Table 4.2 also shows that drought stress caused a dramatic drop in the leaf area (LA) of the second fully expanded leaf (30%), as well as the specific leaf dry weight (SLW) (6.5%), and the dark-adapted leaf epidermal conductance (g_{dark}) (32%).

In contrast, leaf relative water content (LRWC) was significantly higher in the plants that had been exposed to cyclic drought stress (3.4%), as was the rehydrated leaf water content/leaf fresh weight ratio (RLWC/LFW) (4.5%).

4.5.2 *Effects of water stress on WUE and its components*

There were no significant treatment, cultivar, or treatment x cultivar interaction effects on water use efficiency (WUE), though both of its components, namely TDW and plant water use (WU), were reduced by approximately the same amount (50%) when the plants were exposed to cyclic water deficit in comparison to the well watered condition (Table 4.2).

4.5.3 *Recovery of A_N following drought stress*

As can be seen in Table 4.3, 24 hours after re-watering, the net CO_2 assimilation rate (A_N) of second fully expanded leaf was significantly reduced, about 21% and 25% at light levels of 250 and 1200 μmol m^{-2} s^{-1}, respectively, in comparison to the control. This reduction in A_N was paralleled by changes in several parameters underlying A_N. At 250 μmol m^{-2} s^{-1} PPFD, there were significant reductions in both g_s (36%) and g_m (33%). The reduction in C_C (26%) led to a large reduction in V_c/V_o (26%), while the reduction in C_i was relatively minor (7%). In contrast, Φ_{II} and J_e did not show any difference in comparison with the control.

At 1200 μmol m^{-2} s^{-1} PPFD, there were similar trends for A_N and its component limitations as seen at the lower PPFD level, with a 25% decrease in A_N accompanied by considerable reductions in g_s (31%), g_m (21%), C_c (20%), and the V_c / V_o ratio (19%), while there was a slight drop in Φ_{II} (9%). In addition, across the light levels there was a significant decrease in R_d (37%) while a slight increase in α (2%) was observed with exposure to water stress.

4.5.4 *The relationship between A_N and potential limitation factors*

Pooling all data together showed good correspondence between A_N and the other components including C_c, g_s, and g_m at both light levels of 250 μmol m^{-2} s^{-1} (Figures 4.5; 4.6; 4.7) and 1200 μmol m^{-2} s^{-1} (Figures 4.8; 4.9; 4.10). In contrast to the strong relationship between A_N and C_C, no relationship was found between A_N and C_i (Figures 4.11 and 4.12). This was because leaves with very low g_s values generally had relatively high apparent C_i values (Figures 4.13 and 4.14).

4.5.5 *Magnitude of gas phase diffusive resistance components*

To compare the relative importance of the two main gas phase diffusive resistance components, stomatal and mesophyll resistances, the relative mesophyll diffusive limitation (l_m) was calculated as mentioned previously. Its relationship with A_N under two different treatments, drought and water replete, are shown in Figures 4.15 and 4.16. These figures show that at both light levels the relative magnitude of the mesophyll resistance, as a fraction of the whole diffusive resistance from stomata to carboxylation sites in chloroplast stroma, was as high as 80% in some leaves, 24-h after relieving the drought stress.

4.6 DISCUSSION

In the present study, to compare the ability of six Ontario-adapted commercial soybean cultivars to recover their photosynthetic capacities following a simulated severe water stress, two cycles of controlled soil dry down were applied over a period of two weeks in a greenhouse. The water stress treatment reduced plant growth, with significant reductions in both shoot and root dry matter. On the other hand, there was no significant effect of water shortage on whole plant WUE because both WUE components, TDW and WU, were reduced by the same amount (about 50%). This is in contrast to Walden's finding (2009) of a decrease in WUE under drought conditions in comparison with water replete conditions. The culture system used for the current experiment was different from soil medium used by Walden (2009), so different water holding capacity and / or fertility of these two media could affect the severity of drought stress and its effect on WUE. Similarly, because SDW and RDW both declined by the same amount, R/S was unaffected by the water deficit treatment (Table 4.1). However, the water stress treatment caused a steep decline in g_{dark} ($p< 0.0001$) (Table 4.1), which confirmed previous findings by Walden (2009). With respect to Walden's finding (2009) that g_{dark} is a predictor of g_s, so when the plant is adapting to water shortage conditions through stomatal closure which causes a decrease in g_s, g_{dark} was also reduced.

Interestingly, the LRWC in the leaves exposed to water stress showed a significant increase in comparison to the control. Moreover, the same substantial change was found in the LWC/LFW and the RLWC/LFW ratios, while SLW significantly dropped (Table 4.1). It can be concluded that the leaves that experienced the two week

cyclic drought stress had significantly less DW per unit area and per unit fresh weight than the control.

The rate of leaf photosynthesis was significantly lower in plants recovering from water stress than in control plants. Despite these large differences, Φ_{II} (Table 4.2) only showed a negligible decrease (9%), and only at the higher light level. This result showed that the severe water stress treatment did not significantly impact the capacity of the photosynthetic light reactions, as also reported by Reddy et al. (2004), Gale et al. (2009), Galle et al. (2007), and Galmes et al. (2007a).

In terms of components limiting photosynthetic recovery, compared to control plants, plants exposed to water stress had a significant residual reduction in g_s (Table 4.2) as has been shown before (e.g. by Cornic, 1994; Flexas et al., 2004; and Flexas et al., 2006a). It generally means that photosynthesis could be limited via water deficit-induced stomatal closure. In the same way, water stressed plants showed large decreases in g_m. These results are in line with previous studies, where a decrease of g_m has been observed during water stress (confirming findings by Ennahli and Earl, 2005; Gale et al., 2009; Grassi and Magnani, 2005; Monti et al., 2006; Galmes et al., 2007b). In addition, chloroplast CO_2 concentrations were strongly reduced (Table 4.2). However, gas phase leaf internal CO_2 concentrations (C_i) were only slightly reduced (Table 4.2). This implies that it was the decrease in g_m, not g_s, that was the most important effect reducing photosynthesis during recovery from water stress in this experiment. Furthermore, g_s, g_m, and C_c all showed strong correlation with A_N, while C_i did not show any meaningful

correspondence with A_N at either PPFD level (Figures 4.11 and 4.12). As shown in Figures 14.13 and 14.14, when C_i was plotted versus g_s the same pattern emerged at both PPFD levels; under the water shortage, as Ennahli and Earl (2005) reported, the A_N/C_i relationship tended to be erratic; in other words there was high calculated C_i at the low level of g_s induced by water deficit. In fact, the high C_i at low g_s implies that g_s was not limiting to CO_2 assimilation in those leaves with low g_s values. Another possibility is that there were greater biochemical limitations at the level of the chloroplast, but if that were the case a change in the A_N/C_C relationship would also be expected, and that was not apparent (refer to Figures 4.5 and 4.8).

Moreover, regarding the relationships shown in Figures 4.15 and Figure 4.16, the relationship between A_N and l_m showed that g_m is the major persistent photosynthetic limitation following recovery from drought stress in soybean, as was reported before by a few researchers (e.g. Ennahli and Earl, 2005) although some researchers identified reduction in leaf internal CO_2 concentration (C_i) following the stomatal closure as the major reason for leaf photosynthetic rates suppression under mild or moderate water stress (reviewed by Chaves, 1991; Cornic, 2000; Flexas et al., 2004). These present data support the idea that g_m can respond to drought stress in much the same way as g_s, as suggested by Flexas et al. (2007).

On the other hand, regarding the lack of correlation between A_N and C_i at low g_s, it should be considered that C_i estimates tend to be unreliable at very low g_s, which might exaggerate the apparent magnitude of nonstomatal limitations to photosynthesis. This

overestimation could be because of two reasons. First, the estimation of C_i from gas exchange measurements relies on the assumption that there is the same gas phase diffusive pathway for CO_2 and water vapour. When the plant is exposed to severe water shortage and stomatal conductance is very low, this assumption leads systematically to overestimation of C_i, because the non-stomatal (cuticular) water vapour exchange becomes a non-negligible fraction of the total (Boyer, et al., 1997). Second, non-uniform stomatal closure sometimes occurs during water stress, and this too leads to overestimation of C_i from gas exchange measurements (Downton et al., 1988; Meyer and Genty, 1998).

In conclusion, these results indicate that increased resistance to CO_2 diffusion between the substomatal cavity and chloroplasts, that is, reduced g_m, constitutes a major component of the persistent limitation to photosynthesis in soybean following recovery from water stress. Also, because significant genetic diversity was found for g_m in this experiment (Table 4.1), g_m must be considered as a potentially important determinant of variation for leaf photosynthesis and possibly crop productivity in soybean.

Table 4.1. The genotype effect and genotype LSMEANS for second fully expanded leaf leaflet area (LA), dark-adapted leaf conductance (g_{dark}), dark respiration (R_d), stomatal conductance (g_s) and CO_2 concentration in the chloroplast (C_C) at 250 µmol m^{-2} s^{-1} PPFD and g_s, C_C and mesophyll conductance (g_m) at 1200 µmol m^{-2} s^{-1} PPFD of six Ontario-adapted soybean cultivars. Values are means across the two watering treatments.

				PPFD 250		PPFD 1200		
Cultivar	LA (cm^2)	g_{dark} (mmol $m^{-2}s^{-1}$)	R_d (µmol m^{-2} s^{-1})	g_s (mmol $m^{-2}s^{-1}$)	C_C (ppm)	g_s (mmol $m^{-2}s^{-1}$)	C_C (ppm)	g_m (mmol m^{-2} s^{-1})
26-02R	70	22.4	0.85	103	150	187	136	260
RCAT Corbett	68	20.8	0.91	81	125	118	101	163
RCAT Matrix	56	19.5	0.83	112	158	156	132	270
OAC Bayfield	55	14.8	0.98	116	159	137	115	200
OAC Lakeview	71	18.7	0.71	88	127	128	105	180
RCAT Pinehurst	59	22.4	0.91	115	149	112	97	130
P-value	0.0004	<0.0001	0.02	0.02	0.004	0.02	0.0002	0.03
$LSD_{0.05}$	9	2.23	0.15	25	21	45	193	75

Table 4.2: The treatment effect and LSMeans for TDW, SDW, RDW, R/S, WU, WUE, LA, LRWC, SLW, LWC/LFW, RLWC/LFW, and g_{dark} in a water replete treatment (control) and in a cyclic drought treatment (drought) for six Ontario-adapted soybean cultivars. The P-value given is for the treatment main effect.

	Treatment	LSMean	SE	P value
Total dry weight (TDW) (g)	control	19.32	0.62	<0.0001
	drought	9.62	0.68	
Shoot dry weight (SDW) (g)	control	13.11	0.32	<0.0001
	drought	6.68	0.32	
Root dry weight (RDW) (g)	control	6.21	0.32	<0.0001
	drought	2.94	0.32	
Root : shoot dry weight ratio (R/S)	control	2.36	0.05	0.98
	drought	2.36	0.05	
Plant water use (WU) (L)	control	4.54	0.13	<0.0001
	drought	2.23	0.13	
Water use efficiency (WUE) (g L^{-1})	control	4.28	0.11	0.37
	drought	4.27	0.11	
Leaf area (LA) (cm^2)	control	72.85	1.90	<0.0001
	drought	53.49	1.98	
Leaf relative water content (LRWC)	control	0.87	0.01	0.0066
	drought	0.90	0.01	
Specific leaf weight (SLW) (g cm^{-2})	control	49.73	1.67	0.007
	drought	43.12	1.67	
Leaf water content : leaf fresh weight (LWC/LFW)	control	0.77	0.005	<0.0001
	drought	0.82	0.005	
Rehydrated leaf water content/leaf fresh weight (RLWC/LFW) (g^{-1})	control	0.88	0.006	<0.0001
	drought	0.92	0.006	
Dark adapted epidermal conductance (g_{dark}) ($mmolm^{-2}s^{-1}$)	control	23.1	1..1	<0.0001
	drought	15.7	2.0	
PAR leaf absorptance (α)	control	0.88	0.003	0.0081
	drought	0.89	0.003	
Dark respiration (R_d) ($\mu mol\ m^{-2}\ s^{-1}$)	control	1.06	0.031	<0.0001
	drought	0.67	0.029	

Table 4.3: The treatment effect and LSMeans for leaf-level measurements made at two PPFD levels, for a water replete treatment (control) and during recovery from a cyclic drought treatment (drought) for six Ontario-adapted soybean cultivars. The P-value given is for the treatment main effect.

		PPFD 250 μmol m^{-2} s^{-1}			PPFD 1200 μmol m^{-2} s^{-1}		
	Treatment	LSmean	SE	P value	LSmean	SE	P value
Net CO_2 assimilation rate (A_N) (μmol m^{-2} s^{-1})	control	7.9	0.3	<0.0001	16.2	0.8	0.0009
	drought	6.2	0.3		12.2	0.8	
Gross CO_2 assimilation rate (A_g) (μmol m^{-2} s^{-1})	control	9.0	0.3	<0.0001	17.3	0.8	0.0003
	drought	6.9	0.3		12.9	0.8	
Stomatal conductance (g_s) (mmol m^{-2} s^{-1})	control	125	5	<0.0001	165	9	0.0003
	drought	80	4		114	9	
Internal CO_2 concentration (C_i) (ppm)	control	260	4	0.0011	205	5	0.10
	drought	242	4		193	5	
Quantum efficiency of photosytem II (Φ_{II})	control	0.67	0.006	0.22	0.33	0.009	0.025
	drought	0.66	0.005		0.30	0.009	
Linear electron flux (J_e) (μmol m^{-2} s^{-1})	control	74	1	0.69	177	5	0.071
	drought	73	1		164	5	
Carboxylation rate : oxygenation rate ratio (Vc/Vo)	control	1.95	0.05	<0.0001	1.49	0.04	<0.0001
	drought	1.45	0.05		1.20	0.05	
Chloroplast CO_2 concentration (C_c) (ppm)	control	166	4	<0.0001	127	4	<0.0001
	drought	123	4		102	4	
Mesophyll conductance (g_m) (mmol m^{-2} s^{-1})	control	95	10	0.0060	225	15	0.030
	drought	64	10		177	15	

Figure 4.1: Plants growing in the greenhouse in pots without drainage holes, with caps to prevent evaporation from the soil; medium was 2/3 sand 1/3 peat-based potting mix. Yellow traps were used to protect plants against greenhouse pests.

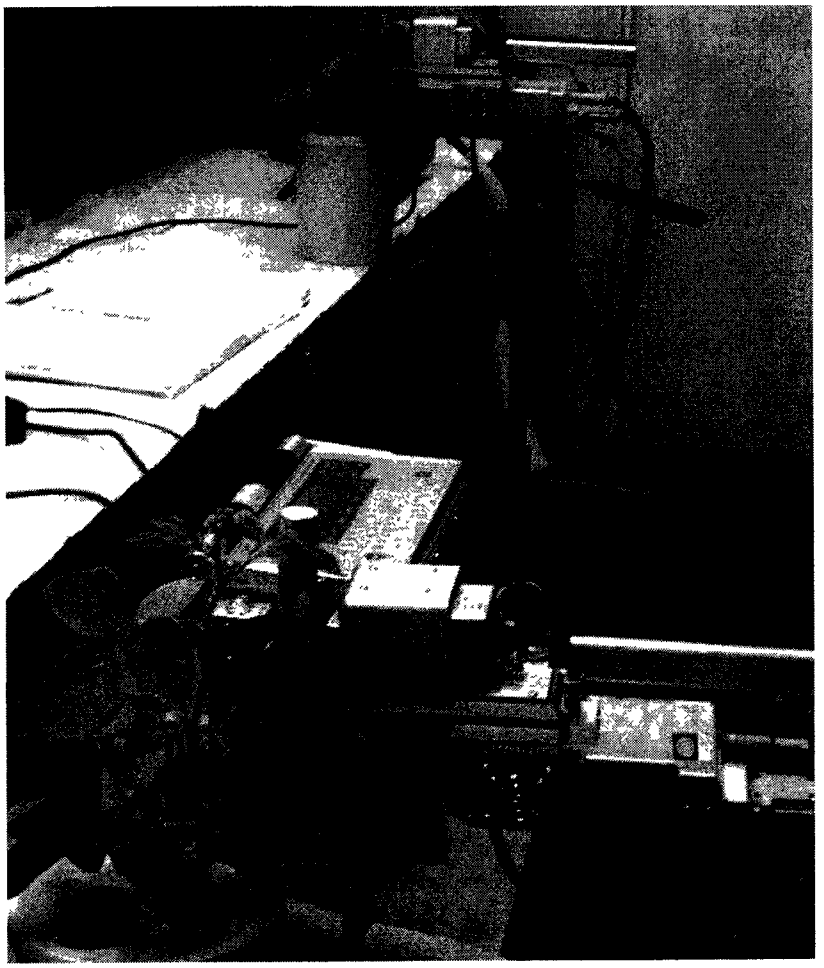

Figure 4.2: The LI-6400 XT portable photosynthesis system equipped with LED-based fluorescence / light source attachment (Model 6400-40).

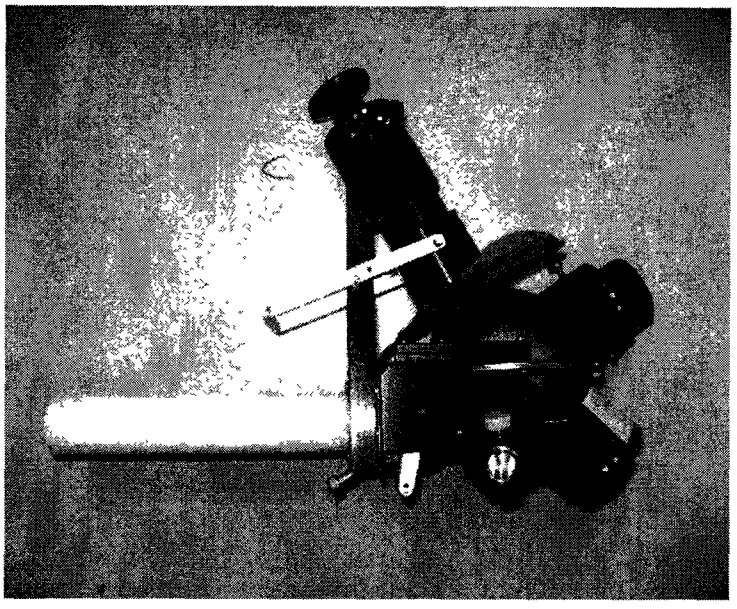

Figure 4.3: LI-1800-12B external integrating sphere (LI-COR) to measure reflectance and transmittance of leaves between 400 and 700 nm.

