Characterization of drought tolerance traits in nodulated soybeans: The importance of maintaining photosynthesis and shoot biomass under drought-induced limitations on nitrogen metabolism

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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 trifoliate leaf stage under water-replete conditions. When water was withheld at the third trifoliate 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.

KEYWORDS: photosynthesis; symbiotic nitrogen fixation; drought tolerance; carbon-nitrogen interactions; water use efficiency

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 (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 avoided 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 overlap 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 (Vos et al. 2005; Ding 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 N_2-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 et al. 2003; Chaves and Oliveira, 2004; 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 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 a lso 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. 1995; Serraj et al. 1999).

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 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. Genotypic variation in drought sensitivity of shoots and below-ground (roots and root nodules) organs was investigated in three soybean lines: 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).

Materials and methods

Plant material and growth conditions

Seeds of commercial soybean (*Glycine max* L. Merr.) cultivars Prima 2000, A5409RG, and Jackson 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 [17.5 cm x 20 cm diameter (top) 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/m^3. 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 twice per week.

All experiments were performed in controlled environment chambers, with a 13 h photoperiod at a light intensity of 600 mmol m^{-2} s^{-1} (with supplemental light for 3 h provided by metal-halide lamps) and a day/night temperature of 25^0C / 17^0C and 60 % relative humidity. Eighty soybean plants in total per experiment were grown to the third trifoliate 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.

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 trifoliate leaves between 4 and 6 h after the start of the photoperiod using an infra-red gas analysis (CIRAS-1, PP Systems Hitchin Herts, UK). Measurements were made at 25^0C and an irradiance of 700 µmol photons m^{-2}s^{-1} and a CO_2 concentration of 350 ± 10 µmol 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 CO_2 assimilation rates and stomatal conductance values as described previously (Soares-Cordeiro et al. 2009).

Leaf water potential

The leaf water potential values were determined 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 (Valenzuela-Vazquez et al. 1997).

Water content of vermiculite

Vermiculite core (11 cm) 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 weight 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 $80^{o}C$ for 24 h. Vermiculite water contents (SWC) were calculated as the difference between the first and second measurements as: SWC (%) = [fresh weight-dry weight/dry weight] x 100.

Biomass

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 $80^{o}C$ for 48 h.

Nitrogenase activity measurement

Nitrogenase activity was determined in the crown and lateral root nodules using the acetylene reduction assay essentially as described by Turner and Gibson (1980).

Statistical analysis

CO_2 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 (ANOVA) 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 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.

RESULTS

Photosynthesis, stomatal conductance and water use efficiencies

Under water-replete conditions plants of the three cultivars had similar rates of photosynthesis (11.1±0.47 µmol m^{-2} s^{-1} for Prima 2000, 12.1±0.47 for A-5409RG and 10.3±0.42 for Jackson (Figure 1). Photosynthesis (CO_2 assimilation) rates decreased as a result of the imposition of drought in all three cultivars (Figure 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 1B; about day 4) than in Jackson (at day 9) or Prima (day 11; Figures 1A and 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 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 (P< 0.05) after which this parameter decreased by about 50% in bothA-5409RG and Jackson but not in Prima by day 18 (Figure 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 1A).

Under water-replete conditions Prima had the highest stomatal conductance values (Figure 3A) and Jackson the lowest (Figure 3C) values. Stomatal conductance decreased in all the cultivars when the plants were deprived of water (Figures 3). A further analysis

of the of the responses of photosynthetic parameters to drought revealed a significant cultivar-drought interaction with respect to photosynthetic CO_2 assimilation rates, Fv/Fm ratios, and stomatal conductance but not in relation to the intracellular CO_2 concentration within the leaves (Table 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). 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). 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).

The leaf water potential values were similar in all cultivars under water-replete conditions (Figure 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 4B).

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 5A) and Jackson had a lower, but not significantly ($P >$

0.05), root biomass value than the other two cultivars under these conditions (Figure 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 5A). A similar trend was observed for root biomass (Figure 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 weight basis on day 18 of the experiment under water-replete conditions (Figure 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 5A). After 18 d of drought, Prima had the greatest shoot and root biomass (Figure 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 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 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 5C). The shoot/root ratios measured on a fresh weight (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 weight (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 5C). A-5409RG had

the highest and Prima the lowest shoot/root ratios expressed either on fresh weight or dry weight basis after 18 d of drought (Figure 5C).

