

**ROOT PROPERTIES AND PROLINE AS POSSIBLE INDICATORS
FOR DROUGHT TOLERANCE IN SOYBEAN**

By

OBED JOHN MWENYE

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Promoter: Dr. Rouxléne van der Merwe

Co-promoter: Prof. Leon D. van Rensburg

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Dedication

I dedicate this Thesis work to my wife, Trintus, who has been a constant source of support and encouragement; To my daughter and son, Isabella and Thanthwe, I am truly thankful for having you in my life, I believe you will take this mantle to another level.

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List of abbreviations and SI units

ABA	Abscisic acid
Abs	Absorbance
ANOVA	Analysis of variance
BC	Before Christ
BL	Breeding line
cm	Centimetre
CV	Coefficient of variation
CV	Commercial cultivar
DAFF	Department of Agriculture, Fisheries and Forestry
°C	Degrees Celsius
DNA	Deoxyribonucleic acid
DW	Dry weight
ENSO	El Niño/Southern Oscillation
ET	Evapotranspiration
FAOSTAT	Food Agriculture and Organisation Statistics
FC	Field capacity
FD	Field
Flr	Flowering growth stage
FW	Fresh weight
g	Gram
g	Relative centrifugal force
G	Genotype
GH	Glasshouse
GMP	Geometric mean productivity
GS	Growth stage
GxE	Genotype by environment interaction
GxT	Genotype by trial interaction
GxWLIS	Growth stage by water-limited-induced-stress interaction
GxWLISxGS	Growth stage by water-limited-induced-stress by growth stage interaction

GSxG	Growth stage by genotype interaction
ha	Hectare
HI	Harvest index
IITA	International Institute of Tropical Agriculture
K	Potassium
kg	Kilogram
	Litre
LSD	Least significance difference
m	Metre
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
nmol	Nanomolar
μmol	Micromolar
MP	Mean productivity
Mpa	Megapascal
MRI	Magnetic resonance imaging
N	Nitrogen
NE	No effect
nM	Nanometre
NAMC	National Agricultural Marketing Council
P	Phosphorus
PAW	Plant available water
PC	Principal Component
PCA	Principal Component Analysis
%	percentage
Pd	Pod development growth stage
Prl	Proline
PVC	Polyvinyl chloride
R	Reproductive growth stage
RCBD	Randomised completely block design

RDW	Root dry weight
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SADC	Southern Africa Development Community
SDW	Shoot dry weight
SL	Shoot length
SR	Slow release
SSA	Sub-Saharan Africa
SSI	Stress Susceptibility Index
T	Trial
TOL	Stress tolerance
TRL	Tap root length
TxG	Trial by genotype interaction
TxGS	Trial by growth stage interaction
UFS	University of the Free State
v/v	Volume per volume
Ve	Vegetative growth stage
Vol	Volume
WHC	Water holding capacity
WLIS	Water-limited-induced-stress
WLISxGS	Water-limited-induced-stress by growth stage interaction
WUE	Water use efficiency
Y	Seed mass
YI	Yield index
YSI	Yield stability index
Zn	Zink

ABSTRACT

Use of an efficient selection tool in breeding for complex traits such as soil water-limited-induced-stress (WLIS) tolerance is of paramount importance in order to reduce the genotype by environment interaction effects that affect the heritability of the trait and reduce the associated cost implications of testing in multiple environments. Hence this study was carried out in order to determine the response of drought tolerant and -sensitive soybean genotypes to soil WLIS conditions at different growth stages. Furthermore, the study evaluated seedling root properties and proline accumulation as possible selection criteria for soil WLIS in soybean. The association between proline accumulation and yield-based drought tolerance indices was investigated.

Results showed that different soil WLIS levels had varied effects on the morphological traits measured. The severe (30% deficit irrigation) and moderate (50% deficit irrigation) soil WLIS treatments drastically affected the morphological traits. Soil WLIS stress imposed during pod-development growth stage affected the seed yield of soybeans much more than drought stress imposed during the flowering and vegetative growth stages. Soil WLIS reduced soybean seed yield, 100-seed mass, seed mass, seed number per plant, number and mass of pods per plant, plant height, node number and plant biomass among the morphological traits measured. Significant differences were observed among genotypes for seed yield and seed mass per plant in response to soil WLIS. Seed mass mean difference is the relative performance of a genotype in a soil WLIS in contrast to a non-stress environment. Seed mass mean difference proved to be a useful index for soil WLIS tolerance, as tolerant genotypes were discriminated from the sensitive ones. Tolerant genotypes showed lower reduction in seed mass per plant after severe WLIS, compared with that of sensitive genotypes.

Soybean genotypes showed varied seedling shoot- and root morphology in response to soil WLIS. Seedling root traits with potential use as soil WLIS tolerance indicators include tap root length to shoot ratio and deep rooting ability. Drought tolerant genotypes showed deep rooting ability and larger root-to-shoot ratios compared to the drought sensitive genotype. This is a coping mechanism of drought tolerant genotypes to soil WLIS. Furthermore, the adopted

‘deep-pot’ system that was used to phenotype the roots of the soybeans was effective in studying the root system and is recommended for similar studies.

Similarly, soil WLIS had a significant effect on proline accumulation amongst the soybean genotypes. Proline concentration tended to increase with an increase in soil WLIS. It was observed that proline concentration tended to increase much more in the tolerant genotypes than the sensitive genotypes. This was true for both the controlled glasshouse (pot and lysimeter) and field experiments. Results also showed that proline accumulation under soil WLIS conditions correlated positively with yield potential (Y_p and Y_s) as well as the tolerance indices geometric mean productivity (GMP), mean productivity (MP) and tolerance index (TOL). This suggested that proline is a useful index in screening for drought tolerance in soybean. The proline determination essay proved to be easy and cost-effective as multiple samples from different sites can be evaluated at the same time since it only requires few leaf samples to be carried out.

Key words: Drought, *Glycine max*, agronomical traits, proline accumulation, roots, tolerance index

CHAPTER 1

General introduction

Soybean (*Glycine max* L. Merrill) is an important source of high-quality vegetable oil and protein in the world (Maestri et al. 1998). The seed contains approximately 38% - 42% protein and 18% - 23% oil at maturity (Dornbos and Mullen 1992; Clemente and Cahoon 2009). Soybean products are extensively used as less caloric food products with lower lipid but higher protein content. The products are fit for the nutritional needs of all age-groups and also among the resource poor communities (Nascimento et al. 2010). Given the potential of soybean as a raw material in livestock feed industry, the demand for soybean products in Southern Africa has increased tremendously (Kolapo 2011).

Drought stress is a major constraint to soybean production and yield stability (Manavalan et al. 2009). Drought is initiated when the crop demand for water exceeds the supply (Blum 2011). It is increasingly becoming an important issue as a result of the climate change phenomenon, as agricultural production is being adversely affected through increased aridity and warmer temperatures (Godfray et al. 2010; Blum 2011). Drought stress reduces yield, and affects the quality and quantity of protein and oil as well as the biological nitrogen fixation system in soybeans (Manavalan et al. 2009). Vital processes that are affected by water-limited-induced stress (WLIS) include cell growth, wall synthesis, protein synthesis, protochlorophyll formation, nitrate reductase level, abscisic acid (ABA) accumulation, cytokinin level, stomatal opening, CO₂ assimilation, respiration, proline accumulation and sugar accumulation (Hsiao 1973). However, the level of the effect or crop responses to drought stress varies depending on the species, duration of the stress and stage of development of the plant (Hsiao 1973; Lakso 1985; Mastorilli et al. 1995; Cakir 2004).

Drought stress is a wide-spread problem seriously influencing soybean yields (Buckland et al. 2000), but development of tolerant cultivars is hampered by the lack of effective selection criteria (Sio-Se et al. 2006). Conventional direct selection for yield in dry environments is often inefficient due to (i) associated cost implications as multiple trial sites are desired; (ii) large seasonal variation in weather and (iii) generally a large genotype x environment (GxE)

interaction, resulting in low heritability for yield (Richards 2006). Hence the need to come up with even more efficient, low-cost phenotype-based techniques that would rapidly identify drought tolerant genotypes with minimal cost as opposed to extensive multi-location field trials.

