

CHAPTER TWO

CHARACTERIZATION OF DROUGHT TOLERANCE TRAITS IN NODULATED SOYBEANS

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I was responsible for conducting the trial by setting up the experiments and analyzing the data. I was trained by Simon Driscoll/Leeds University to carry out and analyze the infra-red gas analysis study (CIRAS-1, PP Systems Hitchin Herts, UK) and FluorPen fp100 system (Photon Systems Instruments, Brno, The Czech Republic). After I analysed and wrote the draft article, the co-author Prof Christine Foyer helped me in editing the publication.

2.1 Abstract

Drought is the single most important factor limiting soybean (*Glycine max* L. Merr.) yields in the field. The following study was therefore undertaken to identify phenotypic markers for enhanced drought tolerance in nodulated soybeans. Leaf and nodule parameters were compared in three genotypes: Prima 2000, glyphosate-resistant A5409RG and Jackson, which had similar shoot biomass and photosynthesis rates at the third trifoliolate leaf stage under water-replete conditions. When water was withheld at the third trifoliolate leaf stage, photosynthesis, nodule numbers, nodule biomass and symbiotic nitrogen fixation (SNF) were greatly decreased. Significant cultivar-drought interactions were observed with respect to photosynthesis, which also showed a strong positive correlation with nodule SNF, particularly under drought conditions. Prima leaves had high water use efficiencies and they also maintained high photosynthetic electron transport efficiencies under long term drought. Moreover, Prima had the highest shoot biomass under both water-replete and drought conditions. A-5409RG was the most drought-sensitive genotype showing early closure of stomata and rapid inhibition of photosynthesis in response to drought. In addition to classifying the genotypes in relation to drought tolerance, the results demonstrate that the ability to sustain shoot biomass under nitrogen limitation is an important parameter, which can be easily applied in germplasm screening for drought tolerance in soybean.

2.2 Introduction

Grain legumes, such as peas, beans and soybean are rich in protein, starch, fibre and other essential nutrients, and are valuable in the production of human nutrition and animal feed. The presence of nitrogen-fixing bacteria in specialized organs (nodules) on legume roots means that grain legumes, such as soybean, which has a capacity for nitrogen fixation in the range of 49-450 kg/ha N (Herridge et al., 2008; Wani et al., 1995) can also provide an inexpensive method of natural nitrogen fertilization (Van Heerden et al., 2007). The spatial deployment of roots determines the ability of a plant to secure edaphic resources (De Dorlodot et al., 2007). Root architecture plays an important role in nutrient acquisition with considerable impact on nitrogen use efficiency (NUE) (Garnett et al., 2009). While a correlation between the QTLs for nitrogen uptake and the QTLs for root architecture traits has been suggested (Coque et al., 2008), the involvement of the root traits in NUE is complicated by difficulties encountered when determining the contributions of root systems under field conditions (Garnett et al., 2009). The adverse effects of drought can often be successfully minimized by changing carbon allocation patterns to allow for the formation of a deep root system before the onset of water limitation (Lopes et al., 2011). Thus, it is perhaps not surprising that there is an association between QTLs for root features and plant productivity in terms of yield, water use or nutrient capture (Steele et al., 2007).

The ability to maintain high rates of photosynthesis is an important determinant of the ability of a crop plant to maintain growth during nitrogen limitation (Ding et al., 2005; Vos et al., 2005). Photosynthesis also sustains nodule growth and symbiotic nitrogen fixation (SNF) in legumes

(Voisin et al., 2003). Sucrose produced by photosynthesis in the shoot provides the energy and carbon skeletons required for SNF, ammonia assimilation and the export of amino acids and other nitrogenous compounds. Thus, a significant proportion of the carbon fixed during photosynthesis is allocated to nodule and SNF is a strong sink for photo-assimilates (Silsbury, 1977). The fixed nitrogen originating from SNF in the nodules is supplied to the rest of the plant, via the xylem, as organic N compounds, principally amides and amino acids (Pate et al., 1984). While the nodules of amide-transporting plant species (*Vicia*, *Pisum* and *Lupinus*) contribute relatively more carbon to shoots than do the nodules of ureide-transporting legumes, such as soybean, the transport of assimilates to and from the shoots is dependent on the presence of N₂-fixing nodules and removal of nodules resulted in a large decrease in the transport of the carbon fixed either by photosynthesis or by the nodules via phosphoenol pyruvate carboxylase (Vance et al., 1985).

Grain legume production is severely restricted by drought (Grzesiak et al., 1996; Sincik et al., 2008; Sinclair et al., 2007), which poses a significant threat to food security (Boyer, 1982). About one-third of the world's agricultural land currently suffers from chronically inadequate water availability (Boyer, 1982; Flexas et al., 2006a; Ghannoum, 2009) and this situation is predicted to become progressively worse (Chaves and Oliveira, 2004; Chaves et al., 2003; Jury et al., 2007). The plant hormone methyl jasmonate has been found to enhance the drought tolerance of soybean under conditions of nitrogen fertilization (Anjum et al., 2011). However, very little information is available on the effects of such compounds on nodulated soybeans, which depend on SNF for nitrogen. The legume/*Rhizobium* symbiosis is de-stabilized by drought, which like other stresses causes premature nodule senescence (Matamoros et al., 1999). SNF activity is rapidly inhibited

by water deprivation, which causes changes in nodule morphology and metabolism (Fernandez-Luquen~F. et al., 2008). Drought-induced inhibition of nitrogenase activity is caused by several mechanisms including carbohydrate depletion and feedback regulation by nitrogen accumulation (Serraj et al., 1999). Like chilling stress (Van Heerden et al., 2008), drought can also adversely affect the oxygen diffusion barrier that is crucial to the effective operation of the nodule (Serraj et al., 1999). Oxygen-based limitations on nitrogenase activity occur in soybean nodules even under ambient conditions (Hunt et al., 1989). The permeability of the nodule to oxygen is also influenced by many other factors including water movements into or out of intercellular air spaces (Minchin, 1997; Purcell and Sinclair, 1994; Serraj et al., 1999; Serraj et al., 1995).

Adaptation to drought is a quantitative trait controlled by many different genes. While molecular-genetic approaches are increasingly used to characterise the complex network of drought-related traits in crop species (Manavalan et al., 2009), relatively few applications for DNA marker technologies have so far emerged in practical breeding programs (Lopes et al., 2011). Of the many reasons for the relatively slow uptake of new technologies poor or inadequate phenotyping remains an important constraint (Lopes et al., 2011). Breeding for high crop yields in drought-prone environments is complicated by the year-to-year variability in the amount and temporal distribution of available soil water and the low heritability of drought-resistance traits under these conditions (Lopes et al., 2011). While molecular approaches provide essential candidate gene sequences that allow dissection of QTLs or transgenic approaches to drought tolerance, such approaches require accurate high throughput phenotyping in the field (Araus et al., 2011; Manavalan et al., 2009). The following study was undertaken in order to define physiological

markers that could be useful in future breeding programmes for selection of drought-tolerant soybean genotypes and might also be the target for the development of a molecular marker.