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 3). 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 3). 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 6A; $R^2 = 0.67$), stomatal conductance (Figure 6B; $R^2 = 0.79$) and leaf intracellular CO_2 concentrations (Figure 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 7A; $R^2 = 0.68$) and leaf water potential (Figure 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.

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 plants under drought stress. The findings of the present study, in which genetic variation in drought sensitivity was characterized in three nodulated soybean varieties, allow us to draw the following conclusion:

1. Considerable genetic variation 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 consistent with the extensive literature on this subject (Cornic 2000; Flexas et al. 2004; Lawlor 2002). Genetic 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). While the present study revealed some genotypic variation in the responses of stomatal conductance to drought treatments, 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 (Table 2). These data show that the ability to maintain high leaf water use efficiencies is a useful trait for soybean breeding programs selecting for improved drought tolerance.

The stability of photosynthesis under conditions of water deprivation is also considered be 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. 2006a; 2006b; Warren 2008; Lawlor and Tezara 2009), the maximal rate of

carboxylation of ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBiSCO), was shown to correlate well with photosynthetic capacity in soybean (Gilbert et al. 2011). Considerable genotypic variation was observed in the responses of different photosynthetic parameters to short and long term drought (Figures 3 and 4). The data presented in Figure 1 demonstrate that Jackson was able to maintain high rates of photosynthetic CO_2 assimilation over a long period (up to 14 days) of drought exposure (Figure 1). In the first stages of water deprivation, Jackson maintained high stomatal conductance values and photosynthesis (Figure 3). These features are consistent with the classification of Jackson as a drought-tolerant genotype (Chen et al. 2007; Sall and Sinclair 1991).

Photorespiration is considerably increased upon stomatal closure in C_3 plants (Noctor et al. 2002). 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 values (Foyer et al. 2009). While photosynthetic CO_2 assimilation rates declined following the onset of drought stress, the Fv/Fm data suggest that photorespiratory carbon flow was able to maintain photosynthetic electron transport rates and protect the photochemical reactions, in all but the most severe drought conditions. Prima leaves had significantly higher Fv/Fm ratios than either A-5409RG or Jackson at the end of the period of water deprivation, demonstrating that Prima is able to protect the photosynthetic electron transport processes in the leaves more effectively under drought.

2) Symbiotic nitrogen assimilation shows a strong correlation with photosynthesis

SNF is decreases in soybean nodules by stress-induced inhibition of nitrogenase activity (King and Purcell 2001; 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 (Figure 5). 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 in was equally inhibited by long term (day 18 of the experiment) drought in all genotypes.

3) Significant genotypic variation 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 (Table 3). Furthermore, nodule numbers and biomass were in general equally

affected by drought in all cultivars (Table 3). 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. The data presented show that there is 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).

4) The genotypes are ranked as Prima>Jackson> A-5409RG in terms of drought tolerance

Based on the findings reported here, we classify Prima as 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, we consider that genotypes like Prima are suitable for cultivation in areas with a longer growing season but suffering drought during the growth period. Shoot biomass accumulation in beams shows a strong correlation with seed yield under both well-watered and drought conditions (Shenkut and Brick 2003). Shoot biomass is therefore likely to indicative of seed yields and thus could be an important trait in the

selection of soybean germplasm for drought tolerance. Prima exhibits highest biomass might also therefore be predicted to give the highest seed yields under drought conditions, as we have observed field experiments (authors own unpublished data).

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 (Sall and Sinclair, 1991; 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 CO_2 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 our 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 CO_2 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.

5) The ability to maintain high shoot biomass production under conditions of drought-induced nitrogen deficient 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 underpinning 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).

References

Ainsworth E.A., C.R. Yendrek, J.A. Skoneczka and S.P. Long, 2011: Accelerating yield potential in soybean: Potential targets for biotechnological improvement. Plant Cell, Environ. DOI: 10.1111/j.1365-3040.2011.02378.x

Anjum S.A., Wang L., Farooq M., Khan I., Xue L, 2011: Methyl jasmonate-induced alteration in lipid peroxidation, antioxidative defence system and yield in soybean under drought. J. Agrom. Crop Sci. 197, 296-301.