Significant progress has been made in identifying the physiological and genetic basis of traits for improving yield in water-limited environments and quantifying their impact on yield improvement in soybean (Ludlow and Muchow 1990; Manavalan et al. 2009; Sadok and Sinclair 2011). Proline accumulation and root traits contribute to plants' survival and productivity especially under WLIS environments (Manavalan et al. 2009). Proline is a common osmolyte that accumulates in drought-stressed plants (Gupta 2006). Proline accumulation has been associated with drought stress tolerance in several crop plants (Ashraf and Foolad 2007). Root traits have also been strongly associated with drought tolerance in soybean (Sadok and Sinclair 2011). Extensive and deep rooting systems are recognised as the most important traits for improving drought tolerance in crops (O'Toole and Bland 1987). However, the impact of utilising proline accumulation and/or root traits to discriminate drought-tolerant and -sensitive genotypes in soybeans produced in South Africa has not been well documented.

The aim of the study was to determine the response of drought tolerant and -sensitive soybean genotypes to soil WLIS conditions at different growth stages. Specific objectives of the study included:

- (i) Screening soybean genotypes for drought stress tolerance and drought sensitivity using agronomical and physiological traits,
- (ii) determining the effect of soil WLIS at different growth stages on proline concentration, yield and yield components of soybean genotypes that differs in sensitivity to drought stress,
- (iii) determining seedling shoot- and root growth responses among soybean genotypes to drought stress and
- (iv) evaluating associations among agronomical traits, drought tolerance indices and proline accumulation in selection for drought tolerance in soybean.

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CHAPTER 2

The roles of proline and root traits on selection for drought-stress tolerance in soybeans

2.1 Soybean: Botany, economic importance and production trends in South Africa

Soybean is an annual legume that belongs to the legume family *Fabaceae* (Tefera 2011) and genera *Glycine*. The genera consists of two sub genera; *Glycine* and *Soja* but the cultivated soybean belongs to the sub genera *Soja*. It is a strictly self-pollinating legume with $2n=40$ chromosomes (Hymowitz and Newell 1981). It is an erect, branching plant with trifoliate leaves. The soybean is classified as a simple fruit as it is derived from a single ovary and can be produced from single seeds contained within a pod of the plant (Gill and Vear 1980; Hymowitz and Newell 1981).

Soybean originated from south east China, from a wild form of *Glycine soja* (Sieb. Et. Zuss) (Gill and Vear 1980). Evidence of cultivation exists in the region dating back to 4000-5000 years B.C. (Janick et al. 1974). It is also mentioned in early Chinese literature dating 2838 BC where it is referred to as one of the 'sacred grains' vital for the Chinese empire (Gill and Vear 1980). Later on, the crop spread to Japan, Korea and Mongolia, where among other uses, the seeds were either cooked or ground, while the bean flour was used for preparation of a variety of products such as oil and milk (Gill and Vear 1980). Soybean was first introduced to Europe and North America in the 18th century (Janick et al. 1974). However, large-scale official introduction into USA did not occur until the early 1900s. Until 1954, China led the world soybean production. Since then, USA has become the world largest producer (Liu 2005). Soybeans were first introduced into South Africa in 1903 (Du Toit 1942) for production of hay of considerable feeding value for livestock (Hall 1930). Production was mainly recommended in the Natal region (present day KwaZulu-Natal Province) because of its good rainfall pattern (Hall 1930; Dlamini et al. 2014).

World soybean harvest has reached a record 308 million tonnes (2014 production data) (FAOSTAT 2015). The USA, Brazil, Argentina, China and India accounted for over 90% of the

world output. South Africa with 948, 000 tonnes, is the major producer of soybean on the African continent, followed by Nigeria with 679, 000 tonnes, Zambia (214, 179 tonnes), Malawi (110, 000 tonnes), Zimbabwe (90, 000 tonnes), Egypt (25, 000 tonnes) and the Democratic Republic of Congo (22, 000 tonnes) (FAOSTAT 2015). Increased domestic and global demand of soybeans over the past decade and global market trends continues to pull the production steadily upwards (Ash and Dohlman 2006).

Soybean and its related products are used in various forms in South Africa. High protein meal and soybean oil are the most prominent products used (Joubert 2011; DAFF 2014). High protein meal is an essential ingredient in the manufacturing of feed for the poultry and pork industries. Soybean oil is used in the industrial sector for manufacturing of various products (Joubert 2011). The South African soybean market is the largest and most vibrant in the sub-Saharan Africa (SSA) region; and production is dominated by commercial farmers (NAMC 2011). The total value of soybean produced in South Africa was estimated at R1.1 billion in 2009. Total imports of soybean and soybean related products amounted to R4.35 billion in 2010; with exports valued at R672, 7 million (mainly high protein meal, refined soybean oil and crude protein oil) (Joubert 2011). This suggests that South Africa remains a net importer of soybeans and soybean related products (NAMC 2011; DAFF 2014). The soybean subsector is a vital component of the South African agricultural economy, with potential to increase its market share (Dlamini et al. 2014). Recently, there has been a growing demand for soybean products for human consumption in South Africa, because of the associated health benefits; i.e. lowering of cholesterol and combating of heart diseases, as well as provision of high quality proteins for vegetarians (DAFF 2014).

Soybean production in South Africa is mainly concentrated in Mpumalanga (42%), Free State (27%), KwaZulu-Natal (13%) and Limpopo (8%) (DAFF 2014). Total soybean production in South Africa has reached a record 948, 000 tonnes from a total area of 502, 900 hectares, representing an average yield of 1.88 tonnes per hectare under dry land conditions (NAMC 2011; DAFF 2014; CEC 2015). Soybean production in South Africa has stagnated and yields continue to fluctuate; this is despite of increased allocation of area of production (DAFF 2014). This has mainly been attributed to unfavourable weather conditions, especially drought and high temperatures which continues to negatively impact crop production in most production

regions (DAFF 2012), forcing most commercial farmers to supplement the water deficit with irrigation. Since water is a scarce resource in South Africa (Gbetibouo and Hassan 2005), drought often poses as a major limitation on soybean production.

2.2 Drought stress

Drought in agricultural terms is defined as “a reduction in water availability below the optimal level required by a crop during each different growth stage, resulting in impaired growth and reduced yields” (FAO 2016). Furthermore, agricultural drought relates to an imbalance in the water content of the soil during the growing season which, although influenced by other variables such as the crop water requirement, the water holding capacity and degree of evaporation is also largely dependent upon rainfall amount and distribution (Buckland et al. 2000). During the last decade, the frequency and impact of drought on the agricultural sector has increased in South Africa (Ngaka 2012). This is a result of the effects of climate change due to the greenhouse gas emissions and the recurrence of the El Niño/Southern Oscillation (ENSO) (Lizimu et al. 2014), which often affects seasonal temperature and precipitation; characteristic of most of the Southern African agricultural production regions (Ngaka 2012; DAFF 2014).

South Africa’s average annual rainfall of 450 mm per year is well below the world’s average of 860 mm, while evaporation is comparatively high. Rainfall is also distributed unevenly across the country, with humid, subtropical conditions in the east having as high as 1000 mm rainfall and dry, desert conditions in the west with less than 100 mm (Behnein 2008) suggesting that the country is largely semi-arid to arid. As such, Gbetibouo and Hassan (2005) emphasised the need for more research in agricultural production technologies and methods that are more water-efficient in South Africa.

2.2.1 Effects of drought stress on soybean yield and yield components at various growth stages

Drought stress affects yield and its components in soybean production and its effect is dependent on the duration and intensity of the stress, as well as the timing of the stress with respect to the growth stages of the plant. Soybean yield and its components are least sensitive to WLIS during the vegetative growth stage (Tables 2.1 and 2.2).