In this part of the study it has been investigated if plant traits related to above ground performance, such as stomatal conductance, photosynthesis and biomass is directly related to parameters for nitrogen acquisition in the nodules and can be used for soybean cultivar performance evaluation under drought.

2.3 Materials and methods

2.3.1 Plant material and growth conditions

Seeds of different soybean (*Glycine max* L. Merr.) cultivars Prima 2000, a commercial variety registered in South Africa, A5409RG, a commercial variety used in South Africa that harbours a glyphosate-resistance gene, and Jackson a variety that has nominally been classed as drought-tolerant (Chen et al., 2007; Sall and Sinclair, 1991) obtained from Pannar Seed South Africa (Greytown, South Africa) were inoculated (0.5 g per pot) with a cell powder of the *Bradyrhizobium japonicum* strain WB74-1 (Soygro bio-fertilizer Limited, South Africa). Seedlings were grown in large pots with the volume of 218.2 cm³ [17.5 cm x 20 cm diameter and 13.1 cm (bottom)] in fine grade vermiculite (Mandoval PC, South Africa), which has a particle size of 0.5-3 mm and a loose bulk density of 100 kg/m³. This medium consists of thin, flat flanks containing microscopic layers of water (Dupré Minerals Ltd, England). Vermiculite was used specifically in these experiments to provide an N-free medium to facilitate maximal nodule formation under water-replete conditions. Vermiculite has been previously been shown to be the best growth medium for the production of nodules with high SNF activities (Van Heerden et al., 2007) because it avoids problems encountered with soil nutrients that interfere with nodule formation and impair SNF. The plants grown under water-replete conditions were watered twice daily with distilled water and supplied with N-free Hoagland nutrient solution (Hoagland and Arnon, 1950) twice per week.

The experiment was conducted in controlled environment chambers at Forestry and Agricultural biotechnology Institute (FABI), University of Pretoria (-25° 45' 20.64"S, 28° 14' 8.16"E) during summer season of 2009. The climatic condition of growth condition was, day/night temperature of 25°C / 17°C and 60% relative humidity, 13 h photoperiod at the average light intensity of photosynthetically active radiation of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The light intensity was measured using PAR 2 Meter with SW 11L sensor (S.W & W.S. Burrage, United Kingdom. The indicated PAR is the average of incoming solar radiation measured from 10 am to 3 pm. Furthermore, the supplemental light with a capacity of 350 $\mu\text{mole m}^{-2} \text{s}^{-2}$ was supplied with high pressure sodium lamps from 4:00-7:00 pm. The environmental condition in the growth phytotron was monitored regularly to ensure the adequate growth conditions maintained. Eighty soybean plants in total per cultivar were grown to the third trifoliolate leaf stage. At this point, half of the plants were maintained under water-replete conditions and half were subjected to drought stress by withholding water and nutrient solution for 18 consecutive days. Pots containing plants under water-replete conditions or subjected to drought randomized throughout each chamber. Further, for all other plant performance traits measurements four individual plants were measured for each water treatment.

2.3.2 Photosynthesis measurements and calculation of instantaneous water use efficiency (IWUE) values

Photosynthetic gas exchange measurements were performed daily on the central leaflet in each case of attached third and fourth trifoliolate leaves between 10:00 am to 12:00 am throughout the measurement period using an infra-red gas analysis (CIRAS-1, PP Systems Hitchin Herts, UK)

obtained from University of Leeds. Measurements were made at 25⁰C and an irradiance of 700 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ and a CO₂ concentration of $350 \pm 10 \mu\text{mol mol}^{-1}$ were used.

Chlorophyll *a* fluorescence measurements were performed using a FluorPen fp100 (Photon Systems Instruments, Brno, and The Czech Republic). For measurement, leaf material was dark-adapted for 20 min by adapting only the part of the leaf which was measured using the leaf clip.

Instantaneous water use efficiency (IWUE) values were calculated as the ratios between CO₂ assimilation rates and stomatal conductance values as described previously (Soares-Cordeiro et al., 2009a).

2.3.3 Leaf water potential

The leaf water potential values were determined from 11:00 -12:00 am during the course of the experimental period using a pressure bomb model 3005 (ICT International, Australia) on the central leaflet that was cut from a fully expanded trifoliate leaf and placed into a chamber with cut ends protruding through the specimen holder. Nitrogen gas was then applied to the leaflet until a drop of sap was observed at the cut end. The pressure required to force a drop of sap from the sample is considered as equivalent to the force with which water is held to plant tissues by forces of adsorption and capillarity (Mario Valenzuela-Vazquez et al., 1997).

2.3.4 Water content of vermiculite

Vermiculite core samples was taken using a cylindrical cork borer (1.4 cm diameter and 11 cm length) every second day. These cores reflect vermiculite water contents to more than half way down the root systems. The fresh mass of the sample was measured immediately using a Model B-502-S Metter Toledo balance (Switzerland). The samples were then placed into a drying oven (Type U 40, Mommert, Germany) at a temperature of 80°C for 24 h. Vermiculite water contents (SWC) were calculated as the difference between the first and second measurements as: $SWC (\%) = [(fresh\ mass - dry\ mass) / dry\ mass] \times 100$.

2.3.4 Biomass

Four individual plants (replicates) were harvested and used for destructive biomass measurements. For biomass determination either all vegetative above-ground plant parts (shoot biomass) or all below-ground roots (root biomass) were harvested. Nodule biomass was determined separately after removing the plant roots. Dry biomass of shoots and roots was determined after exposure of plant parts in a drying oven (Type U 40, Mommert, Germany) to a temperature of 80°C for 48 h.

2.3.5 Nitrogenase activity measurement

Nitrogenase activity was determined using the acetylene reduction assay essentially as described by Turner and Gibson (1980). All crown and lateral nodules of four individual plants for each cultivar were harvested and after the mass as well as nodule number recorded, the nodules were assayed for acetylene reduction. Nodules were placed in an airtight small flask of 43 ml capacity and ethylene production was determined after 10 minutes incubation with 4 ml acetylene and injecting 1ml of gas from each flask into a gas chromatograph Varian 3900 (Varian inc., USA). The oven temperature was maintained at 80°C, FID detector: 200, 1177:180°C, Gas flow: air (300), H₂ (30), N₂ carrier gas (25) and running time was 4.8 minute. For calibration, a standard curve was made by injecting different level of ethylene.

2.3.6 Statistical analysis

CO₂ assimilation, stomatal conductance, leaf water potential and vermiculate water contents during the duration of the experiment were analysed using Sigma plot 2001, Version 7.0 (1986-2001 SPSS Inc.). The analysis of variance was performed using a JMP[®] 8.02 statistical package (SAS Institute Inc., Cary, NC, USA). Statistical significance between parameters determined on water-replete and drought treated plants was determined using the LSmeans Student's t-test. The data was further analysed by a bi-variate platform fitting analysis using JMP[®] 8.02 statistical package (SAS Institute Inc., Cary, NC, USA) software.

