CHAPTER SIX

ADAPTATION OF SOYBEAN CULTIVARS TO DROUGHT STRESS UNDER FIELD CONDITIONS: ROLE OF ROOT ARCHITECTURE, BIOMASS PARTITIONING, WATER USE EFFICIENCY AND SYMBIOTIC NITROGEN FIXATION

6.1 Abstract

Field experiments were conducted using three soybean cultivars (Jackson, A5409RG, Prima 2000) which were also used in a previous greenhouse study under well-watered and drought stress condition. The main objective was to evaluate root and shoot traits under drought stress to determine the physiological basis of differences in growth and seed yield. Drought stress was induced at one month after establishment and measurements were made at three growth stages (flowering, mid-pod filling and at harvest). Measurements, included root architectural and morphological traits, biomass partitioning, leaf chlorophyll content, water use efficiency (WUE) and symbiotic nitrogen fixation (SNF). WUE and SNF were measured as carbon isotope discrimination and ^{15}N natural abundance ($\delta^{15}N$), respectively. Prima 2000 and Jackson performed better under drought when root morphology and architectural traits, CID and $\delta^{15}N$ were used as performance parameters. Jackson as an early maturing cultivar was superior to other two cultivars in partitioning a greater proportion of biomass to seed under drought stress. However, higher values of total dry mass as well as seed yield under drought were observed with the longer maturing Prima 2000 when compared to the other two soybean cultivars. Among the three cultivars, longer maturing and transgenic A5409RG was least adapted to drought stress. There was also a significant correlation of CID (WUE) with $\delta^{15}N$ (SNF) as well as root traits and seed yield under drought. Results also indicate that lower values of CID under drought could contribute to higher CO_2 assimilation resulting in better N_2 fixation. Use of root architectural traits, such as diameter and branching density, and morphological traits, such as root length, surface area and volume, might be useful not only to evaluate genotypic differences in response to drought but also to improve genetic adaptation of soybean to drought stress.

6.2 Introduction

Genetic variability exists in the soybean germplasm for root architectural traits, such as root angle (Zhao et al., 2004) root diameter and rooting depth (Ao et al., 2010), and morphology traits such as root length (Zhao et al., 2004), root volume, area, and length (Ao et al., 2010). Further, it was also shown that the differential performance of soybean cultivars was related to root length, density and dry mass that contributes to improved water absorption under drought (Garay and Wilhelm, 1982). The ability of the plant to extract greater amounts of available soil water under drought conditions through the deep root architecture has been shown to contribute to improved growth and seed yield (Garay and Wilhelm, 1982; Zhao et al., 2004). However, the methodology to evaluate root architecture under field conditions needs to be improved and its contribution to performance of soybean needs to be defined. Therefore, more detailed information is needed on root architecture and morphology traits.

Since measurement of water use efficiency (WUE) using carbon isotope discrimination provides information about the longer-term plant performance associated with CO_2 fixation, CID can be used as a surrogate for WUE. Previous results indicated that carbon is incorporated into the plant tissue, transported and metabolized for a substantial time during the entire growth period of the plant (Evans et al., 1986; Johnson et al., 1990; Shaheen and Hood-Nowotny, 2005). Therefore, the CID technique has been used in several crops for performance evaluation under abiotic stress to demonstrate a possible relation between photosynthetic assimilation and water utilization (Farquhar et al., 1989). Stresses investigated include drought and salinity in wheat (Shaheen and Hood-Nowotny, 2005) and waterlogging and salinity in clover and puccinellia (Mostajeran and

Rengel, 2007). Although little information is available on the use of CID in soybean improvements, the potential of CID as a performance measurement for WUE in soybean has been suggested by Kumarasinghe et al. (1992) and White et al. (1996). Ultimately, using CID for performance evaluation in the field might help to better understand the response of soybean cultivars to drought under field conditions. For field-grown legumes, measurement of ^{15}N natural abundance is one of the widely used methods to assess N fixation ability. The principle behind this technique is that the concentration of ^{15}N in biologically fixed N is lower than that of N from other sources and is based on small difference in ^{15}N concentration (Shearer and Kohl, 1986). The importance of the use of ^{15}N abundance for measurement of SNF performance in soybean field experiments has been proposed (George T. et al., 1996; Kumarasinghe et al., 1992). Using non-fixing legumes or cereals (weeds) for studying natural abundance of nitrogen in legumes is a common procedure and has been used in soybean and clover with ryegrass and marigold (Kohi et al., 1980) as well as in pea with barley (Holdensen et al., 2007) and when ten different annual legumes were compared and wheat was used as a control in field studies (Unkovich and Pate, 2000).

The hypothesis of this study was, differential variation in root architecture and morphology traits, including shoot and productivity markers for soybean cultivar grown under field exists and these genetic differences can help for performance evaluation under water-limited condition. Further, it has been also hypothesized that similar in common bean water use efficiency as measured by carbon isotope discrimination as well as symbiotic nitrogen fixation determined by ^{15}N natural abundance is associated with productivity as well as root traits to use as selection marker under drought studies. Moreover, this study was also aimed to verify if performance of soybean cultivars for drought stress under filed condition will be comparable as the output found under control condition and plant traits used for performance evaluation would be the similar with common bean.

6.3 Materials and methods

6.3.1 Plant material and Experimental procedure

Experiments were conducted during the 2010 cropping season (February to May) at the hosting institute of Ukulima Root Biology Center (URBC), operated by Natural Conservation Thrust, Limpopo Province, South Africa ($24^0$32.002'S, $28^0$07.427'E and 1237m above sea level). Other experimental area description as indicated at chapter four. Soybean (*Glycine max* L. Merr.) cultivars which exhibit different background were used in this study: A5409RG, a glyphosate resistant transgenic cultivar, Prima 2000, a commercial cultivar grown in South Africa, and Jackson, considered to be a drought-tolerant cultivar (Chen et al., 2007; Sall and Sinclair, 1991). Since a non-fixing soybean cultivar was not available as a reference/control for isotope studies, two non-fixing common bean lines (DOR 364-NN and BAT 477-NN) were used as controls. The two lines were grown in the same field using an identical experimental design.

Before the commencement of the experiment, a soil analysis for both macro and micro-nutrients was conducted by Alpha Agric PLC soil analysis laboratory, Nylstroom, South Africa. Based on the results, 4 kg/ha boron, 1 kg/ha zinc sulfate and 25 kg/ha potassium sulfate were applied. Before land preparation 3L/ha of Roundup, a systemic, broad-spectrum herbicide were applied to kill all the weeds on the field. Before planting the pre-emergence herbicides Unimoc EC

(800ml/ha) and Imazethaphyr (400ml/ha) were also sprayed to control both grasses and broadleaf weeds. After planting, hand-weeding was performed as needed. The nematicide oxadate (3L/ha) was applied to prevent nematode infestation for up to a month after planting.

6.3.3 Planting and experimental layout

The experiment was conducted in a randomized complete block design with two water regimes, with well watered and water-limited. Each cultivar was planted in five rows with the spacing of 75 cm between rows and 10 cm between plants. Row length was 4 m and plot size was 12 m^2. The central three rows were used for data collection, while the two outer rows served as borders. One seed per hole was planted at 5 cm depth using a Jab planter which is specially designed to plant with uniform depth.

Before the initiation of drought stress, all the treatments were grown with adequate water supply by applying 8 mm water/day using pivot sprinkler irrigation. Drought stress was started 30 days after planting by turning off the sprinkler nozzles. Moisture stress was applied for 28 days, although rain fell on the 7^{th}, 19^{th} and 26^{th} days after planting with a total of 34 mm after which data collection started.

6.3.4 Measured parameters

6.3.4.1 Chlorophyll content

Three plants per plot of each variety (nine plants per water regime treatment) were sampled at the beginning and at the end of the drought stress treatment using the central leaflet with same age of the 3rd and 4th trifoliate leaf. Chlorophyll content of leaves was measured non-destructively using the Chlorophyll Meter SPAD-502 (Konica Minolta Sensing, Inc., Japan) and chlorophyll content of three individual SPAD chlorophyll meter readings (SCMR) per plot were averaged.

6.3.4.2 Root architecture

Root architectural measurements were made for the two main root types (Figure 6.1) of soybeans after one month of drought exposure. Three plants per plot and per replication (nine plants per cultivar) for each water regimes were sampled using the "Shovelomics"(Lynch, 2011; Trachsel et al., 2011) technique. Subsequently, tap and lateral root thickness (diameter) was measured by multiple measurements 2 cm away from the origin of these roots or attachment using an electronic digital caliper 5HA 1890 Model (Omni- Tech). The branching density of tap and lateral root was determined by counting the lateral root/root hairs emerging within 2 cm root segment of the tap root and for three randomly selected lateral roots.