Table 2.1 The effect of drought stress on yield at different growth stages of soybean

Author	Location	Type	Climate/ rainfall	Irrigation method/ strategy	Stress intensity/ Duration	Yield (kg ha ⁻¹)	Percentage yield reduction by growth stage*				
							Vn	R 1-2	R 3-6	R 7-8	All growth stages (Vn-Rn)
Cox and Jolliff (1986)	Oregon state, USA	Field	-	Deficit irrigation Line source	Well-watered kept above -0.05 Mpa	Dryland: 400 Deficit: 2400 Control: 3290	-	-	-	-	87% 27% -
Eck et al. (1987)	Texas, USA	Field	Semi-arid 360 mm,	Deficit irrigation	40-80% less of Well-watered	370-3130	-	12-28%	27-88%	8%	-
**Dornbos et al. (1989)	Iowa, USA	Greenhouse plastic pots	-	Deficit irrigation	50-75% less of well-watered, for mild and severe	Control 46.2 Mild: 34.0 Severe: 23.7	-	-	38% 58%	-	-
Specht et al. (2001)	Nebraska, USA	Field	-	Deficit irrigation Sprinkler	0, 20, 40, 60, 80, 100% replenishment of the crop ET	Yield: 933-2085	-	-	-	-	50%
Karam et al. (2005)	Tal Amara, Lebanon	Field weighing lysimeters	Semi-arid 592 mm	Deficit irrigation 700-800 mm	R2, 5 7 (7 days)	2300-3500	-	15%	35%	15%	
Dogan et al. (2007)	Sanliurfa, Turkey	Field	Semi-arid 450 mm	Deficit irrigation 440-690 mm	8-12 days	1955-3684	-	13%	19-47%	-	-
Kobraee et al. (2011)	Iran	Field	Semi-arid 478 mm	Deficit irrigation (R1, R3, R6)	-	1688-3173	-	45%	30-47%	-	-
Candogan et al. (2013)	Turkey	Field	Temperate	Deficit irrigation at 0-90 cm profile	-	2070-3760					12-45%
AL-Jobori and Salim (2014)	Iraq	Greenhouse, pots (10 kg)	Semi-arid	Deficit irrigation	-	-	-	-	-		- 20-47%
Kobraee et al. (2014)	Kermanshah, Iran	Field experiment		Deficit irrigation	-	1637-2274		29%	22-28%		-

* Vn = vegetative phase, R1-2 = early reproductive phase i.e. initial to full bloom, R3-R6 = mid reproductive phase i.e. pod initiation to full seed setting, R7-8 = late reproductive stage i.e. beginning maturity to physiological maturity (Fehr et al. 1971), ** measured in g plant⁻¹.

Table 2.2 The effect of drought stress at different growth stages on the yield components of soybean

Authors	Yield components	Effect of drought stress on yield components by growth stage*				
		Vn	R 1-2	R 3-6	R 7-8	All growth stages
Cox and Jolliff (1986); Dornbos et al. (1989); Foroud et al. (1993a)	Dry matter	-	-	14-24%	-	18-78%
Cox and Jolliff (1986); Dornbos et al. (1989); Foroud et al. (1993a)	Number of pods	-	-	18-41%	-	20-77%
Eck et al. (1987); Dornbos et al. (1989); Foroud et al. (1993a)	Seed number	-	20%	10-51%	2-3%	-
Cox and Jolliff (1986); Specht et al. (2001)	Total seed weight	-	-	14-25%	-	6-25%
Cox and Jolliff (1986); Eck et al. (1987);	Individual seed weight	-	119%	111%	12%	25%
Eck et al. (1987); Specht et al. (2001); Foroud et al. (1993a)	Plant height	-	18%	6-23%	5%	25%
Eck et al. (1987)	Seasonal WUE (kg m ³)	-	105%	10-37%	NE	-
Dornbos et al. (1989)	Photosynthetic rate	-	-	71%	-	-
	Leaf resistance	-	-	168%	-	-
	Reproductive period	-	-	17-27%	-	-

* Vn = vegetative phase, R1-2 = early reproductive phase i.e. initial to full bloom, R3-R6 = mid reproductive phase i.e. pod initiation to full seed setting, R7-8 = late reproductive stage i.e. beginning maturity to physiological maturity (Fehr et al. 1971), NE = no effect, WUE = water use efficiency.

Drought stress during the vegetative growth stage has less effect on yield, but triggers an early switch of plant development from the vegetative to the reproductive phase (Desclaux and Roumnet 1996). Plants have enough time to recover and compensate once the stress is withdrawn, hence the reduced impact on grain yield.

Drought stress during the early reproductive stages [early flowering to full bloom (R1-R2)], reduces soybean yield by 12-45% (Table 2.1). When drought stress is coupled with high temperatures during this growth stage, the effect on yield is extreme (Kobraee et al. 2011). Yield reduction during the early reproductive stage is explained by high flower abortion and consequently a decrease in seed number. However, seed weight increases as assimilates are concentrated on the fewer seeds produced (Eck et al. 1987). Hence, the reduction in grain yield is not as substantial, except for cases where drought stress is prolonged.

Soybean yield is extremely sensitive to drought stress during the mid-reproductive stage [R3-R6 (pod initiation-seed filling)] (Table 2.1). Drought stress during R3-R6 reduces grain yield extensively with reported reductions of 19-88% (Eck et al. 1987; Dornbos et al. 1989; Karam et al. 2005; Dogan et al. 2007; Kobraee et al. 2011; Kobraee et al. 2014). Substantial grain yield reductions (88%) have been recorded at seed filling (R5-R6) (Eck et al. 1987). Similarly, the yield components are extremely sensitive to soil-water deficit in this growth stage (Table 2.2). In addition, the reproductive period, is further shortened (Eck et al. 1987) and less time is left for plant recovery.

Soybean yield and its components are less sensitive to drought in the late reproductive growth stage (Tables 2.1 and 2.2) as compared to the early reproductive growth stage. Drought stress imposed throughout the growth cycle results in substantial yield losses (12-80%) (Table 2.1). This is attributed to the drastic effect on the dry matter, number of pods (77%), individual seed weight (6-25%) and plant height (25%) (Table 2.2).

Variation in yield reduction, due to drought stress imposed during different growth stages (Table 2.1), could be attributed to (i) the different drought stress coping mechanisms (escape, avoidance and tolerance) among the genotypes and (ii) the different stress intensities used. Researchers need to properly characterise the drought coping mechanisms that are involved

in the different genotypes. The effect of drought is dependent on the intensity of the stress. The higher the intensity, the more the yield is reduced. The intensity of stress is often expressed as percentage of soil-plant available water (PAW) and in most cases, ranges from 20-80% at a specified soil depth (Table 2.1). Yield is reduced when the PAW is depleted to 40-60%, but substantially reduced when depleted to as low as 20-30% (Eck et al. 1987; Dornbos et al. 1989; Specht et al. 2001). Drought stress can also be imposed by withdrawing of irrigation water for a specified period (Table 2.1). Soybean yield is reduced in a 4-8 days' irrigation withdraw period at the flowering and seed development growth stages (Karam et al. 2005; Dogan et al. 2007). The impact is more severe with a 12-14 day withdrawal period (Dogan et al. 2007); however, the severity is highly dependent on the prevailing weather conditions (high temperature and low humidity), which increases the effect of drought stress in the soybean plants.

Limited studies have been done on the differential response of determinate and indeterminate soybean genotypes to drought stress. Desclaux and Roumet (1996) suggested that the two types of soybean genotypes differ mostly in their strategy of partitioning assimilate between the main stem and branches under drought stress. Indeterminate genotypes tend to preserve the ability to produce reproductive organs on its main stem by predominant resource partitioning to the main axis (80% of the photosynthates), whereas in the determinate genotypes, assimilates are partitioned preferentially to the branches (Desclaux and Roumet 1996). This agrees with Atti et al. (2004) who found that indeterminate soybean genotypes maintained its proportional allocation of assimilates to reproductive structures under chronic water deficit during the reproductive stage. Hence more studies are needed to ascertain the actual differences between the two types of soybean genotypes in terms of their yield and yield components, as well as sensitivity to soil water deficit stress at different growth stages.

2.2.2 Effects of drought stress on soybean seed protein and oil composition

Seed protein and oil content in soybeans are differentially affected by drought stress. An inverse relationship between total oil and protein content is evident (Table 2.3). Protein content increases with increased drought induced stress (Dornbos and Mullen 1992; Bellaloui and Mengistu 2008).