Araus J.L., C. Sánchez, and G.O. Edmeades, 2011: Phenotyping maize for adaptation to drought. Monneveux P. and Ribaut J.M. (eds), Drought phenotyping in crops: from theory to practice. Generation Challenge Programme, pp. 259-282.

Boyer J.S., 1982: Plant productivity and environment. Science 218, 443-448.

Chaves M.M., J.P. Maroco, and J.S. Pereira, 2003: Understanding plant responses to drought – from genes to the whole plant. Functional Plant Biol. 30, 239-264.

Chaves M.M. and M.M. Oliveira, 2004: Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J. Exp. Bot. 55, 2365-2384.

Chen P., C.H. Sneller, L.C. Purcell, T.R. Sinclair, C.A. King, and T. Ishibashi, 2007: Registration of soybean germplasm Lines R01-416F and R01-581F for improved yield and nitrogen fixation under drought stress. J. Plant Regulation 1, 166-167.

Coque M., A. Martin, J.B. Veyrieras, B. Hirel, and A. Gallais, 2008: Genetic variation for N-remobilization and postsilking N-uptake in a set of maize recombinant inbred lines. 3. QTL detection and coincidences. Theor. Appl. Genetics 117, 729-747.

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

Ding L., K.J. Wang, G.M. Jiang, D.K. Biswas, H. Xu, L.F. Li, and Y.H. Li, 2005: Effects of nitrogen deficiency on photosynthetic traits of maize hybrids released in different years. Annals Bot. 96, 925-930.

Dorlodot S., B. Forster, L. Pages, A. Price, R. Tuberosa, and X. Draye, 2007: Root system architecture: opportunities and constraints for genetic improvement of crops. Trends Plant Sci. 12, 474-481.

Fernandez-Luquen F., L. Dendooven, A. Munive, L. Corlay-Chee, L.M. Serrano-Covarrubias, and D. Espinosa-Victoria, 2008: Micro-morphology of common bean (Phaseolus vulgaris L.) nodules undergoing senescence. Acta Physiol. Plant. 30, 545–552.

Flexas J., J. Bota, F. Loreto, G. Cornic, and T.D. Sharkey, 2004: Diffusive and metabolic limitations to photosynthesis under drought and salinity in C_3 plants. Plant Biol. 5, 1-11.

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

Flexas J., M. Ribas-Carbó, J. Bota, J. Galmés, M. Henkle, S. Martínez-Cañellas, and H. Medrano, 2006b: Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. New Phytol. 172, 73-82.

Foyer C.H., A. Bloom, G. Queval, and G. Noctor, 2009: Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. Ann. Rev. Plant Biol. 60, 455-484.

Garnett T., V. Conn, and B.N. Kaiser, 2009: Root based approaches to improving nitrogen use efficiency in plants. Plant Cell Environ. 32, 1272-1283.

Ghannoum O., 2009: C4 photosynthesis and water stress. Annals Bot. 103, 635-644.

Gilbert M.E., M.A. Zwieniecki, and N.M. Holbrook, 2011: Independent variation in photosyntheitc capacity and stomatal conductance leads to differences in intrinsic water use efficiency in 11 soybean genotypes before and during mild drought. J. Exp.Bot. doi:10.1093/jxb/erq461.

Grzesiak S., W. Filek, S. Pienkowski, and B. Nizio, 1996: Screening for drought resistance: evaluation of drought susceptibility index of legume plants under natural growth conditions. J. Agron. Crop Sci. 177, 237-244.

Herridge D.F., M.B. Peoples, and R.M. Boddey, 2008: Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311, 1-18.

Hunt S., B.J. King, and D.B. Layzell, 1989: Effects of gradual increases in 0_2 concentration on nodule activity in soybean. Plant Physiol. 91, 315-321.

Jury W.A., and H.J. Vaux Jr, 2007: The emerging global water crisis: managing scarcity and conflict between water users. Adv. Agron. 95, 1-76.

King C.A., and L.C. Purcell, 2001: Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Sci. 41, 1099-1107.

King C.A., and L.C. Purcell, 2005: Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. Plant Physiol. 137, 1389-1396.

Lawlor D.W., 2002: Limitations to photosynthesis in water-stressed leaves: stomata *vs*. metabolism and the role of ATP. Annals Bot. 89, 871-885.