6.3.4.3 Root morphology analysis

After one month of exposure of plants to drought, three soil cores per plot were taken in each water regime for analysis of the root morphology. The steel corer lined with a plastic tube (60 cm length and 42 mm diameter) (Giddings Machine Company Inc, USA) was driven into the soil in between two plants. Upon extracting the core, roots were washed out of the soil, scanned with a root scanner (Epson Perfection V 700 Photo /V 750 Pro (Seiko Epson Corporation 2005) and the root images were analyzed using the winRHIZO 2008a program (Regent Instruments Canada Inc., Canada) to determine root morphology.

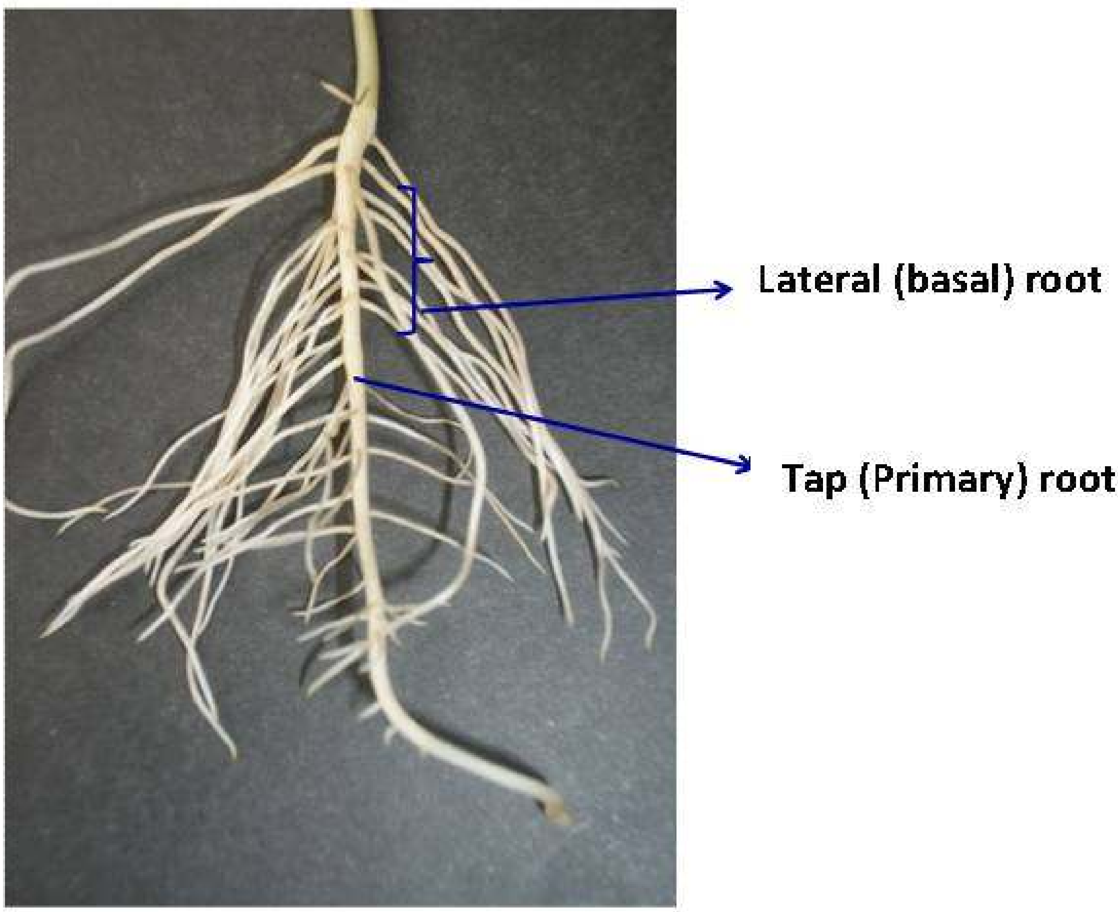

Figure 6.1 Schematic representation of soybean root system architecture.

6.3.4.4 *Biomass partitioning and seed yield measurement*

At flowering and mid-pod filling stage six representative individual plants per plot for each water regime were harvested and plant parts were separated into leaves, stems and pods (at mid-pod filling stage). Dry mass of plant parts was determined after oven drying at 60^0C for 48 h (TERM-O-MAT LABOTEC, South Africa). For determining seed yield of each plot, two rows of 3 m length (2.25 m^2 area) were used, after discarding a border of 0.5 m on both extremes of the rows. Yield per plot and per hectare was computed. Furthermore, all plants from one row (3 m length) were counted and harvested independently and then the pod wall and seed were separated carefully by splitting by hand. Samples were dried in an oven at 60^0C for 48 h and the dry mass was determined. Data were used to calculate the pod harvest index (PHI): (seed mass)/ (seed mass + pod wall mass).

6.3.4.5 *Nodule size, carbon isotope discrimination and ^{15}N natural abundance*

Root nodule size was determined by placing the root nodules on a board with a sketch of the diameter (mm) of nodules. For analysis of CID and ^{15}N natural abundance, the plants samples previously used dry mass determination of above ground parts (both leaf and stem) were ground to fine powder using a grinder (A 11 basic Analytical Mill, IKA® Works, Inc, Germany). The three plants per plot were bulked and ground to make up one replicate, and three replications were used. The samples were analyzed using Isotope ratio Mass Spectrometer (Thermo electron, Bremen, Germany) at Cape Town University, Department Archaeology. Carbon isotope discrimination was calculated from δ^{13}C of each plant sample (Farquhar et al., 1989).

Natural abundance of $\delta^{15}N$ was calculated using the formula previously reported (Shearer and Kohl, 1986; Unkovich et al., 1994; Valles-De La Mora et al., 2003). Furthermore, percentage of the nitrogen fixed by the legumes from the atmosphere was calculated as described before (Bergersen and Turner, 1983; Shearer and Kohl, 1986) (Peoples et al., 2009). The equations for calculation for CID, natural abundance of $\delta^{15}N$ and other SNF parameters is as described in chapter five at section 5.3.2.2.

6.3.5 Statistical analysis

Data were analyzed using JMP® 9.0 statistical package (SAS Institute Inc., Cary, NC, USA). Analysis of variance was used to determine the significance level and treatment comparison via LSmeans Student's t-test was used to evaluate the cultivars for the measured traits. Multivariate Pearson's correlation analysis was used for determining the relationship (correlation) between measured traits.

6.4 **Results**

6.4.1 Chlorophyll content

SPAD chlorophyll meter readings (SCMR) of all cultivars were similar at the start of the experiment and for well-watered treatment after three weeks of drought stress measurement were (40-42 SCMR). Relative to the non-stress treatments, drought stress significantly reduced the chlorophyll content for cultivar A5409RG (10%), however, the reduction for other two cultivars was low (data not shown). Accordingly, under drought stress, Prima 2000 had significantly higher ($P<0.05$) chlorophyll content than A5409RG (Figure 6.2).

6.4.2 Root architecture and morphology

Significant interactions were revealed for water treatment and cultivar for two ways ANOVA analysis for root morphology traits (Appendix 9). However, none of root architectural traits were shown significant difference for water level vs. cultivar interaction (data not shown). For examining the complete story the main effect of root performance traits further evaluated for one way analysis of variance and treatment comparison. Accordingly, under well-watered conditions, all the root morphology traits (root length, area, volume, number of root tips and average diameter) were not significantly different between tested cultivars (Table 6.1). On the other hand, under drought conditions marked significant differences ($P<0.05$) were observed among the cultivars for the root morphology parameters except average root diameter. Drought significantly enhanced parameters of root morphology by 57, 36, 27, and 59% in root length, area, volume and number of root tips, respectively compared to well-watered condition. Prima 2000 and Jackson had significantly enhanced ($P<0.05$) root elongation, surface area and volume, relative to the A5409RG and these two cultivars had higher root length (up to 60%) and a 2 to 3-fold higher root total surface area and root volume than A5409RG which actually displayed a decrease in all root parameters (Table 6.1).

