PHYSIOLOGICAL TRAITS AFFECTING DROUGHT TOLERANCE IN ONTARIO-ADAPTED SOYBEAN

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ABSTRACT

PHYSIOLOGICAL TRAITS AFFECTING DROUGHT TOLERANCE IN ONTARIO-ADAPTED SOYBEAN

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Under water limited conditions, increased water use efficiency by crop plants is beneficial. Using Ontario-adapted soybean (*Glycine max* (L.) Merr.) this study examined the genetic variability for both water use efficiency and a potential surrogate trait, dark adapted leaf conductance. The genetic variability of root elongation rate under osmotic stress, a trait likely to be beneficial in drought conditions, was also measured. Significant genetic variation was found in the Ontario germplasm for all three traits. This variation provides potential for breeding more drought tolerant soybean varieties using material already adapted to Ontario. A strong correlation was found between the water use efficiency and dark adapted leaf conductance traits, so the physiological basis of that relationship was examined. Dark adapted leaf conductance was found to be highly predictive of leaf conductance and leaf internal carbon dioxide concentration in light-adapted leaves.

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CHAPTER 1. GENERAL INTRODUCTION AND OBJECTIVES

1.1 GENERAL INTRODUCTION

In 2008, approximately 1.2 million hectares of soybean were planted in Canada, of which 850 thousand ha were in Ontario (Statistics Canada, 2008). Over the past decade, Ontario has experienced lower than average precipitation and it is predicted that the frequency of droughts will increase in the future (Ontario Ministry of Natural Resources, 2008). Since insufficient soil water availability can negatively impact yield, the development of drought tolerant soybean varieties would be beneficial to soybean growers. Breeding for drought tolerance traits in soybean may reduce yield loss in years with insufficient precipitation. To achieve this, an increased knowledge of the physiological traits that enhance a plant's ability to cope with low water availability would be advantageous.

For any given amount of precipitation, some varieties will produce more growth than others either because they are able to access available soil water more effectively, or because they are able to use water more efficiently. Past research has shown that there is substantial genetic variability for traits such as water use efficiency in the available soybean germplasm (Mian et al. 1996; Hufstetler et al. 2007), however there is no knowledge of the variability of these traits among commercial varieties grown in Ontario. There is also limited knowledge of the physiological relationship between some of these traits (Farquhar et al. 1982; Paje et al. 1988; Hufstetler et al. 2007).

This research examines three physiological traits that may increase drought tolerance. The first is root elongation rate under osmotic stress, which may allow roots to continue growth and development under water stress conditions and access water deeper in the soil profile (Sharp et al. 2004). The second is water use efficiency (WUE), the amount of biomass produced per unit soil water transpired. The third is dark-adapted leaf conductance (g_{dark}); this is a measure of the propensity of leaves to lose water under dark conditions when the stomates are expected to be closed. A recent study (Hufstetler et al. 2007) demonstrated a strong correlation between these last two traits, but the mechanism of this relationship remains unknown. This thesis will further explore this correlation and examine the potential of the use of dark-adapted conductance as a surrogate measurement for water use efficiency. This would be very beneficial for large germplasm screening efforts, as the labour intensity of the WUE trait has limited its potential in the past.

1.2 RESEARCH OBJECTIVES

1. Develop an efficient measurement technique to screen for g_{dark}.
2. Determine the genetic variability of g_{dark} in Ontario-adapted soybean varieties.
3. Determine the strength of the correlation between g_{dark} and WUE in Ontario-adapted soybean varieties.
4. Determine the physiological basis of the correlation between g_{dark} and WUE.
5. Determine the genetic variability among Ontario-adapted soybean varieties for the root elongation rate under osmotic stress, and its ability to recover following relief from osmotic stress.

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CHAPTER 2: LEAF EPIDERMAL CONDUCTANCE - MEASUREMENT TECHNIQUE DEVELOPMENT AND SCREENING OF ONTARIO VARIETIES

2.1 ABSTRACT

A surrogate measurement of water use efficiency (WUE) in soybean [*Glycine max* (L.) Merr.] would be beneficial to large breeding efforts, since measurement of WUE itself is very labour intensive. The previously reported correlation between WUE and another trait, minimum epidermal conductance (g_e), was tested using three different measurement techniques: (i) leaf gas exchange using attached leaves, measured with an open-flow system, (ii) leaf gas exchange of freshly detached leaves, measured with a closed recirculating system, and (iii) leaf gas exchange of detached, wilted leaves, measured with a closed, recirculating system. All of the measurements were performed on dark-adapted leaves of seven soybean genotypes. Significant genotype effects were found for g_e regardless of the measurement system, but g_e measured with the closed system on freshly detached leaves had the highest correlation coefficient with WUE. The measurement on freshly detached compared to wilted leaves produced different values and the correlation between the g_e of the wilted leaves and WUE was not significant. Therefore, it is suggested that the measurements on wilted leaves be referred to as g_e and the measurements on the freshly detached leaves as g_{dark}, since they are apparently distinct traits. A screening of g_{dark} was performed on sixty-three Ontario-adapted genotypes and ten soybean genotypes previously known to differ for both g_{dark} and WUE. Significant variation was found amongst the Ontario genotypes. There was no significant difference between the g_{dark} values of the lowest Ontario lines and the lowest g_{dark} overall. Significant differences were also found between parents of mapping populations.

2.2 INTRODUCTION

Soybean yields will always be lower, on average, in years with inadequate precipitation (Doss et al. 1974), but there is reason to believe that yield losses associated with drought stress can be reduced through variety improvement (Condon et al. 2004). One method for improving drought tolerance is to improve water use efficiency (WUE; the quantity of dry matter produced per unit soil water transpired) (Farquhar et al. 1989). Genetic variability has been found for WUE in several crop species including peanut (*Arachis hypogaea* L.; Wright et al. 1994), cotton (*Gossypium spp.*; Saranga et al. 1998), wheat (*Triticum aestivum* L.; Ehdaie and Waines) sorghum (*Sorghum bicolor* (L.) Moench; Donatelli et al. 1992), alfalfa (*Medicago sativa* L.; Johnson and Tieszen 1994), and soybean (*Glycine max* (L.) Merr.; Mian et al. 1996; Hufstetler et al. 2007).

WUE is a difficult and labour intensive trait to measure in the greenhouse and extremely challenging to measure under field conditions. Therefore its use in large screening efforts is limited, although surrogate traits such as carbon isotope discrimination can sometimes be used (Farquhar et al. 1989, Condon et al. 1990, Saranga et al. 1998, Martin et al. 1999, Xue et al. 2002). Using 23 soybean varieties, a recent study (Hufstetler et al. 2007) determined that there was a strong negative correlation ($r=-0.74$ $p<0.0001$) between WUE over the whole growth period of the plant and an instantaneous measurement of minimum leaf epidermal conductance (g_e, the leaf transpiration rate when stomata are presumed to be closed, divided by the leaf to air vapour concentration difference). This relationship was determined using soybean leaves that were still attached to the plant and had been dark-adapted for at least 36 hours. It

was measured using a commercially available open-flow leaf gas exchange measurement system. A nearly identical correlation between WUE and g_e was discovered in cotton ($r=-0.75$, $p<0.0001$) (Fish and Earl 2009) using similar methods. This relationship may allow g_e to act as a surrogate for WUE, which would be beneficial to large screening efforts due to the relative ease of g_e measurements compared to WUE. The g_e trait may also prove to be complimentary to the most commonly used surrogate trait, carbon isotope discrimination (Farquhar et al., 1989).

Apart from its association with WUE, g_e is also a trait of physiological interest on its own, as crop varieties with low g_e often survive longer under periods of drought (Sinclair and Ludlow, 1986; Smith et al. 2006). Under severe water deficit when stomatal closure is maximized, g_e determines the rate of water loss from leaf tissues and therefore the rate of progression towards a critically low leaf water content (Huftsetler et al. 2007). Sinclair and Ludlow (1986) identified three phases of plants' response to successive drying. The first phase occurs when water is freely available in the soil and both stomatal conductance and water vapour loss are maximal. During the second phase, stomatal conductance declines in response to the rate of water uptake from the soil being unable to match the potential transpiration rate. The third phase begins when stomates are no longer able to compensate for the low rate of water uptake and so the stomates close completely and stomatal conductance is at a minimum. During this phase the maintenance of plant water balance depends on epidermal conductance. The third phase continues until either the soil water level increases or the plant dies.

The term g_e refers to the sum of the stomatal and cuticular diffusive pathways acting in parallel (van Gardingen and Grace 1992; Muchow and Sinclair 1989) and is used when there is uncertainty as to whether stomata are completely closed and the water loss through stomatal pores is quantitatively negligible compared to the water diffusion across the cuticle (Kerstiens 1996). A reduction in g_e and an associated increase in WUE could enhance growth and productivity of Ontario soybean varieties under water limited conditions. However, there is currently no information on how Ontario-adapted soybean varieties rank for these traits compared to other sources of soybean germplasm, or even whether or not there is any variation for the g_e trait among Ontario soybean varieties.

Genetic variation for g_e has been observed in several crop species including rice (*Oryza sativa* L.; O'Toole et al. 1979), maize (*Zea mays* L.; Dube et al. 1975), oats (*Avena sativa* L.; Bengston et al. 1978), sorghum (Muchow and Sinclair 1989), cotton (Quisenberry et al. 1982; Fish and Earl 2009), and soybean (Paje et al. 1988; Hufstetler et al. 2007). All of these studies except Hufstetler et al. (2007) and Fish and Earl (2009) measured g_e using the weight loss of detached leaves under controlled environmental conditions over time. In this method the conductance was calculated using the following equation:

$$g_e = (\Delta FW/t)\ (1/A)\ (1/\ (e_l - e_a))$$

where ΔFW is the change in fresh weight over time t, A is the leaf area and $(e_l - e_a)$ is the water vapour concentration gradient between the leaf interior and the ambient air.

Sinclair and Ludlow (1986) observed three distinct phases in the transpiration decline data for detached leaves. The first was a phase of relatively rapid water loss which was interpreted as the period during which stomates are closing. The second phase occurred during a period where the stomates were fully closed, but the assumption of saturated vapour pressure inside the leaf was still valid. The rate of water loss during this phase was constant and slower than during the first phase. Sinclair and Ludlow (1986) demonstrated that the stomates were fully closed by flushing the leaf chamber with CO_2. A decrease in g_e was not observed and it was therefore concluded that g_e during this phase represented epidermal conductance when stomata were closed. The final zone had curvilinear water loss which was interpreted as a consequence of decreasing vapour pressure inside the leaf due to severe dehydration. It was the second zone, with the constant water loss and completely closed stomates, which was used for the g_e calculations in that study.

It is not clear how g_e measured according to Hufstetler et al. (2007) and Fish and Earl (2009) (dark-adapted, attached leaves in both cases) is related to g_e measured in past studies using detached leaves which were allowed to dry down over time. Also, to date, only g_e measured on attached, dark-adapted leaves has been shown to correlate with WUE, and it is not known if g_e measured using detached leaves would be more or less strongly correlated with WUE.

In the present work there were three main research objectives. The first was to determine how g_e measured on dark-adapted, attached leaves (method from Hufstetler et al., 2007; Fish and Earl 2009) is quantitatively related to g_e of detached, drying leaves (traditional g_e measurement). The second objective was to determine which of these alternate measurements of g_e is most strongly correlated with whole plant WUE. The final main objective was to use the g_e measurement method most strongly correlated with WUE to investigate the extent of genetic variation for g_e in Ontario-adapted soybean varieties, and to determine how the range in g_e among Ontario varieties compared to that observed for other soybean germplasm.

In this work, most measurements of g_e were made using a custom designed, closed (recirculating) leaf gas exchange measurement system, since we hypothesized that such a system would provide more accurate estimates of the very low transpiration rates required for g_e determinations. To test this hypothesis, we directly compared measurements made with this system against measurements made with a commercially available open-flow system.

2.3 MATERIALS AND METHODS:

2.3.1. *Closed System Gas Exchange Measurement Equipment*

The measurement chamber was a 0.7-L PVC plastic chamber with a removable air tight lid. Inside the chamber was a small 12-V DC fan for internal air circulation, and a rack constructed of stainless steel and nylon line to support the leaf sample. The chamber was connected to an LI-840 gas analyser (Licor Inc., Lincoln NE) by 3 mm ID plastic tubing. The gas analyser was connected to an air pump (model TD-2NA(4), Brailsford and Co. Inc., Antrim NH) and then back to the chamber through the same type of tubing. The pump stroke was adjusted to provide a flow rate of approximately 0.75 L min^{-1}, as measured by a rotameter. This was a closed re-circulating system, so when a leaf was sealed in the chamber the system water vapour concentration increased over time (due to transpiration), as did the CO_2 concentration (due to respiration). The chamber was also equipped with a T-type thermocouple to measure the temperature within the chamber. Preliminary experiments demonstrated that air circulation provided by the chamber fan was sufficient to ensure a boundary layer conductance around the sample that was at least two orders of magnitude higher than the leaf conductance. Due to the high boundary layer conductance, the very low transpiration rates, and the fact that measurements were made in darkness, it was assumed that the leaf temperature was the same as the measured air temperature. System pressure, CO_2 concentration and water vapour concentration reported by the LI-840, as well as air temperature reported by the thermocouple analogue / digital interface (Model OMR-6017 connected to model OMR-6018, Omega Engineering Inc. Stamford CT) were recorded on a personal computer running custom designed software. The software permitted system CO_2 and H_2O

concentrations to be displayed graphically as they were measured. The leaves were detached from the plant in order for them to be measured with this system. Preliminary experiments (data not shown) that compared transpiration rates from leaves with and without first sealing the cut petioles with paraffin wax showed that water loss from the petioles was negligible. Accordingly, for all subsequent experiments petioles were not sealed. Following each measurement, the leaf area was determined with a leaf area meter (model LI-3100, Licor).

2.3.2. *g_e calculations for the closed system*

For each leaf sample, data were collected at 5-s intervals, for a period of approximately 120 s after sealing the chamber. As shown in Figure 2.1, the increase in $[H_2O]$ slowed during the course of the measurement, due to the increase in the chamber humidity reducing the leaf–to-air vapour pressure differential. A second order polynomial regression was therefore fit to the $[H_2O]$ / time data; all such curve fits gave R^2 values in excess of 0.98, and were generally above 0.99. The slope of the line at the mid-point time was calculated as the first derivative of the fitted curve at that time. The transpiration rate (E) in mol H_2O s^{-1} was then calculated as

$$E = \text{slope} \times n / A,$$

where slope is the slope of the $[H_2O]$ / time curve at the midpoint time in mol H_2O mol^{-1} air s^{-1}, A is the leaf area in m^2, and n is the "molar volume" of the measurement system, calculated as

$$n = PV / RT,$$

where P is the system pressure in kPa at the midpoint time of the measurement, V is the system volume in L, R is the gas constant (8.3144 kPa L mol^{-1} K^{-1}), and T is the Kelvin temperature inside the chamber at the midpoint time of the measurement. Epidermal conductance (g_e), with units of mol m^{-2} s^{-1} was then calculated as

$$g_e = E / (W_i - W_a)$$

where W_a is the water vapour concentration inside the chamber at the midpoint time of the measurement (i.e., the $[H_2O]$ value at that time predicted by the fitted second order polynomial), and W_i is the leaf internal water vapour concentration, estimated assuming that the leaf interior air was at the saturation vapour pressure (e_s):

$$e_s = 0.61121e^{(17.502 * t) / (240.97 + t)}$$

(Buck, 1981), where t is the leaf temperature in °C. W_i is equal to e_s / P.

2.3.3. *Experiment 1: g_e as a function of time since leaf detachment*

The purpose of this experiment was to determine whether a constant g_e is achieved in detached leaves, and if so, at how many hours since detachment from the plant.