Lawlor D.W., and W Tezara, 2009: Causes of decreased photosynthetic rate and metabolic capacity in water-deficient leaf cells: a critical evaluation of mechanisms and integration of processes. Annals Bot. 103, 561-579.

Lopes M.S., J.L. Araus, P.D.R. Van Heerden, and C.H. Foyer, 2011: Enhancing drought tolerance in C_4 crops. J. Exp. Bot. doi:10.1093/jxb/err105.

Manavalan, L.P., K.G. Satish, P.T. Lam-Son, and H.T. Nguyen, 2009. Physiological and molecular approaches to improve drought resistance in soybean. Plant Cell Physiol. 50, 1260–1276.

Matamoros M.A., L.M. Baird, P.R. Escuredo, D.A. Dalton, F.R. Minchin, I. Iturbe-Ormaetxe, M.C. Rubio, J.F. Moran, A.J. Gordon, and M. Becana, 1999: Stress-induced legume root nodule senescence. Physiological, biochemical, and structural alterations. Plant Physiol. 121, 97–111.

Minchin F.R., 1997: Regulation of oxygen diffusion in legume nodules. Soil Biol. Biochem. 29, 881-888.

Noctor G., S. Veljovic-Jovanovic, S. Driscoll, L. Novitskaya, and C.H. Foyer, 2002: Drought and oxidative load in wheat leaves: a predominant role for photorespiration? Annals Bot. 89, 841-850.

Pate J.S., G.A. Atkins, D.B. Layzell, and B.J. Shelp, 1984: Effects of N_2 deficiency on transport and partitioning of C and N in a nodulated legume. Plant Physiol. 76, 59–64.

Purcell L.C., and T.R. Sinclair, 1994: An osmotic hypothesis for the regulation of oxygen permeability in soybean nodules. Plant Cell Environ. 17, 837-843.

Sall K., and T.R. Sinclair, 1991: Soybean genotypic differences in sensitivity of symbiotic nitrogen fixation to soil dehydration. Plant and Soil 133, 31-37.

Serraj R., P. Fleurat-Lessard, B. Jaillard, and J.J. Drevon, 1995: Structural changes in the innercortex cells of soybean root nodules are induced by short-term exposure to high salt or oxygen concentrations. Plant Cell Environ. 18, 455-462.

Serraj R., S. Bona, L.C. Purcell, and T.R. Sinclair, 1997: Nitrogen accumulation and nodule activity of field-grown 'Jackson' soybean in response to water deficits. Field Crops Res. 52, 109-116.

Serraj R., T. Sinclair, and L. Purcell, 1999: Symbiotic N_2 fixation response to drought. J. Exp. Bot. 50, 143-155.

Shenkut A.A., and M.A. Brick M.A., 2003: Traits associated with dry edible bean (*Phaseolus vulgaris* L.) productivity under diverse soil moisture environments. Euphytica 133, 339–347.

Silsbury J.H., 1977: Energy requirement for symbiotic N_2 fixation. Nature 267, 149–150.

Sincik M., B.N. Candogan, C. Demirtas, H. Buyukcangaz, S. Yazgan, and T. Goksoy, 2008: Deficit irrigation of soya bean (Glyine max (L) Merr.) in a sub-humid climate. J. Agron. Crop Sci. 194, 200-205.

Sinclair T.R., L.C. Purcell, C.A. King, C.H. Sneller, P. Chen, and V. Vadez, 2007: Drought tolerance and yield increase of soybean resulting from improved symbiotic N_2 fixation. Field Crops Res. 101, 68-71.

Soares-Cordeiro A.S., S.P. Driscoll, T.K. Pellny, E. Olmos, M.C. Arrabaça, and C.H. Foyer, 2009: Variations in the dorso-ventral organization of leaf structure and Kranz anatomy coordinate the control of photosynthesis and associated signalling at the whole leaf level in monocotyledonous species. Plant Cell Environ. 32, 1833-1844.

Steele K.A., D.S. Virk, R. Kumar, S.C. Prasad, and J.R. Witcombe, 2007: Field evaluation of upland rice lines selected for QTLs controlling root traits. Field Crops Res. 101, 180-186.

Tschoep H., 2010: The response of growth and primary metabolism to a mild but sustained nitrogen limitation in *Arabidopsis thaliana*. PhD dissertation. Mathematisch-Naturwissenschaftliche Fakultät, University of Potsdam, Germany.