Table 2.3 The effects of drought stress on soybean seed protein and oil content

Author	Location	Type of experiment	Irrigation strategy	Protein content (%)			Oil content (%)		
				Drought stress levels			Drought stress levels		
				Control	Moderate	Severe	Control	Moderate	Severe
¹ Dornbos and Mullen (1992)	USA (Iowa)	Glasshouse, pot experiment	Deficit irrigation (100, 75, 50%)	Yr1: 40.3%	42.3%	44.9%	21.3%	20.3%	19.2%
				Yr2: 37.4%	39.5%	42.4%	23.3%	22.3%	20.4%
				Temp 29°C: 38.7%	39.2%	43.7%	24.3%	23.5%	21.8%
				35°C: 41..5%	45.2%	47%	23.5%	20.8%	17.6%
Foroud et al. (1993b)	Canada (Alberta) Semi-arid	Field experiment (Non-weighing lysimeters)	Deficit irrigation	39.50%	38.65%	39.0%	18.85%	19.35%	19.15%
Specht et al. (2001)	USA	Field experiment	Deficit irrigation	46%	45%	44.8%	17.5%	18.00%	18.1%
Bellaloui and Mengistu (2008)	USA (Mississippi)	Field experiment	Deficit irrigation	Cult.1: 41.1%	42.3%	41.9%	20.6%	20.9%	21.2%
				Cult. 2: 40.7%	41.0%	42.2%	21.1%	20.7%	20.1%
² Masoumi et al. (2011)	Iran Arid	Field experiment	Deficit irrigation	-	-	-	495.36	231.22	115.80
³ Bellaloui et al. (2011)	USA	Greenhouse (pot) experiment	Water deficit	39.8 %	-	41.0 %	20.4%		18.5%
Kobraee et al. (2014)	Kermanshah, Iran	Field experiment	Deficit irrigation	37.66%	37.96%	38.54%	20.28%	18.01%	17.86%

¹ The experiment explored the combined effect of temperature and drought stress; ² Seed oil content shown as kg ha⁻¹, and ³ contents shown as g kg⁻¹.

Soybean seed oil content decreases with increased drought stress (Dornbos and Mullen 1992; Masoumi et al. 2011). High temperatures significantly increase the effect of drought stress on both protein and oil content (Dornbos and Mullen 1992). On the other hand, contrasting results from above have been observed (Foroud et al. 1993b; Specht et al. 2001; Bellaloui and Mengistu 2008); which could be as a result of genotypic or GxE effects to the processes by which carbon flux in soybean is primarily partitioned to form protein and oil during embryogenesis. Thus, more investigation is needed on differences among soybean cultivars' sensitivity to oil and protein content under drought stress conditions.

Even though the general trend indicates that protein increase with stress, Specht et al. (2001) have suggested that soybean protein content may also decrease with increased soil WLIS; however, the proportion of increase or reduction is more of a function of reduced total yields. It would also be more informative to investigate how drought stress affects the fatty acid (palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid) profiles of the soybean oil. Changes in fatty acid profiles determine the health and dietary quality, and industrial utilisation of soybean oil (Clemente and Cahoon 2009).

2.2.3 Effects of drought stress on nitrogen fixation in soybean

Nitrogen (N_2) fixation is highly sensitive to drought stress (Ladrera et al. 2007) and this, in turn, affects the yield and protein quality of soybean. Drought stress affects N_2 -fixation in soybean and legume crops by reducing the acetylene reduction activity, increasing ureide concentration in shoots, reducing carbon and oxygen availability to bacteroids, and alteration in amino acid metabolism (Table 2.4). However, researchers have discovered soybean genotypes with N_2 -fixation insensitivity to drought stress (Serraj and Sinclair 1996; Sinclair et al. 2000).

Table 2.4 Effects of water-limited-stress on nitrogen fixation in soybean

Effects of water stress on nitrogen fixation system	Author
Reduction in acetylene reduction activity	Sprent (1972); Serraj and Sinclair (1996); Serraj et al. (1999)
Increased ureide concentration in the shoots	Serraj and Sinclair (1996); Sinclair et al. (2000); Streeter (2003)
Reduced carbon availability to bacterioids	Ladrera et al. (2007)
Oxygen limitation to bacterioids	Serraj and Sinclair (1996)
Carbon shortage and alteration in amino acid metabolism	Serraj and Sinclair (1996)

2.3 Mechanisms plants use to cope with drought stress

In nature, plants have developed various mechanisms to cope with drought stress (Xoconostle-Cázares et al. 2011). These may be classified into three groups: drought escape, drought avoidance and drought tolerance (Turner et al. 2001). According to Manavalan et al. (2009) drought escape allows the plant to complete its life cycle during the period of sufficient water supply before the onset of drought.

Drought avoidance, on the other hand, involves strategies which help the plant maintain high water status during periods of stress, either by efficient water absorption from roots or by reducing evapotranspiration from aerial parts. The third mechanism on how plants use to cope with drought is, tolerance, and it allows the plant to maintain turgor and continue metabolism even at low water potential, e.g. by protoplasmic tolerance or synthesis of osmoprotectants, osmolytes or compatible solutes (Nguyen et al. 1997).

2.4 Free proline accumulation as a drought-tolerance selection criterion in stressed plants

Plants accumulate compatible solutes as a physiological response to osmotic stress induced by drought, salinity, heat and cold (Blum 2011). Proline is among the compatible organic

solutes which are associated with plant adaptation to adverse conditions (Delauney and Verma 1993). Under drought conditions proline accumulates in the cytosol, where it is thought to play an osmotic adjustment and protectant role in the cytoplasm. It helps in stabilisation of proteins, membranes and sub-cellular structures and protects cellular functions by scavenging reactive oxygen species (Kishor et al. 2005). Proline accumulation is therefore associated with plants' ability to tolerate drought stress at the cellular level (Gupta 2006), as evident in its rate of accumulation in stressed plants (Fulda et al. 2011; Caus et al. 2013; Shen et al. 2015).

Free proline accumulation is a heritable trait (Hanson et al. 1979) and can be used to screen genotypes for drought tolerance (Esack et al. 2015) by examining the genotypes' capacity to accumulate proline under stress conditions. This is evident in Table 2.5 where genotypes of known drought sensitivity were ably discriminated as either drought tolerant and/or sensitive using proline accumulation under varying drought stress levels in plants. The results are summarised according to the crop, plant tissue used for proline analysis, calorimetric method used, genotypes assessed and the level of drought stress.

Genotypic variation in free proline accumulation is evident between known drought tolerant and sensitive cultivars of soybean (Masoumi et al. 2011), chick pea (Mafakheri et al. 2010), maize (Moussa and Abdel-Aziz 2008), cow pea (Hamidou et al. 2007) and tobacco (Van Rensburg et al. 1993) under varying drought stress levels. Proline accumulated with increased drought stress in both sensitive and tolerant cultivars. However, proline accumulates to higher concentrations in tolerant compared to sensitive genotypes. Similar results have been reported for Alfalfa (*Medicago sativa*) (Irigoyen et al. 1992) and spring wheat cultivars (Van Heerden and De Villiers 1996) in which proline accumulation is significantly correlated with increasing drought stress and consequently associated with drought tolerance.

The association of proline accumulation (under drought stress) with cultivars of known tolerance makes it possible for proline to be used as a biochemical marker for drought stress tolerance in crops. However, to make the assay more efficient and consistent there is a need to draw a threshold of proline accumulation above which a cultivar would be deemed tolerant.

Table 2.5 Proline accumulation under soil water deficit stress in different crops

Author	Crop		Plant tissue used	Proline assay used	Proline accumulation at drought stress levels		
Masoumi et al. (2011)	Soybean	Cultivar	Leaf	Bates et al. (1973)	Proline accumulation at different irrigation levels ($\mu\text{mol g}^{-1}$ FW)		
					S1	S2	S3
		L17 ⁱ			9.49	10.79	11.25
		Clean ⁱ			8.99	9.10	11.70
		M9 ⁱ			6.34	9.29	12.58
		T.M.S			5.03	6.40	7.75
		Williams			7.29	11.92	13.62
Mafakheri et al. (2010)	Chickpea	Cultivar	Leaf	Bates et al. (1973) as elaborated by Nayyar and Gupta (2006)	Proline accumulation at different irrigation levels ($\mu\text{mol g}^{-1}$ FW)		
					Vegetative	Anthesis	
		Bivanie ⁱ			8.28 ab	7.36 b	
		ILC482 ⁱ			9.45 a	8.29 ab	
Moussa and Abdel-Aziz (2008)	Maize	Cultivar	Leaf	Bates et al. (1973)	Proline (mM g^{-1} FW) accumulation at different drought stress levels		
					S1 (0)	S2 (-10)	S3 (-20)
		Giza 2 ⁱ			1.80 \pm 0.180	2.40 \pm 0.144	5.82 \pm 0.523
		Trihybrid 321			2.24 \pm 0.089	2.81 \pm 0.112	3.96 \pm 0.396

ⁱ Cultivars with known drought tolerance; S1, S2 and S3 are stress levels based on deficit irrigation. FW = fresh weight.