Regardless of the water regime, Jackson exhibited significantly higher ($P<0.05$) tap root diameter than the other two cultivars (Table 6.2). However, lateral root diameter (thickness) was significantly higher ($P<0.05$) for cultivar A5409RG under well-watered and drought conditions compared to Jackson and Prima (Table 6.2). Although moisture stress enhanced branching density of both tap and lateral root in all three cultivars, branching density differed among cultivars in the drought treatment. Relative to well watered treatment drought stress increased tap root branching by 29% 53%, and 57% for A5409RG, Jackson, and Prima 2000 respectively. Likewise, lateral root branching raise by 42%, 76%, 67% for A5409RG, Jackson, and Prima 2000 respectively (data not shown). These result and the output at Figures 6.3 and 6.4 shows, both under well-watered and drought conditions, Jackson and Prima 2000 had a higher tap and lateral root branching density when compared to A5409RG. Compared to previous computer simulation of root systems in soybean (Zhao et al., 2004) (Figure 6.5). A5409RG, Jackson and Prima 2000 exhibited shallow, deep and intermediate root architecture, respectively.

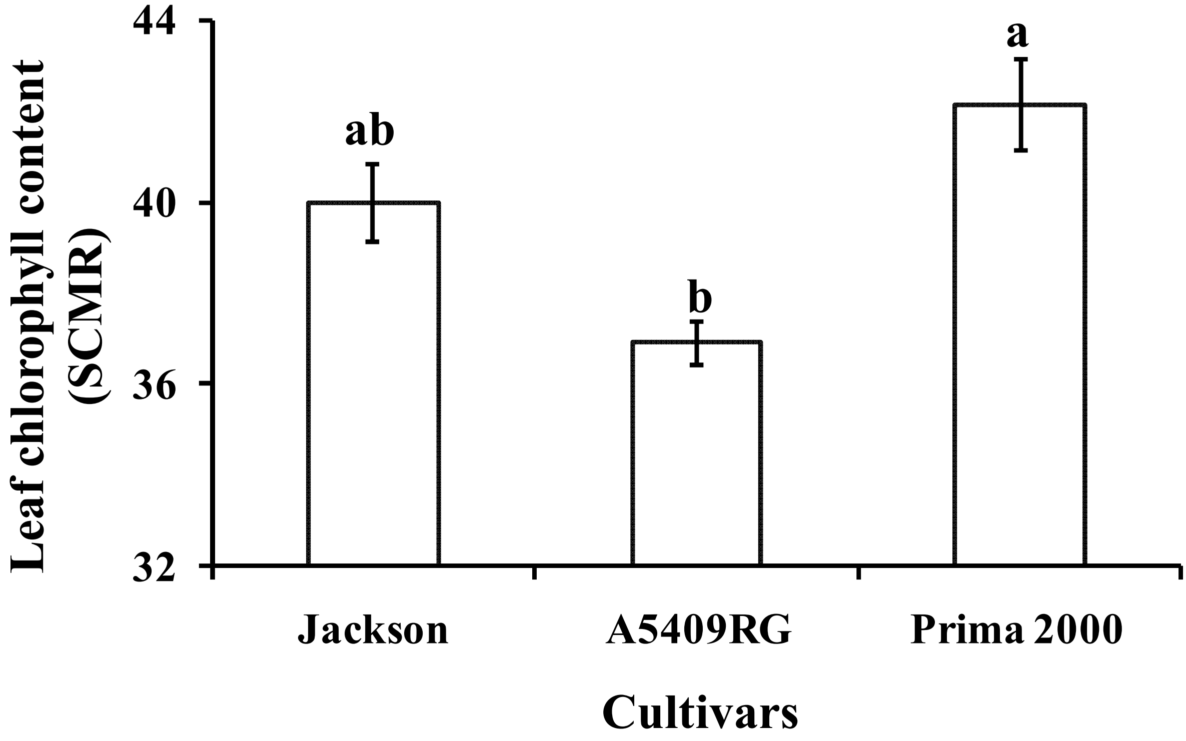

Figure 6.2 Leaf chlorophyll content (SCMR) of three soybean lines measured after three weeks of drought stress (drought block). Results are means ± SEM of three plants per plot and three repetitions. Means with the same letter above the bars are not significantly different as tested by LSmeans Student's t-test (P=0.05).

Table 6.1 Performance of root morphology traits of three soybean cultivars under well-watered and drought growth conditions. The root image was taken by a root scanner and analysis was made by using the winRHIZO 2008a software after 28 days of drought.

Cultivars	**Root length (cm)**	**Surface area (cm^2)**	**Root volume (cm^3)**	**Root tip number**	**Average diameter (mm)**
Well-watered					
A-5409RG	56.61±10.08	8.34±1.27	0.10±0.016	180.61±21.42	0.49±0.032
Jackson	56.91±11.72	8.05±1.48	0.09±0.018	190.50±24.89	0.46±0.037
Prima 2000	51.55±11.39	6.50±1.43	0.07±0.017	168.75±24.19	0.43±0.036
Significance	ns	ns	ns	Ns	ns
Drought					
A-5409RG	41.01±0.38b	4.74±2.31b	0.05± 0.03b	177.79±58.09b	0.40±0.068
Jackson	98.56±0.39a	11.05±1.79a	0.12±0.02a	301.44±44.99ab	0.48±0.052
Prima 2000	120.46±0.39a	15.45±1.83a	0.16±0.02a	377.79±45.92a	0.54±0.054
Significance	**	**	**	*	ns

Significance level was determined using ANOVA (**P<0.001, *P<0.05, and ns P>0.05) and difference between treatment means was determined using the LSmeans Student's t-test. Means followed by the same letter within the column are not significantly different. The result is the mean ± SEM of three replicates for each treatment using soil cores up to 60 cm soil depth.

Table 6.2 The performance of three soybean cultivars using mean separation for root architecture traits of tap and lateral root diameter (thickness) under well-watered and drought condition

Cultivars	Tap root diameter (mm)		Lateral root diameter (mm)	
	Well- watered	**Drought**	**Well- watered**	**Drought**
A-5409RG	2.53±0.15c	2.35±0.40c	4.14±0.33a	4.21±0.12a
Jackson	4.48±0.46a	4.34±0.25a	2.52±0.17b	2.15±1.13b
Prima 2000	3.78±0.12b	3. 63±0.51b	2.46±0.13b	2.23±0.16b
Significance	**	**	**	**

Significance level was determined using ANOVA ($^{**}P<0.001$) and difference between treatment means was determined using the LSmeans Student's t-test. Means followed by the same letter within the column are not significantly different. The result is the mean ± SEM of six representative plants per plot exposed to 28 days of drought.