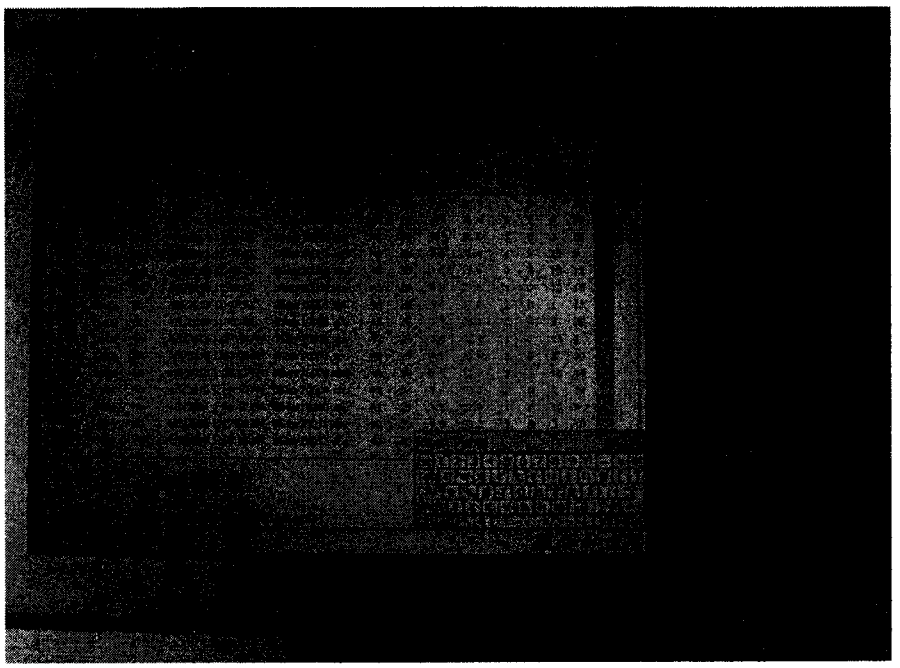

Figure 4.4: The reflectance spectrometer (Unispec DC, PP Systems, Harverhill, MA) used in combination with the LI-1800-12B integrating sphere.

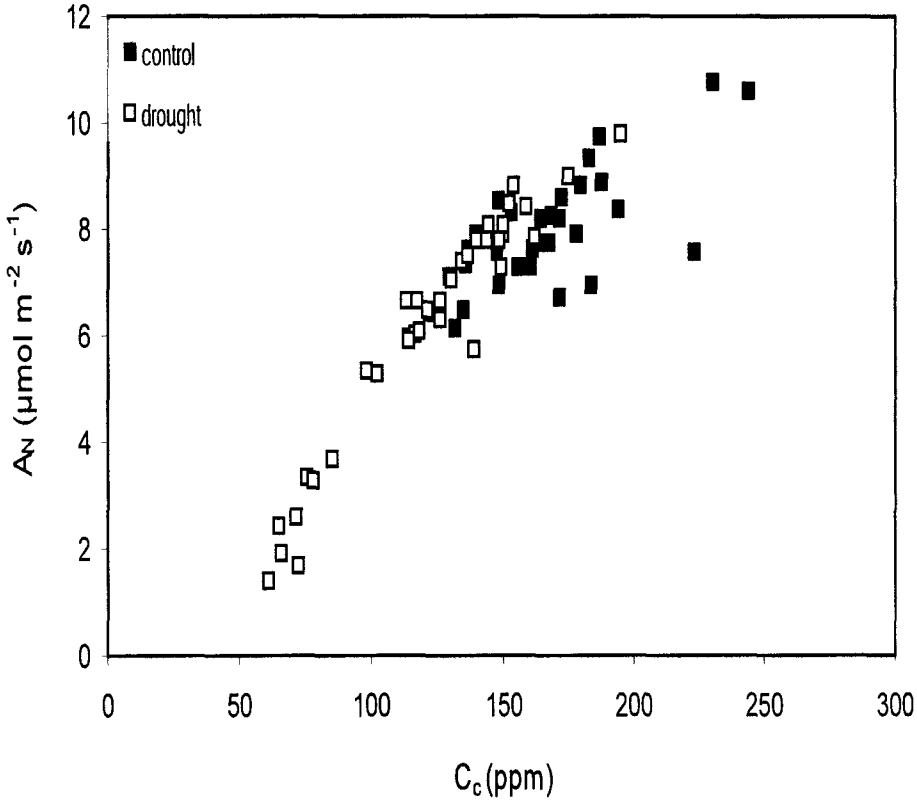

Figure 4.5: The relationship between net CO_2 assimilation rate (A_N) and chloroplast CO_2 concentration (C_c) under a PPFD level of 250 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

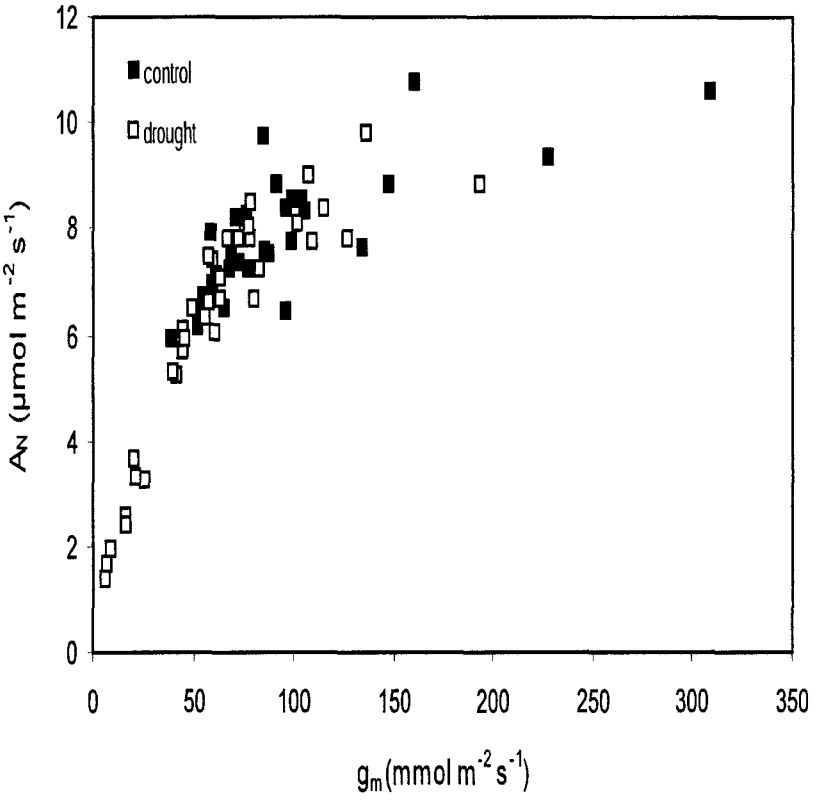

Figure 4.6: The relationship between net CO_2 assimilation rate (A_N) and mesophyll conductance (g_m) under a PPFD level of 250 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

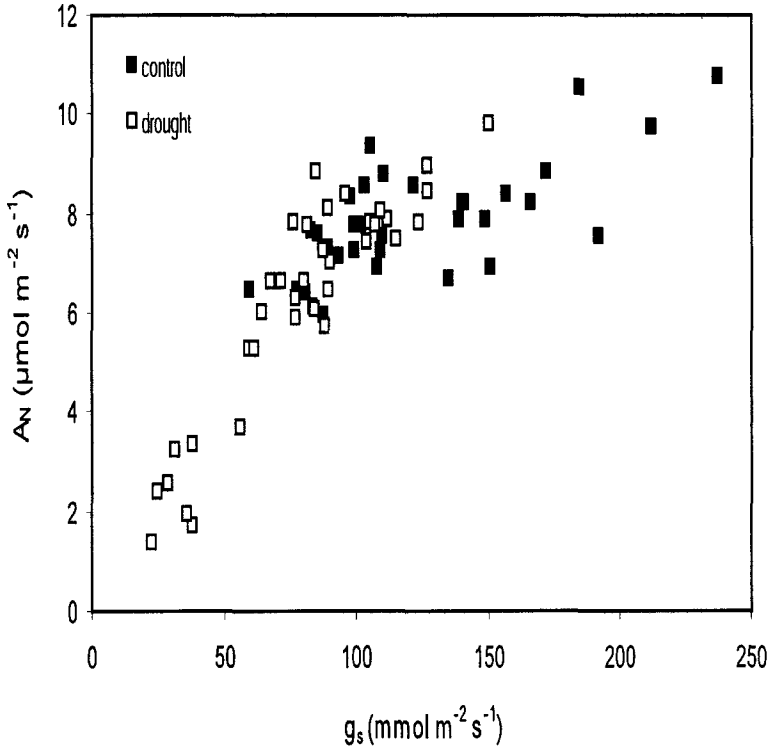

Figure 4.7: The relationship between net CO_2 assimilation rate (A_N) and stomatal conductance (g_s) under a PPFD level of 250 μmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

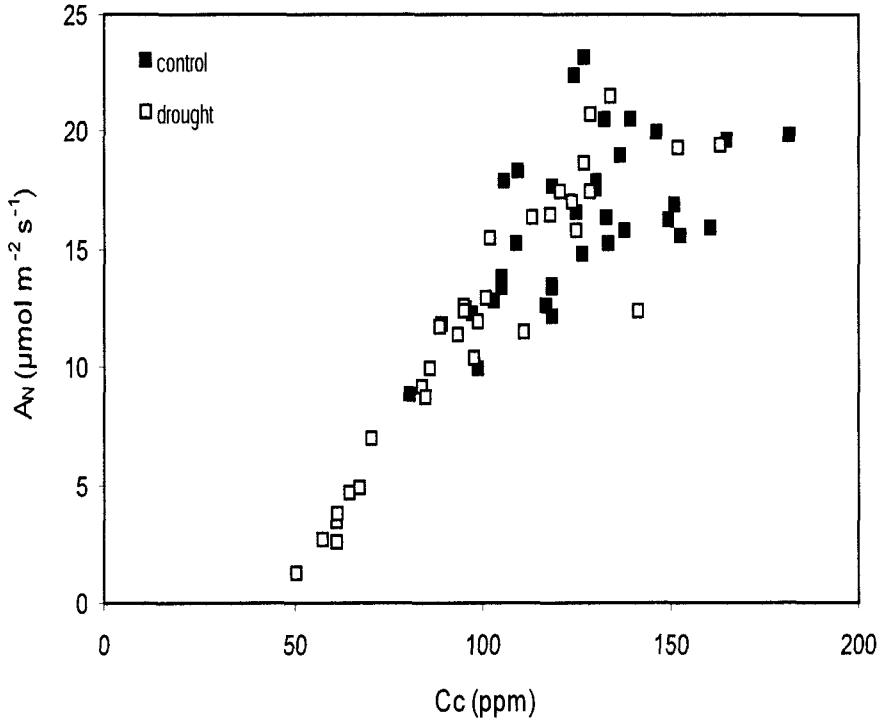

Figure 4.8: The relationship between net CO_2 assimilation rate (A_N) and chloroplast CO_2 concentration (C_c) under a PPFD level of 1200 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment(□). Data were recorded 24 h after relieving the drought stress by re-watering.

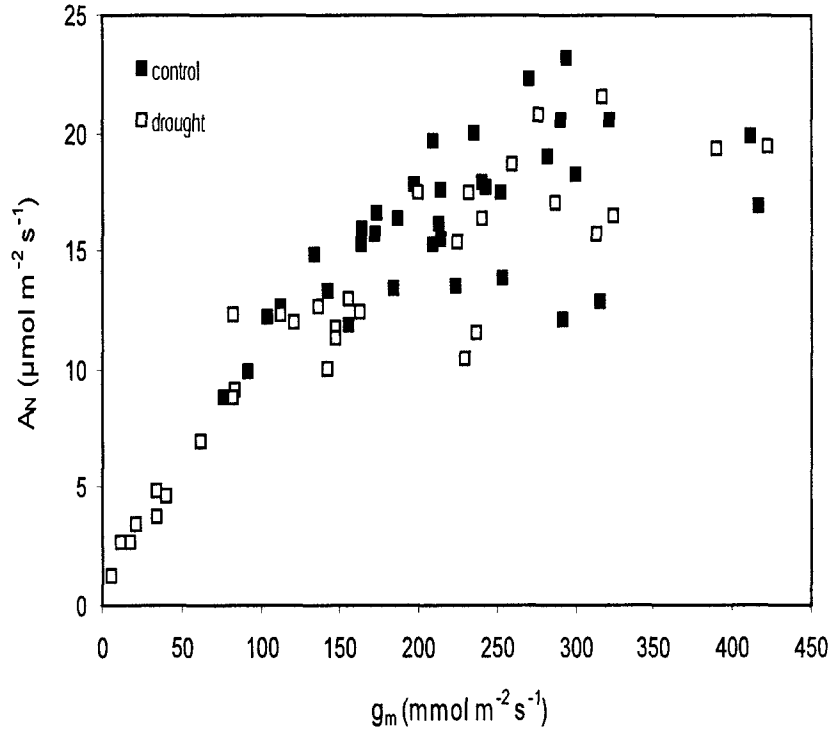

Figure 4.9: The relationship between net CO_2 assimilation rate (A_N) and mesophyll conductance (g_m) under a PPFD level of 1200 μmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

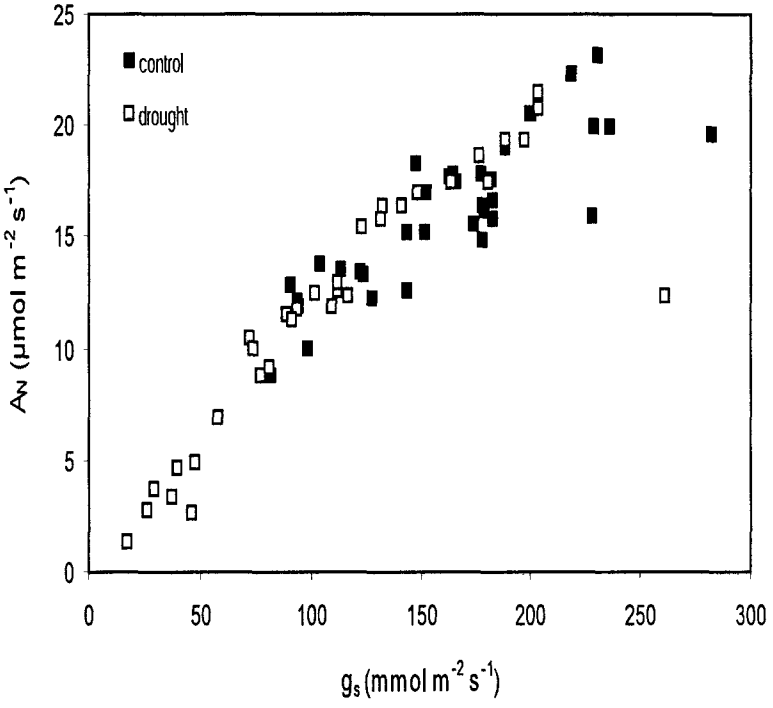

Figure 4.10: The relationship between net CO_2 assimilation rate (A_N) and stomatal conductance (g_s) under a PPFD level of 1200 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

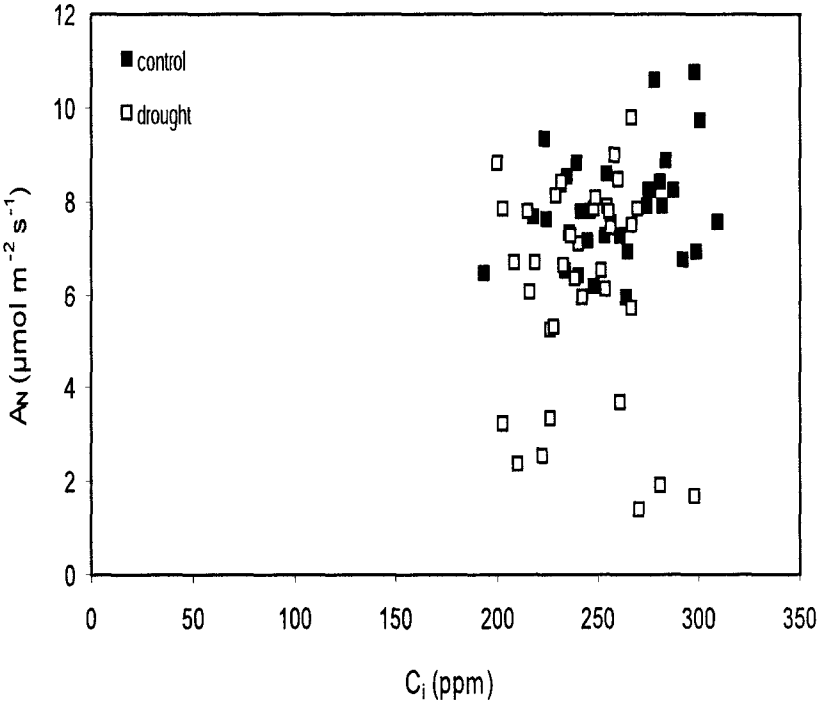

Figure 4.11: The relationship between net CO_2 assimilation rate (A_N) and sub-stomatal (internal) CO_2 concentration (C_i) under a PPFD level of 250 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

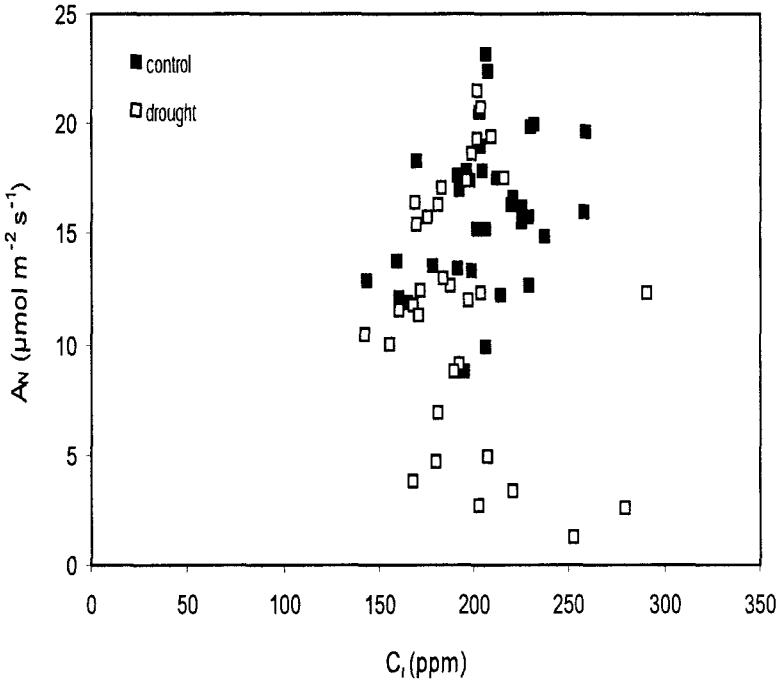

Figure 4.12: The relationship between net CO_2 assimilation rate (A_N) and sub-stomatal (internal) CO_2 concentration (C_i) under a PPFD level of 1200 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