Turner L., and H. Gibson, 1980: Measurement of nitrogen fixation by indirect means. In Methods for Evaluating Biological Nitrogen Fixation. (ed. F.J. Bergersen), pp. 111-138. Wiley, Chichester.

Vance C.P., K.L.M. Boylan, C.A. Maxwell., G.H. Heichel, and L.L. Hardman, 1985: Transport and partitioning of CO_2 fixed by root nodules of ureide and amide producing legumes. Plant Physiol. 78, 774–778.

Van Heerden P.D.R., M. De Beer, D.J. Mellet, H.S. Maphike, and W. Foit, 2007: Growth media effects on shoot physiology, nodule numbers and symbiotic nitrogen fixation in soybean. South African J. Bot. 73, 600-605.

Van Heerden, P.D.R., G. Kiddle, T.K. Pellny, P.W. Mokwala, U. Schlüter, K. Kunert, and C.H. Foyer, 2008: Roles for the regulation of respiration and the oxygen diffusion barrier in soybean in the protection of symbiotic nitrogen fixation from chilling –induced inhibition and shoots from premature senescence. Plant Physiol. 148, 316-327.

Valenzuela-Vazquez M., A. Escobedo-Mendoza, J.L. Almanza-Sandoval, and. A. Ríos-Torres, 1997: Pressure bomb. Final report: New Mexico State University.

Voisin A.S., C. Salon, C. Jeudy, and F.R. Warembourg, 2003: Root and nodule growth in *Pisum sativum* L. in relation to photosynthesis, analysis using 13C-labeling. Annals Bot. 92, 557–563.

Vos J., P.E.L. Putten, and C.J. Birch, 2005: Effect of nitrogen supply on leaf appearance, leaf growth, leaf nitrogen economy and photosynthetic capacity in maize (Zea mays L.). Field Crops Res. 93**,** 64-73.

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

Wani S.P., O.P. Rupela, and K.K. Lee, 1995: Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes. Plant and Soil 174, 29-49.

Table 1. Analysis of variance (ANOVA) comparisons of the effects of drought on photosynthetic CO_2 assimilation (A), stomatal conductance (G), Fv/Fm ratios and intracellular CO_2 concentrations (Ci) in the soybean cultivars: Prima 2000, A-5409RG and Jackson. Data for days 0, 7, 14 and 18 of the experiment were used in this analysis.

Variation	d.f.	Means square			
		A	G	Fv/Fm	Ci
Cultivar (C)	2	20.09	6.74	0.070	15272.4
		*	**	**	**
Drought (D)	1	1065.98	68.12	0.091	376790.8
		**	**	**	**
C*D	2	63.98	4.95	0.0156	1878.6
		*	**	*	ns
Experimental error	117	5.88	0.244	0.002	1536.4
Total d.f.	122				

* $P < 0.05$, and ** $P < 0.01$
d.f. degrees of freedom

Table 2. 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 ±SEM of four independent replicates.

| Cultivar | Instantaneous water use efficiencies (IWUE) $(\mu mol\ CO_2\ per\ mol\ H_2O)$ | | | |
| | 8 days | | 18 days | |
	Water-replete	Drought	Water-replete	Drought
Prima 2000	49.8±3.1b	186.4±9.2a	73.7±13.7a	156.0±14.0a
A-5409RG	56.5±3.4b	150.7±8.9b	63.2±0.9ab	59.6±13.1b
Jackson	72.4±2.9a	171.1±10.2ab	57.6±l.6b	73.9±4.7b
Significance	**	*	*	**

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

Table 3. Comparison of nodule numbers, nodule biomass (fresh weight) and symbiotic nitrogen fixation (SNF) in three soybean cultivars at 8 d and 18 d under either water-replete or drought conditions. Each data point is the mean ±SEM of four independent replicates.