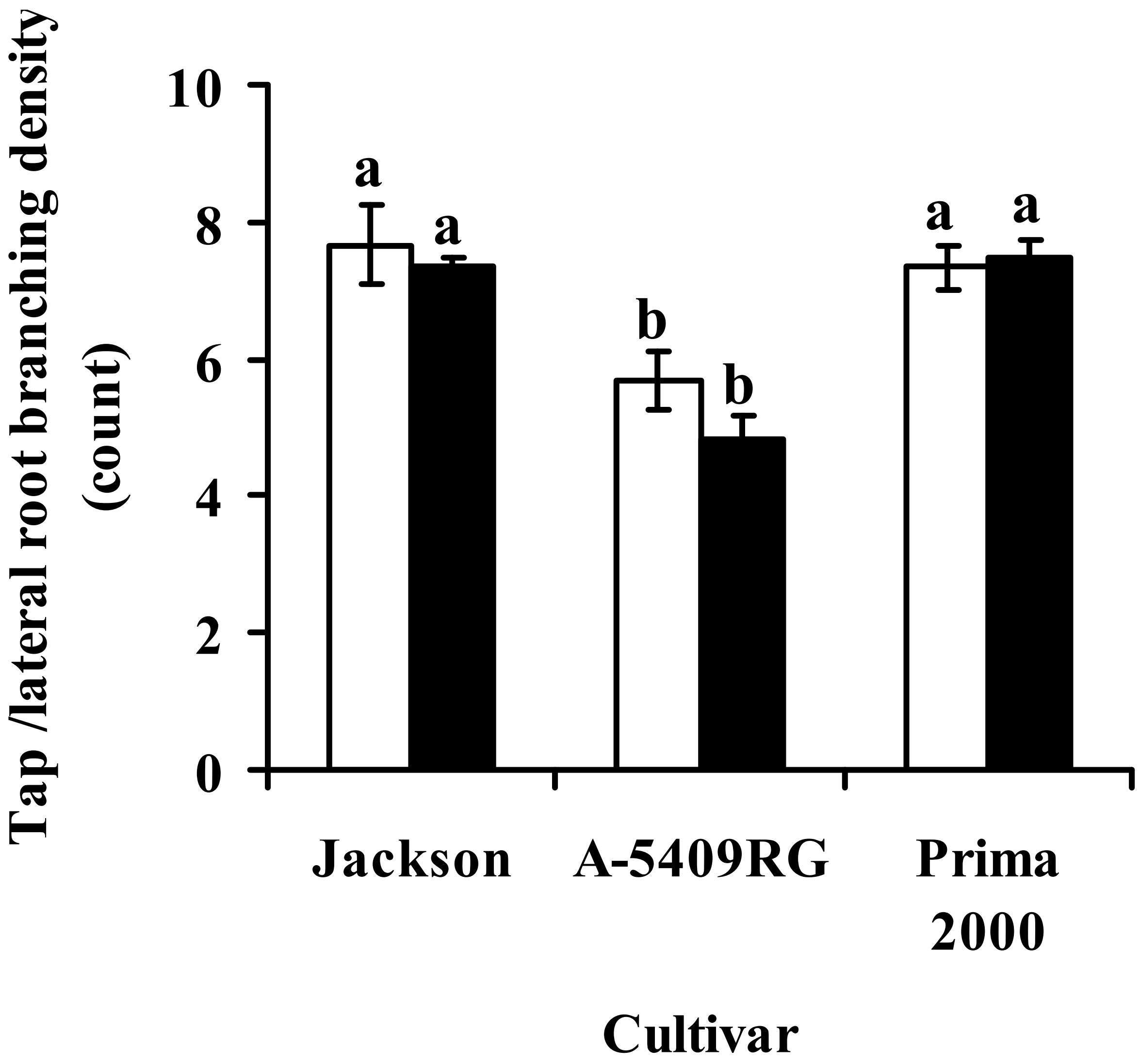

Figure 6.3 The performance of three soybean cultivars for root architecture traits tap root branching (open bars) and lateral root branching (closed bars). Values shown are means ± SEM of three repetitions of three individual plants per plot for each cultivar (nine plants) after one month of drought exposure. The significance letters obtained by the analysis using mean separation LSmeans Student's t-test (P = 0.05).

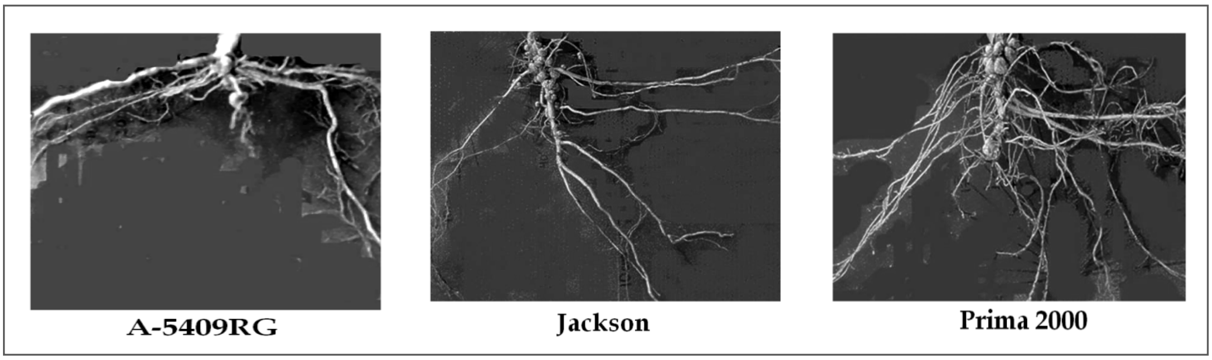

Figure 6.4 A comparison of root phenotypes in three soybean cultivars grown under drought. Photos were taken one month after exposure to drought in the field.

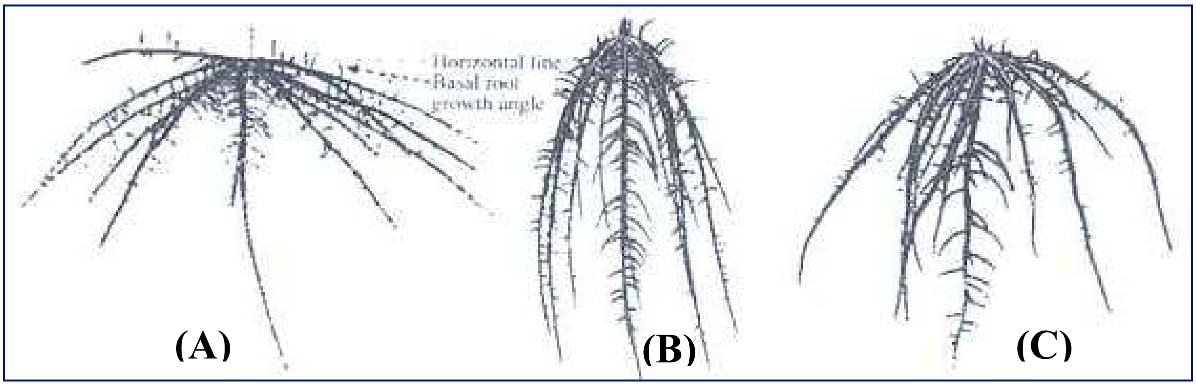

Figure 6.5 Computer simulation of three representative root images representing shallow (A), deep (B) and intermediate (C) roots of soybean obtained in field experiment (Zhao et al., 2004).

6.4.3 Plant biomass, phenology and seed yield

Two way analysis of variance for plant biomass as measured at flowering reveal significant different for cultivar and water level interaction only for leaf dry mass. However, all plant biomasses measured at mid pod filling stage (leaf, pod and total) except stem biomass and seed yield revealed significant difference for cultivar X water level interaction (Appendix 10). For further observation and comparison of the tested cultivars under study, treatment comparison as well was one way ANOVA was conducted. As a result, under drought and well-watered growth conditions, the three cultivars had significantly different ($P<0.05$) leaf, stem and total biomass both at the flowering and mid pod filling stage (including pod mass) (Table 6.3). Under both water regimes, Prima 2000 had significantly higher ($P<0.05$) dry mass (leaf, stem, pod) than the two other cultivars. Drought caused a significant reduction of total biomass at flowering by 21%, 25% and 40% for Prima, Jackson and A5409RG, respectively.

Jackson was the earliest maturing cultivar maturing at 81 days under drought and 90 days under well-watered growth condition. The other two cultivars exhibited similar maturity date of 98-101 days under drought and 115-118 days under well-watered conditions (data not shown).

Under well-watered condition, while the three soybean cultivars revealed non-significant difference for pod harvest index (PHI) (Figure 6.6). Prima 2000 and A5409RG were had higher seed yield (4 to 4.4 t/ha) than Jackson (2.1 t/ha) (Table 6.3). However under drought stress, significant cultivars difference were existed for PHI with Prima 2000 and Jackson exhibited higher Pod harvest index than A5409RG (Figure 6.6), nevertheless, Prima 2000 were had a significantly higher (P<0.05) seed yield (2.4 t/ha) than the two other cultivars, Jackson (1.7 tones/ha) and A5409RG (1.9 t/ha) (Table 6.3). Further, when seed yield accumulation per day was calculated under well-watered condition,, Jackson revealed lower but under drought condition both Jackson (21 kg/ha/day) and Prima 2000 (24 kg/ha/day) exhibited higher seed yield accumulation per day than A5409RG (19 kg/ha/day) (Table 6.4). Leaf biomass accumulation per day between flowering and mid-pod filling stage under drought condition were higher for Jackson and Prima 2000, nevertheless, under well-watered condition the three cultivars exhibited similar performance. However, regardless of the water regime, stem biomass accumulation per day was similar for the tested cultivars (Table 6.4).

Table 6.3 Performance of three soybean cultivars for biomass at flowering (Fl) and mid pod filling stage (MPF) and seed yield under well-watered and drought conditions.

Cultivar	Leaf dry mass at Fl(g)	Stem dry mass at Fl(g)	Total biomass Fl (g)	Leaf dry mass at MPF(g)	Stem dry mass at MPF(g)	Pod dry mass MPF(g)	Total biomass MPF(g)	Seed yield (kg/ha)
Well-watered								
Jackson	5.88±0.13b	5.08±0.27b	10.97±0.37c	22.86±0.32b	25.1317±0.32b	10.40±0.29b	58.39±0.63b	2076.07±117.0b
A5409RG	6.59±0.30ab	5.78±0.24ab	12.37±0.22b	28.66±0.45a	28.9917±0.44a	13.29±0.48a	70.94±0.66a	4060.38±83.51a
Prima 2000	7.27±0.23a	6.43±0.16a	13.71±0.32a	29.86±1.22a	28.2050±1.25a	13.87±0.40a	71.93±1.68a	4425.12±123.6a
Significance	**	**	**	**	**	**	**	**
Drought								
Jackson	4.05±0.93b	4.21±0.17b	8.25±0.18b	21.69±0.22b	21.16±0.43b	9.38±0.13b	52.23±0.46b	1709.63±18.28b
A5409RG	3.77±0.13b	3.65±0.07b	7.42±0.15b	23.15±0.49b	24.40±0.51ab	7.85±0.38c	55.40±0.96b	1868.21±28.23b
Prima 2000	5.96±0.26a	4.88±0.20a	10.84±0.34a	26.47±0.63a	26.22±2.13a	11.20±0.35a	63.82±2.57a	2365.04±35.08a
Significance	**	**	**	**	*	**	**	**

Data represent the mean ± SEM of three replications under both well watered and drought conditions. Biomass at flowering and mid pod filling stage was taken on six representative individual plants per plot. Different letter within a column denote a significant difference (P<0.05).