2.4 Results

2.4.1 Photosynthesis, stomatal conductance and water use efficiencies

Under water-replete conditions plants of the three cultivars had similar rates of photosynthesis ($11.1 \pm 0.47 \mu\text{mol m}^{-2} \text{s}^{-1}$ for Prima 2000, 12.1 ± 0.47 for A-5409RG and 10.3 ± 0.42 for Jackson (Figure 2. 1). Photosynthesis (CO_2 assimilation) rates decreased as a result of the imposition of drought in all three cultivars (Figure 2.1). The patterns of drought-induced decreases in photosynthesis varied between the genotypes, for example, a 50 % inhibition of photosynthesis occurred earlier in A-5409RG (Figure 2.1B, about day 4) than in Jackson (at day 9) or Prima (day 11, Figures 2.1A and 2.1C). Prima also had significantly higher rates of photosynthesis ($P < 0.05$) after exposure to long term (at day 18) drought compared to Jackson and A-5409RG (Figure 2.1).

The Fv/Fm ratios were used to determine whether there were any photoinhibitory effects on the electron transport system. However, they were similar in all cultivars under water-replete conditions over 18 d (data not shown) and Fv/Fm ratios remained high in dark-adapted leaves of all three cultivars until day 10 after which this parameter decreased by about 50% in both A-5409RG and Jackson but not in Prima by day 18 ($P < 0.05$) (Figure 2.2). These data suggest that damage to the photo-electron transport chain had occurred in A-5409RG and Jackson after prolonged exposure to drought. However, Prima was able to avoid damage to the electron transport system despite inhibition of carbon assimilation (Figure 2.1A).

Under water-replete conditions Prima had the highest stomatal conductance values (Figure 2.3A) and Jackson the lowest (Figure 2.3C) values. Stomatal conductance decreased in all the cultivars when the plants were deprived of water (Figures 2.3). A further analysis of the responses of photosynthetic parameters to drought revealed a significant cultivar-drought interaction with respect to photosynthetic CO₂ assimilation rates, Fv/Fm ratios, and stomatal conductance but not in relation to the intracellular CO₂ concentration within the leaves (Appendix 1).

Jackson had significantly higher ($P < 0.05$) instantaneous water use efficiency (IWUE) values than Prima or A-5409RG under water-replete conditions (Table 2.1). While IWUE values were similar in all genotypes following short term (8 day) drought, Prima had a significantly higher ($P < 0.05$) IWUE than Jackson or A-5409RG after the long term (18 day) drought treatments (Table 2.1). After long term water deprivation, only Prima was able to maintain high IWUE values, whereas in Jackson and A-5409RG IWUE had decreased to levels similar to those observed in the water-replete controls (Table 2.1).

The leaf water potential values were similar in all cultivars under water-replete conditions (Figure 2.4A, control) but decreased progressively in all three cultivars when plants were deprived of water. However, the leaf water potential was significantly higher in Jackson following short term drought than in the other cultivars. The water contents of the vermiculite were similar in all cultivars under water-replete conditions but the values declined progressively after the plants were deprived of water, similar trends being found in all three genotypes (Figure 2.4B).

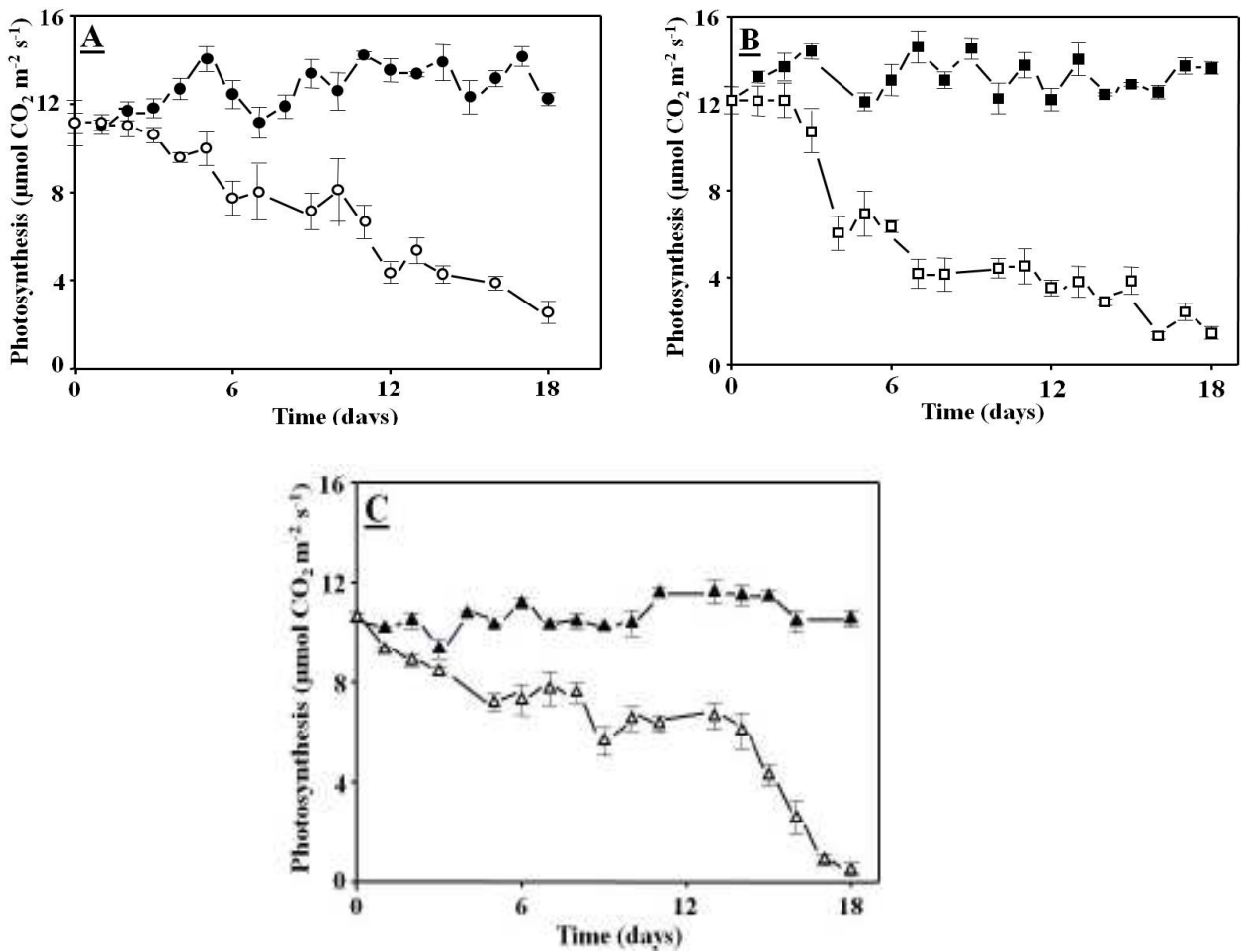


Figure 2.1: Comparison of photosynthetic CO_2 assimilation in Prima 2000 (A), A-5409RG (B) and Jackson (C) leaves. Plants were grown under well-watered (closed symbols) and drought (open symbols) conditions for 18 d. Each data point is the mean \pm SE from 4 individual plants.