Twelve soybean plants of the variety PS56RR were grown in a greenhouse at the University of Guelph, Guelph ON during the fall of 2006 in 2.5-L white plastic pots with drainage holes, containing LA Sunshine Soil Mix 4 Aggregate Plus (SunGro, Bellevue WA) with the air temperature set at approximately 25°C during the 16 hours of daylight and at 20°C during the night. The daylength was extended using a mixture of metal halide and high pressure sodium lamps, which provided a supplemental photosynthetically active photon flux density (PPFD) of approximately 600 μmol $m^{-2}s^{-1}$ at the tops of the plants. Watering to soil saturation was performed every third day initially and then every second day and eventually every day as the plants grew larger and used more water. Fertilization was performed once per week. Each pot received 50 mL of a 0.8% (m/v) solution of 20:20:20 plus micronutrients fertilizer (Plant Products, Brampton ON). When all of the plants were between the 9 and 10 leaf stage they were moved into the dark at 20°C where they were left to acclimate for a minimum of 36 hours. This time period was determined to be sufficient to maximize stomatal closure and to prevent circadian rhythms causing stomatal opening in response to time of day (Hufstetler et al. 2007). After the 36-h dark adaptation period the youngest fully expanded leaf of each of the plants was measured in the dark using the closed re-circulating gas exchange system. The first measurement was immediately after the detachment of the leaf, and then subsequent measurements were taken on the same leaf at times 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h and 12 h since detachment. After the 12-h measurement, the area of each leaf was measured using a leaf area meter (model LI-3100, Licor). Standard error of the g_e estimate was calculated based on data from 12 plants, and g_e was graphed versus time since detachment.

2.3.4. *Experiment 2: measurement technique comparison*

This experiment compared three g_e measurement techniques: i) closed re-circulating system, freshly detached leaves, ii) closed re-circulating system, wilted leaves, and iii) LI-6400 measurement of an attached leaf, to determine which is the most strongly correlated with water use efficiency (WUE; $g_{dry\ matter} / L_{H2O}$). Seven soybean varieties were selected for this experiment: the Ontario-adapted commercial variety OAC Bayfield, plus six lines (Boggs, Hutcheson, N97-9756, PI407859-2, Tokyo, and Young) which were previously shown by Hufstetler et al. (2007) to represent a broad range for both WUE and g_e. The plants were grown essentially in the same manner as in Mian et al. (1996) and Earl (2002) and under the same greenhouse conditions as Experiment 1. The experimental design was a randomized complete block design with six repetitions planted sequentially.

The plants were all grown in 2.5-L white plastic pots with no drainage holes, containing a soil mixture, which consisted of 2/3 sand and 1/3 commercial soil mix. The commercial soil mix (Meadowville Gardens, Guelph ON) was composed of one part top soil: one part peat moss: one part manure. The pots were filled with an equal weight of soil, as were two additional pots. These additional pots had holes drilled in the bottom and a piece of nylon screen across the bottom of the pot to prevent soil loss. The two pots with holes were watered to oversaturation and then weighed once water had stopped dripping from the holes in the bottom of the pot. Each pot was weighed three times to ensure that a constant weight had been achieved; this value minus the empty pot weight was the soil saturation weight. The soil was then removed from the two pots and dried in

metal trays for 48 hours at 80° C. The soil was weighed and this value was used as the dry weight. The dry weight of the soil was subtracted from the saturation weight to determine the amount of water held by the soil at 100% soil water holding capacity. The mean of the values from the two pots was used as the overall soil water holding capacity for this experiment. The pots were watered to 80% relative soil water content (RSWC; 100% is equivalent to the soil water content at saturation), accounting also for pot weight, and maintained between 65% and 80% throughout the growing period using a gravimetric watering system. The watering system was composed of an electronic balance connected to a computer running custom software. A barcode reader was used to identify each pot and the desired weight, which had been set in the computer software. Through 3mm diameter tubing connected to a solenoid valve the plants received water up to the desired weight. The customized software then recorded the difference in the weight before and after watering. The pots were weighed every day or second day in order to maintain the desired weight. The minimum soil water content (65%) is well above the soil water content where soybean plants first start to reduce their water use in this soil type (Sinclair 1998; Earl 2003); thus, plants were considered to be water-replete at all times.

Four seeds were planted per pot and then thinned to one seedling per pot at the first leaf stage. At this point the pots were capped with fitted white lids with a 1 cm diameter hole for the seedling to grow through and a 1.5 cm hole through which the pot was watered. The pots were all watered with 50 mL of 8g/ L 20:20:20 plus micronutrients fertilizer (Plant Products, Brampton ON) on both the planting day and the

capping day. The day the pots were capped was the first day that the water use data was recorded. All water additions were recorded by the computer software. At the end of the experiment the shoots were harvested, roots were washed free of soil, and all plant parts were placed in the dryer for a minimum of 48 hours at 80°C, after which the dry weights were measured. Water use for each plant was calculated as total water additions since capping the pot, plus the difference between the pot weight at capping and the pot weight at harvest.

Just prior to the dry weight harvest, at the 9 or 10 leaf stage the plants were placed in the dark for a minimum of 36 hours, after which g_e was measured using each of three different techniques. First the g_e was measured on attached leaves using an LI-6400 Portable Photosynthesis System (LiCor) with a flow rate of 200 μmol s^{-1}, PAR of 0, the block temperature set to 25°C and a reference side [CO_2] of 400 ppm. The centre leaflet from the youngest fully expanded leaf and from the leaf two mainstem nodes below were measured. Then a different leaflet from the same two leaves was detached and each measured in the closed re-circulating system, as described for Experiment 1. A different leaflet was used in case pressure from the LI-6400 chamber gasket caused bruising, which might affect the results. Finally, another measurement was taken with the closed re-circulating system 4 hours after the leaflet had been detached from the plant. This specific time after detachment was chosen based on the results of Experiment 1, since it seemed to correspond to the time period after detachment when g_e was constant. The leaf area of each of the leaflets was measured following the 4-h measurement.

The data were analysed using PROC MIXED (SAS statistical software version 9.0) to determine if there were significant differences ($p<0.05$) amongst the genotypes for each of the traits measured. The random effects were the rep in which the plant was situated and rep x genotype. The fixed effects were: genotype, leaf, and genotype x leaf. A PROC CORR correlation analysis was then performed between genotype means for WUE and the g_e data from the different measurement techniques to determine which of the techniques produced g_e estimates that were mosst closely correlated with WUE.

2.3.5. *Experiment 3: Screening soybean germplasm for variation in g_e*

Thirty Ontario-adapted soybean varieties were chosen, which included thirteen Round-Up Ready varieties and seventeen conventional (non-RR) varieties selected randomly from across the various soybean heat unit zones in the province. Also included were ten lines from Hufstetler et al. (2007) which represented the known range for the g_e trait. Finally, 23 parents of existing Ontario mapping populations were included for potential future quantitative trait loci analysis. Therefore, a total of 63 soybean lines and varieties (Table 2.1) were screened in this experiment. The soybean plants were grown in the same manner as in Experiment 1. The model was a randomized complete block design. Complete blocks including each of the lines were planted sequentially, approximately a week apart, in order to facilitate measurement.

Approximately 35 days after planting, between the 8 and 11 leaf stage depending on the variety, the g_e measurement was taken in the dark using the closed re-circulating system on leaflets that had just been freshly detached from the plant (based on results

from Experiment 2), following a dark-adaptation of at least 36 h. The leaflets selected were from the leaf below the youngest fully expanded leaf and the leaf two positions below the youngest fully expanded leaf.

The results were analysed using PROC MIXED in SAS Statistical Analysis Software 9.0 in order to determine whether or not there was significant variation amongst the genotypes and amongst the two leaf positions for the g_e trait. The rep and rep x genotype effects were considered random and the genotype, leaf, and genotype x leaf effects were considered fixed. A protected two-tailed $LSD_{0.05}$ was calculated from the standard error of the LSMEANS in order to determine where significant differences amongst genotypes occurred. Because there were few missing data, for simplicity the mean SE across variety LSMEANS was used to calculate the $LSD_{0.05}$.

2.4 RESULTS

2.4.1. *Experiment 1: g_e as a function of time since leaf detachment*

During the first 4 hours after the leaf was detached from the plant there was a large decrease in the mean g_e from 17.6 mmol $m^{-2}s^{-1}$ to 6.1 mmol $m^{-2}s^{-1}$ (Figure 2.2). After this time between 4 hours and 6 hours since detachment there was a plateau in the g_e values, and then after 6 hours since detachment the g_e values began to decrease again over the next 6 hours from a mean g_e of 6.5 mmol $m^{-2}s^{-1}$ to 2.8 mmol $m^{-2}s^{-1}$. Since at 4 hours from detachment a plateau of g_e values was apparent and the leaves were visually wilted, this was the time used for wilted leaf measurements in Experiment 2.

2.4.2. *Experiment 2: Comparison of g_e measurement techniques*

The results of the LI-6400 measurements of the dark-adapted attached leaves demonstrated that there was a significant genotype effect ($p<0.001$), but not a significant leaf position effect ($p=0.57$). The same results were observed using the closed system with freshly detached dark-adapted leaves (significant genotype effect ($p<0.0001$) and a non-significant leaf effect ($p=0.24$)), and wilted leaves in the closed system ($p=0.02$; $p=0.38$ respectively). There was no significant leaf x genotype interaction when the leaves were measured using the LI-6400 ($p=0.49$), but there was a significant interaction using the closed system measurement technique for both the freshly detached ($p<0.001$) and wilted ($p=0.02$) leaves. Therefore the correlation analyses were conducted for both the mean g_e values across leaf positions (Table 2.2; Figure 2.3) and for the leaf positions individually (data not shown).

The analysis of WUE demonstrated that there were significant differences amongst the genotypes measured ($p=0.001$). The correlation between whole plant WUE and g_e was strongest for the freshly detached leaves measured with the closed system, for all three leaf combinations. When the mean of both leaves was used, only the correlation between the closed-system g_e using freshly detached leaves was significantly correlated with WUE ($r=-0.86$, $p=0.013$). The LI-6400 measurements had a good correlation coefficient ($r=-0.71$) but this correlation was not quite statistically significant ($p=0.076$). The wilted leaf measurements had no correlation with WUE ($r=-0.39$, $p=0.385$).

The strongest correlation of all was observed between the lower leaf g_e and whole plant WUE ($r=-0.88$, $p=0.009$), when g_e was measured using the closed system and freshly detached leaves. The correlation between the LI-6400 g_e measurements and WUE was also significant ($r=-0.81$, $p=0.028$) when only lower leaves were considered, but the correlation coefficient was not quite as high. The correlation between the lower leaf g_e and WUE when g_e was measured using wilted leaves in the closed-system was again not significant ($r=-0.63$, $p=0.13$).

None of the measurements of the upper leaves were significantly correlated with whole plant WUE, however, the correlation coefficient for the closed-system, freshly detached leaf measurement ($r=-0.54$, $p=0.207$) was higher than either the measurement taken using wilted leaves ($r=0.16$, $p=0.806$) or the measurements of attached leaves with the LI-6400 ($r=-0.45$, $p=0.309$). Since the correlation with whole plant WUE was always

highest for the freshly detached leaves using the closed system, this measurement technique was chosen for Experiment 3.

2.4.3. *Experiment 3: g_e screening*

Significant differences in g_e were found amongst genotypes ($p<0.0001$) as well as between the two leaf positions ($p<0.001$). There was also a significant genotype x leaf interaction ($p=0.04$). Across leaf positions the Agriculture Canada line AC Colibri had the highest g_e (39.5 mmol $m^{-2}s^{-1}$). This was significantly higher than the next highest genotype OT91-3 (22.3 mmol $m^{-2}s^{-1}$). The lowest measured g_e was genotype N98-7265 (6.6 mmol $m^{-2}s^{-1}$), but the lowest Ontario genotype, RCAT Matrix (7.7 mmol $m^{-2}s^{-1}$) was not significantly different from this lowest g_e value. There was a large range of g_e values for the genotypes measured (33.0 mmol $m^{-2}s^{-1}$) which is a difference of 83.4% (Δ g_e / highest g_e * 100) and a 31.9 mmol $m^{-2}s^{-1}$ range (80.6%) for the Ontario varieties (Table 2.3).

As there was a leaf x genotype interaction the genotype effects were also compared by the individual leaf positions. On average the lower leaf had significantly higher ($p<0.0001$) g_e (13.4 mmol $m^{-2}s^{-1}$) compared to the upper leaf (12.0 mmol $m^{-2}s^{-1}$). When looking at the lower leaf AC Colibri had the highest g_e value (45.4 mmol $m^{-2}s^{-1}$), and Hutcheson had the lowest g_e (6.9 mmol $m^{-2}s^{-1}$). Among Ontario genotypes AC Hime had the lowest g_e (7.9 mmol $m^{-2}s^{-1}$). There was a range of 83.1% overall and 79.7% for Ontario varieties.

The genotype with the lowest g_e of the upper leaf was N98-9265 (6.2 mmol $m^{-2}s^{-1}$), but the lowest Ontario genotype was RCAT Matrix (6.4 mmol $m^{-2}s^{-1}$). The highest g_e value was again AC Colibri (33.7 mmol $m^{-2}s^{-1}$). The range of g_e values including all genotypes for the upper leaf was 81.6% and the range for Ontario genotypes was 81.2%. The lower leaf demonstrated a greater range of g_e values, for both the total range and the range among Ontario varieties, compared to the upper leaf.

Only one of the pairs of mapping population parents demonstrated a significant difference for mean g_e across both leaves (Table 2.3). This same cross (AC Colibri x OT91-3) was also the only significantly different cross for the mean g_e of the upper leaf and the lower leaf. AC Colibri and OT91-3 were the two genotypes with the highest g_e values overall.

2.5 DISCUSSION

The change in g_e over time observed using the closed gas exchange system was consistent with the three phases of apparent g_e observed by Sinclair and Ludlow (1986) using the detached leaf weight loss over time method. There was an initial phase where there was high but declining rate of water loss, which is consistent with stomatal closure restricting conductance in response to a decrease in leaf water status. This was followed by a plateau in conductance, which corresponds to the phase where stomatal closure is maximal and the constant rate of water loss is determined by a combination of epidermal and possibly residual stomatal conductance. The conductance during the plateau phase was considerably lower than the original conductance value measured immediately after detachment. At the commencement of the plateau phase the leaves began to visually wilt. Finally, there was another decrease in g_e which coincided with considerable wilting and corresponds to the phase which Sinclair and Ludlow (1986) interpreted to be a consequence of decreasing vapour pressure inside the leaf due to tissue desiccation (Figure 2.2).

All three measurement techniques detected genotype differences ($p<0.05$), but the effect was stronger for the dark-adapted attached leaves and the dark-adapted freshly detached leaves compared to the wilted leaves. However, when the data from both leaves were combined, only g_e measured with the closed system on freshly detached leaves had a significant correlation with WUE ($r=-0.86$, $p=0.01$). This demonstrates the value of the closed re-circulating measurement system and justifies its use in further experiments.

The g_e of wilted leaves did not significantly correlate with WUE (r=-0.39, p=0.39). Therefore, even though the term "minimum epidermal conductance" has been used for both dark-adapted leaves (Hufstetler et al. 2007) and for leaves wilted using the weight-loss method (Paje et al. 1988, Muchow and Sinclair 1989), the present study clearly shows that the two traits are in fact different and only g_e of the dark-adapted leaves (either attached or detached) is of interest in comparison with WUE. For the remainder of the discussion, and we suggest henceforth, these two traits will be considered distinct. The term "g_e" will refer to conductance of leaves that have been detached from the plant for a period of time and measured using the weight loss method or via gas exchange of wilted leaves, and g_{dark} will refer to the conductance of attached or freshly detached leaves that have been dark adapted.