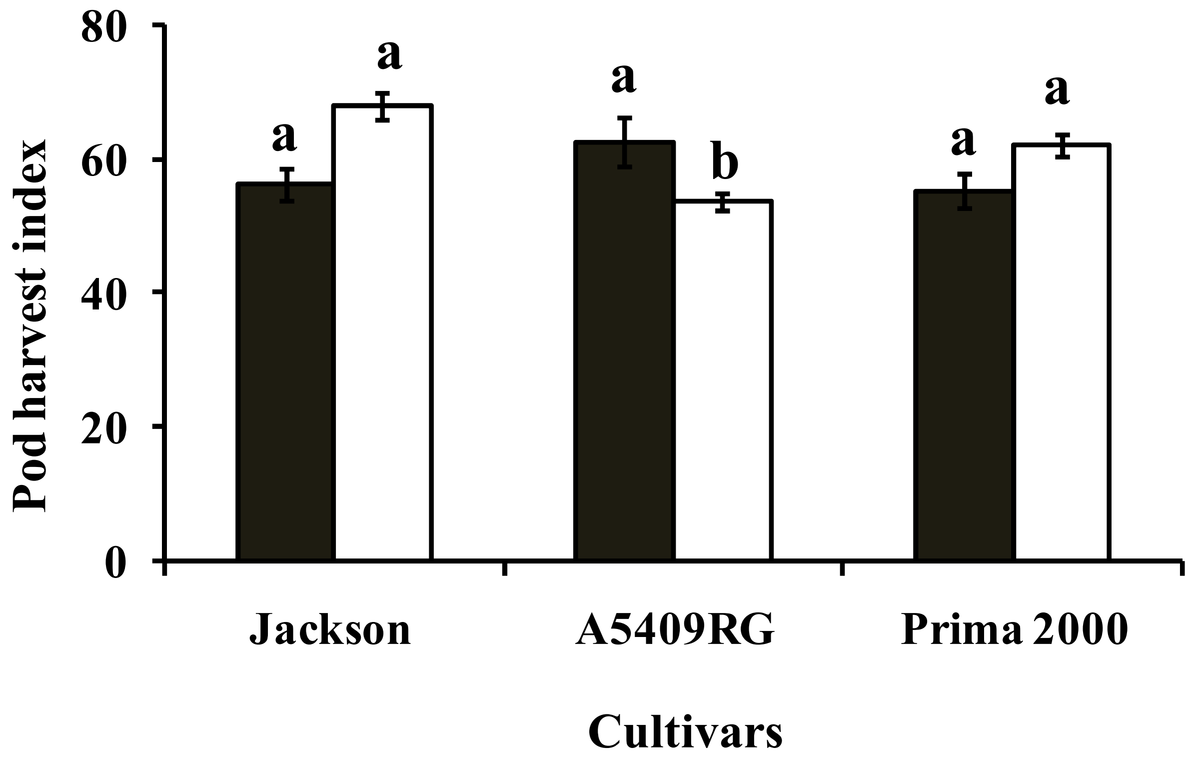

Figure 6.6 Pod harvest index of three soybean cultivar measured at harvest, closed bars indicates for well-watered and open bars for drought stressed treatments. Results are means ± SEM of three repetitions per cultivars. Means with the same letter above the bars are not significantly different as tested by LSmeans Student's t-test (P=0.05).

Table 6.4 Seed yield and biomass accumulation of three soybean cultivars per day. This has been done by dividing seed yield with maturity day, biomass accumulation per day for leaves and stems between days of flowering (Fl) and mid-pod filling (MPF) of the cultivars, by dividing the difference of biomasses with the difference of the days between flowering and maturity.

Cultivars	Yield (kg/ha)/day	Biomass accumulation per day between Fl and MPF stage	
		Leaf (g/day)	Stem (g/day)
Well-watered			
Jackson	25.63	0.33	0.39
A5409RG	40.81	0.32	0.33
Prima 2000	44.47	0.33	0.31
Drought			
Jackson	21.11	0.35	0.33
A5409RG	18.78	0.28	0.30
Prima 2000	23.77	0.30	0.31

6.4.4 Nodule size, carbon isotope discrimination /^{15}N natural abundance

The nodule size of the three soybean cultivars was not significantly different under non-stress conditions (data not shown). However, under drought nodule size differed significantly between cultivars and Jackson and Prima 2000 had larger nodules (4.6 mm) than A-5409RG (3.7 mm) (Figure 6.7).

Analysis for two ways ANOVA for CID determined for both shoot and seed was not significant for the interaction of water treatment and cultivar. As a result for assessing the cultivar performance one way analysis of variance and treatment comparison was conducted. Thus, *c*arbon isotope discrimination under well-watered conditions was significantly different between the tested soybean cultivars and reference non-fixing common bean lines, however, within soybean cultivars there was no significant difference (Table 6.5). Nevertheless, under drought condition, CID was significantly different ($P<0.05$) among the tested soybean cultivars as well as with the reference lines. As a result among the tested soybean cultivars Prima 2000 expressed the lowest CID value followed by Jackson. Regardless of the water regime, the non-fixing reference bean lines had higher CID values than the soybean cultivars (Table 6.5). Nevertheless, seed CID of soybean cultivars was not significantly different under either water regime (data not shown).

Seed $\delta^{15}N$ was not significantly different among tested soybean cultivars under both well-watered and drought conditions (data not shown). However, the performance of the tested soybean cultivars and non-nodulating reference beans for $\delta^{15}N$ values determined for shoot revealed significant differences under both water regimes. There was no significant difference

(P>0.05) in $\delta^{15}N$ among soybeans under well-watered conditions, but under drought, Jackson and Prima 2000 had lower $\delta^{15}N$ values (-2.28‰ to-2.55‰) than A-5409RG (-1.19‰) or the reference bean lines (1.65‰ to 2.83‰) (Table 6.5). Further, a significant difference (P<0.05) was found between soybean cultivars and reference bean lines for shoot %N under both well-watered and drought conditions. Under well-watered condition, shoot %N ranged from (3.7-4.2%) for soybean and for the reference bean lines between 1.5-1.9%, although there was no significant difference (P>0.005) among the tested soybean cultivars. Under drought, Jackson and Prima 2000 had lower $\delta^{15}N$ values and the highest shoot %N (average 4%). This was significantly different (P<0.05) to A-5409RG (3%) and the reference bean lines (on average 1.6%) (Table 6.5).

The three soybean cultivars had comparable performance under well-watered conditions for percent of nitrogen derived from atmosphere (%Ndfa). However, under drought Prima 2000 and Jackson had significantly higher (P<0.05) (65-70%) %Ndfa than A5409RG (55%) (Table 6.6). Regardless of the water regime, the amount of nitrogen fixed per hectare was higher for Prima 2000 (146 kg/ha with well-watered and 106 kg/ha with drought conditions) than the other two cultivars tested, (117 kg/ha for Jackson and 100 kg/ha for A5409RG) with well-watered and (80 kg/ha, for Jackson and 55 kg/ha, for A5409RG) with drought conditions (Table 6.6)