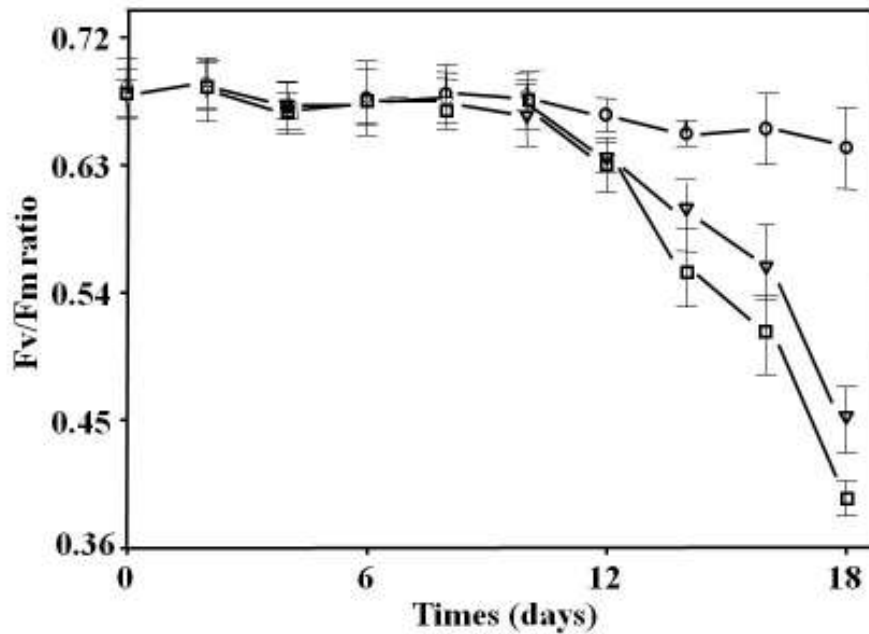


Figure 2.2: Effects of water deprivation on Fv/Fm ratios of leaves of Prima 2000, A-5409RG and Jackson. Plants were grown under drought conditions over 18 d. Circles: Prima 2000, squares A-5409RG, and triangles: Jackson. Each data point is the mean \pm SE from 4 individual plants grown under drought conditions. Control values under water-replete conditions were almost identical over 18 d for all three cultivars with a maximal difference between day 0 and day 18 of less than 5%.

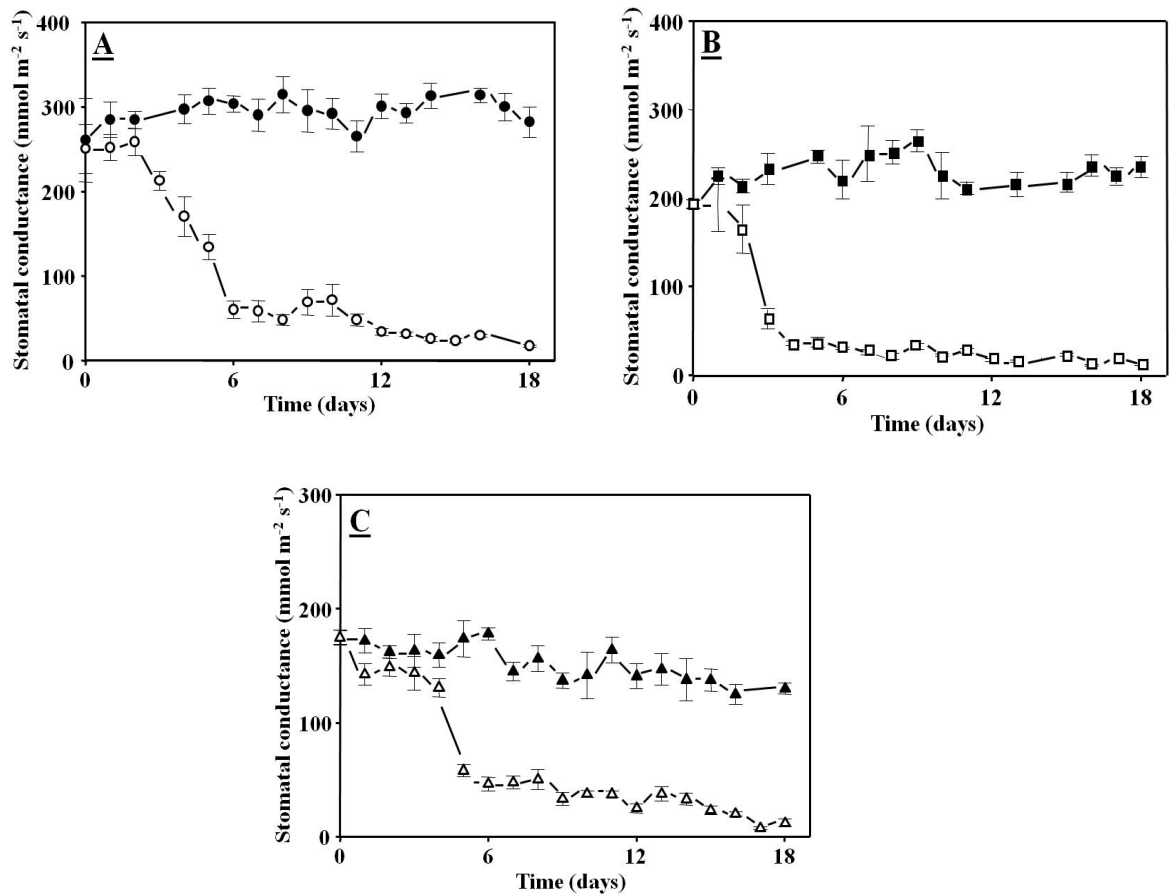


Figure 2.3: Comparison of stomatal conductance values in Prima 2000 (A), A-5409RG (B) and Jackson (C) leaves. Plants were grown under water-replete (closed symbols) and drought conditions (open symbols) for 18 d. Each data point is the mean \pm SE from 4 individual plants.

Table 2.1 A comparison of the instantaneous water use efficiency (IWUE) values of leaves of plants of the three soybean cultivars measured under water-replete or drought conditions on day 8 and day 18 of the experiment. Each data point is the mean \pm SEM of four independent replicates.

Cultivar	Instantaneous water use efficiencies (IWUE) ($\mu\text{mol CO}_2$ per mol H_2O)			
	8 days		18 days	
	Water-replete	Drought	Water-replete	Drought
Prima 2000	49.8 \pm 3.1b	186.4 \pm 9.2a	73.7 \pm 13.7a	156.0 \pm 14.0a
A-5409RG	56.5 \pm 3.4b	150.7 \pm 8.9b	63.2 \pm 0.9ab	59.6 \pm 13.1b
Jackson	72.4 \pm 2.9a	171.1 \pm 10.2ab	57.6 \pm 1.6b	73.9 \pm 4.7b
Significance	**	*	*	**

Different letters in a column denote significant differences (* $P < 0.05$ significant, and ** $P < 0.01$ highly significant).