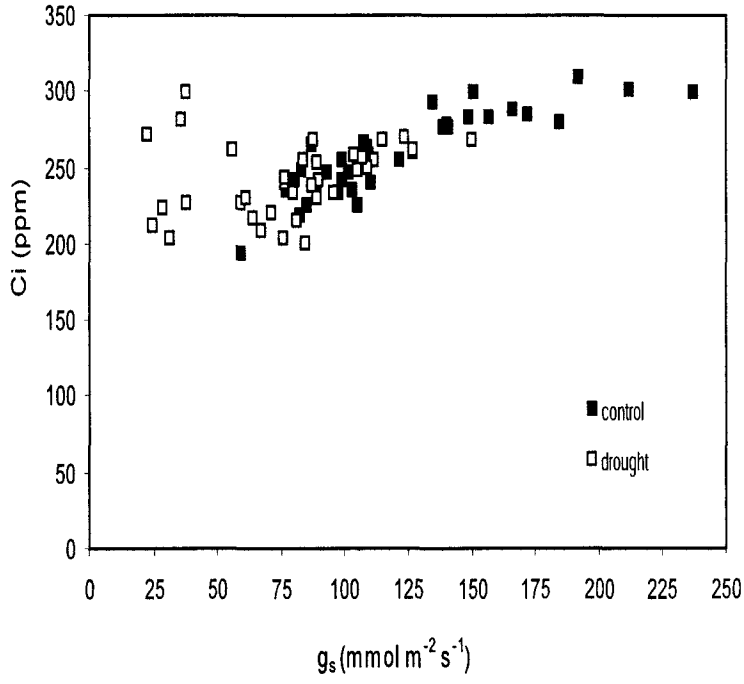

Figure 4.13: The relationship between internal CO_2 concentration (C_i) and stomatal conductance (g_s) under a PPFD level of 250 μmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

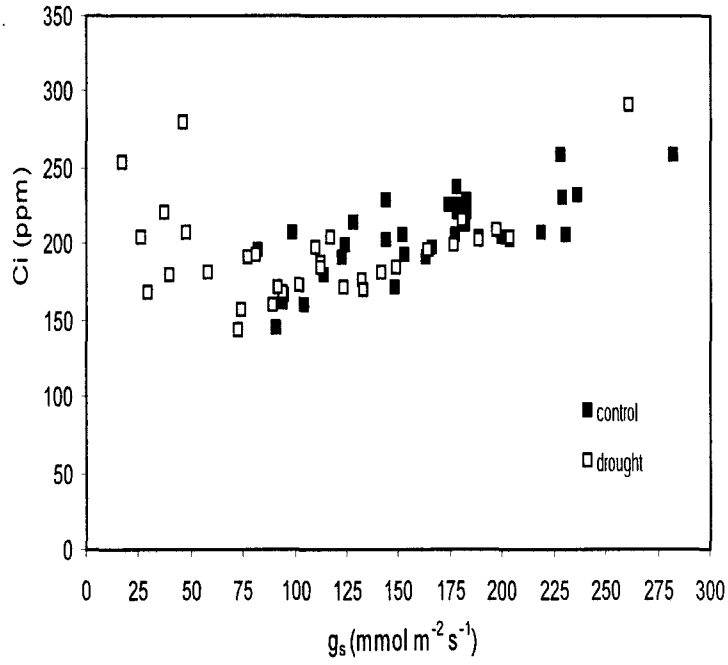

Figure 4.14: The relationship between internal CO_2 concentration (C_i) and stomatal conductance (g_s) under a PPFD level of 1200 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

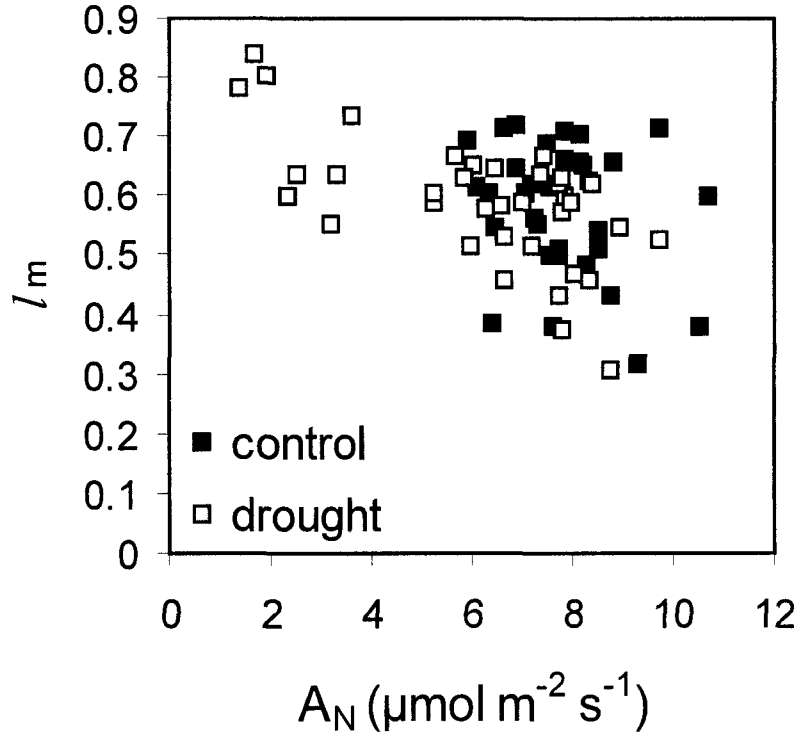

Figure 4.15: The relationship between carbon assimilation rate (A_N) and relative mesophyll resistance (lm) under a PPFD level of 250 μmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

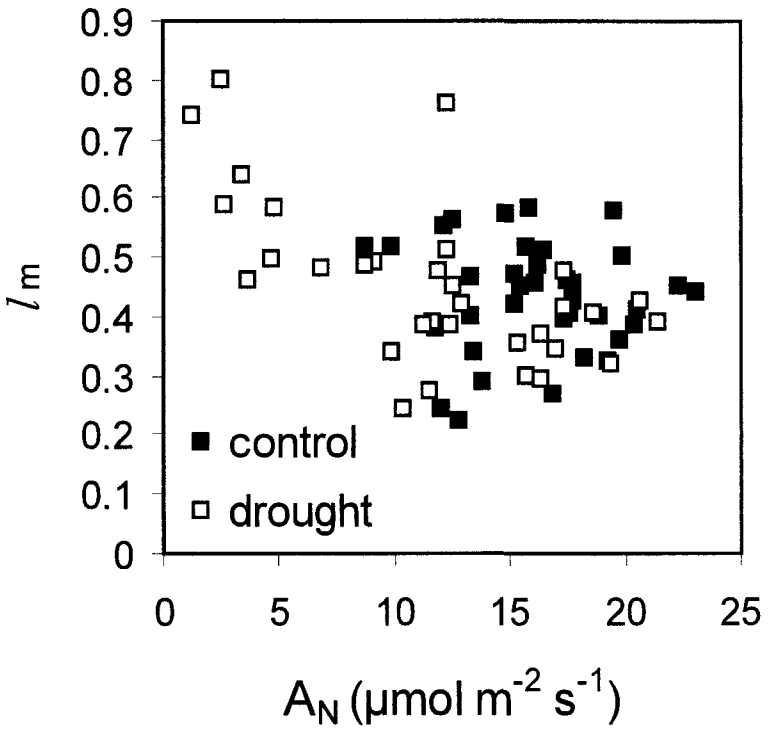

Figure 4.16: The relationship between carbon assimilation rate (A_N) and relative mesophyll resistance (*l*m) under a PPFD level of 1200 µmol m^{-2} s^{-1} across the six Ontario-adapted soybean varieties grown under control treatment (■) and cyclic drought stress treatment (□). Data were recorded 24 h after relieving the drought stress by re-watering.

CHAPTER 5

Traits Related to Water Use Efficiency in Soybean (*Glycine max* L. Merr.) – Do Greenhouse Screens Predict Field Results?

5.1 ABSTRACT

Dark-adapted leaf conductance (g_{dark}) is a trait shown to be negatively correlated with water use efficiency (WUE, amount of crop dry matter produced per unit soil water transpired) in soybean. Six soybean cultivars were grown under natural, rain-fed conditions in the field. In the greenhouse, the same six cultivars were grown under both continuously water-replete and cyclic drought stress conditions, to see which of these would best correlate with field results. In addition to g_{dark}, WUE was measured (greenhouse only), as well as leaf-level gas exchange traits associated with WUE (net CO_2 assimilation rate (A_N), stomatal conductance (g_s), leaf internal CO_2 concentration (C_i), chloroplast CO_2 concentration (C_C), and mesophyll conductance (g_m)). In the field, stomatal length (L_S) and stomatal density (D_S) were also measured. Although there was significant genetic variation for both g_{dark} and D_S in the field ($p < 0.0001$), these parameters were not correlated. WUE and g_{dark} were significantly negatively correlated to each other ($r = -0.85$, $p = 0.03$) in the well watered condition in the greenhouse. Field g_{dark} was significantly correlated with greenhouse g_{dark} ($r = 0.87$, $p = 0.03$) in the drought condition, greenhouse g_s in the drought condition ($r = 0.89$, $p = 0.02$), and also with greenhouse C_i ($r = 0.86$, $p = 0.03$) across the treatments. In addition, greenhouse g_{dark} in the drought condition was correlated with field g_s ($r = 0.84$, $p = 0.03$). Greenhouse A_N in the water replete condition and field A_N were significantly correlated ($r = 0.81$, $p = 0.05$). Field C_i was correlated with greenhouse C_i in the drought condition ($r = 0.83$, $p = 0.03$) and across the treatments ($r = 0.93$, $p = 0.01$). In general it was concluded that greenhouse measurements made under the drought treatment were most predictive of genotypic variation for these traits in the field.

5.2 INTRODUCTION

Water use efficiency (WUE), the amount crop dry matter produced per unit of water vapour transpired, constitutes one of the most important traits controlling plant productivity under water-limited conditions. A better understanding of the physiological bases of water use efficiency and its genetic diversity is the first prerequisite to understand how to improve it, through biotechnology or traditional breeding methods. However, WUE measurement is rather difficult to carry out in the field, which limits its application as a selection criterion in plant breeding.

Recently, an easily-measured leaf trait, g_{dark}, has been shown to be predictive of WUE in greenhouse experiments. For instance, in greenhouse studies Fish and Earl (2009; cotton), Hufsteletler et al. (2007; soybean) and Walden (2009; soybean) found a strong negative correlation between WUE and dark-adapted leaf epidermal conductance (g_{dark}). Also in greenhouse screens, significant variation for g_{dark} has been found among the commercial soybean germplasm adapted to Ontario (Walden, 2009). However, to date there is no information on whether greenhouse screens for g_{dark} accurately predict how soybean genotypes differ for this trait under field conditions.

The physiological basis of the correlation between g_{dark} and WUE is only partially understood. It appears that high g_{dark} correlates with high stomatal conductance (g_s) and leaf internal CO_2 (C_i) of leaves during the day (e.g. Walden, 2009). This is consistent with the negative correlation between g_{dark} and WUE since well-established theory indicates that leaf-level WUE – the ratio of net CO_2 assimilation (A_N) to transpiration – is

negatively correlated with C_i). However, it is still unclear why g_{dark} would predict either g_s or C_i. Some studies which have shown that water deficit leads to a change in stomatal density (D_S) (McCrea and Davis, 1974; Cutler et al., 1977) and stomatal length (L_S) (Cutler et al., 1977; Quarries and Jones, 1977; Spence et al., 1986), indicating this may enhance the adaptation of plants to drought (Cutler et al., 1977; Spence et al., 1986). Such leaf morphological traits may affect leaf gas exchange quite markedly (Woodward, 1987; Nilsson and Ashman, 2007). Therefore, one possibility is that leaves with high stomatal density or large stomata are "leakier" at night (due to the fact that stomata are poorly cuticularized), thus increasing g_{dark}, and that these high stomatal densities or larger stomatal sizes are also associated with higher g_s and C_i (D_s: e.g. Zhenzhu and Guangsheng, 2008; Gizt III et al., 2005; Muchow and Sinclair,1989; L_s: e.g. Paje et al., 1988; Walden, 2009). Indeed, there are examples in other species of stomatal density being correlated with minimum leaf epidermal conductance (Muchow and Sinclair, 1989), although, it should be noted that g_{dark}, measured on dark-adapted but freshly detached leaves, is already known to be a different trait from minimum epidermal conductance which is measured on leaves that have started to wilt (Walden, 2008; Fish and Earl, 2009).

5.3 RESEARCH OBJECTIVES

1. To determine if genotype differences in g_{dark} identified in greenhouse experiments predict genotype differences for this trait in the field.
2. To determine if stomatal density or stomatal size explain genotype differences in g_{dark} under field conditions.

5.4 MATERIALS AND METHODS

5.4.1 *Greenhouse study*

Data from the same greenhouse study described in Section 4.4 of the previous chapter were compared to the field data. Six commercial soybean cultivars adapted to Ontario (26-02R; OAC Bayfield; RCAT Corbett; OAC Lakeview; RCAT Matrix; RCAT Pinehurst) were grown under both water-replete (control) and cyclic water stress treatments, and then combined leaf gas exchange / chlorophyll fluorescence measurements were made on second youngest fully-expanded leaves. Destructive harvests were conducted to determine whole-plant dry matter-based WUE. For further details on culture conditions, treatments, and measurements, refer to Section 4.4.

5.4.2 *Field study*

5.4.2.1 *First year (2008)*

The same six Ontario-adapted soybean varieties listed above were planted by a corn planter at the Elora Research Farm (43° 38' 27.76" N, - 80° 24' 20.43" W) in plots 5 m long, each consisting of seven 18-cm rows, with four complete replications in a RCB design on June, 6 2008. Seeding rate was 50 m^{-2}. Weed control was via glyphosate (Roundup) applied preplant at 2 L ha^{-1}, and Basagran Forte at the V2 stage, also at 2 L ha^{-1}.

On three different dates (49, 70 and 95 days after planting) two plants from each plot were cut off at ground level (Figure 5.1). Stems were immediately re-cut under water

to prevent xylem embolisms, and the cut ends were kept under water while the plants were transported to a dark room kept at 20°C.

After approximately 40 h of dark adaptation, H_2O vapour exchange measurements for the calculation of g_{dark} were made on two freshly detached leaves per plant (the second and the third youngest fully expanded leaves) using a closed gas exchange system, as described in Section 3.4.2.1.

5.4.2.2 *Second year (2009)*

The same field experiment was established again at the Elora Research Farm on May 22, 2009 using the same methods as in 2008, except that no herbicide applications were made and weeds were controlled via hand weeding as required (Figure 5.2). Leaf gas exchange measurements were made *in situ* (54, 78 and 116 days after planting, before senescence) on the second youngest fully expanded leaf of one plant per plot (Figure 5.3). Measurements were made between 10 am and 5 pm with an LI-6400XT portable photosynthesis system, fitted with a 6400-01, red/blue LED light source (Figure 5.4). Leaf temperature was controlled at 25°C using the system's Peltier coolers. The reference side CO_2 concentration was set to 380 $\mu L\ L^{-1}$, and the sample side flow rate was 250 $\mu mol\ s^{-1}$. The PPFD level was set to 1200 $\mu mol\ m^{-2}\ s^{-1}$.

At the end of the day, plants used for gas exchange measurements were cut off at ground level and transported to the dark room for g_{dark} measurements, exactly as was

done in 2008. The leaflet used for g_{dark} measurements was a different leaflet from the same leaf used for leaf gas exchange measurements in the field.

After g_{dark} measurement on the dark-adapted leaflet, two impressions of the leaflet, one impression per each leaf surface, were taken using Extrude Medium impression material (Kerr Dental, Orange CA) which formed a mold. One peel was taken from each mold using clear nail polish. Next, each peel was examined under a magnification of 200x (Axiophot, Zeiss, Germany) so that stomata were visible among the epidermal cells, and a digital photograph was taken of a 0.02 mm^2 area. Then using Image J imaging software (U.S. National Institutes of Health) the number of stomata in this area was used to calculate the stomatal density (D_S) (mm^{-2}). Then, the lengths of ten stomata randomly selected from the same digital photograph were measured and the results were combined to give an estimated mean length of stomatal opening (L_S) for that leaflet.

5.4.3 *Data Analysis*

An analysis of residuals was used to identify observations that were outliers for the field data; the internal studentized residuals were computed for each observation. Then the observations with internal studentized residuals having an absolute value higher than the critical value at a Type I error rate of 0.05 for Lund's test of studentized residuals were declared outliers and removed (Bowley, 2008). The data were analyzed using PROC GLM in SAS (Version 9.2, SAS Institute, Cary, NC, USA). Analysis of variance to detect genotype and treatment main effects was conducted as described in

Section 4.3. As discussed in Chapter 4, there were no significant treatment x genotype interactions for any of the measured parameters. To investigate the hypothesis that greenhouse measurements could predict field results, a correlation analysis using PROC CORR in SAS was performed amongst all parameters measured in greenhouse and field. In 2008, genotype LSMEANS for g_{dark} measured in the field consisted of the average of two leaves per plant, two plants per plot, four plots per genotype and three measuring days, for a total of 48 estimates per genotype. In 2009, all field gas exchange data including g_{dark} were collected for one leaf per plant, one plant per plot, four replications and three measuring days, for a total of 12 estimates per genotype.

5.5 RESULTS

5.5.1 *Variation for traits measured*

Across the two watering treatments in the greenhouse, there was significant genotypic variation for g_{dark} ($p < 0.0001$), C_C ($p = 0.0002$), g_m ($p = 0.003$), and g_s ($p = 0.02$) (Table 5.1). In the control treatment, significant variation was found only for g_{dark} and g_s (Table 5.2) and in the drought treatment only for g_{dark} (Table 5.3). In addition, WUE was not significantly different amongst cultivars across two treatments, but it showed significant variation within individual treatments (Table 5.4).

In the field, diversity among cultivars for g_{dark} was substantial in each year (data not shown) and the average of two years for this trait also showed large genotype differences ($p < 0.0001$) (Table 5.5). As can be seen in Table 5.5, substantial genotypic variation in the field was also recorded for C_i ($p = 0.004$) and D_S ($p < 0.0001$).

5.5.2 *Correlation among traits in the greenhouse*

Table 5.6 provides an overview of correlation coefficients among different parameters measured in the greenhouse. As can be seen in this table for the genotype means across the treatments (Table 5.6 A), A_N was correlated with g_m and C_C ($r = 0.98$, $p = 0.005$, Figure A1; $r = 0.96$, $p = 0.003$, Figure 6.3, respectively) and C_C and g_m were strongly related to each other ($r = 0.98$, $p = 0.0005$, Figure A4); there was also significant correlation between C_i and g_s ($p = 0.83$, $p = 0.04$, Figure A2).

Since most of the measured parameters were strongly affected by treatments (refer to previous chapter), the correlation analyses amongst all parameters were also conducted separately by treatment. Although under water replete conditions WUE was correlated with g_{dark} ($r = -0.85$, $p = 0.03$, Figure A9) and g_s was correlated with g_m ($r = 0.87$, $p = 0.02$, Figure A8), under drought conditions these two correlations were not found ($r = 0.23$, $p = 0.65$, Figure A33; $r = 0.74$, $p = 0.09$). On the other hand, some correlations between traits were found under both treatments (Tables 5.6B and 5.6C); i.e., A_N was correlated to g_s, C_C and g_m in control conditions ($r = 0.94$, $p = 0.01$, Figure A5; $r = 0.86$, $p = 0.03$, Figure A6; $r = 0.97$, $p = 0.002$, Figure A10, respectively) and in drought conditions ($r = 0.92$, $p = 0.01$, Figure A12; $r = 0.99$, $p = 0.0001$, Figure A13; $r = 0.94$, $p = 0.01$, Figure A16, respectively) though A_N just under the drought treatment was significantly correlated to g_{dark} ($r = 0.83$, $p = 0.04$, Figure A14). There was significant correlation between g_m and C_C in both water replete and drought experiments ($r = 0.80$, $p = 0.05$, Figure A11; $r = 0.93$, $p = 0.01$, Figure A17, respectively) as was also the case for g_s and C_C ($r = 0.98$, $p = 0.008$, Figure A7; $r = 0.93$, $p = 0.01$, Figure A15, respectively). However, only under water deficit condition g_{dark} and A_N were correlated ($r = 0.83$, $p = 0.04$, Figure A14); conversely g_s was significantly correlated to g_m only under well watered conditions ($r = 0.87$, $p = 0.002$, Figure A8).