Cultivar	Number		Biomass (g)		SNF (µmol/h/plant)	
	8 d	18 d	8 d	18 d	8 d	18 d
Well watered						
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
Significance	**	**	***	*	**	**
Drought						
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
Significance	**	*ns*	**	*ns*	**	*ns*

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

Figure 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.
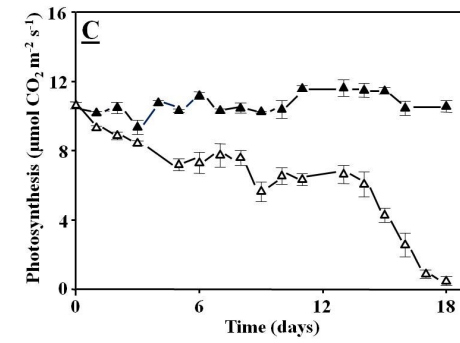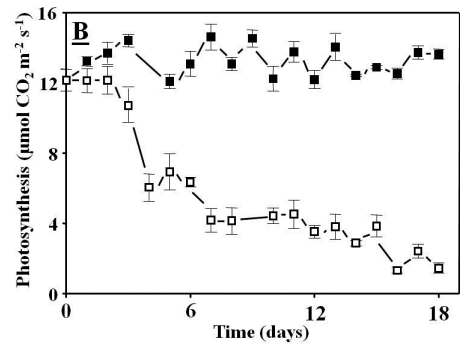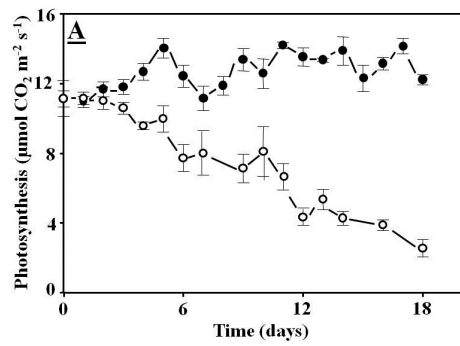
Figure 2. Effects of water deprivation on Fv/Fm ratios of leaves of Prima 2000, A-5409RG and Jackson. Plants were grown under drought conditions over 18 d. Circles: Prima 2000, squares A-5409RG; and triangles: Jackson. Each data point is the mean ± SE from 4 individual plants grown under drought conditions. 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%.
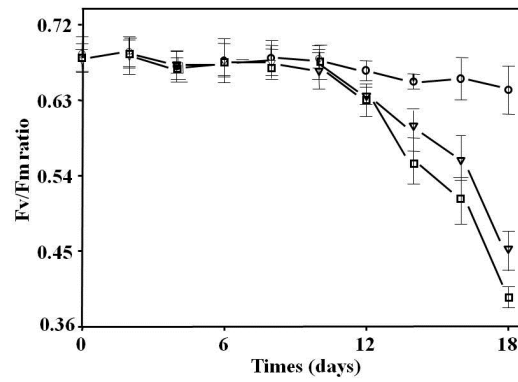