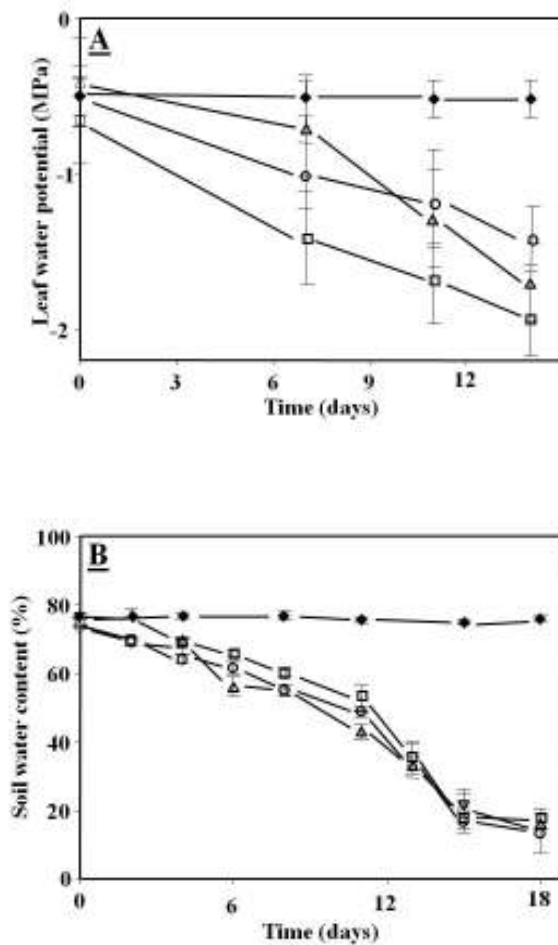


Figure 2.4: Effects of water deprivation on leaf water potential (MPa) values (A) in Prima 2000, A-5409RG and Jackson and vermiculite (Soil) water content as a percentage of the dry soil mass (B). Plants were grown under drought conditions (open symbols) over 18 d. Circles: Prima 2000, squares A-5409RG and triangles: Jackson. Control values (closed diamond) represent the pooled data from all three cultivars using 4 individual plants of each cultivar grown under water-replete conditions. Each data point is the mean \pm SE from 4 individual plants grown under drought conditions.

2.4.2 Plant biomass and shoot to root ratio

All three cultivars had similar shoot biomass under water-replete conditions at the start of the experiment (Figure 2.5A) and Jackson had a lower, but not significantly ($P > 0.05$), root biomass value than the other two cultivars under these conditions (Figure 2.5B). Under water-replete conditions, Prima further showed the greatest increase in shoot biomass accumulation over the 18 d experimental period while Jackson showed the smallest increase over this period (Figure 2.5A). A similar trend was observed for root biomass (Figure 2.5B) but there were no significant differences ($P > 0.05$) in the shoot/root ratios of the three cultivars determined either on a fresh or a dry mass basis on day 18 of the experiment under water-replete conditions (Figure 2.5C). Drought treatment led to a significant decrease ($P < 0.05$) in shoot biomass accumulation at 18 d period of the experiment when compared to water-replete treatment for this period (Figure 2.5A). After 18 d of drought, Prima had the greatest shoot and root biomass (Figure 2.5A, B). Values for Prima shoot biomass were approximately 1.7-times higher under-water replete and 2.4-times higher under drought conditions than those of Jackson at day 18 (Figure 2.5A). Root biomass was 1.5-times significantly ($P < 0.05$) higher in Prima than in Jackson but not significantly different ($P > 0.05$) in Prima and Jackson after 18 d of drought in Prima than Jackson after 18 days of well-watered conditions, but not significantly different ($P > 0.05$) in Prima and Jackson after 18d of drought with the lowest values obtained in A-5409RG.

The drought treatment resulted in a change in biomass partitioning between roots and shoots with a decrease in the ratio under drought and a great proportion of biomass being partitioned to the roots under drought conditions in all three cultivars (Figure 2.5C). The shoot/root ratios measured

on a fresh mass (FW) basis fell from values of 2-3 under water-replete conditions to values of 1-2 after 18 d of drought. The shoot/root ratios measured on a dry mass (DW) basis fell from values of 3-5 under water-replete conditions to values of less than 0.8-1.5 after 18 d of drought (Figure 2.5C). A-5409RG had the highest and Prima the lowest shoot/root ratios expressed either on fresh mass or dry mass basis after 18 d of drought (Figure 2.5C). Furthermore, wilting or leaf rolling was observed in Jackson and A-5409RG but not in Prima under drought stress conditions.

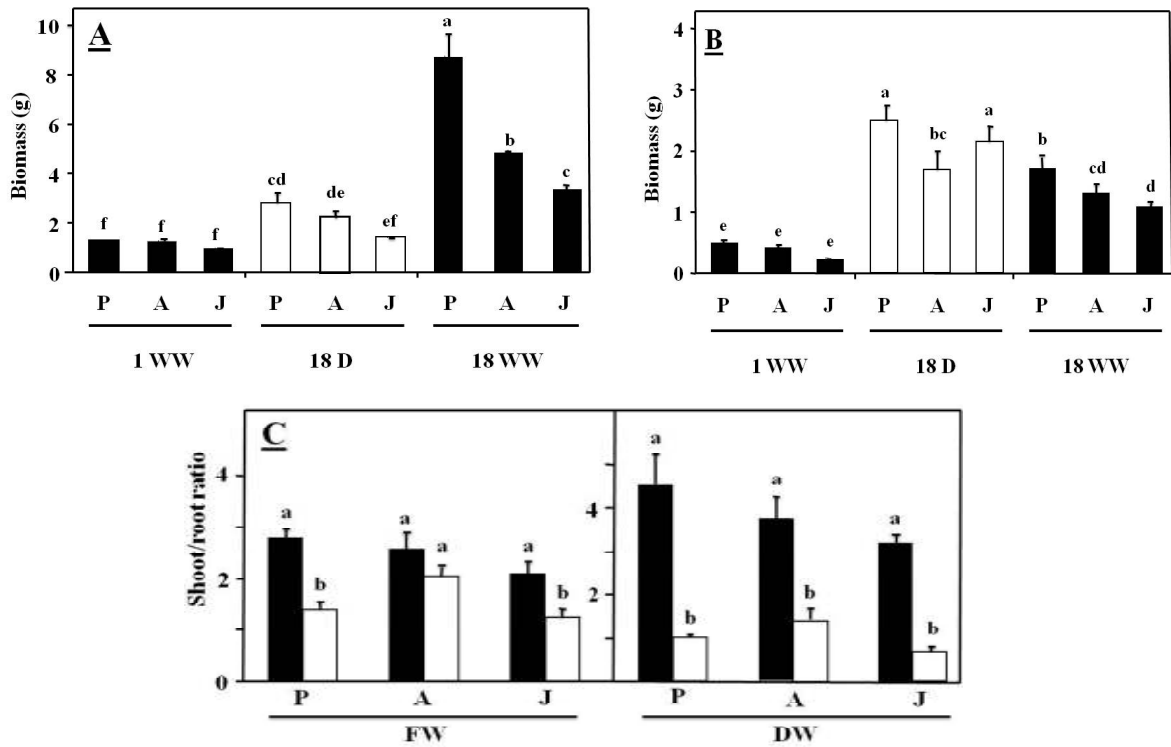


Figure 2.5: Effects of drought on shoot and root biomass and on shoot/root ratio. Shoot biomass (dry mass, A), root biomass (dry mass, B) and shoot/root ratio (fresh mass and dry mass, C) were compared in Prima 2000 (P), A-5409RG (A) and Jackson (J) under water-replete conditions at day 1 (1WW) and day 18 (18WW) and following drought treatment at day 18 (18D). Shoot/root ratios are expressed either on a fresh mass (FW) or dry mass (DW) basis after 18 d exposure to either water-replete (closed columns) or drought conditions (open columns). Each data point is the mean \pm SE from 4 individual plants. Different letters denote significant differences.