5.5.3 *Correlation amongst traits in the field*

The overview of correlation coefficients amongst different parameters measured in the field is shown in Table 5.7. In the field, there was the strongest correlation between g_{dark} and g_s ($r = 0.95$, $p = 0.003$, Figure A19) followed by the correlation

between g_{dark} and C_i ($r = 0.82$, $p = 0.04$, Figure A20). Moreover, g_s and C_i were correlated ($r = 0.80$, $p = 0.05$, Figure A18). Although there was significant variation for stomatal density amongst genotypes ($p < 0.0001$) no significant genotype effect was observed for stomatal length ($p = 0.37$) and neither of these two traits showed correlation with other traits or between themselves (Table 5.7).

5.5.4 *Correlations between the field and the greenhouse*

Turning to relationships between field parameters and the greenhouse parameters calculated from the analysis of the cultivar means across the two treatments in the greenhouse (Table 5.7A), greenhouse C_i and g_s were significantly correlated to C_i measured in the field ($r = 0.93$ $p = 0.01$, Figure A24; $r = 0.94$, $p = 0.01$, Figure A21, respectively). In addition, there was strong correlation between greenhouse C_i and field g_s and g_{dark} ($r = 0.94$, $p = 0.01$, Figure A22; $r = 0.86$, $p = 0.03$, Figure A25, respectively), and between greenhouse WUE and field A_N ($r = 0.81$, $p = 0.05$, Figure A23).

Analysing greenhouse parameters by treatment showed that in the greenhouse water-replete treatment, A_N and g_m were substantially correlated with A_N in the field ($r = 0.82$, $p = 0.05$, Figure A26; $r = 0.88$, $p = 0.02$, Figure A27). However, g_{dark} measured under drought conditions in the greenhouse was correlated to both g_s and g_{dark} in the field ($r = 0.84$, $p = 0.03$, Figure A31; $r = 0.87$, $p = 0.03$, Figure A32, respectively) (Table 5.7B and C). As can be seen in Table 5.7C, g_s in the greenhouse under drought conditions was correlated to g_s and g_{dark} in the field ($r = 0.82$, $p = 0.05$, Figure A28; $r = 0.89$, $p = 0.02$,

Figure A29, respectively). Furthermore, greenhouse C_i and field C_i showed considerable correlation ($r = 0.86$, $p = 0.03$, Figure A30).

5.6 DISCUSSION

5.6.1 *Greenhouse*

The strong correlation found between WUE and g_{dark} under control conditions ($r = -0.85$, $p = 0.03$, Table 5.6B) confirmed previous findings by Hufstetler et al. (2007), Fish and Earl (2009) and Walden (2009). However, there was no significant correlation between these two parameters under cyclic water shortage, which is similar to Walden's finding (2009) with 12 Ontario adapted soybeans and a slightly different culture system. Overall, these results confirm that g_{dark} can predict WUE in the greenhouse, but only under well-watered conditions.

In addition, the g_{dark} value was the most environmentally sensitive trait (significant genotype by treatment interaction) among all of the different parameters measured in the greenhouse (Table 5.2 and Table 5.3). It was decreased overall by water shortage, and by comparing these two tables it can be seen that different cultivars showed different responses of g_{dark} to drought conditions. This again is the same as first reported by Walden (2009) in soybean.

Turning to WUE, there was significant genotype effect for WUE in this experiment. Consistent with the findings of Walden (2009), since the cultivars were specifically chosen based on previously measured differences in WUE by Walden (2009).

However, there was no significant cultivar × treatment effect for WUE, which is the same result found by Hufstetler et al. (2007), but different from the findings of

Walden (2009). It the present study, the two components of WUE (plant dry weight and plant water use) were affected similarly by the cyclic water shortage treatment.

Interestingly, g_s was strongly correlated with C_C in both control conditions and the drought treatment, and with g_m under the control condition (Table 5.6B and C). On the other hand g_s did not show significant correlation with C_i. This can be explained by technical challenges associated with C_i measurement (see Earl and Ennahli, 2005). The strong correlation between g_s and g_m under control conditions suggests that g_m was changing in harmony with stomatal closure, as has been reported by others (e.g. Epron et al., 1995; Warren et al., 2003; Yamori et al., 2006). This results in increased resistance against CO_2 diffusion from the intercellular air spaces towards the chloroplast, and thereby increases the C_i when stomata are relatively closed.

A_N was correlated to g_s and C_C in the control treatment ($r = 0.94$, $p = 0.01$; $r = 0.86$, $p = 0.03$ respectively) and drought treatment ($r = 0.92$, $p = 0.01$; $r = 0.99$, $p < 0.0001$, respectively). Comparing these correlation values indicates that A_N was related to C_C much more strongly in the drought condition than in the well watered condition, which could show the relative importance of C_C and g_m in drought condition (confirming findings by Ennahli and Earl, 2005; Gale et al., 2009; Grassi and Magnani, 2005; Galmes et al., 2007b). It should be noted that correlations of g_m with C_C and A_N can arise because of autocorrelation (because they are calculated from one another). By contrast, A_N and C_i are negatively autocorrelated, so positive relationships between them may in reality be stronger than they appear.

5.6.2 *Field*

According to the strong correlation of g_{dark} to g_s and C_i ($r = 0.95$, $p = 0.003$; $r = 0.83$, $p = 0.04$) recorded in the field, g_{dark} appears to be a reliable predictor of g_s and C_i in the field. This is consistent with Walden (2009) who showed that g_{dark} was an accurate predictor of g_s and C_i of the same leaves under steady-state photosynthesis in the greenhouse. In addition, g_s and C_i were somewhat correlated ($r = 0.80$, $p = 0.05$), but again it should be noted that these two parameters are mathematically autocorrelated. There is no autocorrelation between g_{dark} and other leaf-level traits, since g_{dark} is measured independently.

Consistent with the findings of Paje et al. (1988) and Walden (2009) the present study did not show significant relationships between D_S and any leaf gas exchange parameters, including g_{dark}, g_s, and C_i. This contrasts the results of Zhenzhu and Guangsheng (2008) who found that in *Leymus chinensis* (Trin.) Tzvel. D_S was positively correlated with both g_s, and A_N. L_S was also not correlated with any of these traits in the present study, although Walden (2009) reported that L_S was weakly related to gs and C_i. It is notable that all results mentioned above were from greenhouse studies, while the present experiment was done in the field. Overall, it can be concluded that morphological traits (specifically, stomatal density and size) did not have any statistically significant effects on water vapour exchange. This further strengthens the idea that g_{dark} is a predictor of stomatal opening *per se* and, consequently, WUE in the field.

5.6.3 *Do greenhouse experiments predict results under field conditions?*

The relationships between observations in the field and in the greenhouse are presented in Tables 5.8A, B and C. Surprisingly, g_{dark} in the greenhouse drought condition was correlated with g_{dark} and g_s in the field ($r = 0.87$, $p = 0.03$; $r = 0.84$, $p = 0.03$). Although g_{dark} across the treatments did not show a significant relationship with g_{dark} in the field, C_i showed itself as a potential predictor of C_i in the field . It was also interesting that A_N and g_m, showed significant relationships to field A_N only under water replete conditions in the greenhouse. Moreover, across the treatments, cultivars showed the same order of C_i values in the field and in the greenhouse.

In summary, we found that g_{dark} of plants that had been exposed to cyclic drought stress in the greenhouse accurately predicted cultivar rankings for this trait in the field. The cyclic drought stress treatment was also suitable for predicting genotype differences in g_s and C_i in the field. In contrast, cultivar differences in field A_N were best predicted by A_N (and/or g_m) measured under water replete conditions in the greenhouse.

Table 5.1: The genotype effect and genotype LSMEANS for net carbon assimilation rate (A_N), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), dark-adapted leaf conductance (g_{dark}), CO_2 concentration in the chloroplast (C_C) and mesophyll conductance (g_m) of six Ontario-adapted soybean varieties in the greenhouse. Values are means across the two watering treatments.

	A_N	g_s	C_i	g_{dark}	C_C	g_m
Cultivar	μmol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}
26-02R	17.5	187	211	22.4	136	262
OAC Bayfield	14.4	118	191	20.8	115	200
RCAT Corbett	12.4	156	202	14.8	101	157
OAC Lakeview	13.3	137	196	18.7	105	184
RCAT Matrix	15.9	128	194	22.4	132	269
RCAT Pinehurst	11.8	112	200	19.5	97	135
P-value	0.06	0.02	0.67	<0.0001	0.0002	0.003
LSD 0.05	NS	25	NS	2.2	21	37

Table 5.2: The genotype effect and genotype LSMEANS for net carbon assimilation rate (A_N), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), dark-adapted leaf conductance (g_{dark}), CO_2 concentration in the chloroplast (C_C) and mesophyll conductance (g_m) across the six Ontario-adapted soybean varieties in the control (water replete) environment in the greenhouse.

	A_N	g_s	C_i	g_{dark}	C_C	g_m
Cultivar	μmol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}
26-02R	18.9	196	196	22.2	143	287
OAC Bayfield	16.7	176	176	26.0	133	224
RCAT Corbett	14.3	138	138	23.4	113	181
OAC Lakeview	16.8	160	160	19.8	120	260
RCAT Matrix	16.6	180	180	26.5	140	242
RCAT Pinehurst	14.0	138	138	22.4	110	158
P-value	0.80	0.02	0.86	0.0003	0.83	0.19
LSD 0.05	NS	25	NS	3.0	NS	NS

Table 5.3: The genotype effect and genotype LSMEANS for net carbon assimilation rate (A_N), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), dark-adapted leaf conductance (g_{dark}), CO_2 concentration in chloroplast (C_C) and mesophyll conductance (g_m) for six Ontario-adapted soybean varieties in the cyclic drought treatment in the greenhouse.

	A_N	g_s	C_i	g_{dark}	C_C	g_m
Cultivar	μmol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}	ppm	mmol m^{-2} s^{-1}
26-02R	16.1	177	212	22.6	129	237
OAC Bayfield	12.0	98	172	15.6	96	177
RCAT Corbett	10.4	97	206	14.0	88	134
OAC Lakeview	9.8	96	200	9.8	89	109
RCAT Matrix	15.1	131	175	18.2	124	296
Pinehurst	9.7	85	195	16.5	84	112
P-value	0.80	0.86	0.16	0.0003	0.83	0.19
LSD 0.05	NS	NS	NS	3.3	NS	NS

Table 5.4: The genotype effect and genotype LSMEANS for water use efficiency (WUE) across the treatments, in the water replete treatment (WUE_C) and drought treatment (WUE_D) in the greenhouse.

	WUE	WUE_C	WUE_D
Cultivar	$g\ L^{-1}$	$g\ L^{-1}$	$g\ L^{-1}$
26-02R	4.3	4.3	4.3
OAC Bayfield	4.4	4.1	4.6
RCAT Corbett	4.3	4.4	4.2
OAC Lakeview	4.2	4.4	4.1
RCAT Matrix	4.2	4.1	4.2
RCAT Pinehurst	4.3	4.4	4.2
P-value	0.34	0.004	0.004
LSD 0.05	NS	0.2	0.2

Table 5.5: The genotype effect and genotype LSMEANS for net carbon assimilation rate (A_N), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), stomatal density (D_s) and dark-adapted leaf conductance (g_{dark}) of six Ontario-adapted soybean varieties in the field.

	A_N*	g_s*	C_i*	D_S	g_{dark}+
Cultivar	μmol m^{-2} s^{-1}	mmol $m^{-2}s^{-1}$	ppm	mm^{-2}	mmol $m^{-2}s^{-1}$
26-02R	16.4	422	280	201	29.3
OAC Bayfield	14.8	363	266	185	16
RCAT Corbett	14.4	377	276	192	20.8
OAC Lakeview	16.0	362	271	169	15.3
RCAT Pinehurst	16.0	377	270	198	21.7
RCAT Matrix	14.8	385	270	205	19.1
P-value	0.2	0.11	0.004	<0.0001	<0.0001
LSD 0.05	1.7	NS	14	13	5.6

*Average of four replications in 2009, each replication included three recording dates.

+Average of two years (2008 and 2009), each year included three recording dates.

Table 5.6: Correlations between gas exchange measurements: stomatal conductance (g_s), net carbon assimilation rate (A_N), substomatal CO_2 concentration (C_i), dark-adapted leaf conductance (g_{dark}) and water use efficiency (WUE) for six Ontario-adapted soybean varieties (A) across two treatments, (B) for the water replete treatment and (C) for the drought treatment in the greenhouse . The significant correlation coefficients are shown in bold.

(A): Across two treatments

	A_N	g_s	C_i	C_C	g_m	WUE	g_{dark}
A_N	1.00	0.57	0.34	**0.98**	**0.96**	0.66	0.77
		0.24	0.50	**0.0005**	**0.003**	0.16	0.07
g_s		1.00	**0.83**	0.48	0.43	0.17	0.00
			0.04	0.34	0.39	0.74	1.00
C_i			1.00	0.27	0.16	-0.21	0.02
				0.60	0.77	0.69	0.97
C_C				1.00	**0.98**	0.66	0.79
					0.0005	0.16	0.06
g_m					1.00	0.78	0.76
						0.07	0.08
WUE						1.00	0.55
							0.25
g_{dark}							1.00

(B): Water replete treatment

	A_N	g_s	C_i	C_C	g_m	WUE	g_{dark}
A_N	100	**0.94**	0.28	**0.86**	**0.97**	-0.38	-0.05
		0.01	0.59	**0.03**	**0.002**	0.46	0.93
g_s		1.00	0.58	**0.98**	**0.87**	-0.60	0.75
			0.23	**0.0008**	**0.02**	0.21	0.08
C_i			1.00	0.68	0.12	-0.60	0.75
				0.14	0.82	0.21	0.08
C_C				1.00	**0.80**	-0.71	0.44
					0.05	0.12	0.39
g_m					1.00	-0.33	-0.14
						0.52	0.79
WUE						1.00	**-0.85**
							0.03

Table 5.6 continued ...
(C): Drought treatment

	A_N	g_s	C_i	C_C	g_m	WUE	g_{dark}
A_N	1.00	**0.92**	-0.09	**0.99**	**0.94**	0.29	**0.83**
		0.01	0.87	**0.0001**	**0.01**	0.58	**0.04**
g_s		1.00	0.30	**0.93**	0.74	0.12	**0.80**
			0.57	**0.01**	0.09	0.83	**0.05**
C_i			1.00	-0.03	-0.35	-0.46	0.07
				0.95	0.50	0.36	0.90
C_C				1.00	**0.93**	0.17	0.79
					0.01	0.75	0.06
g_m					1.00	0.25	0.71
						0.64	0.11
WUE						1.00	0.23
							0.65

Table 5.7: Correlations between gas exchange measurements: stomatal conductance (g_s), substomatal CO_2 concentration (C_i) and dark-adapted leaf conductance (g_{dark}) for six Ontario-adapted soybean varieties in the field. The significant correlation coefficients are shown in bold.

	L_S	D_S	A_N	g_s	C_i	g_{dark}
L_S	1.00	0.04	-0.25	0.28	0.64	0.41
		0.93	0.64	0.59	0.17	0.43
D_S		1.00	-0.08	0.66	0.30	0.66
			0.88	0.15	0.56	0.16
A_N			1.00	0.45	0.37	0.48
				0.37	0.48	0.34
g_s				1.00	**0.80**	**0.95**
					0.05	**0.003**
C_i					1.00	**0.82**
						0.04

Table 5.8: Correlations between gas exchange measurements: stomatal conductance (g_s), net carbon assimilation rate (A_N), substomatal CO_2 concentration (C_i) and dark-adapted leaf conductance (g_{dark}) for six Ontario-adapted soybean varieties (A) across two treatments (greenhouse average), (B) under water replete conditions and (C) cyclic drought in the greenhouse, versus the field. The significant correlation coefficients are shown in bold.

	Field	Field	Field	Field		Field	Field	Field	Field		Field	Field	Field	Field
(A)	A_N	g_s	C_i	g_{dark}	(B)	A_N	g_s	C_i	g_{dark}	(C)	A_N	g_s	C_i	g_{dark}
A_N	0.77	0.59	0.37	0.68	A_N	**0.82**	0.43	0.31	0.46	A_N	0.66	0.63	0.38	0.76
	0.07	0.22	0.46	0.13		**0.05**	0.39	0.55	0.36		0.16	0.18	0.46	0.08
g_s	0.48	0.73	**0.94**	0.79	g_s	0.74	0.43	0.18	0.50	g_s	0.75	**0.82**	0.66	**0.89**
	0.34	0.10	**0.01**	0.06		0.09	0.39	0.73	0.31		0.08	**0.05**	0.15	**0.02**
C_i	0.33	**0.92**	**0.93**	**0.86**	C_i	0.16	0.41	-0.10	0.48	C_i	0.20	0.58	**0.86**	0.49
	0.53	**0.01**	**0.01**	**0.03**		0.76	0.42	0.85	0.34		0.71	0.22	**0.03**	0.32
C_C	0.73	0.54	0.30	0.67	C_C	0.68	0.40	0.14	0.52	C_C	0.74	0.63	0.41	0.76
	0.10	0.26	0.56	0.14		0.13	0.43	0.79	0.29		0.09	0.18	0.42	0.08
g_m	0.77	0.41	0.24	0.57	g_m	**0.88**	0.33	0.32	0.39	g_m	0.55	0.39	0.14	0.58
	0.07	0.41	0.64	0.24		**0.02**	0.52	0.53	0.45		0.26	0.45	0.79	0.23
WUE	**0.81**	-0.07	-0.04	0.06	WUE	-0.09	0.20	0.33	-0.02	WUE	-0.24	-0.09	-0.27	-0.05
	0.05	0.89	0.94	0.91		0.86	0.70	0.53	0.97		0.64	0.86	0.60	0.93
g_{dark}	0.67	0.37	-0.12	0.36	g_{dark}	-0.26	-0.16	-0.41	0.02	g_{dark}	0.35	**0.84**	0.45	**0.87**
	0.15	0.47	0.81	0.48		0.62	0.76	0.42	0.96		0.50	**0.03**	0.38	**0.03**

Figure 5.1: Cutting the main stem of plants transported to the lab for g_{dark} measurements

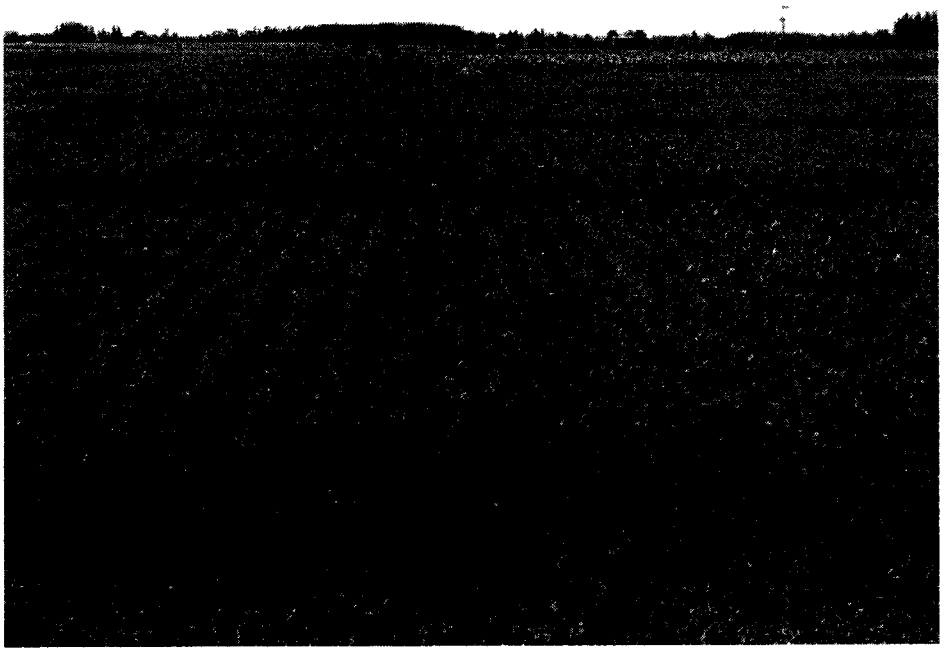

Figure 5.2: Plants growing in field plots at the Elora Research Station, Ponsonby Ontario.