Table 2.5 Proline accumulation under soil water deficit stress in different crops (continued)

Author	Crop		Plant tissue used	Proline assay used	Proline accumulation at drought stress levels						
Van Rensburg et al. (1993)	Tobacco	Cultivar	Leaf	Bates et al. (1973)	Proline (mmol kg ⁻¹ DM) accumulation at different drought stress levels						
					S1 (-0.52)	S2 (-0.77)	S3 (-1.27)	S4 (-1.67)	S5 (-1.97)	S6 (-2.32)	S7 (-2.51)
		TL33			0.6	1.91	3.4	3.72	9.5	18.55	35.66
		CDL28			0.81	1.99	3.3	5.45	5.82	16.26	32.22
		GS46 ⁱ			0.17	1.19	2.12	6.83	14.16	30.2	41.9
		ELSOM ⁱ			0.38	1.59	2.05	11.71	23.72	44.78	47.62
Khamssi et al. (2010)	Chickpea		Leaf	Bates et al. (1973)	Proline accumulation at different irrigation levels (μmol g ⁻¹)						
					S1	S2	S3	S4			
					145.21c	176.3bc	296.8ab	348.34a			
Hamidou et al. (2007)	Cowpea	Cultivars		Bates et al. (1973)	Proline (mg g ⁻¹ DM) accumulation under drought stress levels in glasshouse (GH) experiments						
					Control (GH)	Stress (GH)	Control (GH)	Stress (GH)			
		B21	Leaf		0.29±0.04	1.37±0.75	0.33±0.31	0.44±0.07			
		Go			0.31±0.21	1.52±1.46	0.31±0.04	0.55±0.19			
		KV			0.41±0.23	0.73±0.36	0.42±0.04	1.03±0.26			
		Mo ⁱ			0.55±0.06	2.88±1.2	0.26±0.05	0.89±0.40			
		TN ⁱ			0.32±0.05	3.30±2.01					

ⁱ Cultivars with known drought tolerance; S1 to S7 are stress levels based on deficit irrigation. DM = dry mass; FW = fresh weight.

Use of proline accumulation as a biochemical marker for drought stress sensitivity is still under investigation and additional positive results continue to validate its use as a selection index. Information from the limited research indicates positive association between yield gain under drought stress conditions and proline accumulation. Free proline accumulation showed significant correlations with drought tolerance indices and biological yield in wheat (Singh et al. 1973; Ali Dib et al. 1994; Bayoumi et al. 2008; Farshadfar et al. 2013), rice (Roy et al. 2009) and in tea (*Camellia sinensis*) (Netto et al. 2010).

Given that yield is a quantitative trait and difficult to study, indirect selection via yield components and other related traits such as proline accumulation could be more efficient if these traits are related to yield. Hence, knowledge of the gene action and genetic association of yield with proline accumulation in soybeans needs to be investigated. Kaur et al. (2010) reported that both additive and non-additive gene actions are involved in proline accumulation under drought stress in maize, while Naoroui Rad et al. (2013) and Pourmohammad et al. (2014) suggested that additive gene effects were important in wheat and sunflower respectively. Additional heritability studies of proline accumulation are needed to elucidate the mode of inheritance of the trait in soybean and related crops. Genetic parameters such as, genotypic and phenotypic coefficient of variation, heritability and genetic gain would give a good understanding of the genetic association between yield and proline, which would help breeders and agronomists improve selection efficiency.

On the contrary, Hanson et al. (1979) suggested that, although proline accumulation is a heritable trait, it is simply a symptom of severe internal water stress and has no practical value in breeding for drought. Serraj and Sinclair (2002) noted that the role of proline in plant stress tolerance is barely understood. However, with the current advances in physiology and biotechnology, the role of free proline accumulation in higher plants under stress is being understood (Kishor et al. 2005; Ashraf and Foolad 2007; Szabados and Savouré (2010) and effort is being made to enhance over production of free proline in plants under drought stress (Kishor et al. 2005; Vendruscolo et al. 2007).

Kishor et al. (1995), Zhu et al. (1998), De Ronde et al. (2004) and Vendruscolo et al. (2007) showed that the over production of γ -pyrroline-5-carboxylate synthetase (P5CS) enzyme

(responsible for proline biosynthesis), in transgenic (introgressed with cDNA *P5CS* gene) tobacco, moth bean, rice, soybean and wheat resulted in increased osmoprotection and tolerance. These studies suggested that cDNA *P5CS* gene resulted in high levels of P5CS enzyme and a 10 to 18-fold increase in proline content, which contributed to both salt and water limited stress tolerance through enhanced biomass production i.e. higher levels of shoot and root weight. Results suggested that proline accumulation shows as a biochemical selection criterion in WLIS tolerance. With the discovery of the cDNA *P5CS* and *P5CR* genes, future research could be directed at introgression of the gene, and studying the effect thereof on yield and quality attributes of soybean under drought stress conditions in multi-location trials.

Accumulation of free proline is intrinsic in plant cells as a response to hyper-osmotic stress (Kishor et al. 2005). Researchers have concentrated investigation of free proline accumulation to leaf samples, with limited studies done on roots, root tips and nodules (Table 2.5). Probably the use of leaves makes the proline assay easy and non-destructive, as only a few leaf samples are taken at a growth stage of choice. Comparative studies of proline accumulation in leaves, roots, root tips and nodules of plants subjected to drought stress suggested slight variation in the levels of proline accumulation in these different plant parts. Generally, there is higher free proline accumulation in the leaf samples than the root samples of drought stressed plants (Irigoyen et al. 1992; Sofo and Dichio 2004). Hence there is a need to further explore this variation in leaves and roots for free proline accumulation in response to drought, proline biosynthesis (and/or pathways) and trigger mechanisms involved in these plant parts.

2.5 Root traits as a selection criterion for drought stress tolerance

Root traits affect tolerance levels of crops to WLIS (Ludlow and Muchow 1990; Turner et al. 2001; Kashiwagi et al. 2005; Vadez et al. 2008). Drought tolerance mechanisms in soybean and related crops are closely associated to the rooting system and/or pattern and its response to drought stress (Taylor et al. 1978; Kaspar et al. 1984; Fenta et al. 2014; Nguyen et al. 2014). The root system of a crop acts as the “first line of defence” in as far as drought stress is concerned (Manavalan et al. 2010; Fenta et al. 2014). This is because the root system is responsible for exploring and acquisition of all the water the plant requires from the soil (Malamy 2005). However, information on the improvement of soybean root systems in terms

of response to drought stress is limited, as opposed to above ground plant characteristics. O'Toole and Bland (1987) attributed this to the root systems' concealment in the soil and variable nature, which complicates observation and experimentation. However there is a growing interest in understanding the genetic variability in root parameters associated with drought stress tolerance and their genetic control with the aim of improving crops' ability to withstand drought (O'Toole and Bland 1987) and, furthermore, to explore and develop different techniques of studying the root system (Zhu et al. 2011).

Previous studies on root traits associated with drought stress tolerance in soybean and related crops are summarised in Table 2.6. Root traits associated with drought stress tolerance can be grouped based on their key strategy to match plant physiological functions to water supply. These are (i) adjusting spatial distribution to enhance soil water exploration, access and uptake, (ii) optimising resource partitioning (between shoot and roots) to enhance soil water acquisition and (iii) special sensitivity for soil water uptake under drought stress.

Roots traits that are associated with drought tolerance by adjusting spatial distribution to enhance soil water exploration and access include total root length (cm), tap root length (cm), root length density (mm mm^{-3} or m mm^{-3}), centre of root length density with depth, root dry matter (g), root fresh weight (g), centre of root dry matter with depth, root weight density (g cm^{-3} or kg m^{-3}), total root surface area (m^2), root branching density and root system architecture (Table 2.6).