2.4.3 Nodule parameters and symbiotic nitrogen fixation (SNF)

Prima and A-5409RG had significantly ($P < 0.05$) higher nodule numbers, biomass and SNF under both water-replete and drought conditions than Jackson (Table 2.2). The drought treatment caused a large decrease in nodule numbers in all three cultivars and after 18 d drought treatment, roots had only 11-13% of the nodule numbers when compared to water-replete conditions (Table 2.2). However, after 18 d of drought all three cultivars had similar nodule numbers, biomass and SNF which was not significantly different ($P > 0.05$) between the three cultivars.

Significant positive relationships between nodule SNF and the rate of leaf photosynthesis (Figure 2.6A, $R^2 = 0.67$), stomatal conductance (Figure 2.6B, $R^2 = 0.79$) and leaf intracellular CO_2 concentrations (Figure 2.6C, $R^2 = 0.55$) were observed under water-replete and drought conditions. Significant positive relationships were also observed between SNF and vermiculite water content (Figure 2.7A, $R^2 = 0.68$) and leaf water potential (Figure 2.7B, $R^2 = 0.66$). However, high SNF rates were observed only in a relatively narrow range of high vermiculite water contents and leaf water potential values.

Table 2.2: Comparison of nodule numbers, nodule biomass (fresh mass) and symbiotic nitrogen fixation (SNF) in three soya bean cultivars at 8 and 18 days under either water-replete or drought conditions. Each data point is the mean of four independent replicates.

Cultivar	Number		Biomass (g)		SNF ($\mu\text{mol h}^{-1}/\text{per plant}$)	
	8 d	18 d	8 d	18 d	8 d	18 d
<i>Well watered</i>						
Prima 2000	56.3±1.1a	136±10.9a	1.5±0.03a	3.1±0.3a	1.9±0.1a	1.9±0.9a
A-5409RG	45.5±1.6b	154±5.7a	0.9±0.1b	2.4±0.2a	1.6±0.35a	1.0±0.03b
Jackson	33.3±3.3c	93.2±5.7b	0.6±0.02c	1.3±0.1b	0.6±0.02b	1.0±0.15b
<i>Significance</i>	**	**	***	*	**	**
<i>Drought</i>						
Prima 2000	33.3±1.8a	15.8±3.7	0.56±0.03a	0.16±0.03	0.85±0.07a	0.06±0.04
A-5409RG	23.5±2.1b	20.7±3.7	0.39±0.02b	0.21±0.03	0.31±0.09b	0.03±0.003
Jackson	26.3±1.1b	10.7±1.0	0.48±0.03a	0.13±0.02	0.50±0.01b	0.02±0.016
<i>Significance</i>	**	<i>ns</i>	**	<i>ns</i>	**	<i>ns</i>

Different letters denotes significant differences (* P<0.05 significant, **P<0.01 highly significant, *ns* non-significant).

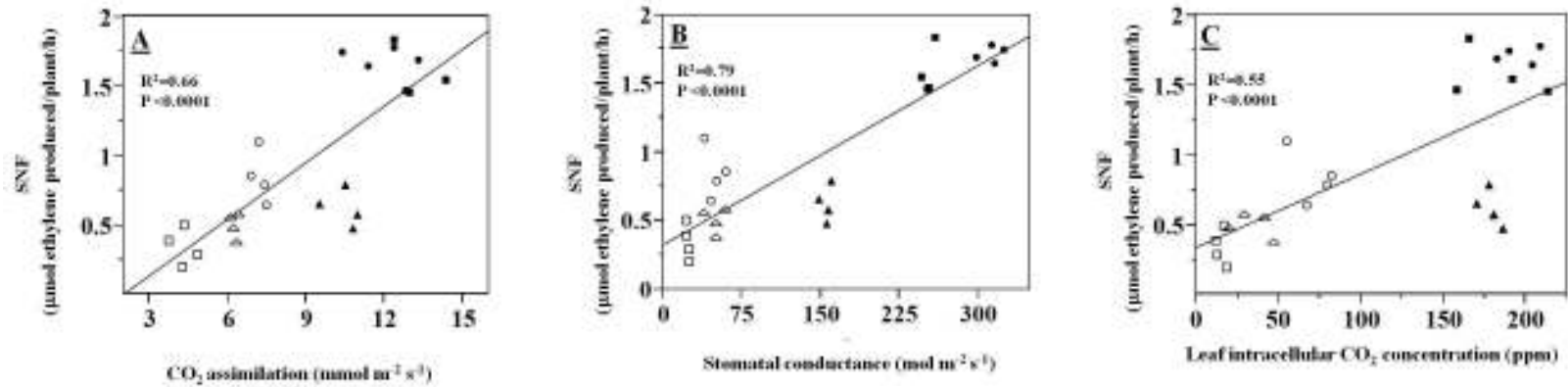


Figure 2.6: Relationships between SNF and CO₂ assimilation (A), stomatal conductance (B) and leaf intracellular CO₂ concentration (C) in Prima 2000 (circles), A-5409RG (squares), and Jackson (triangles) under well-watered (closed symbols) and drought conditions (open symbols). Each data point is the mean from 4 individual plants.

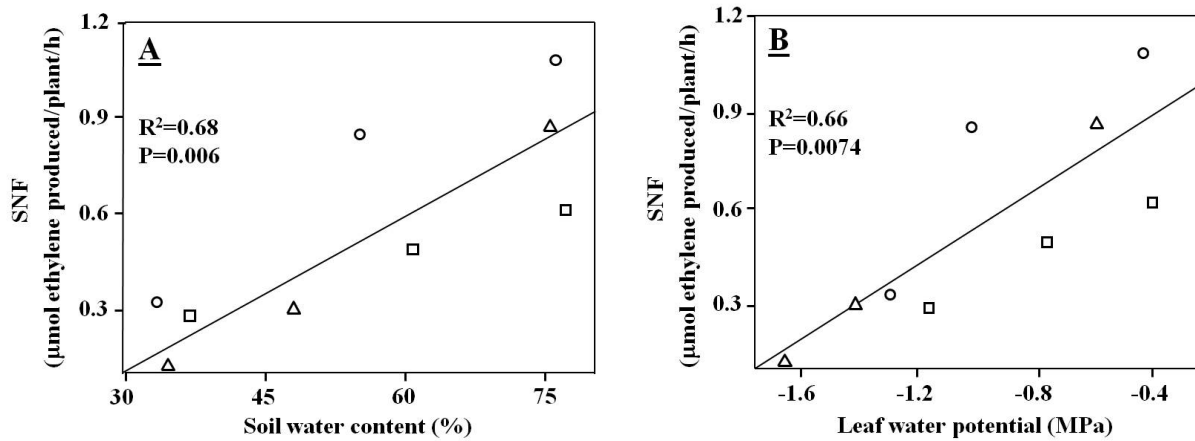


Figure 2.7: Relationships between SNF and soil (vermiculite) water content (A) and leaf water potential (B) in Prima 2000 (circles), A-5409RG (squares), and Jackson (triangles). Data was obtained from plants grown under drought conditions above 30% soil water content or leaf water potentials higher than -1.7. Each data point is the mean from 4 individual plants.