The strength of the correlation between WUE and g_{dark} measured using the LI-6400 and taking the mean across leaf positions, was essentially identical to that found by Hufstetler et al. (2007). This was despite the fact that Hufstetler et al. (2007) measured WUE and g_{dark} on separate plants, whereas in the present work they were measured on the same plants. When looking at just the lower leaf the correlation coefficient was somewhat higher in this experiment (r=-0.86) compared to what was previously found (r=-0.74). Both the results reported by Hufstetler et al. (2007) and in this experiment were fairly consistent with the correlation between WUE and g_{dark} found by Fish and Earl (2009) in cotton (r=-0.75), which was measured using an LI-6400 on attached dark-adapted leaves. The g_{dark} values of upper leaves (youngest fully expanded leaves) were

not significantly correlated with WUE, therefore, a leaf lower on the plant should be used in future studies.

In the measurement technique experiment, the genotype with the highest WUE was the Ontario variety OAC Bayfield. The WUE values measured in this experiment were all higher than those measured by Hufstetler et al. (2007) for the same genotypes. The genotype WUE rankings were also in a different order, with the biggest observed difference in the ranking of the genotype Boggs which went from the highest WUE (3.23 g DM L^{-1} H_2O) in the Hufstetler et al. (2007) study to the lowest WUE in this experiment (Table 2.2). The g_{dark} values measured were also not in the same ranking order as reported by Hufstetler et al. (2007) using any of the different measurement techniques. However, using the LI-6400 and the closed gas exchange system the strong correlation between g_{dark} and WUE was preserved despite the changes in the values and comparative rankings for both of these traits. This consistent correlation points to the need to investigate the physiological and genetic basis of the relationship in order for g_{dark} to be used as a surrogate measurement for WUE in breeding programs.

The screening of Ontario-adapted soybean germplasm demonstrated that there is significant variation for g_{dark} amongst commercially available varieties. The range of 80.6% (1-(lowest g_{dark}/highest g_{dark})) was much higher than has previously been reported for this trait. Hufstetler et al. (2007) found a variation of only 54% in soybean and Fish and Earl (2009) found a range of 68% in cotton. As for WUE, the genotype rankings for g_{dark} values were not consistent across this screening experiment, the previous measuring

techniques experiment, and Hufstetler et al. (2007). However, the rankings in the screening study were much closer to those reported by Hufstetler et al. (2007) than they were to the rankings in the measurement techniques experiment.

The high range of g_{dark} values found in commercially available Ontario-adapted soybean indicates the potential for germplasm improvement using already adapted, high-yielding germplasm. However, the difference in the values of g_{dark} between the two leaf positions amongst these varieties (that is, the leaf x genotype interaction) suggests that caution must be taken when attempting to predict WUE from these g_{dark} values. Also, the lack of consistency in genotype rankings for g_{dark} and WUE across different studies indicates a need to better understand the basis of genotype x environment interactions for these traits.

The significant difference that was found between the mapping population parents AC Colibri and OT91-3 provides the opportunity for quantitative trait loci analysis and to determine whether there is a genetic basis to the correlation between WUE and g_{dark}.

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Table 2.1. Soybean lines and varieties used in Experiment 2 for g_{dark} screening including Ontario-adapted Conventional and Round-up Ready varieties, mapping population parents and varieties shown by Hufstetler et al. (2007) to differ for g_{dark}.

	RR Entries	
	Genotype	**HU Rating**
1	OlexRR	2450
2	25-04R	2500
3	OAC Raptor	2700
4	OAC Rockwood	2700
5	RCAT MatRix	2850
6	RR Rochester	2950
7	RCAT MiRRa	3000
8	RR Respond	3000
9	RCAT 22R1	3150
10	RR Renwick	3150
11	26-02R	2600
12	28-52R	2850
13	30-07R	3000

	Conventional Entries	
	Genotype	**HU Rating**
14	OAC Ayton	2550
15	OAC Carman	2550
16	OAC Bayfield	2650
17	Dundas	2750
18	OAC Wallace	2750
19	OAC Prodigy	2850
20	RCAT Corbett	2850
21	OAC Huron	2900
22	OAC Kent	3050
23	RCAT Pinehurst	3050
24	RCAT Ruthven	3200
25	Colby	2850
26	OAC Lakeview	2700
27	Madison	2700
28	Connor	2600
29	Katrina	2950
30	RCAT Harwich	3050

	Mapping Population Parents
	Genotype
31	AC Brant
32	X 3145-B-B-3-15
33	AC Colibri
34	OT91-3
35	Nattosan
36	NK S08-80
37	OAC Arthur
38	AC 756
39	Harovinton
40	OAC Salem
41	OX939
42	Haro (1-7)
43	OX281
44	Mukden
45	Conrad
46	OX760
47	OX744
48	Williams
49	AC X790P
50	IA2034
51	Westag-97
52	AC Hime
53	Leo

	Lines known to differ for g_{dark}	
	Genotype	**Maturity Group**
54	PI 407859-2	V
55	Tokyo	VII
56	N97-9765	VIII
57	Young	VI
58	Boggs	VI
59	Hutcheson	V
60	PI 416937	V
61	Dillon	VI
62	N98-7265	V
63	N90-7199	VII

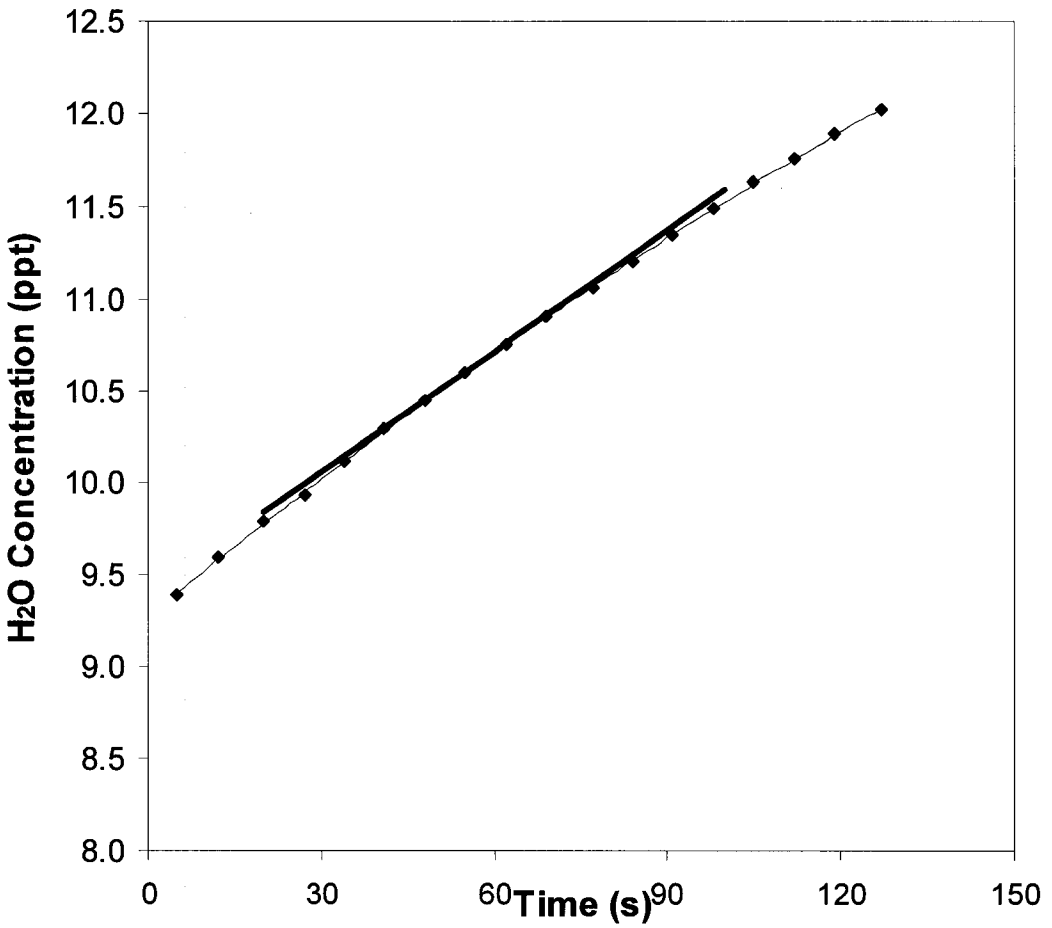

Figure 2.1. Example of the increase in [H_2O] in the sealed chamber during the course of a measurement using the closed gas exchange system. Evapotranspiration was calculated from the tangent line at the midpoint of the measurement.

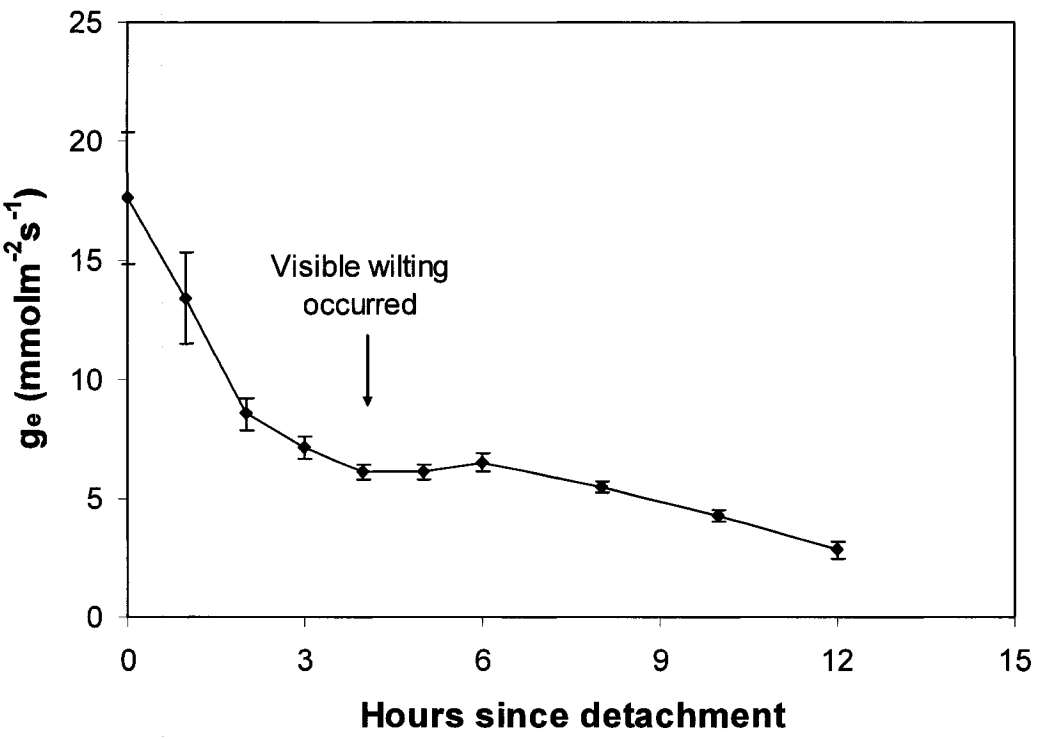

Figure 2.2: Apparent leaf conductance over 12 hours since the detachment of the leaf from the plant. Error bars represent +/- 1 SE, n = 12.

Table 2.2. The mean leaf conductance across two leaf positions measured using three different measurement techniques, and WUE (g L^{-1}) of six genotypes known to differ for these traits, chosen from Hufstetler et al. (2007) and one Ontario variety (OAC Bayfield) (n=6).

		Mean conductance (mmol m^{-2} s^{-1})		
Genotype	WUE (g/L)	LI-6400	Closed system	Closed System Wilted
OAC Bayfield	4.34	24.9	7.5	7.5
PI407859	4.28	28.9	20.9	7.4
Hutcheson	4.18	18.6	5.7	5.7
N97-9765	4.00	24.5	20.6	6.0
Young	3.96	27.3	22.2	7.6
Tokyo	3.84	38.0	30.1	7.7
Boggs	3.73	36.9	39.7	8.2
	$p<0.01$	$p<0.001$	$p<0.0001$	$p<0.05$
	$LSD_{0.05}=0.29$	$LSD_{0.05}=8.1$	$LSD_{0.05}=11.6$	$LSD_{0.05}=1.1$

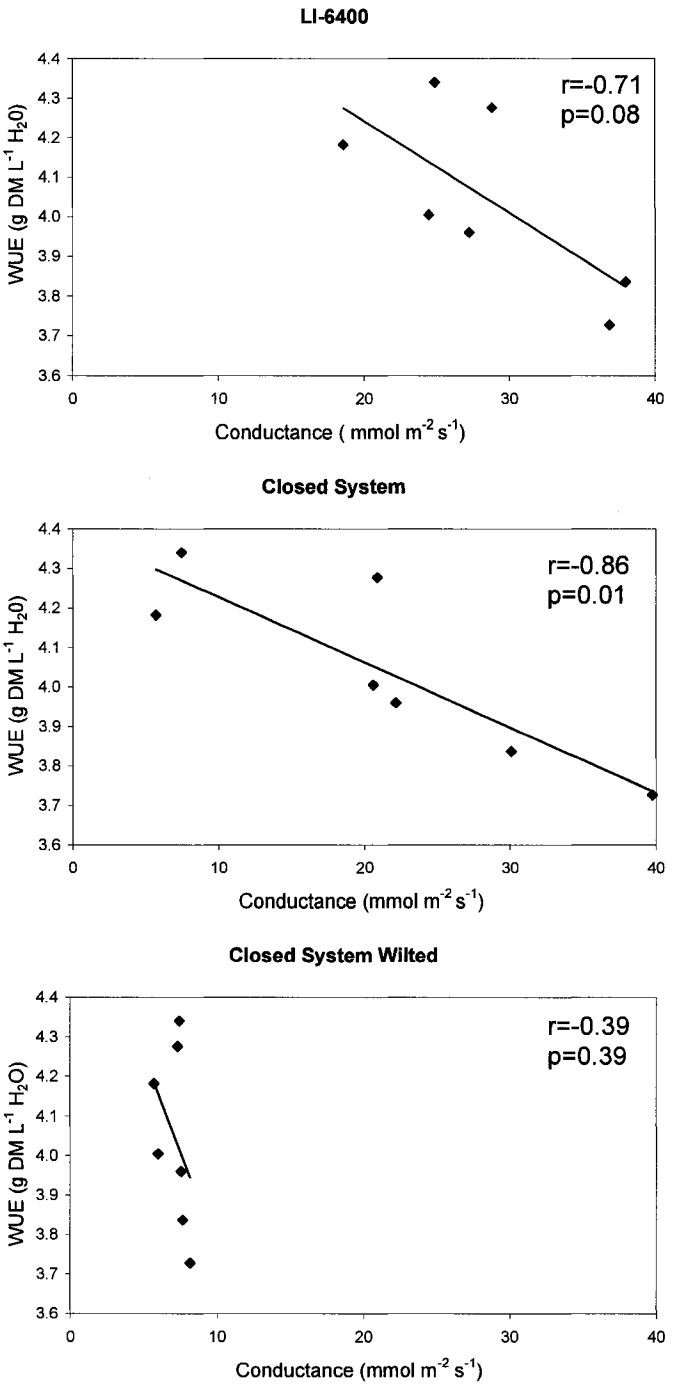

Figure 2.3: Correlation between mean g_e (mmol m^{-2} s^{-1}) and mean WUE (g DM L^{-1} H_2O) across 2 leaf positions of the soybean genotypes previously known to differ for these traits from Hufstetler et al. (2007) and one Ontario variety, with g_e measured using three different techniques. Each point is the mean of six plants of the same genotype.