Drought tolerant soybean, maize, groundnuts, chickpea and tall fescue genotypes demonstrate quick root elongation rates, deeper root, larger root length and weight densities in deeper soil profiles as opposed to sensitive genotypes (Robertson et al. 1980; Stone and Taylor 1983; Garay and Wilhelm 1983; Kaspar et al. 1984; Mastui and Singhi 2003; Kashiwagi et al. 2005; Benjamin and Nielson 2006; Mastuo et al. 2013; Fenta et al. 2014; Pirnajmedin et al. 2015). Root growth angle (measured as the average angle of divergence of lateral roots) also gives an insight into the rooting habit of a genotype. This has been demonstrated by Fenta et al. (2014). A drought tolerant soybean genotype that showed deeper rooting ability was associated with a wide angle of lateral root divergence (60°).

Table 2.6 Root traits with potential for improving drought tolerance in soybean and related crops

Crop	Root trait	Effect on root drought tolerance traits	Author
Soybean, Maize, Groundnuts, Tall fescue	Root length, tap root length, rooting depth (cm, m)	Adjusting roots for soil water exploration and access	Robertson et al. (1980) Stone and Taylor (1983); Pirnajmedin et al. (2015)
Soybean	Root rate elongation rate	Adjusting roots for soil water exploration and access	Kaspar et al. (1984)
Chickpea, Tall fescue, Soybean	Root length density (mm mm ⁻³ or m mm ⁻³) Centre of root length density with depth	Adjusting root distribution for effective soil water access and uptake per soil volume	Kaspar et al. (1984); Kashiwagi et al. (2005); Mastuo et al. (2013); Pirnajmedin et al. (2015)
Cowpea, Field pea and chick pea	Root dry matter (g), root flesh weight (g) Centre of root dry matter with depth	Adjusting root distribution for effective soil water access and uptake per soil volume	Mastui and Singhi (2003); Benjamin and Nielson (2006)
Soybean, Cowpea, Field pea and chick pea	Root weight density (g cm ⁻³ or kg m ⁻³)	Adjusting root distribution for effective soil water access and uptake per soil volume	Garay and Wilhelm (1983); Mastui and Singhi (2003); Benjamin and Nielson (2006)
Soybean	Root surface area (m ²) or (m ² roots m ⁻² soil surface area)	Adjusting roots for access to soil water and uptake	Fenta et al. (2014)
	Lateral and tap root diameter	Determines plant efficiency to water uptake and transport	Fenta et al. (2014)

Table 2.6 Root traits with potential for improving drought tolerance in soybean and related crops (continued)

Crop	Root trait	Effect on root drought tolerance traits	Author
Maize	Allometry (ratio of root-to-shoot length or dry matter)	Biomass partitioning between root and shoot	Zhan et al. (2015)
Soybean	Root penetration angle	Positioning roots to enhance foraging ability of water	Fenta et al. (2014)
Soybean	Root branching density	Positioning roots to enhance foraging ability of water	Fenta et al. (2014)
Maize	Reduced lateral root branching density	Biomass partitioning between root and shoot	Zhan et al. (2015)
Grape vine	Root hydraulic conductivity and/ or aquaporins activity	Soil water extraction and uptake ability; save limited soil water for later use	Vandeleur et al. (2005); Vandeleur et al. (2009)
Soybean, Tall fescue	Root system architecture (spatial distribution of roots)	Positioning roots to enhance foraging ability of soil water	Hudak and Patterson (1996); Fenta et al. (2014)

Such rooting behaviour enhances drought tolerant genotypes' ability to scavenge and access soil water in deeper soil domains that tend to conserve water in times of drought stress. Hence, extensive and deep rooting systems are recognised as the most important traits for improving drought tolerance (O'Toole and Bland 1987) and can therefore be used as indirect selection criteria to improve plants' tolerance to drought.

Drought tolerant genotypes tend to adjust the partitioning of resources (dry matter) towards the root (allometry) under WLIS conditions. This is manifested as a higher root-to-shoot ratio either in terms of dry matter or root length in soybean (Garay and Wilhelm 1983; Mastuo et al. 2013, Fenta et al. 2014) and maize (Zhan et al. 2015) drought tolerant genotypes, a shift in centre of root length and weight density in relation to water availability in cowpea (Mastui and Singhi 2003) and reduced lateral root branching in soybean and maize (Fenta et al. 2014; Zhan et al. 2015). The principle in shift of allometry favouring the roots (dry matter partitioning towards the roots) and/or reduced lateral root branching, lies in the fact that drought tolerant

genotypes optimise resource allocation and utilisation for each unit of water acquired. Drought tolerant genotypes tend to reduce metabolic costs of soil water exploration to improve the efficiency in acquisition of soil water under drought stress (Mastuo et al. 2013).

Additional root characteristics are being identified for drought tolerance breeding purposes. Promising root traits of soybean and legume crops for drought stress tolerance improvement include those that show adjustment for special biochemical and physiological sensitivities (Sadok and Sinclair 2011). These traits include decrease in root hydraulic conductance (Sadok and Sinclair 2011) and aquaporin activity (Vandeleur et al. 2005; Vandeleur et al. 2009). Sadok and Sinclair (2011) suggested the possibility for existence of hydraulic resistance (water flow restriction) in roots of soybean and wheat tolerant genotypes under drought stress as a way of the plants to use water more sparingly, hence slow wilting ability. Genetic variations are evident in both radial and axial root hydraulic conductance in soybeans (Rincon et al. 2003); however, there is a need to establish a strong link between the reduction in root conductance and slow-wilting genotypes and consequently yield gain. Contribution of aquaporins (major membrane intrinsic proteins) activity as the conduits of passive water flow across root membranes under drought stress is promising (Vandeleur et al. 2005). Genetic variation exists in the aquaporins' response to limited water stress in tolerant and sensitive genotypes (Vandeleur et al. 2009). However, limited information is available as to which aquaporins are active and how they respond and confer tolerance to drought stress in soybeans.

Other putative metabolic root traits that have been proposed to associate with tolerance in soybeans and related crops include osmotic adjustment in root tips, which assist in maintenance of root growth under drought, modification of root wall extension properties, growth sustenance effect of ABA and protection of oxidative damage (Yamaguchi and Sharp 2010; Sadok and Sinclair 2011). However, despite the laborious and complexity in studying metabolic root traits being limiting and they have greater potential in selection for drought tolerance, if found to be readily heritable as other root related traits (Kashiwagi et al. 2005).

Different techniques are used to study the root system. These include simple non-destructive and destructive direct root measurement methods such as deep-pot for seedling root system

phenotyping (Manavalan et al. 2010), trench profile technique (direct measurement for root distribution) (Vepraskas and Hoyt 1988), cone-break method for root density measurements (Böhm 1979), constant-temperature water bath for root extension and elongation rate measurements (Stone and Taylor 1983) and cylinder culture system for root distribution investigation (Kashiwagi et al. 2005). A recently developed method to study the root system architecture is infra-red imaging (Fenta et al. 2014). More advanced technologies include 3D imaging for visualising root systems within their natural soil environment non-invasively through X-ray micro computed tomography (μ CT) (Mairhofer et al. 2012) and magnetic resonance imaging (MRI) techniques (Meartzner et al. 2015). Furthermore, complex root and root-soil processes can also be studied through the use of hydroponics and aeroponic culture systems (Vaughan et al. 2011). Recently a high throughput system involving transparent soil for *in situ* 3D imaging of living plants and root-associated microorganisms in the rhizosphere has been introduced (Downie et al. 2012).

However, the usefulness of each technique lies in its ability to phenotype the roots without damaging the tap and lateral roots and value of the data generated. For methods that use artificial soil or rooting media, it is encouraged that the penetration resistance of such media is fully tested and/or standardised to take into account the plasticity nature of the roots under different rooting media (Manavalan et al. 2010). Furthermore, the cost benefit of the study and method employed must be considered to optimise on cost.

For better utilisation of root traits in drought tolerance agronomical and breeding trials, it is suggested that parameters not be studied in isolation, but be looked at from the physiological and biochemical aspects underlying the mechanism in which drought tolerance or avoidance is promoted in crop plants. Another way of circumventing the difficulty in studying the roots may be to study them at seedling stage and correlate early rooting traits with their drought responses and yield ability (Liu et al. 2017).