Table 2.3: The R^2 and P-value obtained from the bi-plot regression curve between CO_2 assimilation, stomatal conductance and intracellular CO_2 concentration with soil/vermiculite water content and leaf water potential. The data represents the average of four individual plants measured at the beginning, first week and day 12 measurements after water stress exposure for both water replete and drought stressed plants of three soybean cultivars.

Trait	Soil/vermiculite water content		Leaf water potential	
	R^2	P-value	R^2	P-value
CO_2 Assimilation	0.82	<0.0001 **	0.75	<0.0001 **
Stomatal conductance	0.68	0.0005 **	0.64	0.0011 **
Intracellular CO_2 concentration	0.79	<0.0001 **	0.77	<0.0001 **

2.5 Discussion

In legumes, as in other crops, targeted approaches to drought tolerance have often concentrated largely on shoot parameters, particularly those associated with photosynthesis, rather than on root traits, such as nodulation, which are increasingly considered to be important targets for yield improvement in legumes under drought stress. The findings of the present study, in which genetic variation in drought sensitivity was characterized in three nodulated soybean varieties, demonstrate that considerable genetic variation first exists in the ability of soybean to maintain high leaf water use efficiencies and photosynthesis during drought: The data presented here concerning the responses of photosynthesis to drought is further consistent with previous findings (Cornic, 2000; Flexas et al., 2004; Lawlor, 2002b). These authors demonstrated that water stress mainly affects the assimilation of CO₂ predominantly through the decline of stomatal conductance of the leaf which might be also due to co-regulation among these plant processes. Differences in stomatal conductance are considered to exert the greatest effect on the intrinsic water use efficiency (WUE_{intr}) in soybean cultivars (Gilbert et al., 2011). The most marked differences were observed in the ability to regulate leaf water use efficiencies. Jackson had the highest IWUE values under water-replete conditions but only Prima leaves had the capacity to maintain high IWUE values following exposure to long-term (18 days) drought conditions. These data show that the ability to maintain high leaf water use efficiencies will be a useful trait for soybean breeding programs selecting for improved drought tolerance.

The existence of highly significant association of gas exchange traits (CO₂ assimilation, stomatal conductance and intracellular CO₂ concentration) with soil water content and leaf water potential

(Table 2.3) demonstrates as these processes are highly affected by plant water status. Further, these finding ascertains as gas exchange traits are stress sensitive processes and stomatal limitation is mainly as a result of water stress. Thus it is a principal determinant process for decreased photosynthesis of plants under water stress, as it has been also stated before by Chaves (1991) and Baker (1996). These also suggests as a cultivar which maintain better leaf turgor would exhibit enhanced performance for gas exchange traits and maintain plant growth as observed in the cultivar Prima in this experiment.

The stability of photosynthesis under conditions of water deprivation is considered being an important aspect of drought tolerance in soybean (Gilbert et al., 2011). While much controversy remains regarding the relative importance of stomatal and non-stomatal limitations of photosynthesis in drought responses (Chaves and Oliveira, 2004; Flexas et al., 2006b; Lawlor and Cornic, 2002; Warren, 2008), under mild water stress although stomatal limitation of photosynthesis plays a principal role non-stomatal limitations or metabolic impairments such as decreased ATP limits RuBP regeneration and content (Lawlor and Cornic, 2002; Lawlor and Tezara, 2009b) thus deceases carboxylation rate of Rubisco. There is also evidence for increased diffusive resistance within the mesophyll, including the chloroplast conductance for CO₂ (Flexas and Medrano, 2002; Flexas et al., 2004; Signarbieux and Feller, 2011).

Further the cultivar Jackson maintained high rates of photosynthetic CO₂ assimilation over a long period (up to 14 days) of drought exposure. In the first stages of water deprivation, Jackson maintained high stomatal conductance and photosynthesis. These features are consistent with the classification of Jackson as a drought-tolerant genotype (Chen et al., 2007; Sall and Sinclair,

1991). Increase in root mass in Jackson and Prima might be responsible for better maintenance of the leaf water potential, and stomatal conductance and therefore photosynthesis rate.

Photorespiration is considered to increase considerably upon stomatal closure in C_3 plants with a decrease in C_i (Foyer et al., 2009). Photorespiration is a high capacity pathway that acts as an alternative electron sink for the protection of the photochemical apparatus when CO_2 assimilation is limited by low C_i (Foyer et al., 2009). However, with increasing water deficit photorespiration decreases in absolute value (but may increase relative to photosynthesis) and C_i does not decrease to the compensation point (Lawlor, 2002a; Lawlor and Cornic, 2002; Lawlor and Tezara, 2009b). This is because RUBP synthesis is decreased and so limits both photosynthesis and photorespiration. While photosynthetic CO_2 assimilation rates decline following the onset of drought stress, F_v/F_m data show that the photosynthetic light reactions are not impaired and suggest that photo-respiratory carbon flow was able to maintain photosynthetic electron transport rates and protect the photochemical reactions. However, alternative sinks for electrons may also consume electrons and reductant.

In the comparison of the soybean cultivars A-5409RG in this study, Jackson maintained high rates of photosynthetic CO_2 assimilation over a long period (up to 14 days) of drought exposure. In the first stages of water deprivation, Jackson maintained high stomatal conductance and photosynthesis. These features are consistent with the classification of Jackson as a drought-tolerant genotype (Chen et al., 2007; Sall and Sinclair, 1991). Increase in root mass which would be an indicative of root depth and surface area, in varieties Jackson and Prima could be

responsible for better maintenance of the leaf water potential as well as stomatal conductance and therefore photosynthesis rate.

Prima leaves had significantly higher F_v/F_m ratios than either A-5409RG or Jackson at the end of the period of water deprivation. This suggests that, although the water potential was maintained for longer for Jackson and Prima, photosystem Prima were less damaged than those two cultivars. This demonstrates that Prima is able to protect the photosynthetic electron transport processes in the leaves more effectively under drought. The maintenance of higher F_v/F_m ratio by Prima suggests that plants of this cultivar have more efficient non-photochemical quenching mechanisms than the others. This protects the photosynthetic machinery from damage. Thus, selecting for more efficient photochemical quenching in drought tolerant plants for example, xanthophyll cycling, which would be important for protecting photosynthetic machinery allowing biomass accumulation under drought conditions and will also allow efficient recovery from drought (Müller et al., 2001).