Table 2.3. The g_{dark} LSMEANS (mmol $m^{-2}s^{-1}$) of the screened soybean varieties sorted by the g_{dark} LSMEANS of the average across leaf positions. The upper leaf refers to the mainstem leaf one below the youngest fully expanded leaf, and the lower leaf refers to the mainstem leaf two below the youngest fully expanded leaf (n = 6).

	g_{dark} LSMEANS (mmol m^{-2} s^{-1})				g_{dark} LSMEANS (mmol m^{-2} s^{-1})		
genotype	Both leaves	Lower leaf	Upper leaf	genotype	Both leaves	Lower leaf	Upper leaf
N98-7265	6.6	6.9	6.2	Tokyo	12.3	13.1	11.4
Hutcheson	6.7	6.9	6.5	OAC Huron	12.3	12.1	12.4
RCAT MatRix	7.7	9.0	6.3	Katrina	12.4	11.5	13.4
Mukden	7.8	7.8	7.8	OAC Kent	12.9	13.6	12.1
Dillon	8.0	7.7	8.3	RR Respond	13.2	15.1	11.3
AC Hime	8.0	7.9	8.2	Westag-97	13.4	14.2	12.7
PI416937	8.1	8.2	8	RCAT MiRRa	13.5	14.0	13
26-02R	8.8	8.8	8.6	RCAT 22R1	13.5	12.7	14.3
OAC Bayfield	8.9	9.3	8.4	Leo	13.5	15.0	12.1
Colby	9.2	9.0	9.4	Madison	13.6	15.4	11.8
Young	9.4	9.6	9.1	Dundas	13.9	15.6	12.2
PI407859-2	9.5	9.6	9.4	N97-9765	14.0	14.0	14.1
RCAT Ruthven	9.9	10.4	9.5	Harovinton	14.2	14.2	14.2
Connor	9.9	11.2	8.7	Conrad	14.2	15.8	12.6
OX939	10.0	9.6	10.4	Haro1-7	14.2	13.9	14.6
RR Rochester	10.1	10.5	9.7	OAC Carman	14.5	14.2	14.8
AC756	10.3	11.0	9.7	RCAT Corbett	14.5	13.5	15.5
OAC Lakeview	10.3	12.4	8.2	IA2034	14.7	16.2	13.2
RR Renwick	10.4	11.2	9.7	25-04R	15.4	15.0	15.8
OAC Rockwood	10.5	10.9	10.1	OAC Arthur	15.5	16.7	14.3
OAC Ayton	10.8	10.6	11.1	28-52R	15.9	16.7	15.2
N90-7199	10.9	10.6	11.1	AC Brant	16.1	17.0	15.3
X3145	10.9	11.3	10.5	Nattosan	16.3	17.7	15
OX281	11.0	12.6	9.3	RCAT Pinehurst	16.4	18.5	14.3
RCAT Harwich	11.1	11.8	10.3	OX744	16.5	16.7	16.4
OAC Salem	11.1	12.1	10	30-07R	17.0	15.5	18.5
ACx790	11.1	11.3	10.8	OX760	17.3	24.0	10.5
OAC Raptor	11.1	11.2	11	NKS08-80	18.3	19.0	17.6
Williams	11.4	11.4	11.3	OT91-3	22.3	22.5	22.2
OAC Prodigy	11.6	12.5	10.6	AC Colibri	39.5	45.4	33.7
Boggs	11.6	12.3	10.9				
OlexRR	11.8	14.0	9.7		$LSD_{0.05}$ = 8.3	$LSD_{0.05}$ = 8.7	$LSD_{0.05}$ = 8.7
OAC Wallace	12.3	12.7	11.8		p<0.0001	p=<0.0001	p=<0.0001

CHAPTER 3: PHYSIOLOGICAL BASIS OF THE CORRELATION BETWEEN DARK-ADAPTED EPIDERMAL CONDUCTANCE AND WATER USE EFFICIENCY

3.1 ABSTRACT

The purpose of this study was to determine whether there was a significant correlation between dark-adapted leaf conductance to water vapour (g_{dark}) and whole plant water use efficiency (WUE) in soybean, and to determine how genotype rankings for these two traits, as well as the correlation between the traits, were affected by water stress. The relationships of g_{dark} and WUE with leaf internal CO_2 (C_i), leaf net carbon assimilation rate (A_n), stomatal conductance (g_s), stomatal density and stomatal size were also examined to try to understand the physiological basis of the correlation between WUE and g_{dark}. These traits were all measured on twelve Ontario-adapted soybean varieties grown in a greenhouse. WUE and g_{dark} were significantly correlated with each other and with C_i, g_s and A_n across treatments and in the water replete treatment. Under water stress conditions g_{dark} and WUE were only significantly correlated with C_i and no longer with each other. The genotype differences for WUE were constitutive across treatments. Stomatal density was not the physical link between WUE and g_{dark}, but there was a significant correlation between g_s and stomatal size. The strong correlation between WUE, g_{dark}, g_s and C_i suggests that the variety differences in WUE were primarily due to differences in conductance. The g_{dark} trait appears to be a reliable predictor of g_s and C_i in illuminated leaves, and this is the physiological basis of its ability to predict WUE.

3.2 INTRODUCTION

When water is a limiting factor, higher water use efficiency (WUE; the quantity of dry matter produced per unit soil water transpired) may lead to increased crop productivity. Genetic variability has been found for WUE in several crop species including peanut (*Arachis hypogaea* L.; Hubick et al. 1988; Wright et al. 1994), cotton (*Gossypium hirsutum* L.; Quisenberry and McMichael 1991, Saranga et al. 1998), sorghum(*Sorghum bicolor* (L.) Moench; Donatelli et al. 1992), barley (*Hodeum vulgare* L.; Hubick and Farquhar 1989) and soybean (*Glycine max* (L). Merr.) (Mian et al. 1996; Hufstetler et al. 2007). However, WUE is a difficult and labour intensive trait to measure in the greenhouse and extremely challenging to measure under field conditions.

The strong correlation observed by Hufstetler et al. (2007) and Fish and Earl (2009) between WUE and another trait, dark-adapted leaf conductance (g_{dark}) merits further investigation due to the relative ease of g_{dark} measurements. This relationship was determined using plants that had been dark-adapted for at least 36 hours and then measured using a commercial open-flow leaf gas exchange measurement system on attached leaves. Fish and Earl (2009) found the significant correlation ($r=-0.75$, $p<0.0001$) using cotton, and Hufstetler et al. (2007) found a nearly identical correlation using soybean ($r=-0.74$, $p<0.0001$). The soybean result was determined under water replete conditions. Earl (2002) determined that the difference in WUE between two soybean varieties was consistent under water replete and cyclic drought stress conditions. This result was confirmed by Fish and Earl (2009) for 22 cotton varieties, converted lines and race stocks. It is important to know if genotypic differences in WUE are constitutive

in nature (i.e., unaffected by water stress), since that implies that selection for WUE – a drought tolerance trait – can meaningfully be made under water replete conditions. This is much easier than trying to impose a defined level of water stress across all entries in a screening trial.

In Chapter 2 the relationship between g_{dark} and WUE was compared using different g_{dark} measurement techniques. The result was that the method with the highest correlation coefficent between WUE and g_{dark} was found when g_{dark} was measured on dark adapted, attached leaves using a closed-recirculating gas analysis system ($r=-0.86$, $p=0.01$). The closed system measured water vapour transpiring from detached leaves in a 0.7-L leaf chamber. The correlation when g_{dark} was measured using an LI-6400 Portable Photosynthesis System on dark-adapted attached leaves ($r=-0.71$, $p=0.08$), when using data from two leaf positions, was nearly the same as observed by Hufstetler et al. (2007) but this relationship was not quite statistically significant, nor was it as strong as the correlation found using the closed recirculating system. The g_{dark} of leaves, which had been detached from the plant for four hours were also measured using the closed-recirculating system. However, g_{dark} of the wilted leaves did not significantly correlate with WUE ($r=-0.39$, $p=0.39$). Therefore, although the term "minimum epidermal conductance" has been used for measurements made both on dark-adapted leaves (Hufstetler et al. 2007) and on leaves wilted using the weight-loss method (Sinclair and Ludlow 1986, Paje et al. 1988, Muchow and Sinclair 1989), the two traits are in fact different and only the dark-adapted, freshly-detached leaves are of interest in comparison with WUE. The strength of the correlation between g_{dark} and WUE, and the potential for

using g_{dark} as a surrogate measurement for WUE was further investigated using Ontario-adapted soybean in the present study.

While the correlation between WUE and g_{dark} has now been demonstrated for both cotton (Fish and Earl, 2009) and soybean (Hufstetler et al., 2007; also Chapter 2 of this thesis), the physiological basis for this relationship remains unclear. Existing theory regarding the physiological basis of genotypic differences for WUE does not predict an obvious role for dark-adapted leaf conductance.

At the leaf level, instantaneous water use efficiency (WUE_l) is the ratio of the net CO_2 assimilation to transpiration. The leaf internal CO_2 concentration (C_i) is calculated as:

$$C_i = C_a - 1.6(A_n/g_s)$$

where C_a is the ambient CO_2 concentration, A_n is the carbon assimilation rate, and g_s is the total gas phase conductance to water (leaf plus boundary layer), assuming the vast majority of water vapour and CO_2 occurs through the stomata (Farquhar et al. 1982). The factor 1.6 is due to the binary diffusivity of water vapour in air being 1.6 fold greater than that of CO_2 in air (Farquhar et al. 1982). Established theory predicts that leaves with a lower internal CO_2 concentration will display higher WUE_l. High WUE_l is correlated with low C_i through the equation:

$WUE_l = A_n/E = [p_a(1-p_i/p_a)]/1.6v$,

where A_n is the net CO_2 assimilation rate of the leaf, E is the transpiration rate of the leaf, p_a is the ambient partial pressure of CO_2, p_i is the intercellular partial pressure of CO_2 and v is the vapour pressure difference between the interior of the leaf and the ambient air (Farquhar et al. 1989). Low C_i results in low p_i and therefore in a high A_n/E ratio and a high WUE_l. Low C_i may arise from either a lower stomatal conductance or from a higher photosynthetic rate at any given C_i (Earl 2002). The latter would allow the stomatal conductance to be reduced while still maintaining the same photosynthetic rate. Previous work by Earl (2002) has shown that the high WUE soybean line Young had a lower C_i than the low WUE line PI416937 and that this difference was due entirely to differences in stomatal conductance. It was subsequently found that Young also had a lower g_{dark} than PI416937 (10.8 compared to 17.0 mmol $m^{-2}s^{-1}$ respectively; Hufsteteler et al. 2007). The recent discovery of the correlation between g_{dark} and WUE (Hufstetler et al. 2007) brings into question whether there is a relationship between C_i and g_{dark} and whether this can help explain the physiological basis of the WUE and g_{dark} relationship.

Previous research has found that the genotypic differences in C_i and WUE (and therefore potentially also g_{dark}) were due to stomatal effects (Earl 2002). These relationships lead to the question of whether g_{dark} is correlated with stomatal size and / or stomatal density. Muchow and Sinclair (1989) found g_e (minimum epidermal conductance) 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, then even when stomata are

closed leaves with high stomatal density would also have high g_e. Paje et al. (1988) found that the correlation between g_e and stomatal density was very low in both well watered leaves (R^2=0.18) and in water-stressed leaves (R^2=0.29) of Australia-adapted soybeans. However, they obtained these results using the weight loss from detached leaves method of measuring g_e, which was demonstrated in Chapter 2 to be a distinct trait from g_{dark}. They also did not look at stomatal size as a factor, only stomatal density. This potential relationship between stomatal density or stomatal size and g_{dark} is examined in the present study using Ontario adapted soybeans. Both the diameter of the stomatal openings and the stomatal densities were compared to the g_{dark} and C_i values measured for 12 genotypes. Another approach to understanding the physiological basis of the relationship between g_{dark} and WUE was to investigate the relationship between g_{dark} and daytime stomatal conductance (g_s) and leaf net carbon assimilation rate (A_n), as both of these two traits are important factors affecting WUE_l.

The objectives of this study were to (1) determine whether differences in WUE and g_{dark} among Ontario-adapted soybean varieties are constitutive across water stress levels (i.e., water-replete vs drought stressed), (2) determine whether there is a significant correlation between whole plant WUE and g_{dark} in Ontario-adapted soybean, and if this correlation is affected by water stress level, (3) determine whether there is a significant correlation between g_{dark} and C_i, which is thought to be the prime determinant of WUE_l, (4) determine whether genotypic differences in g_{dark} (and possibly C_i) are associated with differences in stomatal density, stomatal size, stomatal conductance and / or leaf net photosynthetic rate.

3.3 MATERIALS AND METHODS

3.3.1. *Plant material and water use efficiency*

Twelve Ontario soybean varieties, 6 conventional and 6 glyphosate-tolerant, were selected for this study. The selected varieties were at the extreme high end or the extreme low end of the previous g_{dark} variety screening study (Chapter 2). An effort was also made to choose varieties from varied heat unit ratings.

Table 3.1: Ontario-adapted glyphosate-tolerant (RR) and conventional soybean varieties with either high or low g_{dark} values based on the results of the germplasm screening experiment described in Chapter 2.

Conventional Varieties			Glyphosate-Tolerant Varieties		
		CHU			CHU
Low g_{dark}	OAC Bayfield	2650	Low g_{dark}	RCAT Matrix	2850
	RCAT Ruthven	3200		26-02R	2600
	OAC Lakeview	2700		Renwick	3150
High g_{dark}	RCAT Pinehurst	3050	High g_{dark}	30-07R	3000
	RCAT Corbett	2850		25-04R	2500
	OAC Carman	2550		RCAT 22R1	3150

Plants were grown in the Crop Science Building greenhouse at the University of Guelph between January and March 2008 in 2.5-L white plastic pots without drainage holes. The pots were filled with an equal weight of soil, as were two additional 2.5-L pots. These additional pots had holes drilled in the bottom and a piece of nylon screening across the bottom of the pot to prevent soil loss. The two pots with holes were watered to over-saturation and then were weighed when the pots had reached a constant weight and water had stopped dripping from the pots; this value was the saturation weight. The soil was then removed from the two pots and dried in metal trays for 48 hours at 80° C. At

this point the dry weight of the soil was determined. The dry weight of the soil was subtracted from the saturation weight to determine the amount of water held by the soil at 100% soil water holding capacity. The mean of the values from the two pots was used as the overall soil water holding capacity for this experiment. The soil was a mixture of 2 parts sand to 1 part topsoil, based on the gravimetric method for pot experiments described by Earl (2003). The top soil is a triple mix of one part top soil:one part Peat moss: one part manure (Meadowville Gardens, Guelph).

The greenhouse air temperature was set at approximately 25°C during 16 hours of daylight and 20°C at night. The day length was extended using a mixture of metal halide and high pressure sodium lamps, which provided a supplemental photosynthetically active photon flux density (PPFD) of approximately 600 μmol $m^{-2}s^{-1}$ at the tops of the plants. Four seeds were planted per pot and then thinned to one seedling per pot at the first leaf stage. At this point the pots were capped with fitted white lids, each with a 1-cm diameter hole for the seedling to grow through and another 1.5-cm diameter hole by which the pot was watered. The pots were each watered with 50 mL of 8 g/ L 20:20:20 plus micronutrients fertilizer (Plant Products, Brampton ON) on both the planting day and the capping day. The day the pots were capped was the first day that the water use data were recorded. The pots were watered throughout the growing period using a gravimetric watering system which was composed of a balance connected to a computer running custom software. A barcode reader was used to identify each pot and the desired weight, which had been set in the computer software. Through 3 mm diameter tubing connected to a solenoid valve the plants received water up to the desired weight. The

customized software then recorded the difference in the weight before and after watering. The pots were weighed every day or every other day as needed in order to maintain the desired weight. The amount of water used was added up for the life of the plant until harvest. At the end of the experiment the plants were harvested, including the roots, and placed in a forced air dryer for a minimum of 48 hours at 80°C. Dry weights were measured to determine the mass of dry matter produced per litre of water used from capping of the pots until harvest; i.e., the whole plant water use efficiency (g DM/L H_2O). The plant dry matter at the time of capping was assumed to be negligible. Two pots per repetition contained only soil. These pots were capped and watered using the same methods as the pots containing soybean plants. They were used as a measure of the evaporation occurring from the pots in their respective repetitions. The water loss from the two pots was added up over the life of the plants in their repetition and then the mean was calculated. This mean evaporative water loss was subtracted from the total water use of each plant-containing pot in order to determine a corrected water use for each plant.