2.6 Concluding remarks

Drought stress is a major limiting factor in soybean production. It reduces yield, affects the quantity and quality of seed protein and oil. Soybean yield and yield components are more sensitive to drought stress during flower induction, pod development and seed filling growth

stages. Free proline accumulation and root traits have greater potential as drought tolerance selection criteria in soybeans. Free proline accumulation is a heritable trait. Genotypic variation exists in free proline accumulation between sensitive and tolerant soybean genotypes under drought stress. Potential to enhance the trait in crops through biotechnology exists. The biochemical assay for proline accumulation in plants is simple and relatively cheap and is non-destructive. Root traits associates with drought stress tolerance in soybeans by enhancing access to deeply stored water and optimisation of 'resources' for water exploration and uptake. Several root traits can therefore be used as indirect selection criteria for drought tolerance in soybeans since they associate with plants' ability to escape, avoid and tolerate drought stress. Research on root architecture and is often hampered by the difficulty to observe and study roots. Several techniques have been developed to make root trait studies easier. However, the difficulty and cost in studying plant roots can be off-set by the value of data that is generated. Proline accumulation and root traits therefore can add more value in indirect selection for drought tolerance in soybeans. Understanding the mode of gene action involved in free proline accumulation and root traits are crucial in a breeding programme. Knowledge of the genetic association of these two responses with yield and yield gain under drought stress could assist in improving the selection efficiency for drought stress tolerance in soybean and other crops.

2.7 References

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Chapter 3

Screening soybean genotypes for soil water-limited-induced-stress tolerance using seed mass and proline accumulation

3.1 Introduction

The soybean crop has potential to contribute to South Africa's economy (NAMC 2011; DAFF 2012). This is evident in the increase in demand of soybean based products and raw materials from different industries in the country (NAMC 2011). Despite a record increase in area of production for soybean in the past two decades, net yields have stagnated (DAFF 2015). Among other factors, soil water-limited-induced-stress (WLIS) is the most important abiotic stress factor limiting soybean production in South Africa (Specht et al. 1999; DAFF 2012; Pathan et al. 2014). This is often the case because South Africa is largely a water resource scarce country (Gbetibouo and Hassan 2005); hence the crop experiences soil WLIS at critical periods of the growth cycle.

The land pressure in South Africa forces production of soybeans to extend towards the semi-arid and arid regions of the country; hence crop production relies heavily on supplemental irrigation (DAFF 2012). As such, due to the high costs of irrigation and/or limited water availability (Hensley et al. 2011), there is a great need to improve crop productivity under irrigation. Therefore, genetic improvement for drought tolerance becomes a cost-effective and sustainable approach to deal with soybean yield losses due to soil WLIS conditions (Pathan et al. 2014).

Generally, there is little conscious selection pressure for drought stress in most soybean breeding programmes to deliberately improve drought tolerance (King et al. 2009). As drought is an erratic and unpredictable phenomenon, the objective of most breeders has been to improve the capacity of the plant to tolerate drought stress while maintaining high yield under favourable conditions (Spitters and Schapendonk 1990; Cabello et al. 2013). However, efficient identification of drought tolerant germplasm requires rigorous screening in both favourable and unfavourable environments where drought is common and reduces yield

substantially (Pathan et al. 2014); as well as the use of different intrinsic markers for drought stress tolerance (Manavalan et al. 2009).

Drought tolerance is a complex trait as its mechanisms involve complex interactions among various traits (Mondal et al. 2015). Breeding widely adapted soybean varieties with high grain yield and drought stress tolerance, therefore, requires targeted efforts to integrate a large number of traits with complex inheritance patterns (Reynolds et al. 2012).

Genotypic responses on grain yield and yield components to soil water deficits have been used to screen for drought tolerance in soybeans (Andrian et al. 1991; Foroud et al. 1993; Specht et al. 2001). Yield components that have been used as agro-morphological markers for drought tolerance and/or susceptibility include seed yield, number of seeds and pods per plant, days to maturity, biomass and harvest index (Shadakshari et al. 2014). Further to this, Manavalan et al. (2009) suggested the use of physiological and biochemical traits as markers for drought stress tolerance in soybeans. These include osmotic adjustment (or osmoregulation), i.e. use of proline accumulation in stressed plants (Le Rudulier et al. 1984), stomatal conductance to reduce excessive water loss (Stolf-Moreira et al. 2010), cell turgidity maintenance (Sloane et al. 1990) and reactive oxygen species (ROS) scavenging enzymatic activity of superoxide dismutase, catalase and glutathione peroxidase (Masoumi et al. 2010). The advantage in using physiological and biochemical markers lies in the fact that most of these methods uses rapid calorimetric essays, i.e. proline accumulation, which is simple, quantitative and can be used at different stages of growth of the plant both under field and greenhouse conditions and is non-destructive (Manavalan et al. 2009).

Even though production of soybean has extended to the more arid regions of the country, limited studies have been done in South Africa on the sensitivity of commercial cultivars to drought stress. Furthermore, there is limited knowledge on the effects of drought stress during critical stages on morphological and physiological traits of the commercial soybean cultivars. Hence, the aim of this study was to evaluate eight different soybean genotypes for agronomical and physiological traits that were subjected to different levels of WLIS. The specific objectives of the study were (i) to evaluate seed mass and related agronomical traits in soybean genotypes exposed to different levels of WLIS, (ii) to assess the effect of different

WLIS levels on proline accumulation in soybean genotypes and (iii) to determine the efficacy of using proline accumulation as a physiological marker for drought stress tolerance among commercial soybean genotypes.

3.2 Materials and methods

3.2.1 Plant material, growing conditions and trial design

A pot experiment was conducted in the glasshouse during the 2013/2014 summer season at the University of the Free State, Bloemfontein, South Africa. Eight soybean genotypes, with varying sensitivities to soil WLIS, were used for the study (Table 3.1). These included four breeding lines (BL1, BL2, BL3, BL4) and four commercial cultivars (CV1, CV2, CV3, CV4). Genotypes CV3 and CV4 have previously shown low yield potential in variety trials conducted in drier areas in South Africa and were categorised as sensitive to soil WLIS. Chapmann and Edmeades (1999) and Cerrudo et al. (2018) have also used yielding potential as selection criterion for drought stress tolerance. Drought sensitive genotypes tended to have low yielding potential in the drought stress environments. Seeds were obtained from a PANNAR® SEED (PTY) LTD breeding programme in Greytown, South Africa. Genotypes were coded for confidentiality purposes.

Table 3.1 Soybean genotypes, growth habit and maturity group

Genotype	Growth habit	Maturity group
BL1	Determinate	6.7
BL2	Indeterminate	6.5
BL3	Determinate	6.7
BL4	Indeterminate	7.4
CV1	Determinate	5.6
CV2	Indeterminate	7.0
CV3*	Determinate	5.7
CV4*	Indeterminate	6.2

* Drought sensitive genotypes. BL = breeding line, CV = cultivar.

Plastic planting pots used were 25 cm high, 16 cm in diameter and had a volume of 9 . The pots were filled with 10 kg of air-dried top soil from Kenilworth farm, outside of Bloemfontein.

The soil is loamy aridic usththents (Brainsvlei Amalia 2300). It is reddish brown in colour with a fine sandy texture and with 8-14% clay and 2-4% silt (Soil Classification Working Group 1991). Four seeds per genotype, inoculated with *Bradyrhizobium japonicum* strain WB74, were sown in a pot and seedlings were later thinned to one plant per pot.

At the beginning of the experiment, all pots were water-saturated and allowed to drain freely until there was no change in weight at field capacity (FC). The difference between this weight (FC) and soil dry weight (DW) was used to calculate 100% water holding capacity (WHC) (well-watered). During the first four weeks after planting (four to six-leaf stage), all pots were irrigated and maintained at 90% WHC. Thereafter pots were watered to maintain the desired soil WLIS level [well-watered, moderate (50% WHC) and severe (30% WHC)]. Well-watered pots were weighed every two days and then watered to maintain the full WHC as the difference in weight. Following pot weighing, the stress treated pots were maintained at 50% WHC and 30% WHC, respectively. Plants were applied with micronutrient fertiliser, Hyperfeed (from Hygrotech South Africa), 10 days after emergence (25 g in 10 l of water) and Wonder 3-1-5 (28) SR granule fertiliser (100 g in 10 l of water) and were applied once a week. The greenhouse temperature was controlled at 20°C night and 25°C day temperatures, respectively. Hand weeding and pest control was done when necessary.