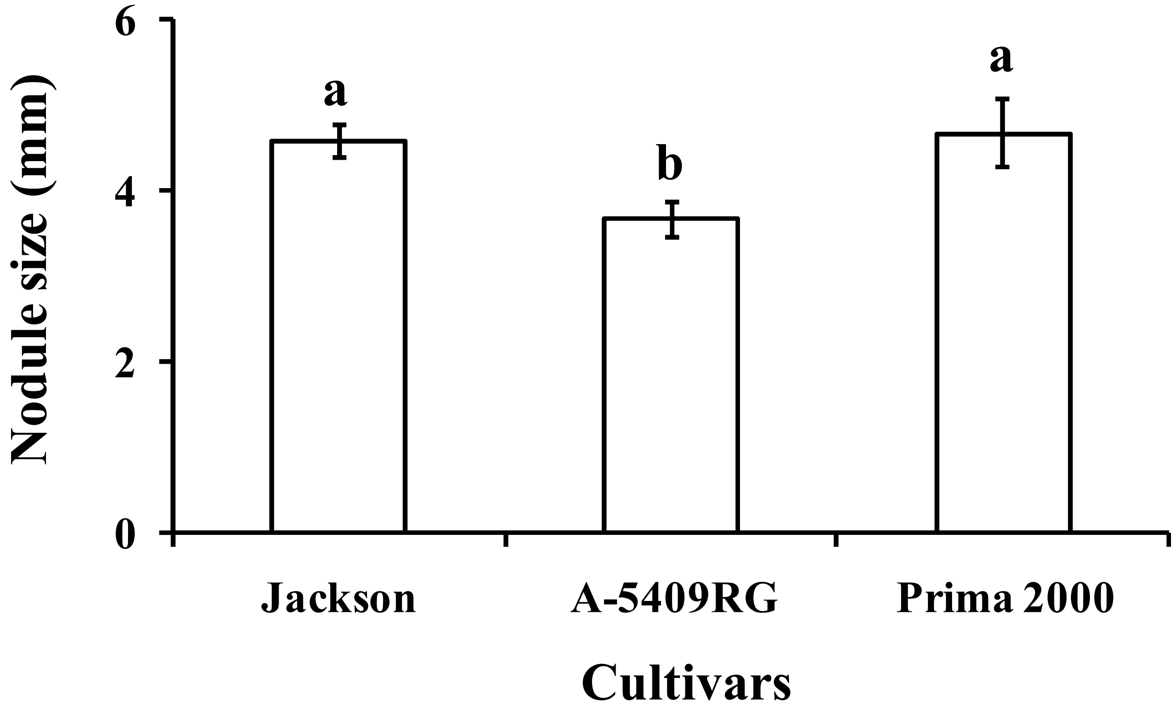

Figure 6.7 Nodules size of three soybean lines grown in the field at drought conditions. Data represent mean ± SEM of four replicates per plot (for twelve individual plants per cultivar) after exposure for one month to drought. Means followed by the same letter on the top of bars are not significantly different ($P<0.05$).

Table 6.5 Performance after 28 days drought stress of three soybean cultivars and two non-fixing reference common bean lines for the analysis of shoot (CID, $\delta^{15}N$ and %N). Result is the mean of three replicates (each replicate from a composite sample of three plant samples) for each soybean cultivar and reference bean line for every water regime.

Cultivars/ lines	**Shoot CID**		**Shoot $\delta^{15}N$**		**Shoot %N**	
	Well-watered	**Drought**	**Well-watered**	**Drought**	**Well-watered**	**Drought**
<u>*N-fixing (soybean)*</u>						
Jackson	21.53±0.33b	20.242 ±0.17bc	-2.727±0.42b	-2.280±0.64bc	3.82±0.35a	4.06±0.08a
A-5409RG	21.291±0.11b	20.854 ±0.02ab	-3.112±0.38b	-1.187±0.57b	3.74±0.19a	3.01±0.3b
Prima 2000	21.293±0.2b	19.513±0.41c	-3.322±0.09b	-2.546±0.29c	4.24±0.08a	3.97±0.1a
<u>*Non-fixing (bean)*</u>						
DOR 364-NN	22.223 ±0.01a	21.337±0.41a	0.066±1.14a	2.834±0.06a	1.78±0.22b	1.88±0.08c
BAT 477-NN	22.293±0.13a	21.677±0.09a	1.735±0.25a	1.650±0.04a	1.45±0.03b	1.34±0.06c
P-value	*0.0059*	*0.0016*	*0.0003*	*<0.0001*	*<0.0001*	*<0.0001*
Significance	**	**	**	**	**	**

Significance level was determined using ANOVA (** P<0.001, and *P<0.05) and difference between treatment means were determined using the LSmeans Student's t-test. Means followed by the same letter within the column are not significantly different.

Table 6.6 Percent nitrogen derived from the atmosphere (%Ndfa) and fixed N (g/plot) for shoot samples of three soybean cultivars using LSmeans Student's t-test mean separation (P=0.05). The result is the mean ± SEM of three replicates (each replicate from a composite sample of three plant samples) for each soybean cultivars and reference bean lines every water regime.

Cultivars	**%Ndfa**		**Fixed N (kg/ha)**	
	Well-watered	**Drought**	**Well-watered**	**Drought**
Jackson	85.113±0.34a	64.47±0.88ab	117.11±1.69b	80.39±6.2b
A-5409RG	81.133±0.21a	54.80±0.30b	100.13±1.86b	54.53±3.1c
Prima 2000	73.852±0.66a	69.66±0.50a	146.18±3.2a	105.6±4.9a
P-value	*0.4811*	*0.0371*	*0.016*	*<0.0001*
Significance	*ns*	*	*	**

Significance level was determined using ANOVA (** P<0.001 * P<0.05 and ns P>0.05) and difference between treatment mean was determined by the LSmeans Student's t-test. Means followed by the same letter within the column are not significantly different.

6.4.5 Association between carbon isotope discrimination and ^{15}N natural abundance traits with seed yield and root traits

Carbon isotope discrimination was highly significant (P<0.01) and positively correlated with $\delta^{15}N$ and also with SNF efficiency traits (shoot %N and %Ndfa) under both water regimes (Table 6.7). Further, there was a significant association of CID with seed yield under both well–watered (r = -0.63, P<0.05) and drought (r = -0.83, P<0.01) conditions (Table 6.7).

The relationship of shoot $\delta^{15}N$ was significantly related with %N under both well-watered (P<0.01, r = -0.94) and drought (P <0.05, r = -0.85) conditions. Further, both %N and %Ndfa had a significant and positive relationship with seed yield under well-watered and drought conditions. Accordingly, the association for %N was (r = 0.8, P<0.01) under both growth conditions and %Ndfa (r = 0.67, P<0.05) under well-watered and (r = 0.95, P<0.01) under drought conditions, respectively. Irrespective of the water regime, a highly significant (P<0.01) association was found between $\delta^{15}N$ and seed yield (r = -0.67, well-watered and r= -0.91, drought) (Table 6.7).

Only a weak correlation of $\delta^{15}N$ with both root architecture and morphology traits was found. Moreover, there was no significant association of CID with root traits under well-watered conditions (data not shown). However, under drought CID was significantly positively associated with lateral root diameter (r = 0.77) but significantly negatively correlated with tap root diameter (r = -0.52). Further, root morphology traits (root length, surface area and volume) had a significant (P<0.05) negative association with CID under drought (Table 6.8).

Table 6.7 Association between shoot CID, $\delta^{15}N$, and % N for (three soybean cultivars and two non-fixing reference common bean lines) of three soybean cultivars. Data are from pooled data from the 3 soybean cultivars of three replicates (each replicate from a composite sample of three plant samples) of plants grown under well-watered and drought conditions.

Traits	Shoot CID	Shoot $\delta^{15}N$	Shoot %N	%Ndfa
Well-watered				
Shoot %C	-0.690*			
Shoot $\delta^{15}N$	0.760**	1.000		
Shoot %N	-0.835**	-0.935**	1.000	
%Ndfa	-0.760*	-1.000**	0.935*	1.000
Seed yield	-0.631*	-0.666**	0.805**	0.666*
Drought				
Shoot %C	-0.521*			
Shoot $\delta^{15}N$	0.821**	1.000		
Shoot %N	-0.795**	-0.849**	1.000	
%Ndfa	-0.917**	-0.717*	0.417ns	1.000
Seed yield	-0.832**	-0.905**	0.838**	0.948**

Correlation is highly significant (** P<0.001), significant (*P<0.05) and non significant (ns P>0.05)

Table 6.8 Correlation between shoot carbon isotope discrimination and root morphology or architectural traits of three soybean cultivars. Data obtained from pooled data of 3 cultivars and from three replicates (each replicate from a composite sample of three plant samples) of plants grown under drought.

Trait	**Trait**	**Correlation coefficient (r)**	**P-value**
	Root architecture		
	Lateral diameter	0.691	0.0159*
	Lateral branching	-0.172	0.7759ns
Carbon isotope	Tap diameter	-0.536	0.0445*
discrimination	Tap branching	0.101	0.7963ns
	Root morphology		
	Length	-0.476	0.0457*
	Surface area	-0.607	0.0324*
	Volume	-0.712	0.0444*

*indicates correlation is significantly different ($P<0.05$), ns indicates correlation is non-significant ($P>0.05$).