There were two watering treatments: 1) water replete (75% relative soil water content (RSWC)), 2) cyclic drought stress. The cyclic drought stress treatment was a controlled dry down from 75% to 15% RSWC (decreasing 10% per day) twice during the experiment. The first (at the 7 or 8 leaf stage) dry down occurred in the second last week of the experiment and the second (9 to 10 leaf stage) was during the final week of the experiment. The plants were re-watered before the measurements were taken as it was

determined in the g_{dark} techniques experiment (Chapter 2) that g_{dark} of wilted leaves was not strongly correlated with WUE.

There were five repetitions grown, with one plant of each genotype in each treatment per repetition. The reps were planted sequentially in order to facilitate measurements. The model was a randomized complete block design. Measurements were taken approximately six weeks after planting when all of the plants had reached between the 10 and 12 leaf stage.

3.3.2 *Gas exchange measurements*

Photosynthetic rate (A_n), leaf internal CO_2 concentration (C_i) and stomatal conductance (g_s) were measured using an LI-6400 Portable Photosynthesis Measurement System (Licor, Lincoln NE) in the greenhouse between 10:00 am and 3:00 pm with a 250 μmol s^{-1} flow rate, 25°C block temperature, sample side CO_2 set at 380 ppm and a photosynthetic photon flux density of 1200μmol s^{-1} flux, provided by the instrument's LED light source. Two leaflets per plant were measured: the centre leaflet one mainstem node below the youngest fully expanded leaf and the centre leaflet one mainstem node below that. The measurements were taken on each leaf once a steady-state C_i had been achieved, approximately five minutes per leaflet. The plants were then transferred into a dark room where they remained for at least 36 hours before dark adapted leaf conductance (g_{dark}) measurements were taken.

The g_{dark} was measured using a closed recirculating system, which was demonstrated in Chapter 2 to be the method producing g_{dark} values with the highest correlation coefficient to WUE. A side leaftlet was selected for measurement in the same two leaf positions as measured using the LI-6400. A different leaflet was selected for the g_{dark} measurement due to concern of potential bruising or damage from the LI-6400. The leaves were measured using the same methods as in Chapter 2.

3.3.3 *Stomatal measurements*

After the dark adapted water loss and leaf area were measured, an impression of the leaf was taken using Extrude Medium impression material (Kerr Dental, Orange CA), which formed a mold. A peel was taken from this mold using clear nail polish which was viewed through an Axiophot (Zeiss, Germany) light microscope at 200x magnification. 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 stomates in this area and the lengths of the openings of six randomly selected stomates were measured per image. An impression was taken of both the top and the bottom of each leaflet measured. The results were then combined to give an average length of stomatal opening and stomatal density per mm^2 for that leaf.

3.3.4 *Statistical Analysis*

The data were analysed using PROC MIXED in SAS statistical software version 9.0. to determine whether there was a significant difference ($p<0.05$) amongst the genotypes for all of the traits measured and whether there was a significant treatment

effect and leaf position effect. A PROC CORR correlation analysis was performed amongst the genotype LSMEANS of all of the traits measured to determine the strengths of the relationships amongst the traits.

3.4 RESULTS

There was a highly significant genotype main effect for WUE ($p<0.0001$), A_n ($p<0.0001$), C_i ($p<0.01$), g_s ($p<0.0001$), g_{dark} ($p<0.0001$), stomatal length ($p<0.0001$) and stomatal density ($p<0.0001$). However, the genotype main effect for the total plant dry weight was not statistically significant ($p=0.07$). A significant treatment effect between water replete and cyclic drought was observed for all the traits measured ($p<0.0001$) except for C_i ($p=0.33$) and stomatal length ($p=0.46$). There was a significant leaf position effect for A_n ($p=0.009$) and stomatal density ($p=0.001$), but not for g_s, g_{dark}, C_i or stomatal length. Since there wasn't a significant difference between leaf positions for the main traits of interest, the mean of the two leaves was used for the rest of the analysis (Table 3.2-3.10).

The mean WUE was significantly higher (3.42 g/L) in the water replete treatment compared to the cyclic drought treatment (3.24 g/L). The total dry matter weight was also higher in the control treatment (21.8 g) than in the drought treatment (11.8 g). The drought treatment plants had significantly lower A_n (9.0 μmol CO_2 m^{-2} s^{-1}) and g_s (89.5 mmol $m^{-2}s^{-1}$) than the plants in the water replete control treatment (A_n=13.4 μmol CO_2 m^{-2} s^{-1}; g_s=157.8 mmol $m^{-2}s^{-1}$). The leaf internal CO_2 mean value was slightly higher in the water replete treatment (199 ppm) than in the drought treatment (195 ppm), but this difference was not statistically significant. The drought treatment did affect the stomatal density (drought= 407 mm^{-2}, control=468 mm^{-2}), but there was not a significant genotype by treatment interaction ($p = 0.76$). There was no significant treatment effect on stomatal length (control =0.89 μm, drought=0.88 μm).

From the analysis of the genotype means across treatments, WUE was most strongly correlated with C_i ($r=-0.73$, $p<0.01$), but it was also significantly correlated with g_s ($r=-0.61$, $p=0.035$) and with g_{dark} ($r=-0.64$, $p=0.025$). Since there were significant treatment effects for most of the variables, the correlations with WUE were also analysed separately by treatment. In the water replete treatment WUE was most strongly correlated with both C_i ($r=-0.71$) and g_s ($r=-0.71$), but the correlation of WUE with g_{dark} ($r=-0.70$) was nearly as strong. The A_n was significantly correlated with WUE, but the correlation was not as strong as the aforementioned correlations (Table 3.11). The drought treatment had very different results compared to the water replete treatment. In this treatment only C_i was significantly correlated with the WUE. All of the other correlations that were present in the control treatment were no longer statistically significant in the drought treatment.

Interestingly, g_{dark} was most strongly correlated with g_s ($r=0.84$, $p<0.0001$) and was also very strongly correlated with C_i ($r=0.83$, $p<0.001$) when the treatment results were combined. There was also a significant correlation with A_n ($r=0.62$, $p=0.03$), but there was no correlation with stomatal length or density. In the water replete treatment g_{dark} was correlated with the same traits as it was when the treatment results were combined, but in the drought treatment g_{dark} only had a significant correlation with C_i (Table 3.11).

The negative correlation of WUE_c (WUE under water replete conditions) with A_{nc} (A_n under water replete conditions) raised the question of whether any of the genotypes

had a significantly higher WUE_c than was predicted by A_{nc} and if any of the genotypes had significantly lower WUE_c than was predicted. A predicted WUE_c was calculated for each plant using the overall regression of WUE_c on A_{nc}. The difference between the predicted and observed WUE_c was calculated and then analysed using PROC MIXED (SAS statistical software 9.0) to determine if there was a significant difference between the two values. Only the variety RR Renwick had a significantly higher WUE_c than was predicted by A_{nc}. The varieties 25-04R and RCAT Corbett both had significantly lower WUE_c than predicted by A_{nc}. None of the other varieties had a WUE_c value that was significantly different from what was predicted (Figure 3.1).

WUE was never significantly correlated with the whole plant dry weight, and g_{dark} was only significantly correlated with plant dry weight under the cyclic drought conditions ($r=-0.58$, $p= 0.049$), but in this treatment g_{dark} was not significantly correlated with WUE.

One surprising result was that although stomatal conductance was not correlated with stomatal density, it was significantly correlated with stomatal opening length in both treatments and across treatments (Table 3.11). Stomatal length was also significantly correlated with C_i in the drought treatment and also across treatments.

3.5 DISCUSSION

The first important research question was whether the previously observed correlation between g_{dark} and WUE (Hufstetler et al. 2007 and Fish and Earl 2009) was also present in Ontario-adapted soybean. A significant correlation was observed across treatments ($r=-0.64$, $p=0.03$), but was stronger under the water replete conditions ($r=-0.70$, $p=0.01$) and the correlation was not significant under the cyclic drought conditions ($r=-0.48$, $p=0.11$). Therefore, when attempting to predict WUE from g_{dark}, the plants should be grown under well watered conditions. This correlation was slightly less than what was previously observed in soybean ($r=-0.74$, $p<0.001$; Hufstetler et al. 2007) and in cotton ($r=-0.75$, $p<0.0001$; Fish and Earl 2009). However, the correlation using Ontario-adapted soybean genotypes under water replete conditions was not as strong as in Chapter 2 where varieties that were known to differ for g_{dark} and WUE from Hufstetler et al. (2007) were used. The plants in both experiments were grown under similar conditions and measured using the same technique. As the g_{dark} measurement was environmentally sensitive (significant genotype by treatment interaction) and the strength of the correlation was based on the watering conditions experienced by the plants, slight differences in water stress conditions may be responsible for the difference in the results between the two experiments. It is also possible that the g_{dark} of Ontario-adapted soybean is actually less correlated with their WUE than is the case with the selection of varieties used in Chapter 2.

Significant differences amongst genotypes for WUE were observed ($p<0.0001$), and these differences were constitutive across the water replete and drought stress

conditions (genotype x treatment interaction was not significant at 0.05). This result was consistent with Earl (2002) who found the difference between the WUE values of two cultivars was essentially the same under both well-watered and cyclic drought conditions. An interesting result was that the WUE observed in this study actually decreased under cyclic drought conditions compared to the water replete treatment. This differed from the results observed by Earl (2002) where WUE was higher in the drought treatment and from Hufstetler et al. (2007) where a significant difference between the WUE in the drought treatment and the water replete treatment was not observed. An explanation for the decrease in the WUE in the cyclic drought treatment could be that under these conditions the stomates are fully closed and that the main component of g_{dark} is epidermal conductance. Therefore, since the stomates are closed, carbon is not being fixed but water is still being lost through the epidermis. When compared to the water replete treatment where the stomates are open during the day and carbon is being fixed, the WUE in the drought treatment is lower due to a larger fraction of water lost compared to carbon fixed. There was also a significant difference amongst genotypes for g_{dark} ($p<0.0001$), but there was a significant genotype x treatment interaction ($p<0.0001$). The drought stress always reduced the g_{dark} values, but the reduction was greater for some genotypes than others (Table 3.8). The effects of water stress on genotype differences in g_{dark} have not been previously reported in soybean.

Consistent with the findings of Paje et al. (1988) who looked at soybeans, but contrary to the results of Gizt et al. (2005) and Muchow and Sinclair (1989) who looked at soybean and cotton respectively, stomatal density was not correlated with g_{dark}, g_s, C_i,

nor WUE. Stomatal length was, however, correlated with g_s and C_{iD} (C_i under water stress conditions) and C_i across treatments. Therefore stomatal size may play an important role in determining stomatal conductance and C_i. These relationships merit further investigation in order to determine whether it is the stomatal size that links g_{dark} to g_s and C_i and WUE.

The g_{dark} was very strongly correlated with both g_s and C_i ($r=0.90$, $p<0.0001$ for both). Therefore, the dark adapted conductance appears to be an accurate predictor of the stomatal conductance and C_i of those same leaves under steady-state photosynthesis. This was a surprising result as there have not been previous reports of dark adapted leaf conductance to water vapour acting as a predictor of leaf gas exchange activity in the light. We considered the possibility that the relationship between C_i and g_{dark} is mathematical rather than physiological. That is, the Ci calculation from the LI-6400 assumes that all measured conductance to water vapour in the light is stomatal, and does not account for water vapour lost through the cuticle. Therefore, leaves with a higher cuticular water loss would end up with erroneously higher estimates of C_i. The upper limit of this error was examined using water replete data and calculating the C_i with and without the g_{dark} component using the equations: $C_i = C_a - An / (0.6\ g_s)$ and $C_i = C_a - An / [0.6\ (g_s - g_{dark})]$ and then comparing the correlation of the two calculated C_i values with g_{dark}. The correlation of g_{dark} with the calculated C_i value with the g_{dark} component included was $r=0.92$, $p<0.0001$ which was a slightly stronger correlation than that produced using the C_i values calculated by the LI-6400. The correlation between g_{dark} and the adjusted C_i value with the g_{dark} component removed from the calculation was

r=0.88, p=0.0001. Therefore, although there was an apparent mathematical component to the relationship between C_i and g_{dark}, observed by the small decrease in the correlation coefficient, the correlation was still significant and had a very high correlation coefficient when the maximum epidermal conductance component was removed from the C_i calculation. This means that the relationship between these two traits is physiological in nature, not an artifact of the assumptions underlying the calculation of C_i. The correlation between the calculated C_{ic} and WUE_c and the adjusted C_{ic} and WUE_c were nearly identical ($r=-0.73$ $p<0.01$ and $r=-0.74$ $p<0.01$ respectively).

C_i was not significantly different in the cyclic drought treatment compared with the water replete treatment ($p=0.33$); this is contrary to other studies where drought led to stomatal closure and a consequent reduction in C_i. Xue et al. (2002) found that water stress conditions induced partial or complete stomatal closure and a decreased C_i. As in our results, Ennahli and Earl (2005) also did not find a difference amongst the well watered and drought condition C_i values in cotton. There are a few possible explanations for why the C_i values were not affected by the drought treatment. The first is that the C_i in the drought treatment could appear artificially high due to the invalidation of the gas exchange-based estimates of C_i. The assumptions of the equation are not met since at low g_s a substantial portion of the conductance is through the epidermis and cuticle and, therefore, not factored into the C_i calculation (Boyer et al. 1997; Ennahli and Earl 2005). A second explanation is that C_i is affected by non-stomatal factors which operate at an optimal C_i and regulate the stomata in order to achieve this C_i level. The C_i value can be affected by the amount and activity of the enzymes in the chloroplast (Condon et al.

1987) which could then regulate the stomata in order to achieve the desired C_i. A final explanation is that since the plants were re-watered about 12 hours before the measurements were taken it is possible that the ratio from which C_i is calculated (A_n/g_s) recovers more rapidly than either of the values independently ($C_i=C_a-1.605(A_n/g_s)$ (Farquhar et al. 1978).

The significant decrease of A_n by the plants that were in the cyclic drought treatment, despite there not being a significant difference in C_i between the two treatments, implies a non-stomatal inhibition of photosynthesis and a lasting mesophyll effect of the drought treatment. If the effects were stomatal it would be expected that within a variety the A_n would be consistent between treatments at a similar C_i value. Ennahli and Earl (2005) also observed a lasting mesophyll effect in cotton, even 48 hours after the plants had been re-watered. The nature of the apparent injury could not be determined from this study.

The variety RR Renwick had a significantly higher WUE_c than what was predicted by A_n. This variety had the highest WUE_c (3.55 gDM $L^{-1}H_2O$) as well as the second highest A_{nc} (13.0 μmol CO_2 s^{-1}) of the varieties measured. This means that this variety is able to fix a higher than predicted amount of carbon per litre of water transpired. This type of knowledge can potentially be used in breeding programs in order to incorporate germplasm with high levels of water use efficiency without compromising the carbon fixation rate.

In conclusion, in this study g_{dark} was an accurate predictor of C_i and therefore WUE. The strong correlation between WUE, g_{dark}, g_s and C_i in this experiment suggests that genotype differences in WUE in the water replete treatment were primarily due to conductance rather than differences in photosynthetic capacity. However, the decrease in A_n at a given C_i suggests lasting mesophyll injury following recovery from water stress. Stomatal density was not the physiological link amongst these traits, but stomatal size may play an important role due to its correlation with g_s. Further investigation into the reason for the relationship between dark adapted conductance and leaf internal CO_2 concentration is needed in order to understand how g_{dark} is such a good predictor of whole plant water use efficiency. A variety with both high carbon fixation rate and high water use efficiency was found (RR Renwick) which may have special potential for breeding highly productive varieties with good drought tolerance.

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Table 3.2: The treatment effect and means for water use efficiency (WUE) and the genotype effect and genotype LSMEANS for WUE across treatments as well as in a control (water replete) treatment (WUE_C) and in a cyclic drought treatment (WUE_D) for twelve Ontario-adapted soybean varieties (n=5).