Figure 5.3: Marking the leaf position used to make gas exchange measurements before cutting the main stem to transport it to the dark room.

Figure 5.4: Measuring leaf gas exchange with the LI-6400 XT in the field (2009).

CHAPTER 6

Conclusion

6.1 *Conclusion*

To compare parents of three existing mapping populations for both WUE and g_{dark}, a greenhouse study was conducted. Among three sets of recombinant inbred line (RIL) population parents, one set was found with significant parental differences for both WUE and g_{dark} ($P < 0.01$); however, the difference in WUE was not considered sufficient to consider phenotyping of the entire RIL population for QTL identification.

A controlled environment experiment was conducted to compare six Ontario-adapted commercial soybean cultivars for their ability to recover photosynthetic capacity following a simulated water stress. Plants were exposed to two cycles of controlled soil dry down over a period of two weeks in a greenhouse. Both shoot dry matter and total plant water use were reduced by the water stress treatment by approximately 50%, with no effect on whole plant water use efficiency. Combined leaf gas exchange and chlorophyll fluorescence analysis was used to quantify component limitations to leaf photosynthesis of second fully expanded leaves 24 h after re-watering. No treatment x cultivar interactions were found for leaf-level measurements, so analysis was combined across cultivars. Compared to control plants, plants that had been exposed to water shortage had reduced stomatal conductance and also lower leaf net CO_2 assimilation rates. However, gas phase leaf internal CO_2 concentrations were only slightly reduced. In contrast, chloroplast CO_2 concentrations were strongly reduced, as was mesophyll conductance to CO_2. It is concluded that increased resistance to CO_2 diffusion between the substomatal cavity and chloroplasts constitutes a major component of the persistent limitation to photosynthesis in soybean following recovery from water stress.

To figure out which of the parameters measured in the greenhouse would best correlate with field results, the same six cultivars were grown in a field experiment, so that genotype means for the three environments (field, greenhouse control, greenhouse cyclic drought) could be compared. In addition to g_{dark}, leaf-level gas exchange traits associated with WUE (A_N, g_s, C_i, C_C, g_m) were measured in the field. WUE was measured in the greenhouse only. WUE and g_{dark} were significantly correlated to each other ($r = -0.85$, $p = 0.03$) in the well water condition in the greenhouse. Field g_{dark} was significantly correlated with greenhouse g_{dark} ($r = 0.87$, $p = 0.03$) only when greenhouse plants were grown under the cyclic drought treatment. Field g_{dark} was also correlated with greenhouse C_i ($r = 0.86$, $p = 0.03$) across the treatments. In addition, greenhouse g_{dark} in the drought condition was correlated with field g_s ($r = 0.84$, $p = 0.03$), and greenhouse A_N in the water replete condition was correlated with field A_N ($r = 0.81$, $p = 0.05$). It can be concluded that greenhouse measurements of g_{dark} in drought conditions can predict genotypic variation for this trait, and for g_s in the field.

The field experiments also verified that g_{dark}, measured on leaves dark-adapted for 36 h, is a good predictor of both g_s and C_i of those same leaves when they are illuminated and undergoing photosynthesis.

Future research should continue to examine any correlation between g_{dark} in the field and greenhouse and also determine the relationship of both greenhouse and field g_{dark} with field WUE.

REFERENCES

Anyia, A.O., J.J. Salski, J.M. Nyachiro, D.J. Archambault, and P. Juskiw. 2007. Relationship of carbon isotope discrimination to water use efficiency and productivity of barley under field and greenhouse conditions. J. Agron. Crop Sci. 193:313-323.

Araus, J.L., G.A. Slafer, M.P. Reynolds, and C. Royo. 2002. Plant breeding and drought in C_3 cereals: what should we breed for? Ann. Bot. 89:925-940.

Arıoglu, H. 1999. Yag bitkileri, yetistirme ve ıslahı. C.U. Ziraat fakultesi genel yayın no: 220. Ders kitapları yayın no. A-70.

Ashley, D.A., and W.J. Ethridge. 1978. Irrigation effects on vegetative and reproductive development of three soybean cultivars. Agron. J. 70:467-471.

Assama, K., A. Sõber, W. Hartung, and Ü Niinemets. 2002. Rate of stomatal opening, shoot hydraulic conductance and photosynthetic characteristics in relation to leaf abscisic acid concentration in six temperate deciduous trees. Tree Physiol. 22:267-276.

Barbouri, M.M., C. R Warren, G.D. Farquhar, G. Forresteri, and H. Brown. 2010. Variability in mesophyll conductance between barley genotypes, and effects on transpiration efficiency and carbon isotope discrimination. Plant Cell Environ. 33:1176-1185.

Bari, A., G. Ayad and R.V. Rao. 2005. Genetic variation of water-use efficiency within crops: prospects and opportunities for managing water scarcity. In: Assessment of plant genetic resources for water-use efficiency (WUE): managing water scarcity. Proceedings of the Bioversity International/INRA/IDRC/AARINENA Workshop for North Africa and West Asia, Marrakech, Morocco, 10-12 October 2005 [92-9043-748-0].

Batchelor, W. D. 1998. Role of water stress in yield variability. Integrated Crop Management 480:3-4.

Begg, J.E., and N.C. Turner. 1976. Crop water deficit. Adv. Agron. 28:161-217. Academic Press, Inc., New York.

Bernacchi, C.J., E.L. Singsaas, C. Pimentel, A.R. Portis, and S.P. Long. 2001. Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant Cell Environ. 24:253-259.

Bogeat-Triboulot, M.B., M. Brosche, and J. Renaut. 2007. Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. Plant Physiol. 143: 876-892.

Bohnert, H.J., D.E. Nelson, and R.G. Jensen. 1995. Adaptations to environmental stresses. Plant Cell 7:1099-1111.

Bongi, G., and F. Loreto. 1989. Gas-exchange properties of salt-stressed olive (*Olea europea* L.) leaves. Plant Physiol. 90:1408-1416.

Bowely, S.R. 2008. A Hitchhiker's Guide to Statistic in Plant Biology (2nd ed.). Any Old Subject Books, Guelph, Ontario.

Boydak, E., M. Alpaslan, M. Hayta, S. Gercek, and M. Simsek. 2002. Seed composition of soybeans grown in the Harran region of Turkey as affected by row spacing and irrigation. J. Agric. Food Chem. 50, 4718-4720.

Boyer, J.S. 1970. Differing sensitivity of photosynthesis to low leaf water potentials in corn and soybean. Plant Physiol. 46:236-239.

Boyer, J.S. 1971. Recovery of photosynthesis in sunflower after a period of low leaf water potential. Plant Physiol. 47:816-820.

Boyer, J.S., S.C. Wong, and G.D. Farquhar. 1997. CO_2 and water vapour exchange across leaf cuticle (epidermis) at various water potentials. Plant Physiol. 114:185–191.

Brendel, O., D.L. Thiec, C. Scotti-Saintagne, C. Bodénès, A. Kremer , and J. M. Guehl. 2008. Quantitative trait loci controlling water use efficiency and related traits in *Quercus robur* L. Tree Gene. Genomes 4:263-278.

Briggs, L.J., and H.L. Shantz. 1914. Relative water requirement of plants. J. Agric. Res. 3: 1-64.

Buck, A.L. 1981. New equations for computing vapour pressure and enhancement factor. J. Appl. Meteor. 20:1527-1532.

Bunce, J. A. 1981. Comparative responses of leaf conductance to humidity in single attached leaves. J. Exp. Bot. 32:629-34.

Centritto, M., F. Loreto, and K. Chartzoulakis. 2003. The use of low [CO_2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. Plant Cell Environ. 26:585-594.

Chaves, M.M. 1991. Effects of water deficits on carbon assimilation. J. Exp. Bot. 42:1-16.

Chaves, M.M., J. Flexas, and C. Pinheiro. 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann. Bot. 103:551-560.

Collard, B.C.Y., M.Z.Z. Jahufer, J.B. Brouwer, and E.C.K. Pang. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica. 142:169-196.

Comstock, J.P. 2002. Hydraulic and chemical signalling in the control of stomatal conductance and transpiration. J. Exp. Bot. 53:195-200.

Condon, A.G., R A. Richards, G J. Rebetzke, and G.D. Farquhar. 2004. Breeding for high water-use efficiency. J. Exp. Bot. 55:2447-2460.

Condon, A.G., R.A. Richards, G.J. Rebetzke, and G.D. Farquhar. 2002. Improving intrinsic water-use efficiency and crop yield. Crop Sci. 42:122-131.

Cooper, R.L., N.R. Fausey, and J.G. Streeter. 1991. Yield potential of soybean grown under a sub-irrigation/drainage water management system. Agron. J. 83: 884-887.

Cornic, G. 1994. Drought stress and high light effects on leaf photosynthesis. In: Baker N.R., J.R. Boyer (eds.) Photo-inhibition of photosynthesis. Oxford: Bios Scientific Publishers.

Cornic, G. 2000. Drought stress inhibits photosynthesis by decreasing stomatal aperture - not by affecting ATP synthesis. Trends Plant Sci. 5:187–188.

Cornic, G., J.L. Legouallec, J.M. Briantais, and M. Hodges. 1989. Effect of dehydration and high light on photosynthesis of two C3 plants (*Phaseolus vulgaris* L. and *Elatostema repens* (Lour) Hall F). Planta 177: 84-90.

Cox W.J., and D. Jolliff. 1986. Growth and yield of sunflower and soybean under soil water deficits. Agron. J. 78:226-230.

Cutler, J.M., D.W. Rains, and R.S. Loomis. 1977. The importance of cell size in the water relations of plants. Physiol. Planta. 40:225-260.

De Costa, W.A.J.M., and K.N. Shanmugathasan. 2002. Physiology of yield determination of soybean (*Glycine max* (L.) Merr.) under different irrigation regimes in sub-humid zone of Sri Lanka. Field Crop Res. 75: 23-35.

Delgado, E., M.A.J. Parry, J. Vadell, D.W. Lawlor, A.J. Keys, and H. Medrano. 1992. Effect of water stress on photosynthesis, leaf characteristics and productivity of field-grown *Nicotiana tabacum* L. genotypes selected for survival at low CO_2. J. Exp. Bot. 43: 1001-1008.

Djekoun, A., and C. Planchon. 1991. Water status effect on dinitrogen fixation and photosynthesis in soybean. Agron. J. 83: 316-322.

Dogan, E., H. Kirnak, and O. Copu. 2007. Effect of seasonal water stress on soybean and site specific evaluation of CROPGRO-Soybean model under semi-arid climatic conditions. Agric. Water Management 90: 56-62.

Doorenbos, J., and A.H. Kassam, 1979. Yield response to water. U.N. Food and Agriculture Organization Irrigation and Drainage Paper No. 33, Rome.

Doss, B.D., R.W. Pearson, and H.T. Rogers. 1974. Effect of soil water stress at various growth stages on soybean yield. Agron J. 66:297-299.

Downton, W.J.S., B.R. Loveys, and W.J.R. Grant. 1988. Non-uniform stomatal closure induced by water stress causes putative non-stomatal inhibition of photosynthesis. New Phytol. 110:503-509.

Earl, H.J., and S. Ennahli. 2004. Estimating photosynthetic electron transport via chlorophyll fluorometry without photosystem II light saturation. Photosynth. Res. 82: 117-186.

Earl, H. J. 2003. A precise gravimetric method for simulating drought stress in pot experiments. Crop Sci. 43:1868-1873.

Earl, H.J. 2002. Stomatal and non-stomatal restrictions to carbon assimilation in soybean (*Glycine max*) lines differing in water use efficiency. Environ. Exp. Bot. 48:237-246.

Earl, H.J., and R. F. Davis. 2003. Effect of drought stress on leaf and whole canopy radiation use efficiency and yield of maize. Agron. J. 95:688-696.

Ehdaie, B., and J.G. Waines, 1993. Variation in water-use efficiency and its components in wheat. I. Well-watered pot experiment. Crop Sci. 33:294-299.

Ehleninger, J.R., J.P. Comstock, and T.A. Cooper. 1987. Leaf- twig carbon isotope ratio differences in photosynthetic-twig desert shrubs. Oecologia 71: 318-320.

Ehleninger, J.R., C.S. Cook, and L.L. Tiezen. 1986. Comparative water use and nitrogen relationships in mistletoe and its host. Oecologia 68: 279-284.

Ehleninger, J.R., S.L. Phillips, and J.P. Comstock. 1992. Seasonal variation in the carbon isotopic composition of desert plants. Functional Ecol. 6: 396-404.

Ehleninger, J.R., P.W. Rundel, and K.A. Nagy. 1986. Stable isotopes in physiological ecology and food web research. Trees 1: 42-45.

Ennahli, S., and H.J. Earl. 2005. Physiological limitations to photosynthetic carbon assimilation in cotton under water stress. Crop Sci. 45:2374-2382.

Environment Canada "Canada's Top Ten Weather Stories Archive", Accessed online: http:// www.ec.gc.ca/ meteo-weather/ default.asp? lang=En& n=3318B51C-1

Ephrath, J.E., A. Marani, and B.A. Bravdo. 1993. Photosynthetic rate, stomatal resistance and leaf water potential in cotton (*Gossypium hirsutum* L.) as affected by soil moisture and irradiance. Photosynthetica 29:63-71.

Epron, D., D. Godard, G. Cornic, and B. Genty. 1995. Limitation of net CO_2 assimilation rate by internal resistances to CO_2 transfer in the leaves of two tree species (*Fagus sylvatica* L. and *Castanea sativa* Mill). Plant Cell Environ. 18:43-51.

Ethier, G.J., and N.J. Livingston. 2004. On the need to incorporate sensitivity to CO_2 transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. Plant Cell Environ. 27:137-153.

Evans, L.T., 1996. Crop Evolution, Adaptation and Yield. Cambridge University Press, Cambridge.

Evans, J.R., T.D. Sharkey, J.A. Berry, and G.D. Farquhar. 1986. Carbon isotope discrimination measured concurrently with gas-exchange to investigate CO_2 diffusion in leaves of higher-plants. Aust. J. Plant Physiol. 13:281-292.

Evans, J.R., and I. Terashima. 1987. Effects of nitrogen nutrition on electron transport components and photosynthesis in spinach. Aust. J. Plant Physiol. 14:59-68.

Evans, J.R., and S. von Caemmerer. 1996. Carbon dioxide diffusion inside leaves. Plant Physiol. 110:339-346.

Evans, J.R., S. von Caemmerer, B.A. Setchell, and G.S. Hudson. 1994. The relationship between CO_2 transfer conductance and leaf anatomy in transgenic tobacco with a reduced content of Rubisco. Australian J. Plant Physiol. 21:475-495.

Fabre, N., I.M. Reiter, N. Becuwe-Linka, B. Genty, and D. Rumeau. 2007. Characterization and expression analysis of genes encoding a and b carbonic anhdrases in Arabidopsis. Plant Cell Environ. 30:617-629.

Farquhar, G.D., J.R. Ehleringer, and K.T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40:503-537.

Farquhar, G.D., M. H. O'Leary, and J. A. Berry. 1982. On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9:121-137.

Farquhar, G. D., and R. A. Richards. 1984 . Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. Aust. J. Plant Physiol. 11: 539-552.

Farquhar, G.D., and T.D. Sharkey. 1982. Stomatal conductance and photosynthesis. Ann. Rev. Plant Physiol. 33:317-345.

Faver, K.L., T.J. Gerik, P.M. Thaxton, and K.M. El-Zik. 1996. Late season water stress in cotton. II. Leaf gas exchange and assimilation capacity. Crop Sci. 36:922-928.

Fish, D.A., and H. J. Earl. 2009. Water use efficiency is negatively correlated with leaf epidermal conductance in cotton (*Gossypium* spp.). Crop Sci. 49:1409-1415.

Flexas, J., J. Bota, J.M. Escalona, B. Sampol, and H. Medrano. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. Functional Plant Biol. 29:461-471.

Flexas, J., J. Bota, J. Galmes, H. Medrano, and M. Ribas-Carbo. 2006a. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol. Plant. 127:343-352.

Flexas, J., J. Bota, F. Loreto, G. Cornic, and T.D. Sharkey. 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. Plant Biol. 6:269-279.

Flexas, J., A. Diaz-Espejo, J. Galmes, R. Kaldenhoff, H. Medrano, and M. Ribas-Carbo. 2007. Rapid variations of mesophyll conductance in response to changes in CO_2 concentration around leaves. Plant Cell and Environ. 30:1284-1298.

Flexas, J., J. Galmes, A. Galle, J. Gulias, A. Pou, M. Ribas-Carbo, M. Tomas, and H. Medrano. 2010. Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement. Australian J. Grape Wine Res. 16:106-121.

Flexas, J., M. Ribas-Carbo, D.T. Hanson, J. Bota, B. Otto, J. Cifre, N. McDowell, H. Medrano, and R. Kaldenhoff. 2006b. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO2 in vivo. Plant J. 48: 427–439.

Flexas, J., and H. Medrano. 2002. Energy dissipation in C3 plants under drought. Functional Plant Biol. 29:1209-1215.

Flexas, J., M. Ribas-Carbo, J. Bota, J. Galmes, M. Henkle, S. Martinez-Canellas, and H. Medrano. 2006c. Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO2 concentration. New Phytol. 172:73-82.