Symbiotic nitrogen assimilation also shows a strong correlation with photosynthesis: SNF decreases in soybean nodules by stress-induced inhibition of nitrogenase activity (King and Purcell, 2001; King and Purcell, 2005; Van Heerden et al., 2008). Significant relationships were observed between SNF and photosynthetic CO_2 assimilation rates and stomatal conductance values under water-replete and drought conditions in this study. The findings demonstrate that SNF is highly sensitive to water availability in the root environment, and to leaf water potential and photosynthesis. An earlier study reported that the decline in SNF related to soil drying was delayed in Jackson relative to another variety (Serraj et al., 1997). In the present study, Prima had

the higher SNF activities and this genotype also accumulated the greatest biomass under both well-replete and drought conditions. Jackson also performed better than A-5409RG under short-term drought conditions but SNF was equally inhibited by long term (day 18 of the experiment) drought in all genotypes.

The value/coefficient R^2 (degree of determination of explained variance) in Figure 2.6 A, B, and C clearly show that the SNF among soybean cultivars is explained by 79, 66, and 55% due to stomatal conductance, CO_2 assimilation and leaf intracellular CO_2 concentration, respectively. The remaining unexplained variance ($1-R^2$) corresponding to that order amounts at 21, 34, and 45%. The magnitude of the unexplained variance are considerable (21, 34, and 45%) showing that the difference in the SNF between cultivars is yet influenced by other additional factors independent to conductance, CO_2 assimilation and leaf intracellular CO_2 concentration. These factors might be a decline in nodule oxygen supply (Denison and Harter, 1995; Minchin, 1997), nodule oxidative stress (damage because of the formation of reactive oxygen species (ROS) (Minchin, 1997) or feedback inhibition of nodule activity due to nitrogen accumulation during water stress (King and Purcell, 2005). These factors also need to be considered in the future during SNF evaluation under drought conditions.

Significant genotypic variation further exists in the control of shoot-root ratios in response to drought. Prima and Jackson were also superior in terms of nodule performance under short-term (first 7 d) drought, in agreement with previous observations for Jackson (Sall and Sinclair, 1991). The better drought tolerance of Jackson has previously been linked to its larger nodule biomass and better supply of photosynthesis to the nodules (King and Purcell, 2001). While cultivar

variations in nodules numbers and nodule biomass were observed in the present study, Jackson had the lowest values for these parameters under most conditions. Furthermore, nodule numbers and biomass were in general equally affected by drought in all cultivars. These data show that Jackson performs no better than the other cultivars in terms of sensitivity to long-term drought.

The concept that the adverse effects of drought can be successfully avoided by changing carbon allocation patterns to allow the formation of a deep root system before the onset of a growth-limiting water shortage is widely accepted. This study has shown drought-induced decrease in shoot to root ratios in all three cultivars together with a reduction in whole plant biomass. However, shoot to root ratios under drought were significantly higher in A-5409RG than Jackson or in Prima. This finding shows that there is considerable genotypic variation in the control of shoot to root ratios in soybean in response drought. A recent study revealed considerable flexibility in the control of shoot to root ratios in *Arabidopsis thaliana* in response to another important environmental signal, nitrate (Tschoep, 2010). In the experiment of low nitrate in *Arabidopsis*, Tschoep (2010) found out plants responded to the low N availability by decreasing the shoot growth due the competition of carbon assimilates with the root and thus alters the shoot: root ratio of the plants. In this soya bean experiment for low water availability plants respond for the stress by adjusting the shoot: root growth and the cultivar that respond quickly and efficiently were found to adjust the stress condition and perform better.

The genotypes are ranked as Prima>Jackson> A-5409RG in terms of drought tolerance. Based on the findings reported here, Prima is relatively drought-tolerant because it maintained high leaf water use efficiencies, high Fv/Fm ratios and had the highest biomass under long term drought

conditions. This genotype was also able to attain a greater root biomass under drought without severely compromising shoot biomass. On this basis, genotypes like Prima are suitable for cultivation in areas with a longer growing season but suffering drought during the growth period. Shoot biomass is likely to be indicative of seed yield due to the findings that shoot biomass accumulation in beans strongly correlates with seed yield (Shenkut and Brick, 2003). Further, in a research conducted in common bean by Shenkut and Brick (2003), the existence of moderate to high heritability of biomass trait and economic seed yield were also ascertained. Therefore, shoot biomass is likely to be indicative of seed yields and thus could be an important trait in the selection of soybean germplasm for drought tolerance.

Determinate cultivars with early flowering properties are considered to be well-suited to arid and semi-arid areas. Jackson is considered to be a drought-tolerant cultivar (Serraj et al., 1997) and shows early flowering and seed maturation (data not shown). This genotype is able to maintain a high rate of photosynthetic CO₂ assimilation over a relatively long period of water deprivation, a trait that would be advantageous in conditions where the soil moisture content is adequate early in the season but rapidly declines due to drought. These findings, together with field observations (data not shown) support for classification of Jackson as a drought-escaping cultivar.

Based on all the parameters measured in the present study, A-5409RG was clearly the most drought-sensitive genotype. This glyphosate-resistant genotype had low water use efficiencies, showed early closure of stomata and a rapid inhibition of photosynthetic CO₂ assimilation in response to drought. Furthermore, this cultivar also had the highest shoot to root ratio under

drought conditions. These observations suggest that A-5409RG would be better suited to areas that do not experience drought or where crops are grown under irrigation.

The ability to maintain high shoot biomass production under conditions of drought-induced nitrogen deficiency might be crucial to sustaining high yields under drought. Prima was able to maintain a higher biomass achieved under drought conditions that result in severe nitrogen deficiency because of impaired SNF. Prima was also able to maintain relatively high rates of photosynthesis under drought conditions and thus maximise carbon gain under conditions of nitrogen deficiency. This trait could contribute to better drought tolerance in soybeans and could be used as a phenotypic marker in breeding programs. While the mechanisms that facilitate rapid growth under limiting nitrogen remain to be elucidated, this trait may be more important in enabling the plants to overcome drought in the field, where nitrogen depletion inevitably accompanies water deprivation.

The characterization of accurate and cost-effective simple phenotypic markers is essential for future legume breeding programs, particularly in Africa. The use of such markers can shorten the selection process for superior plant material with enhanced drought tolerance traits. The present study confirms that in soybean traits related to above ground performance, such as photosynthesis, biomass and stomatal conductance, are directly related to parameters for nitrogen acquisition in the nodules. The ability to maintain vigorous shoot growth under drought-induced nitrogen limitation is an important trait that can be used to select for improved drought tolerance. This trait can be added to the list of potential targets that might be useful in boosting soybean yield gains under optimal as well as drought stress conditions. This list includes improved photosynthetic

efficiency, increased sink strength potential and allocation of C and N to developing pods (Ainsworth et al., 2011).