Treatment	p<0.0001	
	Control	3.42 g L^{-1}
	Drought	3.24 g L^{-1}
Genotype	p<0.0001	
G * T	p=0.40	

Genotype	WUE_C	WUE_D	WUE
	g L^{-1}	g L^{-1}	g L^{-1}
22R1	3.52	3.48	3.50
25-04R	3.16	2.98	3.07
26-02R	3.50	3.20	3.35
30-07R	3.41	3.10	3.26
OAC Bayfield	3.52	3.28	3.40
OAC Carmen	3.26	3.17	3.21
RCAT Corbett	3.27	3.19	3.23
OAC Lakeview	3.54	3.43	3.49
RCAT Matrix	3.42	3.35	3.39
RCAT Pinehurst	3.38	3.16	3.27
RR Renwick	3.55	3.36	3.45
RCAT Ruthven	3.53	3.23	3.38
$LSD_{0.05}$	**0.20**	**0.20**	**0.15**

Table 3.3: The treatment effect and means for plant water use (Water) and the genotype effect and genotype LSMEANS for plant water use across treatments as well as in the control water replete treatment ($Water_C$) and in the cyclic drought treatment ($Water_D$) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	$p<0.0001$	
	Control	6.37 L
	Drought	3.64 L
Genotype	$p<0.05$	
G * T	$p=0.70$	

Genotype	$Water_C$	$Water_D$	Water
	L	L	L
22R1	6.52	3.27	4.90
25-04R	5.95	3.21	4.58
26-02R	6.52	3.34	4.93
30-07R	6.07	3.51	4.79
OAC Bayfield	6.48	3.35	4.91
OAC Carmen	6.92	4.14	5.53
RCAT Corbett	6.68	3.93	5.30
OAC Lakeview	6.12	3.75	4.94
RCAT Matrix	6.26	3.72	4.99
RCAT Pinehurst	6.69	4.23	5.46
RR Renwick	5.82	3.67	4.74
RCAT Ruthven	6.45	3.61	5.03
$LSD_{0.05}$	**0.98**	**0.98**	**0.82**

Table 3.4: The treatment effect and means for total plant dry weight (DW) and the genotype effect and genotype LSMEANS for total plant dry weight across treatments as well as in the control water replete treatment (DW_C) and in the cyclic drought treatment (DW_D) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	p<0.0001	
	Control	21.8 g
	Drought	11.8 g
Genotype	p=0.07	
G * T	p=0.69	

Genotype	DW_C	DW_D	DW
	g	g	g
RCAT 22R1	23.0	11.5	17.2
25-04R	18.8	9.6	14.2
26-02R	22.9	10.7	16.8
30-07R	20.7	11.0	15.8
OAC Bayfield	22.8	11.1	16.9
OAC Carmen	22.6	13.1	17.9
RCAT Corbett	21.9	12.5	17.2
OAC Lakeview	21.7	12.9	17.3
RCAT Matrix	21.3	12.4	16.9
RCAT Pinehurst	22.6	13.3	18.0
RR Renwick	20.7	12.3	16.5
RCAT Ruthven	22.8	11.7	17.2
$LSD_{0.05}$			

Table 3.5: The treatment effect and means for carbon assimilation rate (A_n) and the genotype effect and genotype LSMEANS for carbon assimilation rate across treatments as well as in the control water replete treatment (A_{nC}) and in the cyclic drought treatment (A_{nD}) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	$p<0.0001$	
	Control	13.4 μmol m^{-2} s^{-1}
	Drought	9.0 μmol m^{-2} s^{-1}
Leaf Position	$p<0.01$	
	Upper	12.0 μmol m^{-2} s^{-1}
	Lower	10.4 μmol m^{-2} s^{-1}
Genotype	$p<0.0001$	
G * T	$p<0.01$	

Genotype	An_C	An_D	An
	μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}	μmol m^{-2} s^{-1}
22R1	10.1	8.0	9.1
25-04R	16.8	9.3	13.0
26-02R	11.1	10.4	10.7
30-07R	14.0	5.3	9.7
OAC Bayfield	13.6	10.2	11.9
OAC Carmen	20.7	10.4	15.6
RCAT Corbett	13.1	8.1	10.6
OAC Lakeview	9.3	9.8	9.5
RCAT Matrix	10.5	8.8	9.6
RCAT Pinehurst	13.3	7.9	10.6
RR Renwick	14.7	11.3	13.0
RCAT Ruthven	13.2	9.0	11.1
$LSD_{0.05}$	**4.0**	**4.0**	**2.8**

Table 3.6: The treatment effect and means for leaf internal CO_2 concentration (C_i) and the genotype effect and genotype LSMEANS for leaf internal CO_2 concentration across treatments as well as in the control water replete treatment (C_{iC}) and in the cyclic drought treatment (C_{iD}) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	p=0.33	
	Control	199 ppm
	Drought	195 ppm
Leaf	p=0.20	
	Upper	200 ppm
	Lower	194 ppm
Genotype	p<0.0001	
G * T	p=0.18	

Genotype	Ci_C	Ci_D	Ci
	ppm	ppm	ppm
22R1	206	184	195
25-04R	234	225	229
26-02R	181	184	183
30-07R	186	205	195
OAC Bayfield	198	191	194
OAC Carmen	232	194	213
RCAT Corbett	196	191	193
OAC Lakeview	180	192	186
RCAT Matrix	194	191	193
RCAT Pinehurst	197	193	195
RR Renwick	200	192	196
RCAT Ruthven	189	198	193
$LSD_{0.05}$	**31.4**	**31.4**	**22.2**

Table 3.7: The treatment effect and means for stomatal conductance (g_s) and the genotype effect and genotype LSMEANS for stomatal conductance across treatments as well as in the control water replete treatment (g_{sC}) and in the cyclic drought treatment (g_{sD}) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	p<0.0001	
	Control	157.8 mmol $m^{-2}s^{-1}$
	Drought	89.5 mmol $m^{-2}s^{-1}$
Leaf	p=0.08	
	Upper	135 mmol $m^{-2}s^{-1}$
	Lower	113 mmol $m^{-2}s^{-1}$
Genotype	p<0.0001	
G * T	p<0.001	

Genotype	g_{sC}	g_{sD}	g_s
	mmol $m^{-2}s^{-1}$	mmol $m^{-2}s^{-1}$	mmol $m^{-2}s^{-1}$
22R1	111.7	72.2	91.9
25-04R	280.6	113.7	197.2
26-02R	99.3	95.0	97.1
30-07R	131.8	51.5	91.7
OAC Bayfield	134.5	95.2	114.9
OAC Carmen	371.1	100.0	235.5
RCAT Corbett	135.3	77.2	106.3
OAC Lakeview	91.7	94.7	93.2
RCAT Matrix	103.6	82.6	93.1
RCAT Pinehurst	149.5	75.7	112.6
RR Renwick	156.1	126.7	141.4
RCAT Ruthven	128.0	88.8	108.4
$LSD_{0.05}$	**85.6**	**85.6**	**60.6**

Table 3.8: The treatment effect and means for dark-adapted leaf conductance (g_{dark}) and the genotype effect and genotype LSMEANS for dark-adapted leaf conductance across treatments as well as in the control water replete treatment (g_{darkC}) and in the cyclic drought treatment (g_{darkD}) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	p<0.0001	
	Control	12.5 mmol $m^{-2}s^{-1}$
	Drought	7.3 mmol $m^{-2}s^{-1}$
Leaf	p=0.50	
	Upper	9.5 mmol $m^{-2}s^{-1}$
	Lower	9.9 mmol $m^{-2}s^{-1}$
Genotype	p<0.0001	
G * T	p<0.0001	

Genotype	g_{darkC}	g_{darkD}	g_{dark}
	mmol $m^{-2}s^{-1}$	mmol $m^{-2}s^{-1}$	mmol $m^{-2}s^{-1}$
22R1	12.7	10.2	11.5
25-04R	19.0	14.4	16.7
26-02R	8.1	7.3	7.7
30-07R	9.3	7.1	8.2
OAC Bayfield	9.2	5.5	7.4
OAC Carmen	27.6	9.3	18.4
RCAT Corbett	12.1	6.1	9.1
OAC Lakeview	11.8	4.4	8.1
RCAT Matrix	7.6	5.9	6.8
RCAT Pinehurst	13.1	6.2	9.7
RR Renwick	8.1	5.1	6.6
RCAT Ruthven	7.0	5.4	6.2
$LSD_{0.05}$	**4.7**	**4.7**	**3.3**

Table 3.9: The treatment effect and means for stomatal density (Den) and the genotype effect and genotype LSMEANS for stomatal density across treatments as well as in the control water replete treatment (Den_C) and in the cyclic drought treatment (Den_D) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	<0.0001		
	Control	468.1	mm^{-2}
	Drought	407.0	mm^{-2}
Leaf	p=0.001		
	Upper	421.8	mm^{-2}
	Lower	401.8	mm^{-2}
Genotype	p<0.0001		
G * T	p=0.76		

Genotype	Den_C	Den_D	Den
	mm^{-2}	mm^{-2}	mm^{-2}
22R1	541.5	456.6	499.0
25-04R	446.5	349.5	398.0
26-02R	512.0	495.0	503.5
30-07R	463.0	387.5	425.3
OAC Bayfield	508.0	412.5	460.3
OAC Carmen	451.5	414.0	432.8
RCAT Corbett	455.0	395.0	425.0
OAC Lakeview	450.0	408.0	429.0
RCAT Matrix	491.0	397.0	444.0
RCAT Pinehurst	438.0	393.2	415.6
RR Renwick	443.5	389.8	416.7
RCAT Ruthven	417.5	386.0	401.8
$LSD_{0.05}$	**70.8**	**71.9**	**54.0**

Table 3.10: The treatment effect and means for stomatal diameter (Length) and the genotype effect and genotype LSMEANS for stomatal diameter across treatments as well as in the control water replete treatment($Length_C$) and in the cyclic drought treatment ($Length_D$) of twelve Ontario-adapted soybean varieties (n=5).

Treatment	p=0.46	
	Control	0.892 µm
	Drought	0.884 µm
Leaf	p=0.934	
	Upper	0.889 µm
	Lower	0.888 µm
Genotype	p<0.0001	
G * T	p=0.203	

Genotype	$Length_C$	$Length_D$	Length
	µm	µm	µm
22R1	0.868	0.817	0.843
25-04R	0.916	0.981	0.948
26-02R	0.871	0.870	0.871
30-07R	0.835	0.832	0.833
OAC Bayfield	0.896	0.841	0.869
OAC Carmen	0.961	0.905	0.933
RCAT Corbett	0.868	0.851	0.859
OAC Lakeview	0.871	0.873	0.872
RCAT Matrix	0.850	0.866	0.858
RCAT Pinehurst	0.921	0.944	0.933
RR Renwick	0.893	0.934	0.913
RCAT Ruthven	0.952	0.900	0.926
$LSD_{0.05}$	**0.083**	**0.084**	**0.068**

Table 3.11: Correlations between gas exchange measurements: stomatal conductance (g_s), carbon assimilation rate (A_n), leaf internal CO_2 concentration (C_i) and dark-adapted leaf conductance (g_{dark}) and stomatal traits: stomatal diameter (L) and stomatal density (Den) and water use efficiency (WUE) for twelve Ontario-adapted soybean varieties under both water replete (C) and drought (D) conditions. The correlation coefficients printed in bold are statistically significant ($P<0.05$).

	WUE	WUE_C	WUE_D	g_s	g_{sC}	g_{sD}	g_{dark}	g_{darkC}	g_{darkD}	A_n	A_{nC}	A_{nD}	C_i	C_{iC}	C_{iD}	Den	Den_C	Den_D	L	L_C	L_D
WUE	1																				
WUE_c	**0.93**	1																			
WUE_d	**0.94**	**0.75**	1																		
g	**-0.61**	**-0.65**	-0.47	1																	
g_{sc}	**-0.67**	**-0.71**	-0.53	**0.98**	1																
g_{sd}	-0.02	-0.02	0.02	0.55	0.38	1															
g_{dark}	**-0.64**	**-0.76**	-0.44	**0.84**	**0.89**	0.19	1														
g_{darkc}	-0.57	**-0.70**	-0.36	**0.86**	**0.91**	0.20	**0.96**	1													
g_{darkd}	**-0.61**	**-0.70**	-0.48	**0.58**	**0.61**	0.14	**0.81**	**0.61**	1												
A_n	-0.48	-0.44	-0.41	**0.93**	**0.88**	**0.65**	**0.62**	**0.68**	0.33	1											
A_{nc}	**-0.69**	**-0.63**	**-0.63**	**0.91**	**0.93**	0.34	**0.7**	**0.74**	0.44	**0.91**	1										
A_{nd}	0.22	0.19	0.25	0.40	0.24	**0.89**	0.07	0.14	-0.08	0.56	0.18	1									
C_i	**-0.73**	**-0.78**	**-0.59**	**0.84**	**0.85**	0.36	**0.83**	**0.73**	**0.82**	**0.65**	**0.75**	0.06	1								
C_{ic}	**-0.60**	**-0.71**	-0.40	**0.89**	**0.90**	0.39	**0.89**	**0.83**	**0.78**	**0.74**	**0.77**	0.21	**0.93**	1							
C_{id}	**-0.72**	**-0.63**	**-0.73**	0.47	0.49	0.18	0.45	0.32	**0.61**	0.31	0.48	-0.21	**0.79**	0.51	1						
Den	0.46	0.41	0.46	-0.36	-0.35	-0.21	-0.12	-0.17	0.02	-0.32	-0.44	0.11	-0.46	-0.25	**-0.67**	1					
Den_c	0.39	0.29	0.43	-0.34	-0.32	-0.24	-0.07	-0.17	0.15	-0.36	-0.43	-0.01	-0.28	-0.11	-0.48	**0.92**	1				
Den_d	0.47	0.46	0.42	-0.32	-0.33	-0.15	-0.15	-0.15	-0.12	-0.24	-0.40	0.21	-0.56	-0.35	**-0.75**	**0.93**	**0.72**	1			
L	-0.46	-0.39	-0.45	**0.72**	**0.65**	**0.62**	0.47	0.48	0.31	**0.71**	**0.63**	0.44	**0.61**	**0.58**	0.46	**-0.59**	**-0.66**	-0.44	1		
L_c	-0.31	-0.25	-0.30	**0.69**	**0.66**	0.45	0.47	0.54	0.20	**0.73**	**0.65**	0.43	0.48	0.55	0.21	-0.40	-0.51	-0.23	**0.87**	1	
L_d	-0.50	-0.43	-0.49	**0.61**	0.53	**0.64**	0.38	0.35	0.33	0.57	0.50	0.37	**0.61**	0.50	**0.58**	**-0.63**	**-0.65**	-0.53	**0.92**	**0.61**	1

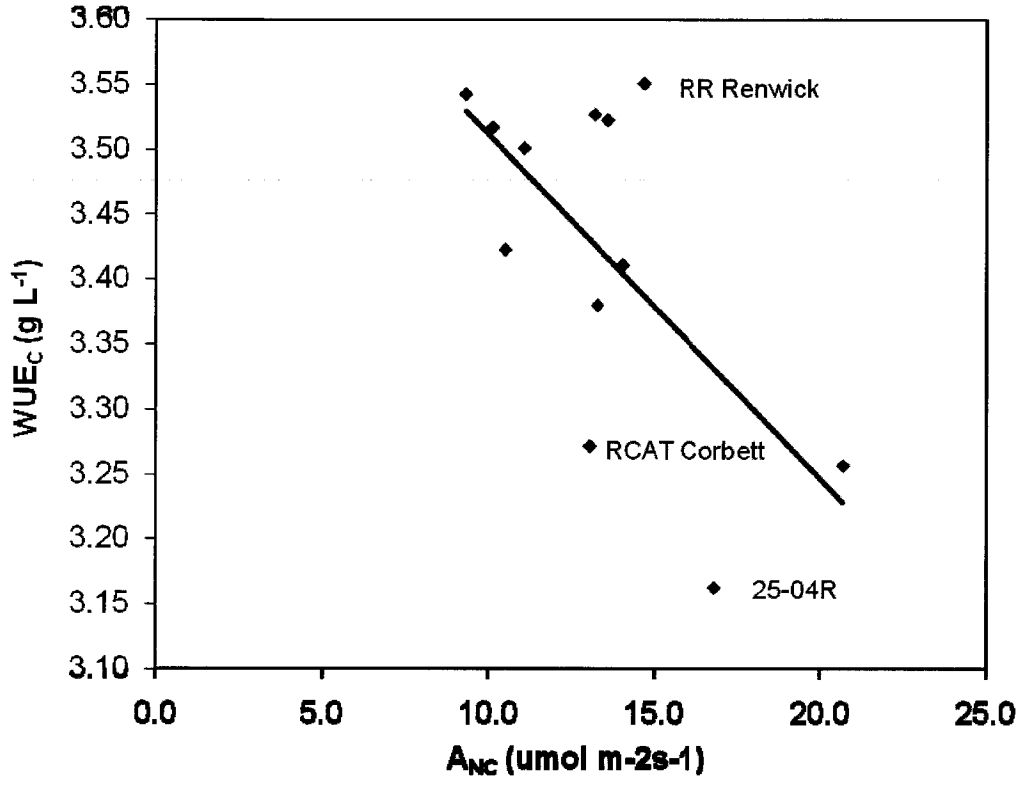

Figure 3.1. The mean leaf carbon assimilation rate under water replete conditions (A_{nc}) versus the water use efficiency under water replete conditions of twelve Ontario-adapted soybean varieties r = -0.63, P<0.05).