6.5 Discussion

In this study drought had a significant effect on both root morphology (Table 6.1) and architectural parameters (Table 6.2, Figure 6.3 and 6.4). As roots are the primary sensor of water deficit, the ability of the plant to adapt to an altered soil environment depends on root developmental plasticity attributes (morphology and architecture) (Lynch et al., 2005). As a result, there was a significant cultivar difference in the response of root traits under drought in this study and the tested cultivars can be classified into three types. A-5409RG had a shallower root system with a less than 40^0 basal/lateral root angle (Figure 6.4) classified as type A (Figure 6.5) (Zhao et al., 2004). Further, plants of this cultivar had a short and slender tap root (Figure 6.4), thicker and few lateral (basal) roots, a lower branching density (Figures 6.3 and 6.4, Table 6.2), smaller root length, surface area and volume (Table 6.1). In contrast, Jackson had a deeper root system with a basal/lateral root angle greater than 60^0, (Figure 6.4) classified as type B (Figure 6.5) (Zhao et al., 2004). This cultivar had a long and thicker tap root system, many basal roots (Figure 6.4 and Table 6.2) and therefore a high branching density (Figure 6.3) as well as a higher root length, surface area and volume (Table 6.1). Cultivar Prima 2000 had an intermediate root system with a basal root angle between $40\text{-}60^0$ (Figure 6.4), classified as type C (Figure 6.5) (Zhao et al., 2004), with a long and deep tap root, numerous basal roots and consequently higher branching density (Figures 6.3 and 6.4), and also higher root length, surface area and volume (Table 6.1).

The two cultivars, Jackson and Prima 2000, were further able to extend their root system to adapt to drought (Tables 6.1 and 6.2, Figures 6.3 and 6.4). However under drought condition, Prima had a significantly higher yield (2.4 t/ha) than Jackson (1.7 t/ha). But estimates of yield per day and leaf biomass accumulation per day between flowering and mid pod filling stage under drought indicates that Jackson and Prima 2000 had greater ability to accumulate photosynthates and the higher values of pod harvest index indicate that these two cultivars also have greater ability to mobilize photosynthates to seed compared to A5409RG. Hence, these shows, plant traits that can help to evaluate the proportion of photosynthates that are partitioned to seed yield might contribute to the selection of more efficient genotypes. Cultivars Prima 2000 and Jackson showed better performance under drought suggesting that biomass accumulation and partitioning towards the developing seeds are key physiological factors in the adaptation to drought. Therefore, although the cultivar with a longer maturation produces more yield than with a short maturation, the rate of daily seed yield accumulation (rate of partitioning) is indispensable for comparing adaptation under drought. Accordingly, these two cultivars could serve as parents for future improvement of seed yield under drought stress. Nevertheless, the low yield of Jackson relative to Prima 2000 was due to the duration to attain maturity for Jackson which was short to convert assimilated carbohydrate and absorbed nutrients to grain yield.

The amount of plant biomass and grain yield depends on the amount of photosynthetically active radiation (PAR) interception by the plant canopy and partitioning of the photosynthetic product to harvestable form (Mayers et al., 1991). This has a direct relationship with the duration of the crop growth (days to attain maturity and relative duration of pod filling). These observations might suggest that productivity under long-term drought is related to phonology in soybean (Lawn and James, 2011). In general, Prima 2000 had a better root architecture (tap and lateral root branching) and morphology traits (root length, surface area, and volume) as well as plant shoot biomass under both water regimes. It appears that there is a good balance between root and shoot growth in this cultivar. Jackson, regardless of the water regime, had shorter shoots and a deeper root system which might contribute to water saving mechanisms during drought stress. Results obtained from this study are in agreement with the observations made by Zhao et al. (2004) from a soybean core collection study that highlighted the need to match root and shoot architectures for efficiently converting assimilated carbon as well as absorbed nutrients and water to harvestable grain yield and for improving drought-tolerance in soybean.

In this study, soybean cultivars Jackson and Prima 2000 with better root performance had also low shoot CID values. Condon et al. (1990); Farquhar and Richards (1984) previously reported that CID is negatively associated with water use efficiency under drought. Lower CID has been considered as a marker for better water use efficiency (Farquhar et al., 1989). Lower CID could be either due to lower stomatal conductance which has been found in rice (Dingkuhn et al., 1989) or greater CO_2 assimilation found in common bean (Ehleringer, 1990) and winter wheat (Morgan et al., 1993). The better performing soybean cultivar Prima had an enhanced root system (root morphology and architecture) under drought in comparison to the susceptible cultivar (A5409RG). This would allow better opening of the stomata and improved CO_2 assimilation. One important factor affecting the guard cell turgor is the leaf water status. Plants with a deep and dense root system (enhanced root system flexibility) under drought helps for better water extraction from the soil allowing capturing more water keeping both shoot turgidity and opening of stomata. As a result, the plant will assimilate more CO_2.

The relatively higher amount of chlorophyll found for Prima 2000 also supports the view that this cultivar is less affected by drought. These characteristics were also found for Prima 2000 in the phytotron studies with better CO_2 assimilation, stomatal conductance and WUE (Fenta et al., 2011). More importantly, the negative association of CID with root morphology traits (length, area, and volume) (Table 6.8) as well as with seed yield under drought (Table 6.7) indicates the importance of maintaining the plant water status through utilization of available water for continuous CO_2 assimilation under water-limited growth conditions. Therefore, these observations suggests, this study support the view that low CID (high WUE) could be due to enhanced photosynthetic assimilation per unit water transpired (transpiration efficiency).

Carbon isotope discrimination was also negatively associated with SNF efficiency (shoot %N and %Ndfa) and positively associated with $\delta^{15}N$, which is a desirable association, as the value of $\delta^{15}N$ is negative. This further ascertains the close intimacy of carbon and nitrogen fixation. Previous findings in beans have also indicated that nitrogen accessibility to the plant increases concurrently with increased WUE (Caemmerer and Farquhar 1984). Farquhar and Richards (1984) have also highlighted the importance of better nitrogen availability for enhanced CO_2 assimilation. In this study, Prima 2000 had better root traits, biomass and productivity as well as higher SNF ability (146 kg/ha, under drought) (Table 6.6) and shoot %N (Table 6.5) in comparison to the other cultivars tested that performed less for the measured traits. This shows that accessibility of nitrogen (absorption from the soil and N fixation from the air) is highly affected by water availability.

Since water is absorbed by the plant mostly through mass flow, which is also highly affected by contact of the root surface area to the soil, the response of the root system under drought is fundamental for drought tolerance and productivity under drought. The water supply to nodules is via the phylum (Walsh et al., 1989). Since drought affects the volumetric flow in the phylum, the relative water content of nodules decreases (Purcell and Sinclair, 1995) reducing the nodule size. Maintaining the water supply of the phylum improves the water status of nodules and facilitates the solute flow from the nodule (Purcell and Sinclair, 1995). This ultimately helps for improved SNF of the plant. Therefore, effect of drought stress on nodule size would be one of the important traits which should be considered during drought screening.

According to multivariate analysis of variance for cultivar and water treatment soybean cultivars exhibited consistent performance for plant performance traits for CID and SNF traits under the two water treatment however, plant biomasses measured at mid pod filling stage (leaf, pod and total) as well as leaf biomass at flowering, and for root morphology traits water stress act as a moderator on affecting the response of soybean cultivars for these traits. These traits especially, biomass and seed yield was found to be noticeable traits for selecting specific cultivar adaptable for specific water regime. As a result, both Prima 2000 and A5409RG can be selected for well-watered condition but, under drought stress Prima 2000 (Table 6.3).

Overall, field experiments confirmed the previous phytotron results of superior performance of Prima 2000 under drought. The study also verified the importance of root system architecture and morphology for providing drought tolerance in soybean. Results further suggest that root architectural traits of tap and lateral roots (thickness and branching density) and morphological traits (root length, surface area and volume) could be used as simple and quick performance evaluation tools in future soybean improvement programs. Also, for the first time, the strong association of CID (WUE) with $\delta^{15}N$ (SNF), root traits as well as seed yield in soybean exposed to drought has been ascertained. This also demonstrates the importance of CID (WUE) as a potent selection criterion for enhanced soybean performance under drought. In addition, research findings suggest that higher performance in CID under drought stress may be due to higher CO_2 assimilation and better N_2 fixation resulting in better root system architecture and morphology of the drought tolerant cultivar Prima 2000 for maintain the water status of the plant for efficient biological activity.

CHAPTER SEVEN

GENERAL DISCUSSION AND FUTURE RESEARCH

The overall aim of this PhD study was to investigate performance of different bean and soybean cultivars under drought by using selected morphological or physiological phenotypic markers (traits), and to investigate the potential of these performance markers under environmentally controlled and/or field growth conditions. In general, results obtained in field experiments were in agreement with the findings in environmentally controlled phytotron experiments. In particular, in this study support has been found for the hypothesis that common bean and soybean have a similar morphological or physiological phenotypic basis of drought adaptation permitting the use of identical performance markers for selection of more drought-tolerant cultivars under both types of growth environments.