Flexas, J., M. Ribas-Carbo´, A. Diaz-Espejo, J. Galmés, and H. Medrano. 2008. Mesophyll conductance to CO2: current knowledge and future prospects. Plant Cell Environ. 31:602-621.

Flowers, M.D., E.L. Fiscus, K.O. Burkey, F.L. Booker, and J.J.B. Dubois. 2007. Photosynthesis, chlorophyll fluorescence, and yield of snap bean (*Phaseolus vulgaris* L.) genotypes differing in sensitivity to ozone. Environ. Exp. Bot. 61:190-198.

Frederick, J.R., C.R. Camp, and P.J. Bauer. 2001. Drought-stress effects on branch and mainstem seed yield and yield components of determinate soybean. Crop Sci. 41:759-763.

Fredrick, J.R., J.T. Woolley, J.D. Hesketh, and D.B. Peters. 1991. Seed yield and agronomic traits of old and modern soybean cultivars under irrigation and soil water deficit. Field Crop Res. 27:71-81.

Fuhrer, J. 2003. Agroecosystem responses to combinations of elevated CO_2, ozone, and global climate change. Agric. Eco. Environ. 97:1-20.

Galle, A., and U. Feller. 2007. Changes of photosynthetic traits in beech saplings (*Fagus sylvatica*) under severe drought stress and during recovery. Physiol. Plant. 131: 412-421.

Galle, A., I. Florez-Sarasa, M. Tomas, A. Pou, H. Medrano, M. Ribas-Carbo, and J. Flexas. 2009. The role of mesophyll conductance during water stress and recovery in tobacco (*Nicotiana sylvestris*): acclimation or limitation? J. Exp. Bot. 60:2379-2390.

Galle, A., P. Haldimann, and U. Feller. 2007. Photosynthetic performance and water relations in young pubescent oak (*Quercus pubescens*) trees during drought stress and recovery. New Phytol. 174: 799-810.

Galmes, J., A. Abadia, J. Cifre, H. Medrano, and J. Flexas. 2007a. Photoprotection processes under water stress and recovery in Mediterranean plants with different growth forms and leaf habits. Physiol. Planta. 130:495-510.

Galmes, J., J. Flexas, R. Save, and H. Medrano. 2007b. Water relations and stomatal characteristics of Mediterranean plants with different growth forms and leaf habits: responses to water stress and recovery. Plant Soil 290: 139-155.

Galmes, J., H. Medrano, and J. Flexas. 2007c. Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. New Phytol. 175:81-93.

Garten, C.T., and G.E. Taylor. 1992. Foliar $\triangle^{13}C$ within a temperate deciduous forest: spatial, temporal, and species sources of variation. Oecologia 90: 1-7.

Gaspar, T., T. Franck, B. Bisbis, C. Kevers, L. Jouve, J.F. Hausman, and J. Dommes. 2002. Concepts in plant stress physiology. Application to plant tissue cultures. Plant Growth Regulation 37: 263-285.

Genty, B., J.M. Briantais, and N.R. Baker. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta 990:87-92.

Gerbaud, A., and M. André. 1987. An evaluation of the recycling in measurements of photorespiration. Plant Physiol. 83:933-937.

Giorgi, F. and P. Lionello. 2008. Climate change projections for the Mediterranean region. Glob. Planet Change 63:90–104. (Cited in: http://www.fs.fed.us/ccrc/ bibliography/ 6_2_regional.shtml)

Gizt, III, D.C., L. Liu-Gizt, S.J. Brizt, and J.H. Sullivan. 2005. Ultraviolet-B effect on stomatal density, water-use efficiency, and stable carbon isotope discrimination in four glasshouse-grown soybean (*Glycine max*) cultivars. Environ. Exp Bot. 53:343-355.

Gomes, F., M.A. Oliva., M.S. Mielke, A-A.F. de Almeida, H.G. Leite, and L.A. Aquino. 2008. Photosynthetic limitations in leaves of young Brazilian Green Dwarf coconut (*Cocos nucifera* L. 'nana') palm under well-watered conditions or recovering from drought stress. Environ. Exp. Bot. 62:195-204.

Grass, I.G., and F. Magnani. 2005. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. Plant, Cell Environ. 28:834-849.

Grassi, G., and F. Magnani. 2005. Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. Plant Cell Environ. 28:834-849.

Guo, S.W., Y. Zhou, N. Song, and Q. Shen. 2006. Some physiological processes related to water use efficiency of higher plants. Agric. Sci. in China 5:403-411.

Hanba, Y.T., M. Shibasaka, Y. Hayashi, T. Hayakawa, K. Kasamo, I. Terashima, and M. Katsuhara. 2004. Overexpression of the barley aquaporin HvPIP2;1 increases internal CO_2 conductance and CO_2 assimilation in the leaves of transgenic rice plants. Plant and Cell Physiol. 45:521-529.

Harley, P.C., F. Loreto, G. Dimarco, and T.D. Sharkey. 1992. Theoretical considerations when estimating the mesophyll conductance to CO_2 flux by analysis of the response of photosynthesis to CO_2. Plant Physiol. 98:1429-1436.

Hatfield, J. L., T. J. Sauer, and J. H. Prueger. 2001. Managing soils to achieve greater water use efficiency: a review. Agron. J. 93:271-280.

Heckathorn, S.A., E. H. DeLucia, and R.E. Zielinski. 1997. The contribution of drought-related decreases in foliar nitrogen concentration to decreases in photosynthetic capacity during and after drought in prairie grasses. Physiol. Plant. 101:173-82.

Heltdt, K.T. 1997. Plant Biochemistry & Molecular Biology. Oxford University Press.

Hsiao, T.C. 1973. Plant responses to water stress. Ann. Rev. Plant Physiol. 24:519-70.

Hubick, K., and G. Farquhar. 1989. Carbon isotope discrimination and the ratio of carbon gained to water lost in barley cultivars. Plant Cell Environ. 12:795-804.

Hufstetler, E.V., H.R. Boerma, T.E. Carter Jr., and H.J. Earl. 2007. Genotypic variation for three physiological traits affecting drought tolerance in soybean. Crop Sci. 47:25-35.

Johnson, R.C., and L. Yangyang. 1999. Water relations, forage production, and photosynthesis in tall fescue divergently selected for carbon isotope discrimination. Crop Sci. 39:1663-1670.

Jones, H.G. 1992. Plants and Microclimate: A Quantitative Approach to Environmental Plant Physiology. 2nd edition, Cambridge University Press, Cambridge.

Jones, H.G., 1985. Partitioning stomatal and non-stomatal limitations to photosynthesis. Plant Cell Environ. 8:95-104.

Julier, B., K. Bernard, C. Gibelin, T. Huguet, and F. Lelièvre. 2010. QTL for water use efficiency in alfalfa. In: Sustainable Use of Genetic Diversity in Forage and Turf Breeding, C. Huyghe (ed.), Springer Science+Business Media B.V.

Karam, F., R. Masaad, T. Sfeir., O. Mounzer, and Y. Rouphael. 2005. Evapotranspiration and seed yield of field grown soybean under deficit irrigation conditions. Agric. Water Management. 75:226–244.

Keeling, C.D., T.P. Whorf, M. Wahlen, and J. van der Plicht. 1995. Inter-annual extremes in the rate of rise of atmospheric carbon dioxide since 1980. Nature 375:660-670.

Korte, L.L., J.E. Specht, J.H. Williams, and R.C. Sorensen. 1983. Irrigation of soybean genotypes during reproductive ontogeny. II. Yield component responses. Crop Sci. 23:528-533.

Kozlowski, T.T., and S.G. Pallardy. 1997. Physiology of Woody Plants. Academic Press, NY.

Kramer, J.K., and J.S. Boyer. 1995. Water Relations of Plants and Soils. Academic Press, California.

Lal, A., M.S.B. Ku, and G.E. Edwards. 1996. Analysis of inhibition of photosynthesis due to water stress in the C_3 species *Hordeum vulgare* and *Vicia faba*: electron transport, CO_2 fixation and carboxylation capacity. Photosynth. Res. 49:57-69.

Lange, O.L., R. Losch, E.D. Schulze, and L. Kappen. 1971. Responses of stomata to changes in humidity. Planta 100:76-86.

Lauteri, M., M. Scartazza, M.C. Guido, and E. Brugnoli. 1997. Genetic variation in photosynthetic capacity, carbon isotope discrimination and mesophyll Ecol. 11:675-683.

Lawlor, D.W., and G. Cornic. 2002. Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant Cell Environ. 25: 275-294.

Lecoeur, J., J. Wery, O. Turc, and F. Tardieu. 1995. Expansion of pea leaves subjected to short water-deficit: cell number and cell-size are sensitive to stress at different periods of leaf development. J. Exp. Bot. 46:1093-1101.

Leuschner, C., K. Backes, D. Hertel, F. Schipka, U. Schmitt, O. Terborg, and M. Runge. 2001. Drought responses at leaf, stem and fine root levels of competitive *Fagus sylvatica* L. and *Quercus petraea* (Matt.) Liebl. trees in dry and wet years. Forest Ecol. Management 149:33-46.

Levitt, J. 1980. Responses of Plants to Environmental Stresses. Academic Press, New York and London.

Liu, F., M.N. Andersen, S. Jacobsen and C.R. Jensen. 2005. Stomatal control and water use efficiency of soybean (*Glycine max* L. Merr.) during progressive soil drying. Environ. Exp. Bot. 54:33-40

Lloyd, J., J.P. Syvertsen, P.E. Kriedemann, and G.D. Farquhar. 1992. Low conductances for CO_2 diffusion from stomata to the sites of carboxylation in leaves of woody species. Plant Cell Environ. 15:873-899.

Long, S.P., P.K. Farage, and R.L. Garcia. 1996. Measurement of leaf and canopy photosynthetic $C0_2$ exchange in the field. J. Exp. Bot. 47:1629-1642.

Loreto, F., P.C. Harley, G. Di Marco, and T.D. Sharkey. 1992. Estimation of mesophyll conductance to CO2 flux by three different methods. Plant Physiol. 98:1437-1443.

Loreto, G., G. Di Marco, D. Tricoli, and T.D. Sharkey. 1994. Measurements of mesophyll conductance, photosynthetic electron transport and alternative electron sinks of field grown wheat leaves. Photosynth. Res. 41:397–403.

Majeau, N., M.A. Arnoldo, and J.R. Coleman. 1994. Modification of carbonic anhydrase by antisense and over-expression constructs in transgenic tobacco. Plant Molecular Biol. 25:377-385.

Martin, B., C.G. Tauer, and R.K. Lin. 1999. Carbon isotope discrimination as a tool to improve water-use efficiency in tomato. Crop Sci. 39:1775-1783.

McCree, K.J., and S.D. Davis. 1974. Effect of water stress and temperature on leaf size and number of epidermal cells in grain sorghum. Crop Sci. 14:751-755.

Meckel, L., D.B. Egli, R.E. Phillips, D. Radcliffe, and J.E. Leggett. 1984. Effect of moisture stress on seed growth in soybeans. Agron. J. 75:1027-1031.

Mederski, H. J., and D. L. Jeffers. 1973. Yield response of soybean varieties grown at two soil moisture stress levels. Agron. J. 65:410-412.

Medrano, H., J. M. Escalona, J. Bota, J. Guias, and J. Flexas. 2002. Regulation of photosynthesis of C_3 plants in response to progressive drought: stomatal conductance as a reference parameter. Ann. Bot. 89: 895-905.

Meyer, S., and B. Genty. 1998. Mapping intercellular CO_2 mole fraction (C_i) in Rosa rubiginosa leaves fed with abscisic acid by using chlorophyll fluorescence imaging. Significance of C_i estimated from leaf gas exchange. Plant Physiol. 116:947-957.

Mian, M.A.R., D.A. Ashley, and H.R. Boerma. 1998. An additional QTL for water use efficiency in soybean. Crop Sci. 38:390-393.

Mian, M.A.R., M.A. Bailey, and H.R. Boerma. 1996. Molecular markers associated with water use efficiency and leaf ash in soybean. Crop Sci. 36:1252-1257.

Miyashita, K., S. Tanakamaru, T. Maitani, and K. Kimura. 2005. Recovery responses of photosynthesis, transpiration, and stomatal conductance in kidney bean following drought stress. Environ. Exp. Bot. 53:205-214.

Miyazawa, S., S. Yoshimura, Y. Shinzaki, M. Maeshima, and C. Miyake. 2008. Relationship between mesophyll conductance to CO_2 diffusion and contents of aquaporin localized at plasma membrane in tobacco plants grown under drought conditions. In: J.F. Allen, E. Gantt, J.H. Golbeck, and B. Osmond (eds.), Photosynthesis. Energy from the Sun: 14th International Congress on Photosynthesis, pp. 805-808.

Monclus, R., E. Dreyer, M. Villar, F.M. Delmotte, D. Delay, J.M. Petit, C. Barbaroux, D. Thiec, C. Brechet, and F. Brignolas. 2006. Impact of drought on productivity and water use efficiency in 29 genotypes of *Populus deltoids* x *Populus nigra*. New Phytol. 169: 765-777.

Muchow, R.C., and T.R. Sinclair. 1989. Epidermal conductance, stomatal density and stomatal size among genotypes of Sorghum bicolor (L .) Moench. Plant Cell Environ. 12:425-431.

Ni, B.R., and S.G. Pallardy. 1992. Stomatal and non-stomatal limitations to net photosynthesis in seedlings of woody angiosperms. Plant Physiol. 99:1502-1508.

Niinemets, Ű., A. Diaz-Espejo, J. Flexas, J. Galmes, and C.R. Warren. 2009. Importance of mesophyll diffusion conductance in estimation of plant photosynthesis in the field. J. Exp. Bot.. 60:2271-2282.

Nilson, SE, and S.M. Assmann. 2007. The control of transpiration. Insights from Arabidopsis. Plant Physiol. 143:19–27.

Nonami, H., Y. Wu, J.S. Boyer. 1997. Decreased growth induced water potential: a primary cause of growth inhibition at low water potentials. Plant Physiol. 114:501-509.

Ogren, E., and G. Oquist. 1985. Effects of drought on photosynthesis, chlorophyll fluorescence and photo-inhibition susceptibility in intact willow leaves. Planta 166:380-388.

Ohashi, Y., H. Saneoka , and K. Fujita. 2000. Effect of water stress on growth, photosynthesis, and photoassimilate translocation in soybean and tropical pasture legume Siratro. Soil Sci . Plant Nutr. 46:417-425.

Paje, M.C.M., M.M. Ludlow, and R.J. Lawn. 1988. Variation among soybean (*Glycine max* (L.) Merr.) accessions in epidermal conductance of leaves. Aust. J. Agric. Res. 39: 363-373.

Pandey, R. K., W. A. T. Herrera, and J. W. Pendleton. 1984. Drought response of grain legumes under irrigation gradient: I. Yield and yield components. Agron. J. 76:549-553.

Parkhurst, D.F. 1994. Diffusion of CO_2 and other gases inside leaves. New Phytol. 126:449-479.

Payne, W.A., P.E. Rasmussen, C. Chan, and R.E. Ramig. 2001. Assessing simple wheat and pea models using data from a long-term tillage experiment. Agron. J. 93:250-260.

Pons, T.L., J. Flexas, S. von Caemmerer, J.R. Evans, B. Genty, M. Ribas-Carbo, and E. Brugnoli. 2009. Estimating mesophyll conductance to CO_2: methodology, potential errors, and recommendations. J. Exp. Bot. 60: 2217-2234.

Pou, A., J. Flexas , M. del M. Alsina, J. Bota, C. Carambula, F.D. Herralde, J. Galmés, C. Lovisolo, M. Jiménez, M. Ribas-Carbó, D. Rusjan, F. Secchi, M. Tomàs, Z. Zsófi, and H. Medrano. 2008. Adjustments of water-use efficiency by stomatal regulation during drought and recovery in the drought-adapted Vitis hybrid Richter-110 (*V. berlandieri* x *V. rupestris*). Physiol. Plant. 134:313-23.

Price, G.D., S. von Caemmerer, and J.R. Evans. 1994. Specific reduction of chloroplast carbonic-anhydrase activity by antisense RNA in transgenic tobacco plants has a minor effect on photosynthetic CO2 assimilation. Planta 193:331-340.

Quarrie, S.A., and H.G. Jones. 1977. Effects of abscisic acid and water stress on development and morphology of wheat. J. Exp. Bot. 28:192-203.

Quick, W.P., M.M. Chaves, R Wendler., M. David, M.L. Rodrigues, J.A. Passaharinho, J.S. Pereira, M.D. Adcock, R.C. Leegood, and M. Stitt. 1992. The effect of water stress on photosynthetic carbon metabolism in four species grown under field conditions. Plant Cell Environ 15:25-35.

Ramanjulu, S., N. Sreenivasulu, and C. Sudhakar. 1998. Effect of water stress on photosynthesis in two mulberry genotypes with different drought tolerance. Photosynthetic. 35:279-283.

Reddy, A.R., K.V. Chaitanya, and M. Vivekanandan. 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. Plant Physiol. 161:1189-1202.

Richards, R.A. 1991. Crop improvement for temperate Australia: future opportunities. Field Crops Res. 26:141-169.

Richards, R.A., G.J. Rebetzke, A.G. Condon, and A.F.V. Herwaarden. 2002. Breeding opportunities for increasing the efficiency of water use and crop yield in temperate cereals. Crop Sci. 42:111-121.

Ritchie, J.T., and B. Basso. 2008. Water use efficiency is not constant when crop water supply is adequate or fixed: the role of agronomic management. Europe. J. Agron. 28:273-281.

Roupsard, O., P. Gross, and E. Dreyer. 1996. Limitation of photosynthetic activity by CO2 availability in the chloroplasts of oak leaves from different species and during drought. Annales des Sciences Forestieres 53:243-54.

Sasaki, H., T. Hirose, Y. Watanabe, R. Ohsugi. 1998. Carbonic anhydrase activity and CO_2-transfer resistance in Zn-deficient rice leaves. Plant Physiol. 118:929-934.

Shantz, H.J., and L.N. Piemeisel. 1927. The water requirement of plants at Akron, Colo. J. Agric. Res. 34:1093-1190.

Sharkey, T. D. 1990. Water stress effects on photosynthesis. Photosynthetica. 24:651.

Sharkey, T.D., C.J. Bernacchi, G.D. Farquhar, and E.L. Singsaas. 2007. Fitting photosynthetic carbon dioxide response curves for C-3 leaves. Plant Cell Environ. 30:1035-1040.

Sinclair, T.R., L. Salado-Navarro, E.N. Moarndi, M.L. Bodrero, and R.A. Martignone. 1992. Soybean yield in Argentina in response to weather variation among cropping seasons. Field Crops Res. 30:1-11.

Siniot, N., and P.J. Kramer. 1977. Effect of water stress during different stages of growth of soybean. Agron. J. 69:274-278.

Smith, J.A.C., and H. Griffiths. 1993. Water Deficits: Plant Responses from Cell to Community. Bios Scientific Publishers, Oxford, UK.

Soolanayakanahally, R.Y., R.D. Guy, S.N. Silim, E.C. Drewes , and W. Schroeder. 2009. Enhanced assimilation rate and water use efficiency with latitude through increased photosynthetic capacity and internal conductance in balsam poplar (*Populus balsamifera* L.). Plant Cell Environ. 32:1821-1832.