CHAPTER 4: VARIATION OF ROOT ELONGATION RATE UNDER OSMOTIC STRESS OF ONTARIO-ADAPTED SOYBEAN

4.1 ABSTRACT

The ability to access soil water deep in the soil profile is beneficial for plants under drought conditions. Therefore plants that are able to maintain their root growth and development under osmotic stress are better able to maintain plant function under drought conditions. Using a hydroponic system, the variation for root elongation rate (RER) under osmotic stress and the recovery of the root elongation rate once the stress had been removed were measured on seedlings of 53 soybean varieties adapted to Ontario, including 10 parents of mapping populations. Seven soybean lines which were previously known to differ for other water use traits were also included in the study. Soybean seedlings were grown for 24 hours in a growth solution. At the end of that time, osmotic stress was produced using an 18.75% polyethyleneglycol (PEG) solution which was pumped into the hydroponic boxes. After another 24 hours the PEG solution was removed and the growth solution was returned to the boxes. The recovery of the root elongation rate was then measured. Significant variation and a large range of values were observed for the relative reduction in RER under stress conditions amongst the 60 genotypes. The variation found provides the opportunity to select for this trait in breeding programs. There was no significant variation for the relative recovery of RER after the stress had been removed. There was, however, a large range of values for this trait. This large range merits further investigation and with a more refined measurement system there is the possibility that significant variation may be found.

4.2 INTRODUCTION

An important factor affecting crop performance under water stress is the ability to maintain root growth and development (Sharp et al. 2004), and therefore access to soil water deeper in the profile, in response to soil water scarcity. Expansion growth of plant cells is driven by turgor pressure which causes irreversible expansion of the cell wall (Spollen and Sharp 1991). Under water limiting conditions roots are unable to maintain this turgor pressure and the root elongation rate decreases (Nonami et al. 1997).

Considerable research has been carried out on maize seedlings to determine the effects of osmotic stress on root elongation rate (RER) (Spollen and Sharp 1991, Voetberg and Sharp 1991, Verslues et al. 1998). Spollen and Sharp (1991) found that when maize seedlings were transplanted into a low water potential (Ψ_w) vermiculite growing medium (-1.6 MPa) the RER was significantly lower than for seedlings in a high Ψ_w medium (-0.02 MPa). Frensch and Hsiao (1994) determined that root elongation of maize seedlings grown in an aerated culture system stopped immediately when either mannitol or KCL were used to induce stress. The root growth re-started approximately 10 minutes after the induction of stress and increased slightly, but only to a rate that was still slower than the RER before the treatment. When the stress was removed, the final growth rate was very similar to the initial growth rate.

Verslues et al. (1998) investigated a culture system using polyethylene glycol (PEG) to induce osmotic stress. They suggested that when studying root behaviour in a low Ψ_w solution culture it is desirable to use a solution that does not interact with the

plant in any way other than to lower the Ψ_w of the medium. PEG, due to its large molecular size ($M_r \geq 6000$), cannot penetrate the cell wall (Carpita et al. 1979). Since PEG does not enter the apoplast, water is withdrawn from both the cell and the cell wall. This mimics dry soil more closely than osmotic solutions containing solutes with a lower molecular radius. Their conclusion was that as long as supplemental oxygenation was provided to the PEG culture system, then it could be used to conduct experiments at low Ψ_w. Ogawa and Yamauchi (2006) examined the effect on a maize cultivar of three different concentrations of PEG solution producing water potentials of -0.13,-0.41, -0.89 MPa, and a control nutrient solution (0.08MPa). They determined that RER decreased within one minute of the onset of the stress and that a greater inhibition of root growth was observed with higher PEG concentrations.

Genetic variation for RER has been previously observed in Arabidopsis (Beemster et al. 2002), chickpea (*Cicer arietinum* L.; Kashiwagi et al. 2005), lentil (*Lens culinaris* subsp. *culinaris*; Gahoonia et al. 2006) and maize (T. Wambach and L. Lukens unpublished). However, Beemster et al. (2002) and Gahoonia et al. (2006) performed their investigations under water replete conditions. Kashiwagi et al. (2005) investigated the RER variation in chickpea under drying soil conditions. The plants were provided with 100% field capacity until the seedlings uniformly emerged and then the plants were not provided with any more water. After 35 days the roots of the seedlings were measured and a significant variation in root length was observed. Due to the time consuming nature of this type of study, large screening efforts remain a challenge. Genetic variability for RER in maize was observed (T. Wambach and L. Lukens

unpublished) using an adaptation of the methods of Verslues et al. (1998) which created the opportunity to examine a large number of genotypes simultaneously. To the best of our knowledge there have not been any studies investigating the effects of a PEG solution culture system on the RER of soybean. The genetic variation in RER for Ontario soybean varieties remains unknown for control conditions, as well as under osmotic stress conditions. Our first objective was to determine the effects of a PEG culture system on RER; specifically, to determine what concentration of PEG would best distinguish the potential genetic variability. The second objective was to quantify the level of genetic variation within Ontario germplasm in the inhibition of RER due to osmotic stress and in the ability to recover initial RER after the stress had been removed. The third objective was to determine whether there were any mapping population parents that differed for the maintenance of RER under osmotic stress, or for the recovery of RER after the stress was removed. This would indicate potential for future discovery of QTL conditioning RER under osmotic stress.

4.3 MATERIALS AND METHODS

4.3.1. *Growth System*

The hydroponic system consisted of 4-L plexiglass boxes with an aquarium aeration tube across the bottom of each box. The aeration tubes were all connected through plastic tubing to an air pump. Each box contained 30 plastic drinking straws suspended in drilled holes from a central plastic crosspiece at the top of the box. Seven holes were pierced through each straw to assist the entrance of oxygen into the straws. A soybean seedling was placed on a hole and the root of the seedlings grew straight down within the straw. The boxes either contained a growth solution which consisted of: 0.98 g/L MES [2(N-morpholino)ethanesulfonic acid]; 0.00038 g/L H_3BO_3; and 0.086 g/L $CaSO_4$, or a polyethylene glycol (PEG) solution which was composed of the growth solution plus a determined concentration of PEG. The hydroponic system was in a growth chamber (Model PGW36, Conviron, Winnipeg MB) with 24 hours/day of darkness, 25 °C and 95% humidity.

4.3.2. *Experiment 1*

The purpose of this experiment was to determine which concentration of PEG solution would slow the growth rate of the roots during the application of stress, but still allow measurable recovery after the stress had been removed. Four concentrations of PEG-6000 solution were used: 0.063 kg PEG/ L H_2O, 0.125 kg PEG/ L H_2O, 0.188 kg PEG/ L H_2O and 0.250 kg PEG/ L H_2O, approximately -0.25, -0.50, -0.75, -1.0 MPa respectively. There were two replications of each concentration for a total of eight boxes.

Eighty seeds of each of the soybean varieties PS56RR and OAC Bayfield were germinated for 72 hours on four layers of wet paper towel in large petri dishes. The germination occurred in the growth chamber with the same conditions as for the hydroponic growth system. The intention was to have five seedlings of each variety in each box, however due to the slower germination of OAC Bayfield compared to PS56RR, there were only enough seedlings to have one OAC Bayfield seedling per box. Therefore each box contained five PS56RR seedlings and one OAC Bayfield seedling.

After the 72-hour germination period, seedlings with roots approximately 5 cm long were transplanted into the holes in the boxes. The length of each root was then measured and this was used as an initial root length (d_1). The roots were all measured at 2-h intervals for the following 12 h to determine whether the roots grew at a constant rate. Twenty-four hours after entering the hydroponic growth system all of the root lengths were measured (d_2) and the initial root elongation rate was calculated as:

$RER_i = d_2\text{-}d_1/24hrs$

The growth solution was then pumped out of the plexi-glass boxes using a clear plastic tube which went to the bottom of the box. One of the four PEG solutions was pumped into each box to create osmotic stress. All of the root lengths were measured again (d_3) in case any of the roots had been moved during the pumping process. Once more the roots were measured at 2-h intervals for 12 h to determine whether there was a constant rate of growth under the stress treatments. The seedlings remained in the stress solution for 24 h

at which time another root length measurement was taken (d_4). The root elongation rate under osmotic stress was then calculated as:

$RERs = d_4 - d_3/24$ hrs

The PEG solution was pumped out and growth solution was pumped back into the boxes. The seedlings were all measured once they were back in the growth solution (d_5) and again measurements were taken every two hours for 12 hours. After 24 hours in the growth solution a final root length measurement was taken (d_6) and the recovery growth rate was calculated as:

$RERr = d_6 - d_5/24$hrs

The root lengths at each measurement time were graphed and then visually assessed to determine which concentration of PEG solution reduced the elongation rate while the stress was applied but still allowed the recovery of the growth rate once the stress was removed.

4.3.3. *Experiment 2*

The purpose of this experiment was to determine whether there was significant variation amongst the soybean varieties for their RER under osmotic stress and in their recovery from the osmotic stress. The experiment was replicated six times, with three replicates growing simultaneously in the growth chamber (group 1) and then the other

three replicates were grown in a separate week (group 2). The replications were divided into the two groups to facilitate measurements. Fifty-three Ontario genotypes, including 10 parents of populations which have been genetically mapped and seven exotic (i.e., non-Ontario adapted) genotypes were selected for this screening process. The exotic genotypes were selected from those used by Hufstetler et al. (2007) and were determined to have differing water use efficiency and dark-adapted leaf conductance. Since to our knowledge there have not been any other screening experiments for these traits using soybean, we used the same varieties as in Chapter 2. The seeds were germinated for either 80 hours (group 1) or 72 hours (group 2) before entering the hydroponic system. The initial germination time led to the roots of some genotypes growing to the bottom of the plexi-glass boxes before the end of the experiment so the second germination time was used for group 2. Seedlings with root lengths between five and seven cm were transplanted into the hydroponic system. Since there was only space for 30 seedlings in a plexi-glass box, each replicate needed to be divided into two boxes. The seedlings were each randomly assigned a location in one of the two boxes within each replicate. The experiment was replicated six times.

For the first 22 hours the seedlings were in the growth solution. After this time the growth solution was pumped out as described above and a solution containing 0.188 kg PEG/ L H_2O (determined from Experiment 1) was pumped into the system to create osmotic stress (~ -0.75 MPa). The seedlings remained in the stress solution for 24 hours and then the PEG solution was pumped out and growth solution was pumped back into the boxes in which the seedlings remained for another 24 hours. The seedlings were in

the growth solution for 12 hours before any measurements were taken. Root length measurements were taken 3 times at 6-h intervals in each of the solutions.

The mean elongation rate was calculated for the roots in each solution. The initial root elongation rate (RER_i) was calculated by subtracting the third measurement in the growth solution from the first measurement in the growth solution, and then dividing by the 12 hour period between measurements. The root elongation rate under osmotic stress (RER_s) was calculated by subtracting the third measurement in the PEG solution from the third measurement in the growth solution and dividing by the 24 hours between measurements. Finally, the recovery root elongation rate once the osmotic stress solutions had been removed (RER_r) was calculated by subtracting the second measurement in the recovery growth solution from the third measurement in the stress solution and then dividing by the 18 hours between measurements. The second measurement in the recovery growth solution was used instead of the third because a large number of roots had reached the bottom of the boxes by the time of the third measurement. PROC MIXED in SAS statistical software 9.0 was used to determine whether there was a significant difference amongst the genotypes for their reduction in root elongation rate due to osmotic stress and in their recovery of elongation rate after the stress was removed. The random variables were group and the box within the group. Genotype was the fixed variable. The relative RER under stress was calculated as RER_s / RER_i, and the recovery of root elongation rate once the stress was removed was calculated as RER_r / RER_i. Statistical analyses were performed on square roots of the ratios, because of the non-uniform distribution of the data; the square root transformation

produced a normal distribution. A protected $LSD_{0.05}$ test was performed using the transformed data to determine where the differences amongst genotypes occurred.

4.4 RESULTS

The 18.8 % PEG concentration was selected as the concentration for the screening experiment as it decreased the growth rate, but still allowed recovery after the stress had been removed. The 25.0% concentration was too intense a stress as the root elongation stopped completely during the stress period (Figure 4.1). The 6.3% and 12.5% PEG solutions did not sufficiently decrease the RER.

The screening of 60 genotypes determined that there was a significant box effect ($p=0.03$) and a significant difference amongst genotypes ($p=0.02$) for the ratio of RER_s/RER_i (Table 4.2). The range of relative reductions was very large with the greatest reduction occurring in genotype Katrina, which only had a growth rate in the stress solution that was 24.9% of the growth rate in the initial growth solution. The relative reduction in growth rate of this Ontario variety was not significantly different from the largest relative reduction in growth rate of the exotic genotypes. The smallest reduction was observed in the genotype AC Brant which only had a growth rate reduction of 9.3% in the stress solution, which was a significantly smaller reduction than the exotic genotype with the smallest reduction in growth rate (Hutcheson). Therefore, the range of reductions in growth rate of the Ontario germplasm was larger than the range of exotic germplasm in this study.

There was no significant box effect ($p=0.10$), nor a significant genotype effect ($p=0.16$) for the ratio of the recovery growth rate to initial growth rate (RER_r/RER_i). There was, however, still a large range of LSMEANS for this ratio with many genotypes

growing at a more rapid rate once the stress had been removed compared to the rate at which they were growing before the stress was applied (Table 4.3). OAC Lakeview had the lowest RER_r/RER_i with a recovery growth rate that was only 48.1% of its initial growth rate. AC Brant had the highest RER_r/RER_i which was 226.7% its original growth rate. There was not a significant difference between the extreme Ontario varieties and the extreme exotic genotypes for this trait.

Two of the mapping population parent crosses differed significantly for RER_s/RER_i (Table 4.2). These crosses were AC Colibri x OT91-3 and AC Brant x X3145-B-B-3-15.