Figure 3. Comparison of stomatal conductance values in Prima 2000 (A), A-5409RG (B) and Jackson (C) leaves. Plants were grown under water-replete (closed symbols) and drought conditions (open symbols) for 18 d. Each data point is the mean ± SE from 4 individual plants.
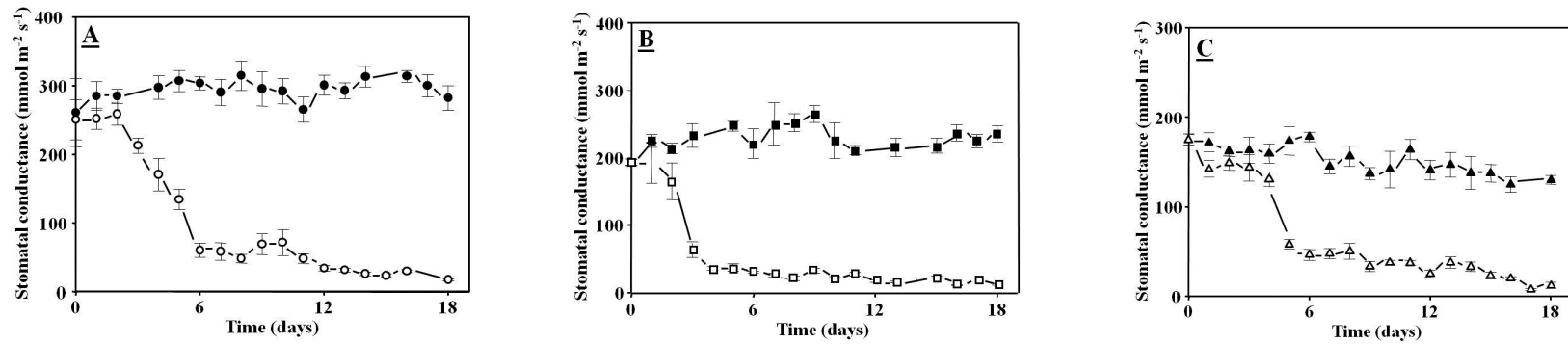
Figure 4. Effects of water deprivation on leaf water potential (MPa) values (A) in Prima 2000, A-5409RG and Jackson and vermiculite (Soil) water content (B). Plants were grown under drought conditions (open symbols) over 18 d. Circles: Prima 2000, squares A-5409RG and triangles: Jackson. Control values (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 ± SE from 4 individual plants grown under drought conditions.
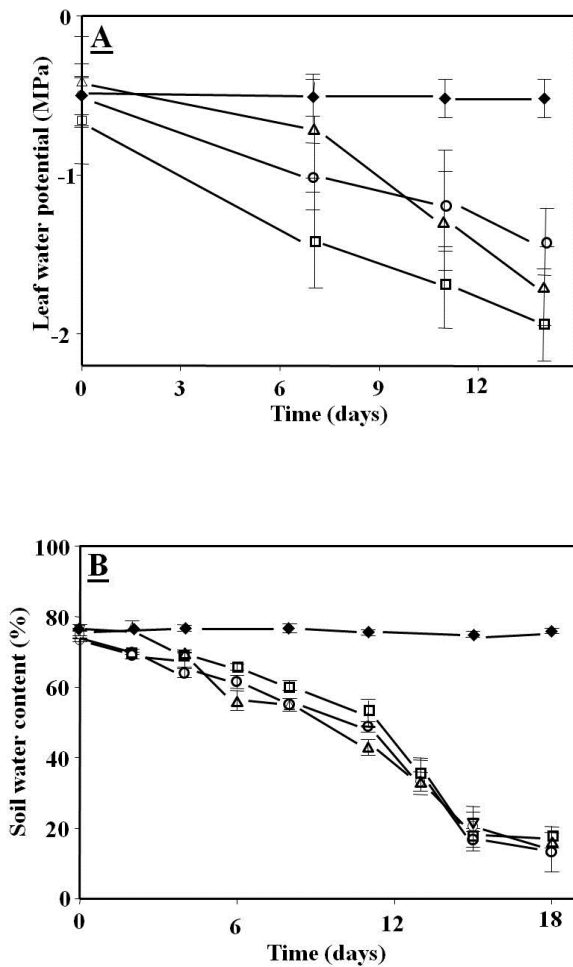
Figure 5. Effects of drought on shoot and root biomass and on shoot/root ratio. Shoot biomass (dry weight; A), root biomass (dry weight; B) and shoot/root ratio (fresh weight and dry weight; C) were compared in Prima 2000 (P), A-5409RG (A) and Jackson (J) under water-replete conditions at day 1 (1WW) and day 18 (18WW) and following drought treatment at day 18 (18D). Shoot/root ratios are