Specht, J.E., J.H. Williams and C.J. Weidenbenner. 1986. Differential responses of soybean genotypes subjected to a seasonal soil water gradient. Crop Sci. 26:922-934.

Spence, R.D., H. Wu, P.J.H. Sharpe, and K.G. Clark. 1986. Water stress effects on guard cell anatomy and the mechanical advantage of the epidermal cells. Plant Cell Environ. 9:197-202.

Stanhill, G. 1986. Water use efficiency. Adv. Agron. 39: 53-85.

Stiller, W.N., J.J. Read, G.A. Constable, and P.E. Reid. 2005. Selection for water use efficiency traits in a cotton breeding program: cultivar differences. Crop Sci. 45:1107-1113.

Stone, P.J., D.R. Wilson, J.B. Reid, and G.N. Gillespie. 2001. Water deficit effects on sweet corn: I. Water use, radiation use efficiency, growth, and yield. Aust. J. Agric. Res. 52:103-113.

Subramanian, V.B., and M. Maheswari. 1990. Stomatal conductance, photosynthesis and transpiration in green gram during, and after relief of water stress. Ind. J. Exp. Biol. 28:542-544.

Syveresten, J.P., J.C. Lloyd, C. McConchie, P.E. Kriedemann, and G.D. Farquhar. 1995. On the relationship between leaf anatomy and CO_2 diffusion through the mesophyll of hypostomatous leaves. Plant Cell Environ. 18:149-157.

Tang, A.C., Y. Kawamitsu, M. Kanechi, and J.S. Boyer. 2002. Photosynthetic oxygen-evolution at low water potential in leaf discs lacking an epidermis. Ann. Bot. 89:861-870.

Terashima, I., and K. Ono. 2002. Effects of $HgCl_2$ on CO_2 dependence of leaf photosynthesis: evidence indicating involvement of aquaporins in CO_2 diffusion across the plasma membrane. Plant Cell Physiol. 43:70–78.

Terashima, I., T. Araya, S. Miyazawa, K. Sone, and S. Yano. 2005. Construction and maintenance of the optimal photosynthetic systems of the leaf, herbaceous plant and tree: an eco-developmental treatise. Ann. Bot. 95:507-519.

Toung, T.P., and S. I. Bhuiyan. 1999. Increasing water-use efficiency in rice production: farm-level perspectives. Agric. Water Management 40:117-122.

Udayakumar, M., M.S. Sheshshayee, K.N. Nataraj, H.B. Madhava, R. Devendra, I.S. A. Hussain, and T.G. Prasad. 1998. Why has breeding for water use efficiency not been successful? An analysis and alternate approach to exploit this trait for crop improvement (Bangalore). Current Sci. 74:994-1000.

Uehlein, N., C. Lovisolo, F. Siefritz, and R. Kaldenhoff. 2003. The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. Nature 425:734-737.

von Caemmerer, S., and G.D. Farquhar. 1981. Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376-87.

von Caemmerer, S., and J.R. Evans. 1991. Determination of the average partial-pressure of CO_2 in chloroplasts from leaves of several C_3 plants. Aust. J. Plant Physiol. 18:287-305.

Walden, A. 2009. Physiological traits affecting drought tolerance in Ontario-adapted soybean. M.Sc. Thesis University of Guelph.

Wallace, J.S. 2000. Increasing agricultural water use efficiency to meet future food production. Agriculture. Eco. Environ. 82:105-119.

Warren, C.R. 2006. Estimating the internal conductance to CO_2 movement. Func. Plant Biol. 33:431-442.

Warren, C.R. 2008. Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. J. Exp. Bot. 59:1475-1487.

Warren, C.R., and E. Dreyer. 2006. Temperature response of photosynthesis and internal conductance to CO_2: results from two independent approaches. J. Exp. Bot. 57: 3057-3067.

Warren, C.R., and M.A. Adams. 2004. Evergreen trees do not maximize instantaneous photosynthesis. Trends in Plant Sci. 9:270-274.

Warren, C.R., and M.A. Adams. 2006. Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and N use in photosynthesis. Plant Cell Environ. 29:192-201.

Warren, C.R., G.J. Ethie, N.J. Livingston, N.J. Grant, D.H. Turpin, D.L. Harrison, and T.A. Black. 2003. Transfer conductance in second growth Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) canopies. Plant Cell Environ. 26:1215-1227.

Widodo, W., J.C.V. Vu, K.J. Boote, J.T. Baker, and L.H.J. Allen. 2003. Elevated growth CO_2 delays drought stress and accelerates recovery of rice leaf photosynthesis. J. Environ. Exp. Bot. 49:259-272.

Woodward, F.I. 1987. Stomatal numbers are sensitive to increases in CO2 from pre-industrial levels. Nature 327:617-618.

Xu, Y., J.R. Coburn, B.E. Gollands, R.C. Pausch, C.F. Fleet, S.R. McCouch, and J.P. Comstock. 2004. Mapping quantitative trait loci for water use efficiency in rice. In: Plant & animal genomes XII conference, San Diego, January 10–14, 2004.

Yamori, W., K. Noguchi, Y.T. Hanba, and I. Terashima. 2006. Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. Plant Cell Physiol. 47:1069-1080.

Yamori, W., K. Noguchi, Y.T. Hanba, and I. Terashima. 2006. Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. Plant Cell Physiol. 47:1069-1080.

Yordanov, I., V. Velikova, and T. Tsonev. 2000. Plant responses to drought, acclimation, and stress tolerance. Photosynthetica 38:171-186.

Zhang, J., and W.J. Davies. 1989. Abscisic acid produced in dehydrating roots may enable the plant to measure the water status of the soil. Plant Cell Environ.12:73-81.

Zhang, J., and W.J. Davies. 1990. Does ABA in the xylem control the rate of leaf growth in soil dried maize and sunflower plants? J. Exp. Bot. 41:1125-1132.

Zhang, N., Y. Zhenliang, Y. Guirui, and W. Jianguo. 2007. Scaling up ecosystem productivity from patch to landscape: a case study of Changbai Mountain Nature Reserve, China. Landscape Ecol. 22:303-315.

Zhang, Z., P. Xu and J. Jia. 1998. Genetics for improving crop water use efficiency. Proceedings of the 4th International Crop Science Congress in Australia. 1-5. Accessed online: http://www.cropscience.org.au/icsc2004/ poster/1/1/426_zhengbin.htm

Zhao, B.I., M. Kondo, M. Maeda, Y. Ozaki, and J. Zhang. 2004. Water-use efficiency and carbon isotope discrimination in two cultivars of upland rice during different developmental stages under three water regimes. Plant Soil 261: 61–75.

Zhenzhu, X., and G. Zhou. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. J. Exp. Bot. 59:3317-3325.

APPENDIX

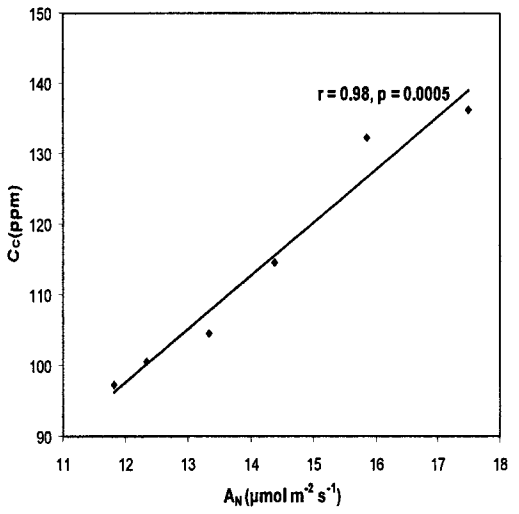

Figure A1. Relationship between cultivar means for C_C and A_N across both treatments in the greenhouse.

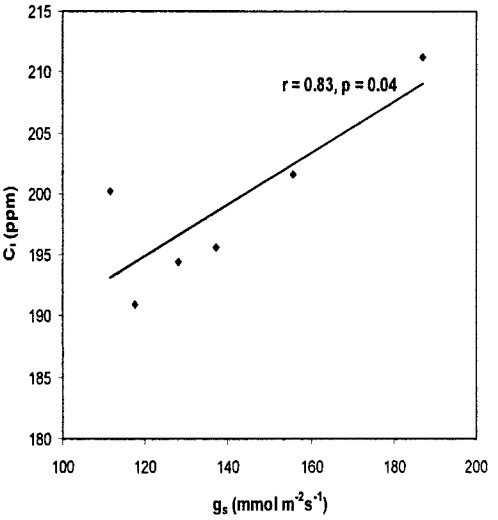

Figure A2. Relationship between cultivar means for C_i and g_s across both treatments in the greenhouse.

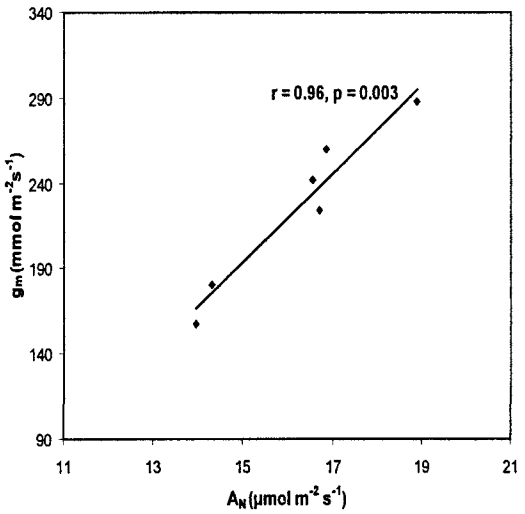

Figure A3. Relationship between cultivar means for g_m and A_N for the across both treatments in the greenhouse.

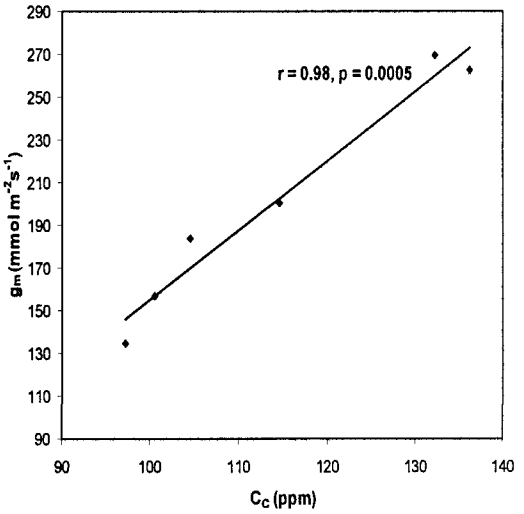

Figure A4. Relationship between cultivar means for g_m and C_c across both treatments in the greenhouse.

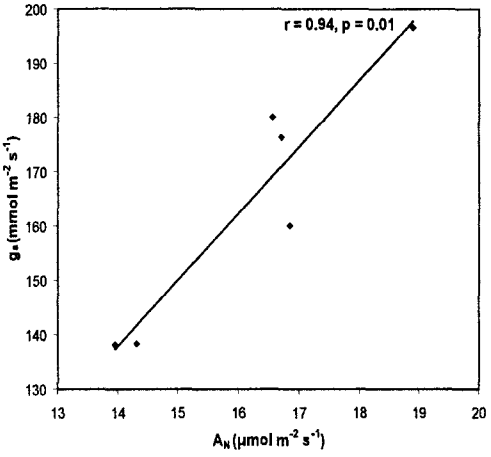

Figure A5. Relationship between cultivar means for g_s and A_N across both treatments in the greenhouse.

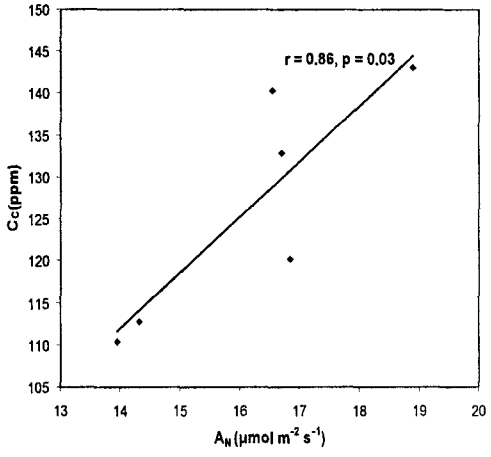

Figure A6. Relationship between cultivar means for C_C and A_N across both treatments in the greenhouse.

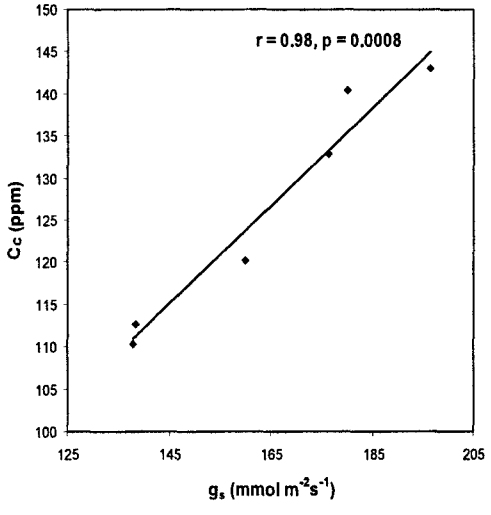

Figure A7. Relationship between cultivar means for C_C and g_s for the control treatment in the greenhouse.

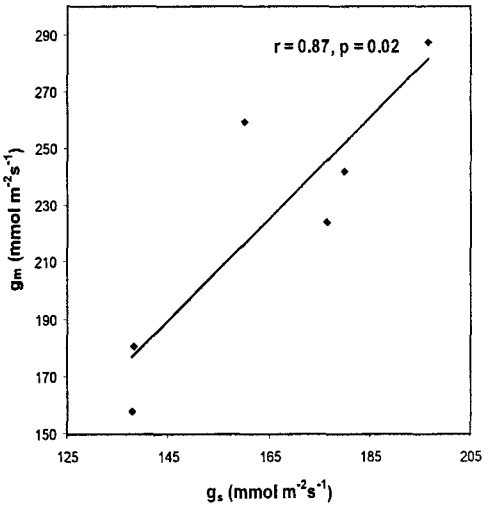

Figure A8. Relationship between cultivar means for g_m and g_s for the control treatment in the greenhouse.

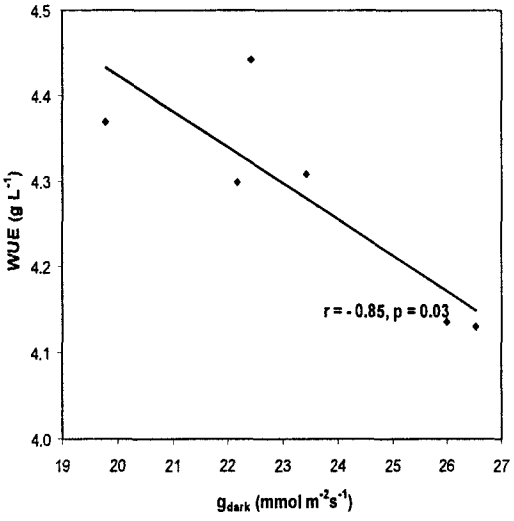

Figure A9. Relationship between cultivar means for WUE and g_{dark} for the control treatment in the greenhouse.

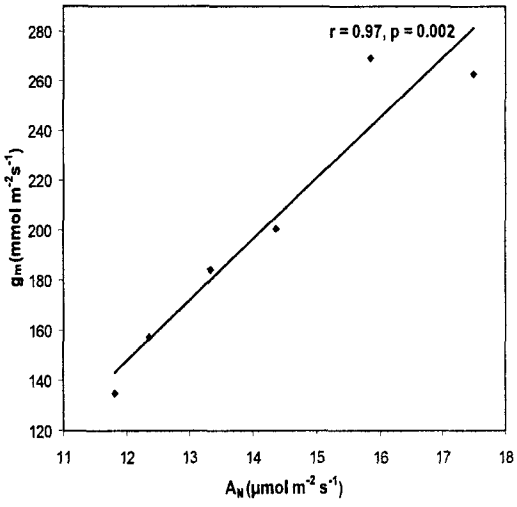

Figure A10. Relationship between cultivar means for g_m and A_N for the the control treatment in the greenhouse.

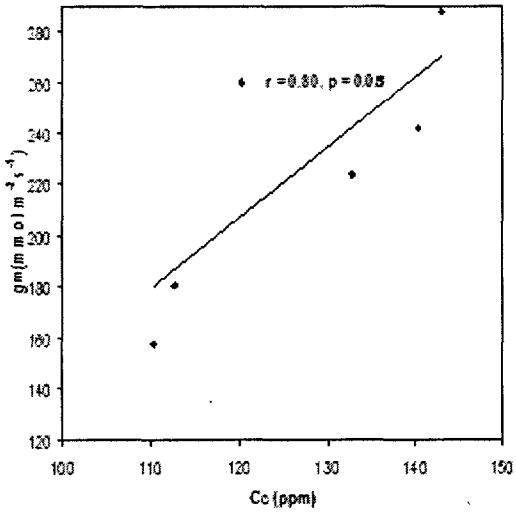

Figure A11. Relationship between cultivar means for g_m and C_C the control treatment in the greenhouse.

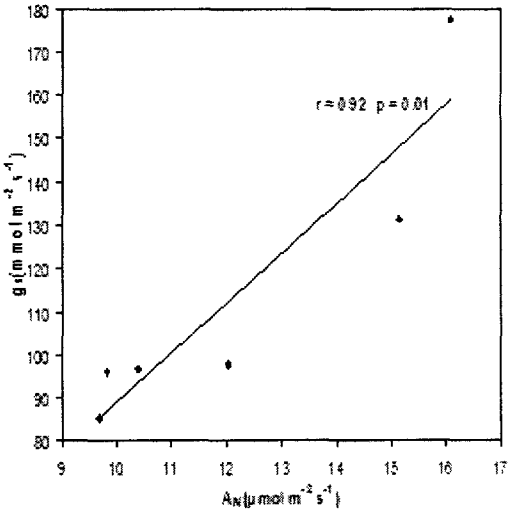

Figure A12. Relationship between cultivar means for g_s and A_N for the drought treatment in the greenhouse.

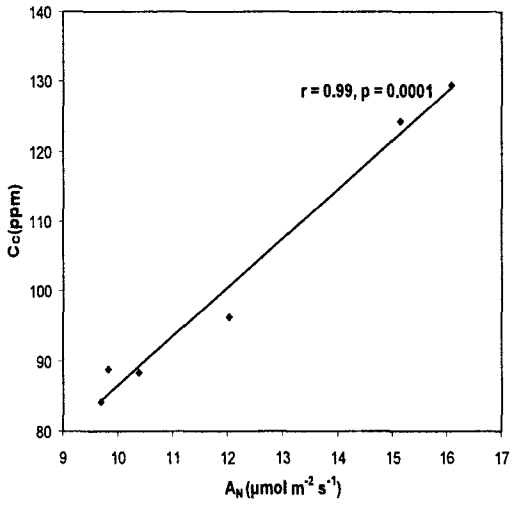

Figure A13. Relationship between cultivar means for C_C and A_N for the drought treatment in the greenhouse.