A novel finding of this study was that of associating better performance of both legumes under drought with both developmental plasticity of the root system and with enhanced photosynthetic carbon assimilation: WUE (water use efficiency), SNF (symbiotic nitrogen fixation) and plant biomass production. Results from field experiments clearly demonstrated that particular root morphology and architectural traits are important for better performance under drought and that these root traits are associated with better seed yield. These field experiments further allowed determining the contribution of SNF to %N, and elucidating the relationship of SNF with WUE. Specifically the root architectural traits (1^{st} whorl angle, basal root number and adventitious root branching density) and root morphological traits (root length, area and volume) were significantly associated with better seed yield under drought. These root traits might be important selection criteria in future bean improvement program to select for drought tolerance.

A further new finding was the direct relationship between both carbon isotope discrimination (i.e., WUE) and SNF, and root morphological (root length, area and volume) as well as architectural (basal root number, 1^{st} as well as 2^{nd} whorl angles) traits in common bean under drought. The capacity to maintain nodule size in common bean under water-limited condition was also associated with these traits. Also, the direct relationship found between CID (WUE) and SNF with PHI and seed yield under drought stress condition has further ascertained the importance of maintaining the water status of the plant through enhanced plasticity of root architecture for better performance under drought as well as for higher SNF ability.

For soybean, the importance of the root morphology (root length, surface area and volume) for drought tolerance was also clearly shown. In addition, the study provided new knowledge about an existing strong association of CID (WUE) with $\delta^{15}N$ (SNF), root traits and seed yield in soybean under drought. More importantly, this research revealed, for the first time, that higher WUE (lower CID value) is related to higher photosynthetic assimilation and better N_2 fixation as well as improved root system architecture and morphology that contribute to maintaining the water status of the plant for efficient biological activity under drought stress.

A further novel aspect of the study was the finding that the ability to sustain shoot biomass under nitrogen limited conditions is important for selecting improved drought tolerant legume germplasm (soybean and common bean in this study). By using correlation and principal component analysis, it was shown in this study that maintaining SNF ability under controlled conditions of drought stress in legumes is strongly associated with their ability for improved CO_2 assimilation and stomatal conductance. Also the importance of the leaf water status and IWUE

(CO_2 assimilation/stomatal conductance) for better performance under drought was verified. Cultivars, such as Prima 2000 (soybean), BAT 477 and BT_34-1-1 (common bean), have the capacity for maintaining IWUE for a longer period which is essential for maintaining better SNF ability for a longer time that affects plant development. Therefore, enhanced CO_2 assimilation through better stomatal conductance together with enhanced root and shoot development are important plant processes for drought-tolerant soybean and common bean for maintaining the balance between the shoot and the root biomass partitioning.

Generally, this study has generated new knowledge about the use of physiological markers (traits) that can be used for legume evaluation under drought suitable for both phytotron and field studies. This includes shoot biomass, WUE (IWUE/CID), nodule size/mass, and SNF (ARA/δ^{15}N). Markers highly appropriate for phytotron studies include determination of biomass, leaf area, gas exchange parameters and leaf water potential. For field evaluation root architectural and morphological traits were found to be important markers that can be used in a legume improvement program. Particularly in common bean measurement of whorl angle, number of basal roots and number as well as branching density of adventitious roots would also be an important addition. Figure 8.1 presents a proposed overall inter-relationship of the performance markers based on the outcome of this study. Finally, a further outcome of this study would be suggesting to modify the previously proposed function of grain yield set by Passioura (1996) with [Y= EUW x WUE x HI] into Y= EUW x WUE x HI x SNF, for legumes with Y representing grain yield, EUW the effective use of water through enhanced root development and HI the harvest index where PHI (the pod harvest index) is an important sub component of HI..

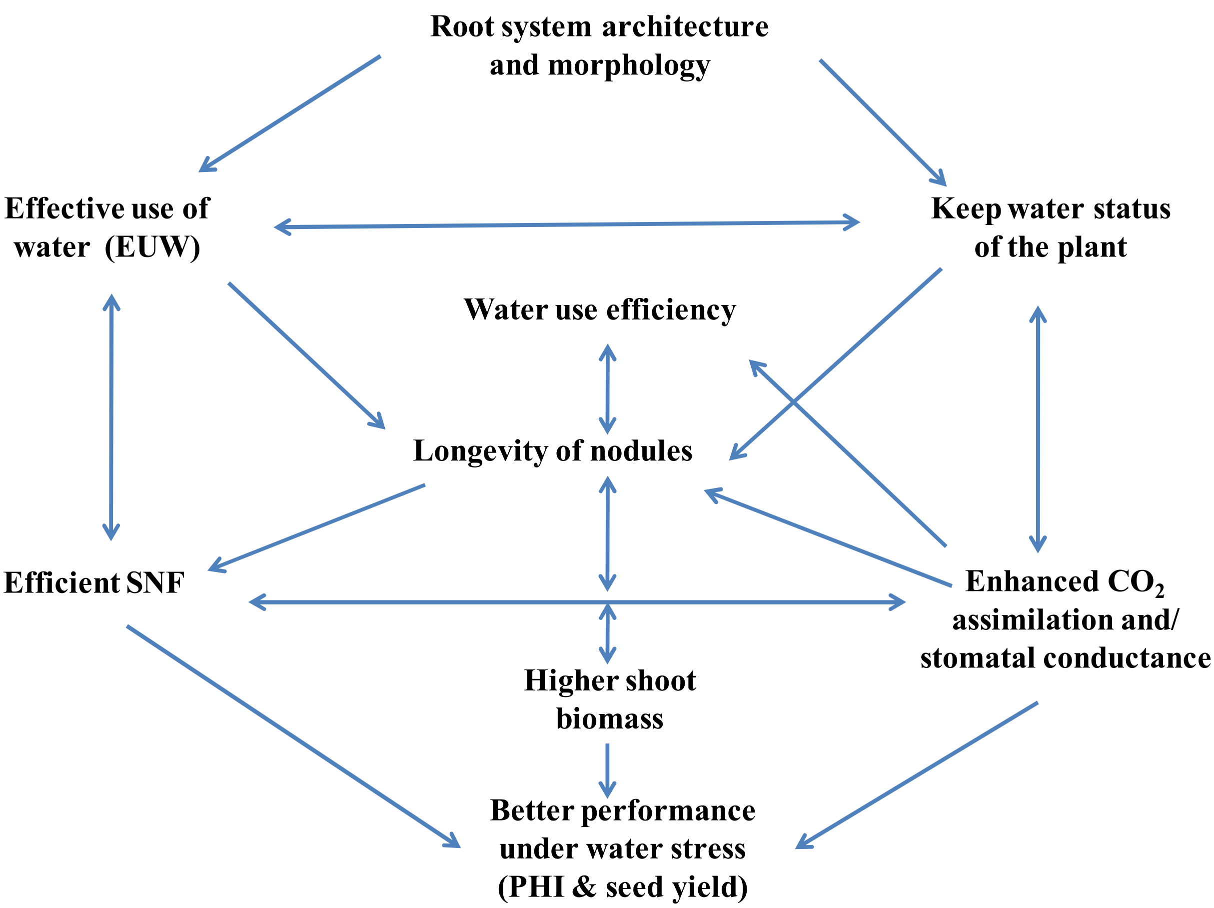

Figure 7.1 Proposed schematic representation inter-relationship of the physiological and morphological performance markers under drought based on results obtained in this study.

Future research actions should focus first on implementing identified markers (traits) into the existing bean program in Ethiopia. The applicability of these markers in a large germplasm screening program should be evaluated and in particular root performance markers, which have not been used in varietal improvement programs, and these should be evaluated for their potential to be easily applicable in such large programs. A second future action should include carrying out crossings with commercial cultivars and studying the heritability of these markers. Wider application to tropical legumes, such as chickpea, cowpea and mungbean, which are of relevance for Ethiopia, should be a third future research action. Finally, a fourth future research action should be the application of these markers for providing superior performance under various other abiotic stresses of relevance to Ethiopia such as low P, aluminium toxic acid soils, and low fertility soils. The root architectural growth is highly affected by the fertility of the soil, especially low P, which is a serious problem for crop production for over 70% of agricultural land throughout the world. Therefore, for identification of legumes for multiple stresses or for better soil P acquisition and for drought would be a major challenge for breeders. Therefore, the need to understand and investigate morpho-physiological plant processes or traits, particularly root system traits responsible for multiple stresses, would be a vital addition to bean breeding programs.