expressed either on a fresh weight (FW) or dry weight (DW) basis after 18 d exposure to either water-replete (closed columns) or drought conditions (open columns). Each data point is the mean ± SE from 4 individual plants. Different letters denote significant differences
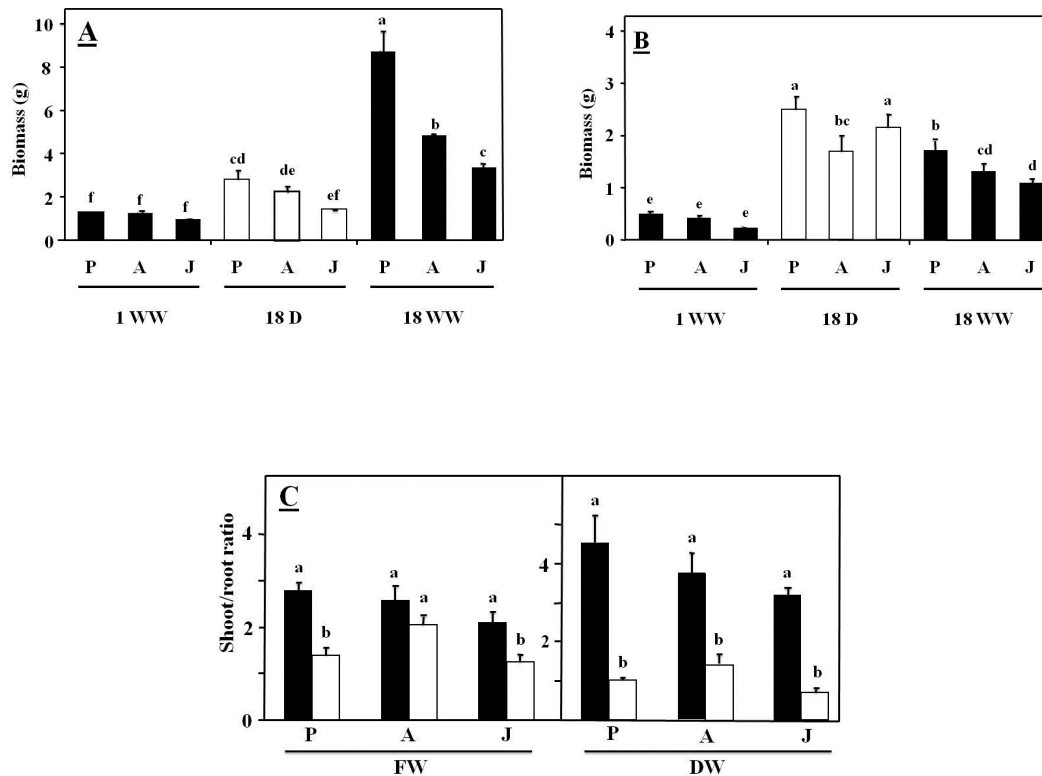
Figure 6. Relationships between SNF and CO_2 assimilation (A), stomatal conductance (B) and leaf intracellular CO_2 concentration (C) in Prima 2000 (circles), A-5409RG (squares), and Jackson (triangles) under well-watered (closed symbols) and drought conditions (open symbols). Each data point is the mean from 4 individual plants.
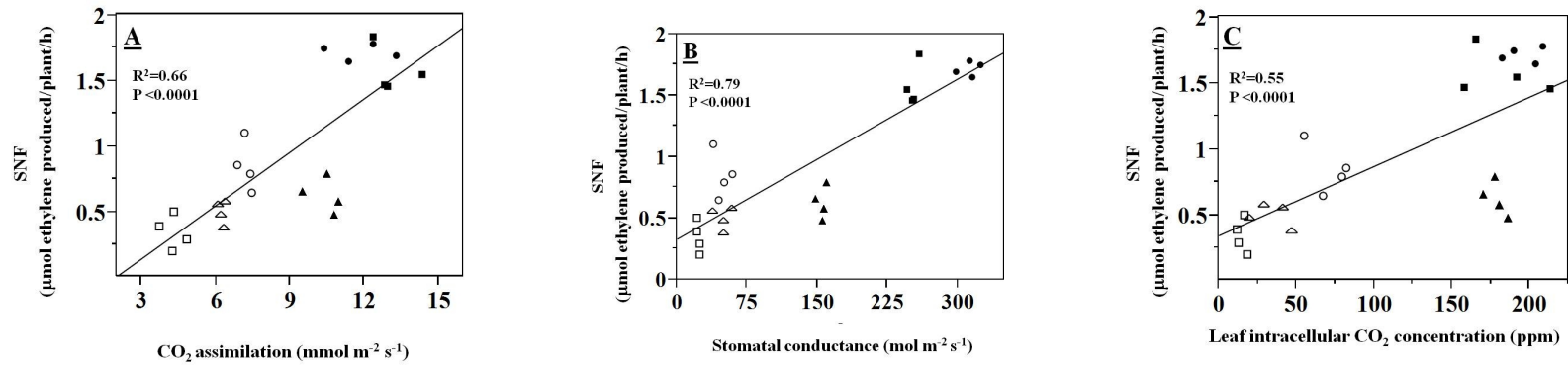
Figure 7. Relationships between SNF and soil (vermiculite) water content (A) and leaf water potential (B) in Prima 2000 (circles), A-5409RG (squares), and Jackson (triangles). Data was obtained from plants grown under drought conditions above 30% soil water content or leaf water potentials higher than -1.7. Each data point is the mean from 4 individual plants.