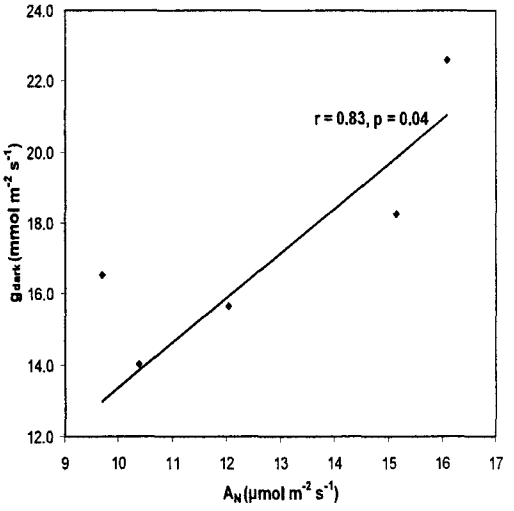

Figure A14. Relationship between cultivar means for g_{dark} and A_N for the drought treatment in the greenhouse.

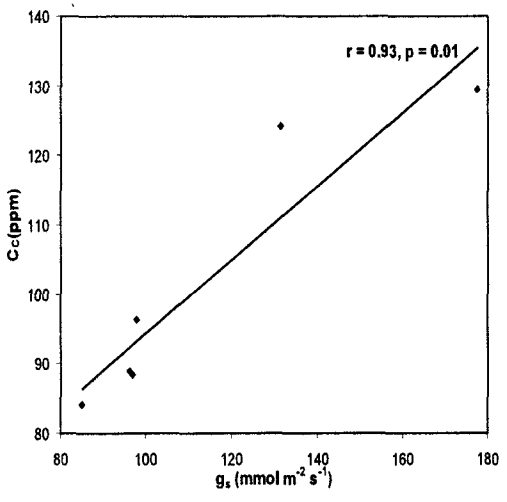

Figure A15. Relationship between cultivar means for C_C and g_m in the drought treatment in the greenhouse.

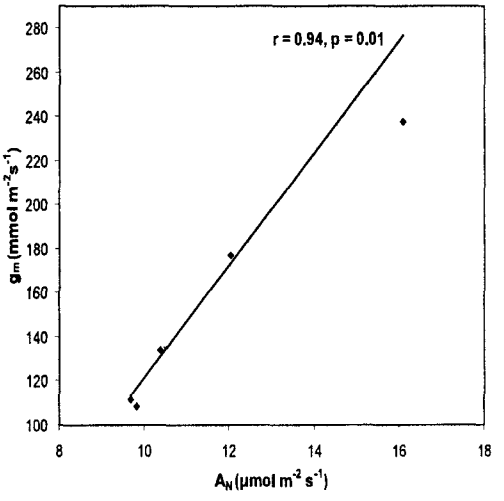

Figure A16. Relationship between cultivar means for g_m and A_N for the drought treatment in the greenhouse.

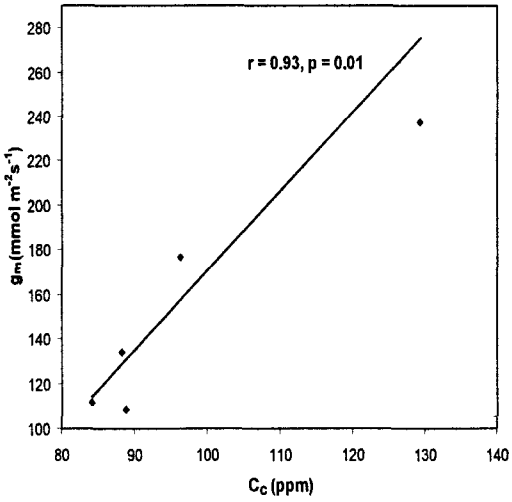

Figure A17. Relationship between cultivar means for g_m and C_C for the drought treatment in the greenhouse.

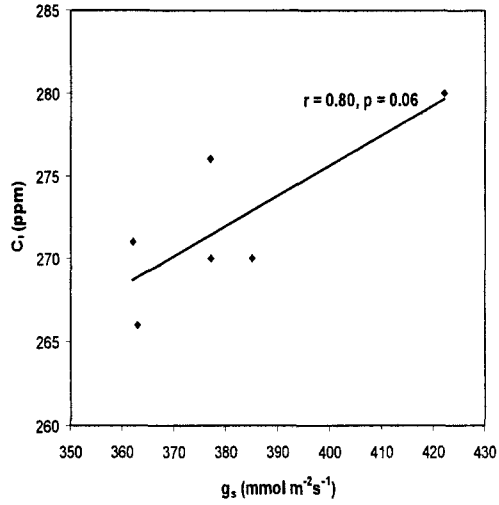

Figure A18. Relationship between cultivar means for C_i and g_s in the field.

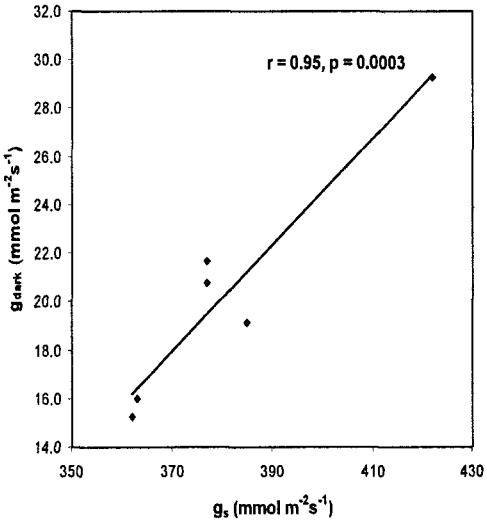

Figure A19. Relationship between cultivar means for and g_{dark} and g_s in the field.

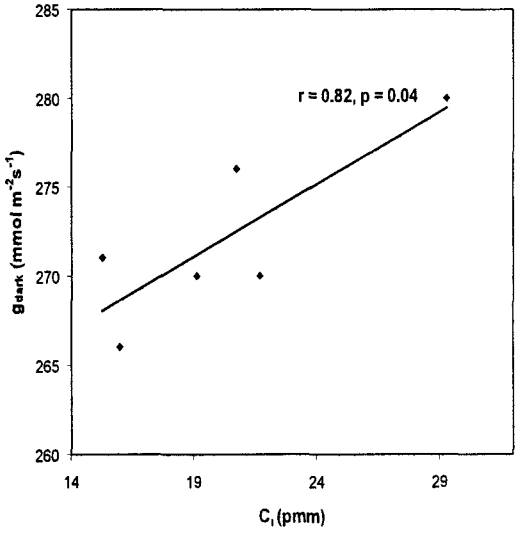

Figure A20. Relationship between cultivar means for g_{dark} and C_i in the field.

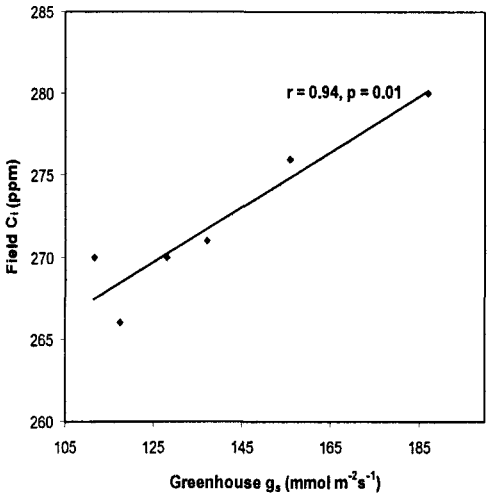

Figure A21. Relationship between cultivar means for C_i in the field and g_s across treatments in the greenhouse.

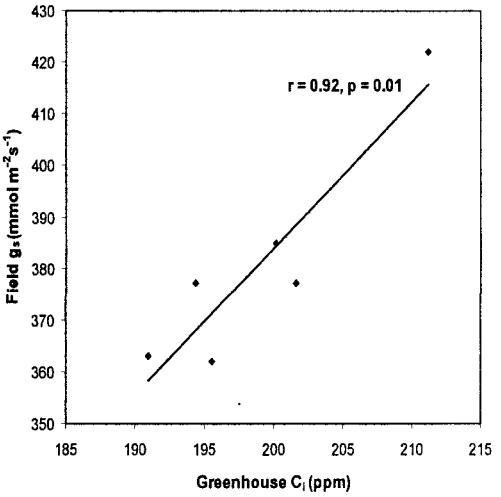

Figure A22. Relationship between cultivar means for g_s in the field and C_i across treatments in the greenhouse .

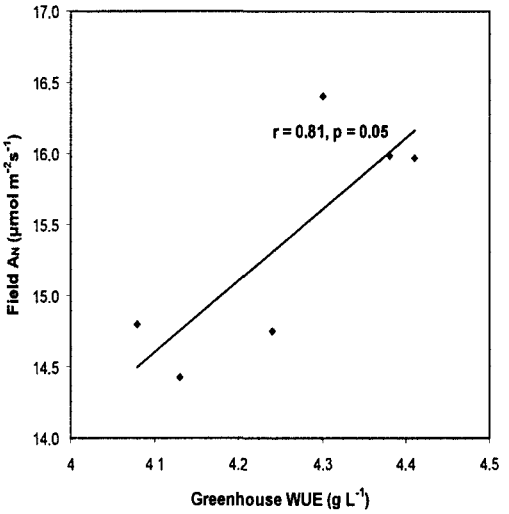

Figure A23. Relationship between cultivar means for A_N in the field and WUE across treatments in the greenhouse.

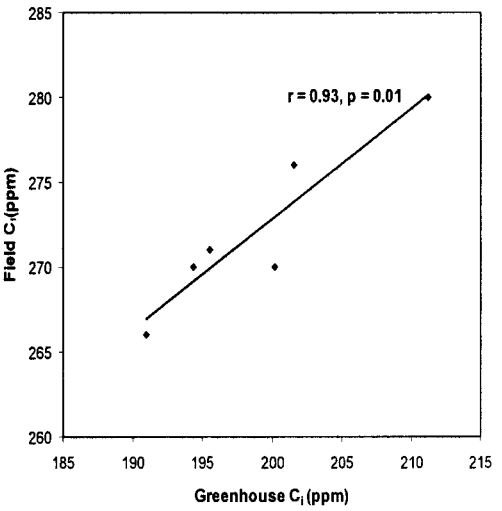

Figure A24. Relationship between cultivar means for C_i in the field and C_i across treatments in the greenhouse.

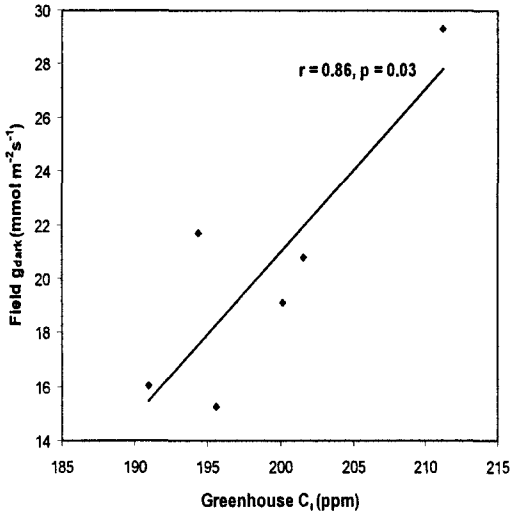

Figure A25. Relationship between cultivar means for g_{dark} in the field and C_i across treatments in the greenhouse.

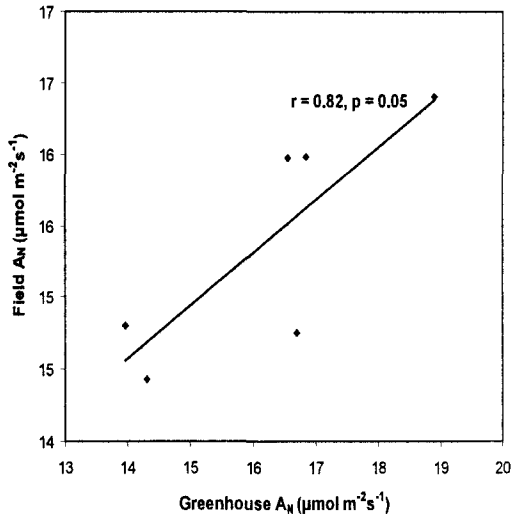

Figure A26. Relationship between cultivar means for A_N in the field and C_i in the control treatment in the greenhouse.

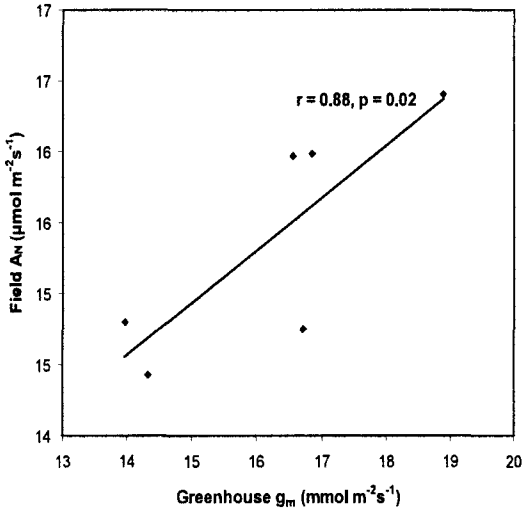

Figure A27. Relationship between cultivar means for A_N in the field and g_m for the control treatment in the greenhouse.

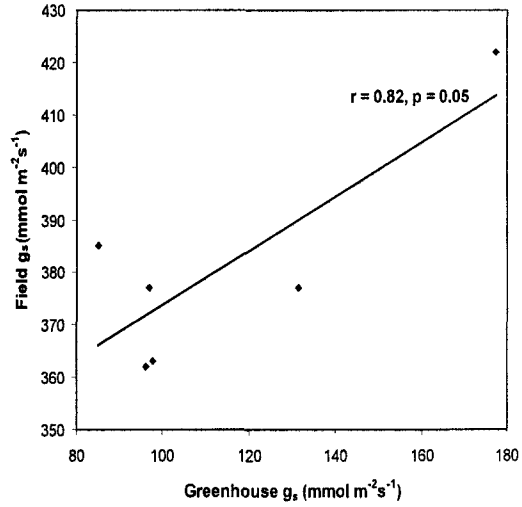

Figure A28. Relationship between cultivar means for g_s in the field and g_s in the drought treatment in the greenhouse.

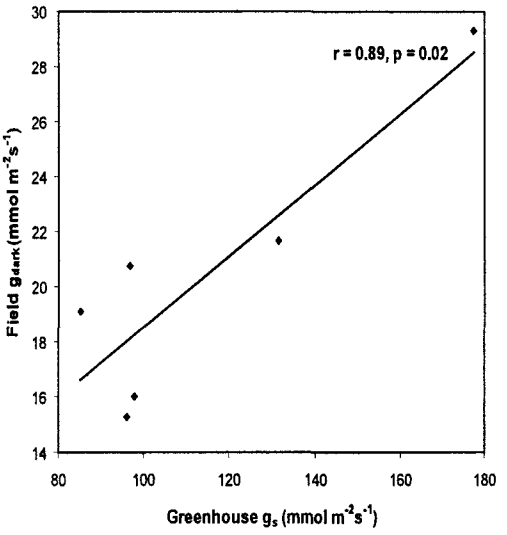

Figure A29. Relationship between cultivar means for g_{dark} in the field and g_s in the drought treatment in the greenhouse.

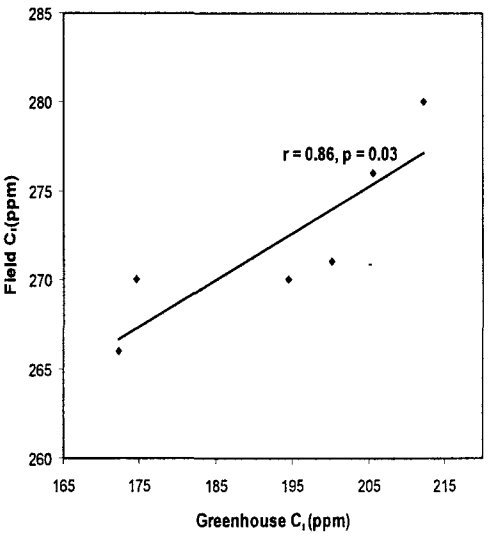

Figure A30. Relationship between cultivar means for C_i in the field and C_i in the drought treatment in the greenhouse.

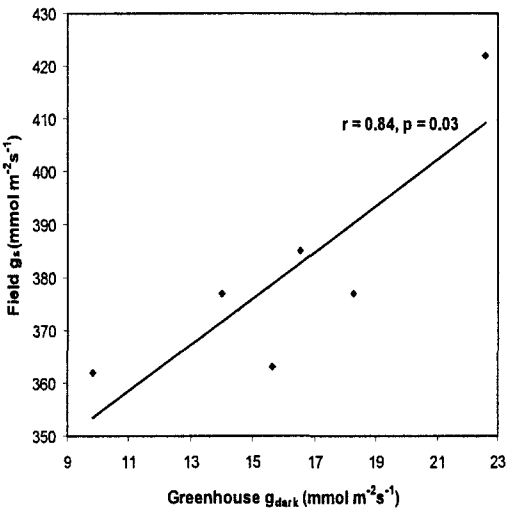

Figure A31. Relationship between cultivar means for g_s in the field and g_{dark} for the drought treatment in the greenhouse.

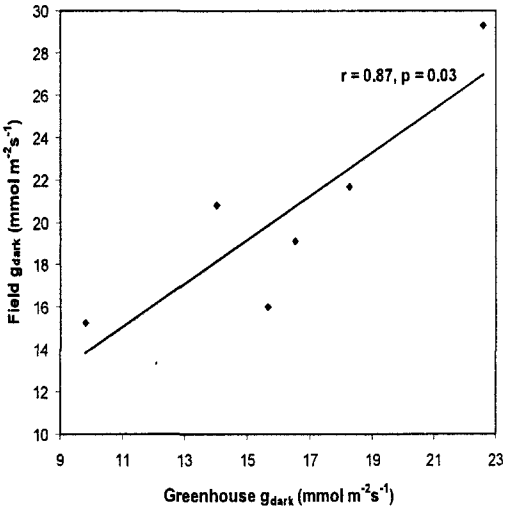

Figure A32. Relationship between cultivar means for g_{dark} in the field and g_{dark} in the drought treatment in the greenhouse.

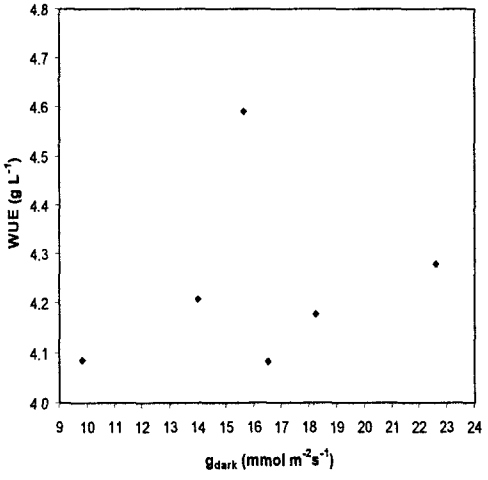

Figure A33. Relationship between cultivar means for WUE and g_{dark} in the drought treatment in the greenhouse.