4.5 DISCUSSION

The different PEG treatments produced different degrees of inhibition of root growth rate. The two lower PEG concentrations (6.25% and 12.5%) did not cause a strong effect and, therefore, there was concern that these stress levels would not effectively differentiate the genotypes in the screening experiment. The 25% PEG concentration was previously used to identify variation in RER in maize under osmotic stress (T. Wambach and L. Lukens unpublished), but this concentration was too high for soybean as it completely stopped the root growth during the stress period and therefore prevented the opportunity to observe differences in the RER_s. The 18.8% PEG concentration (-0.75 MPa) did noticeably reduce the RER when the stress treatment was applied, but it also allowed the roots to recover after the stress had been removed. Therefore, the PEG concentration which produced the -0.75 MPa osmotic potential was selected as the concentration to use in order to determine the genetic variation for reduction in RER under osmotic stress. Consistent with Ogawa and Yamauchi (2006), who used maize, the higher PEG concentrations resulted in a greater inhibition of RER under stress conditions compared to the initial growth rate.

Verslues et al. (1998) said that caution must be used when comparing the results of PEG induced osmotic stress to that caused by vermiculite as at a given Ψ_w a plant will be more stressed in vermiculite than in the PEG solution. A previous study did look at the effects of PEG on root weight using a soybean variety grown in sand and watered with a nutrient solution containing 15% PEG (El-Shourbagy and Malibari 1988). This study by El-Shourbagy and Malibari (1988) found that the fresh weight of the root

decreased substantially under the PEG stress treatment, but that the root dry weight actually increased slightly. However, to the best of our knowledge there are no studies with soybean that look at the effects on RER of osmotic stress using PEG, making these results unique.

Significant variation for inhibition of root elongation rate was observed, and the range of values for this trait in Ontario genotypes was 47% ((Δ RER_s/RER_i) / highest (RER_s/RER_i)* 100). The large range observed in the relative reduction under the osmotic stress treatment provides the potential for breeding for higher root elongation rate under conditions of osmotic stress using Ontario-adapted germplasm. The hydroponic growth system allowed for a large number of genotypes to be measured simultaneously and could be used in large screening efforts for this trait. The significant difference found amongst the mapping population parents AC Colibri and OT91-3 as well as AC Brant and X3145-B-B-3-15 provides the opportunity to perform quantitative trait loci analysis for RER_s/RER_i to determine chromosomal regions contributing to the control of this trait.

Significant variation for RER_r/RER_i was not observed in this study. There are two possible explanations for why this variation was not observed. The first is that the varieties that experienced a more serious reduction in RER under stress conditions were able to recover from the stress so that there wasn't a significant difference in RER_r/RER_i between these genotypes and the ones that were not severely affected. The other explanation is that the measurement technique was not sensitive enough to detect the differences. There was a fair amount of missing data at this stage in the experiment due

to roots growing to the bottom of the box and therefore no longer being measurable. This could have resulted in the seedlings with the higher RER_r not being included in the data, and/or it could result in the seedlings with the higher RER_s being the individuals that grew to the bottom of the box and being removed from the data. Either of these scenarios would reduce the opportunity to observe variation amongst the genotypes in the ratio of RER_r/RER_i.

To our knowledge there haven't been any previous studies comparing the recovery of various genotypes after the stress has been removed. However, Frensch and Hsiao (1994) did observe that maize roots were able to recover their original growth rate after the removal of osmotic stress, which is consistent with what we observed. Over half the genotypes actually had higher root growth rates after the stress had been removed than before (Table 4.3). Frensch and Hsiao (1994) also observed that when the stress was removed there was a sudden steep increase in RER which then decreased to a similar level to what had been initially observed before the onset of stress. They suggest that this may be the result of the RER not being inhibited by cell wall mechanics but rather the result of osmotic adjustment in the root, and then the removal of the stress results in a sudden increase in turgor. Despite no statistical difference (Table 4.3), there was still a large range of mean RER_r/RER_i values observed. This large range merits further investigation and through further refinement of the measurement system there is potential that significant genetic variation may be found for RER_r/RER_i.

4.6 REFERENCES

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Table 4.1. Soybean lines and varieties used in Experiment 2 for RER screening including Ontario-adapted conventional and Roundup Ready varieties, mapping population parents and varieties shown by Hufstetler et al. (2007) to differ for dark-adapted leaf epidermal conductance and WUE.

RR Entries

	Genotype	HU Rating
1	OlexRR	2450
2	25-04R	2500
3	OAC Raptor	2700
4	OAC Rockwood	2700
5	RCAT MatRix	2850
6	RR Rochester	2950
7	RCAT MiRRa	3000
8	RR Respond	3000
9	RCAT 22R1	3150
10	RR Renwick	3150
11	26-02R	2600
12	28-52R	2850
13	30-07R	3000

Conventional Entries

	Genotype	HU Rating
14	OAC Ayton	2550
15	OAC Carman	2550
16	OAC Bayfield	2650
17	Dundas	2750
18	OAC Wallace	2750
19	OAC Prodigy	2850
20	RCAT Corbett	2850
21	OAC Huron	2900
22	OAC Kent	3050
23	RCAT Pinehurst	3050
24	RCAT Ruthven	3200
25	Colby	2850
26	OAC Lakeview	2700
27	Madison	2700
28	Connor	2600
29	Katrina	2950
30	RCAT Harwich	3050

Mapping Parents

	Genotype
31	AC Brant
32	X 3145-B-B-3-15
33	AC Colibri
34	OT91-3
35	Nattosan
36	NK S08-80
37	OAC Arthur
38	AC 756
39	Harovinton
40	OAC Salem
41	OX939
42	Haro (1-7)
43	OX281
44	Mukden
45	Conrad
46	OX760
47	OX744
48	Williams
49	AC X790P
50	IA2034
51	Westag-97
52	AC Hime
53	Leo

Lines known to differ for epidermal conductance

	Genotype	Maturity Group
54	PI 407859-2	V
55	Young	VI
56	Boggs	VI
57	Hutcheson	V
58	PI 416937	V
59	Dillon	VI
60	N98-7265	V

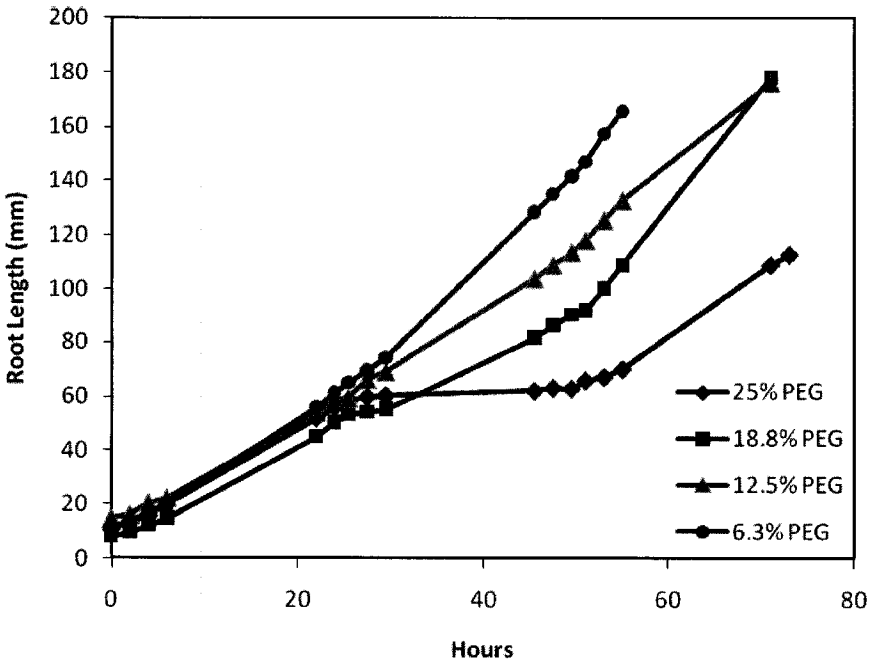

Figure 4.1. The mean length of soybean roots under four different concentrations of polyethylene glycol (PEG) stress, with 7 plants per box and 2 boxes per treatment. The stress was applied 24 hours into the experiment and removed 48 hours into the experiment.

Table 4.2. Transformed (square root) least squares means and untransformed least squares means and standard errors for the ratio of root elongation rate during osmotic stress to initial root elongation rate (RER_s/RER_i) for seedlings of 60 soybean genotypes (n=6).

genotype	SR LSMEAN	LSMEAN	genotype	SR LSMEAN	LSMEAN
	$mm^{1/2}\ h^{-1/2}$	$mm\ h^{-1}$		$mm^{1/2}\ h^{-1/2}$	$mm\ h^{-1}$
Katrina	0.50	0.25	OAC Kent	0.68	0.47
PI416937	0.52	0.27	OX760	0.69	0.47
OlexRR	0.52	0.27	RCAT Harwich	0.69	0.47
OAC Bayfield	0.53	0.28	Dillon	0.70	0.48
OX744	0.54	0.29	28-52R	0.70	0.49
RCAT 22R1	0.54	0.29	Haro1-7	0.70	0.49
OAC Ayton	0.56	0.32	OAC Arthur	0.70	0.50
Nattosan	0.57	0.32	26-02R	0.70	0.50
PI407859-2	0.57	0.33	OX281	0.71	0.50
OAC Raptor	0.58	0.33	Boggs	0.71	0.50
OT91-3	0.58	0.34	ACx790P	0.71	0.50
30-07R	0.60	0.36	OAC Rockwood	0.72	0.51
Connor	0.61	0.37	RCAT Renwick	0.72	0.51
RCAT Pinehurst	0.61	0.37	AC Hime	0.72	0.52
OAC Lakeview	0.61	0.38	OAC Wallace	0.72	0.52
Williams	0.63	0.39	Hutcheson	0.72	0.52
RR Respond	0.63	0.40	RCAT MiRRa	0.73	0.53
AC756	0.63	0.40	Conrad	0.73	0.53
RR Rochester	0.63	0.40	OAC Salem	0.73	0.54
OAC Carman	0.64	0.41	Dundas	0.74	0.55
Young	0.64	0.41	Madison	0.74	0.55
Leo	0.64	0.41	RCAT Corbett	0.75	0.56
Huron	0.64	0.41	Harovinton	0.76	0.58
IA2034	0.64	0.42	OX939	0.77	0.59
RCAT MatRix	0.65	0.42	25-04R	0.78	0.61
Westag-97	0.65	0.42	OAC Prodigy	0.78	0.61
RCAT Ruthven	0.66	0.43	AC Colibri	0.80	0.65
x3145	0.66	0.44	NKS08-80	0.80	0.65
Mukden	0.67	0.45	AC Brant	0.95	0.91
Colby	0.67	0.45	p=0.02		
N98-7265	0.68	0.46	$LSD_{0.05}$	0.20	

Table 4.3. Transformed (square root) least squares means and untransformed least squares means and standard errors for the ratio of root elongation rate during recovery from osmotic stress to the initial root elongation rate (RER_r/RER_i) for seedlings of 60 soybean genotypes.

genotype	SR LSMEAN	LSMEAN	genotype	SR LSMEAN	LSMEAN
	$mm^{1/2}\ h^{-1/2}$	$mm\ h^{-1}$		$mm^{1/2}\ h^{-1/2}$	$mm\ h^{-1}$
OAC Lakeview	0.69	0.48	Westag-97	1.07	1.15
RCAT 22R1	0.75	0.57	RCAT Renwick	1.07	1.15
Connor	0.83	0.70	Dillon	1.07	1.15
OT91-3	0.84	0.70	Williams	1.08	1.16
OlexRR	0.84	0.70	OAC Carman	1.08	1.16
Katrina	0.85	0.72	Harol-7	1.08	1.17
30-07R	0.86	0.74	Conrad	1.08	1.17
PI416937	0.87	0.75	RCAT MatRix	1.09	1.18
RR Respond	0.89	0.80	RCAT Pinehurst	1.09	1.19
AC Colibri	0.90	0.80	NKS08-80	1.10	1.20
OX760	0.91	0.82	RCAT Ruthven	1.10	1.21
Nattosan	0.91	0.83	26-02R	1.10	1.21
OAC Raptor	0.94	0.88	AC 756	1.10	1.22
RCAT Harwich	0.94	0.89	OAC Salem	1.11	1.23
PI407859	0.96	0.91	OAC Rockwood	1.12	1.25
28-52R	0.96	0.92	Colby	1.13	1.27
OAC Ayton	0.96	0.93	Leo	1.15	1.32
OX744	0.97	0.94	IA2034	1.16	1.34
OAC Wallace	0.99	0.98	Harovinton	1.16	1.35
Madison	0.99	0.98	Dundas	1.17	1.36
RR Rochester	0.99	0.98	RCAT Corbett	1.19	1.42
OAC Kent	1.01	1.02	25-04R	1.20	1.43
Young	1.01	1.02	OX939	1.23	1.50
N98-7265	1.01	1.02	Hutcheson	1.23	1.51
RCAT MiRRa	1.02	1.03	OX281	1.25	1.55
OAC Huron	1.02	1.05	x3145	1.25	1.57
Mukden	1.04	1.08	AC Hime	1.27	1.61
OAC Arthur	1.04	1.08	OAC Prodigy	1.34	1.79
ACx790P	1.05	1.10	AC Brant	1.51	2.27
OAC Bayfield	1.05	1.11	p=0.10		
Boggs	1.06	1.13	$LSD_{0.05}$	0.44	

CHAPTER 5: CONCLUSIONS

5.1 CONCLUSION

Sixty soybean varieties were screened for their root elongation rate under osmotic stress which was imposed in a hydroponic culture system. There was significant variation amongst Ontario-adapted soybean varieties for relative root growth in the stress environment. Significant variation was not observed amongst the soybean varieties for the relative recovery of root elongation rate after the osmotic stress had been removed. Further investigation into whether this lack of variation was a result of the need for refinement of the measurement system, or whether there isn't variation for root elongation rate recovery after osmotic stress in Ontario-adapted soybean is required if this screening method is to be used for genetic improvement of this trait.

A new method for measuring dark-adapted leaf conductance to water vapour was developed using a closed recirculating gas exchange system. This technique was compared to other methods using wilted leaves for measuring minimum epidermal conductance to see which method had the highest correlation coefficient with water use efficiency (WUE). It was determined that the conductance from wilted leaves and the conductance from dark-adapted leaves had different levels of correlation with WUE and that the traits could not be considered identical. Currently in the literature both traits are referred to as "minimum epidermal conductance", however, we suggest that the term "dark-adapted leaf conductance" be used in reference to conductance of non-wilted dark adapted leaves.

Significant variation was found amongst Ontario-adapted soybean varieties for dark-adapted leaf conductance (g_{dark}). A subset of the varieties found to have g_{dark} values at both extreme ends of the spectrum were used to determine the suitability of g_{dark} as a surrogate measurement for WUE and to gain understanding of the physiological basis of the correlation between these two traits. A significant correlation was found between g_{dark} and WUE under the control (water replete) treatment, but this relationship was no longer significant under the cyclic drought treatment. Genotype differences in WUE were determined to be constitutive across the water replete and drought environments. Therefore, when using g_{dark} as a screening tool for WUE it is important to perform the measurements on plants which have not experienced water stress.

The g_{dark} trait was also very strongly correlated with leaf internal CO_2 concentration (C_i) which is consistent with current theory predicting that C_i is an important determinant of WUE. The correlation between WUE, g_{dark}, C_i and stomatal conductance suggests that the variety differences in WUE observed are due to stomatal effects. However, stomatal density was not the link between these traits. The stomatal size was correlated with stomatal conductance and photosynthetic rate, but not with WUE. There also appeared to be lasting mesophyll damage resulting from the cyclic drought treatment, which was apparent as a decrease in photosynthetic rate despite C_i being unchanged. Further investigation into the nature of the damage to the photosynthetic capacity by the cyclic drought would be beneficial in order to develop soybean varieties that are able to recover their productivity after a drought stress has passed.

The genetic variability for root elongation rate under osmotic stress and dark-adapted leaf conductance provides the opportunity for future research into the genetic control of these traits as well as the opportunity to integrate these physiological traits into breeding programs and potentially improve drought tolerance in Ontario-adapted soybean. The strong correlation between WUE and g_{dark} also raises questions of whether they are under the control of the same chromosomal regions. Future research should continue to examine the physiological basis of the correlation between WUE and g_{dark} as well as investigate whether there are genetic reasons for this relationship.