The trial was a factorial experiment laid out in a randomised complete block design (RCBD) with three replications. The two factors included were (i) soil WLIS levels (well-watered, moderate and severe) and (ii) genotypes. Each treatment unit consisted of four pots, i.e. four plants per plot.

3.2.2 Agronomical trait measurements

Plant height (cm) and number of nodes per plant were measured at vegetative, flowering and pod development growth stages, respectively. Plant height was measured on the main stem from the soil level to the top of the plant using a tape measure. The number of nodes were counted on the main stem. The following traits were measured at harvest: number and mass (g) of pods per plant, number and mass (g) of seeds per plant and total biomass (g) per plant. Pod and seed mass were respectively determined using a laboratory balance (Mettler PB

3000). Harvest index (HI) was determined as dry seed mass (g) divided by the total above-ground biomass (g) (seed plus shoot).

3.2.3 Extraction and determination of free proline content

At least three fully expanded leaves were harvested at three growth stages (vegetative, flowering and pod development) for all three WLIS levels, weighed and snap freeze-dried in liquid nitrogen. The leaves were kept at -80°C until proline determination. Free proline was extracted and determined using a ninhydrin based method according to Gibon et al. (2000) as modified by Carillo et al. (2011). The freeze-dried leaves were crushed in liquid nitrogen using a mortar and pestle. The material was mixed with an ethanol:water (70:30 v/v) buffer equivalent to 20 times the fresh weight (FW) in volume. The mixture was placed in a 50 ml falcon tube and centrifuged at 12000 g for 5 minutes. After centrifugation, 0.5 ml of the supernatant was added to a reaction mixture of 0.5 ml 1% ninhydrin solution, prepared in glacial acetic acid:water (60:40 v/v) and 0.5 ml ethanol 20% (v/v). After homogenisation of the mixture by vortexing, the mix was heated in the water bath at 95°C for 20 minutes and then centrifuged for 2 minutes at 12000 g. The supernatant was poured into a disposable cuvette and the absorbance was read at 520 nm. The standard curve was prepared using proline standard solution (0.03-0.50 mM). The free proline concentration in the extract was derived using the following equation (Carillo et al. 2011):

$$\text{Proline in } \mu\text{mol g}^{-1} \text{ FW} = (\text{Abs}_{\text{extract}} - \text{blank}) / (\text{slope} * \text{Vol}_{\text{extract}}) / (\text{Vol}_{\text{aliquot}} * 1/\text{FW})$$

Where $\text{Abs}_{\text{extract}}$ is the absorbance determined with the extract, blank (expressed as absorbance) and slope (expressed as absorbance nmol^{-1}) are determined by linear regression, $\text{Vol}_{\text{extract}}$ is the total volume of the extract, $\text{Vol}_{\text{aliquot}}$ is the volume used in the assay, FW (expressed in mg) is the amount of plant material extracted.

3.2.4 Data analysis

Data collected on agronomical traits and free proline content were subjected to analysis of variance (ANOVA). The ANOVA was carried out in order to partition the various sources of variation on agronomical traits and free proline content amongst the soybean genotypes

subjected to varying soil WLIS levels. Means were separated by least significant difference (LSD). Data were analysed using GenStat Release 18 statistical package (VSN International 2015).

3.3 Results

3.3.1 Effects of soil water-limited-induced stress on agronomical traits

Results of the combined ANOVA over three soil WLIS levels and eight genotypes (Table 3.2) showed that WLIS had significant effects on plant height at pod development stage ($P<0.001$), number of nodes per plant at vegetative ($P<0.01$), flowering ($P<0.01$) and pod development growth stages ($P<0.001$) respectively. It also had significant effects on number of pods ($P<0.001$), pod mass ($P<0.001$), seed number and seed mass ($P<0.001$) per plant respectively, biomass ($P<0.001$) and harvest index ($P<0.05$) respectively at harvest. Genotype effects were significant for plant height at pod development stage ($P<0.01$), seed number ($P<0.001$) and seed mass ($P<0.001$) per plant respectively. Genotype x WLIS interaction effects were not significant for any of the agronomical traits measured indicating that genotypes responded similarly to the different WLIS levels.

Soil WLIS reduced plant height, numbers of nodes and pods, pod mass, seed mass, seed number and biomass per plant, respectively (Table 3.3). The effect of soil WLIS was more drastic on the severe treatment compared to the moderate treatment. Severe (49.0 cm) and moderate (60.3 cm) soil WLIS treatments reduced plant height significantly during the pod development growth stage compared to the well-watered treatment (67.3 cm). The number of nodes per plant for plants exposed to severe soil WLIS was significantly lower at all three growth stages (5.1 at vegetative, 11.6 at flowering and 13.0 at pod development, respectively) as compared to the well-watered treatment (6.4, 12.6 and 14.8 for vegetative, flowering and pod development, respectively). Similarly, both severe and moderate soil WLIS reduced the number of pods (34.2 and 58.6) and pod mass (21.6 g and 37.1 g) per plant, respectively compared to the well-watered treatment (67.9 for number of pods and 42.1 g for pod mass per plant, respectively).

Table 3.2 Combined analysis of variance showing mean square values of agronomical traits evaluated across eight soybean genotypes exposed to different soil water-limited-induced-stress levels during three growth stages

Source of variation	Plant height (cm)			Number of nodes plant ⁻¹			Pods plant ⁻¹		Seed plant ⁻¹		Biomass (g)	HI
	Ve	Flr	Pd	Ve	Flr	Pd	Number	Mass (g)	Number	Mass (g)		
Replication	125.5	67.7	124.1	3.4	1.4	2.1	147.8	1309.6	3.9	997.2	6964.2	3270.6
WLIS	37.9	16.9	2040.4***	10.9**	6.1**	23.7***	7288.9***	2733.2***	1308.9***	37645.8***	11074.8***	390.0*
Genotype	45.5	50.2	159.5**	2.5	1.4	3.3	788.0	73.2	39.5***	2759.5***	200.4	183.7
Genotype x WLIS	10.5	14.8	31.1	0.8	1.1	1.2	93.2	21.3	8.5	291.5	184.5	24.1
Residual	25.0	28.8	44.8	1.4	0.1	1.6	138.0	61.3	9.0	567.3	223.1	98.9
CV%	14.6	14.2	11.4	20.7	8.2	9.0	29.9	23.3	17.8	24.1	24.8	33.1

*, **, ***, significant at P<0.05, P<0.01, P<0.001 respectively. WLIS = water-limited-induced stress, Ve = vegetative growth stage, Flr = flowering growth stage, Pd = pod development growth stage, CV = coefficient of variation, HI = harvest index.

Table 3.3 Soil water-limited-induced-stress level mean values of agronomical traits measured on eight soybean genotypes

WLIS	Plant height (cm)			Number of nodes plant ⁻¹			Pods plant ⁻¹		Seeds plant ⁻¹		Biomass (g)	HI
	Ve	Flr	Pd	Ve	Flr	Pd	Number	Mass (g)	Number	Mass (g)		
Well-watered	35.4	38.4	67.3a	6.4a	12.6a	14.8a	67.9a	42.1a	133.1a	23.9a	83.5a	0.27b
Moderate	34.3	38.2	60.3b	5.6b	12.5a	14.6a	58.6b	37.1b	107.4b	18.2b	56.0b	0.35a
Severe	32.9	36.9	49.0c	5.1b	11.6b	13.0b	34.2c	21.6c	55.4c	9.0c	41.2c	0.29b
<i>Mean</i>	<i>34.1</i>	<i>37.9</i>	<i>58.9</i>	<i>5.7</i>	<i>12.2</i>	<i>14.1</i>	<i>53.6</i>	<i>33.6</i>	<i>98.7</i>	<i>16.9</i>	<i>60.3</i>	<i>0.30</i>
LSD _{0.05}	2.9	3.1	1.9	0.7	0.6	0.7	6.8	4.5	13.8	1.7	8.7	0.06

WLIS = water-limited-induced-stress, Ve = vegetative growth stage, Flr = flowering growth stage, Pd = pod development growth stage, HI = harvest index, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

Severe and moderate soil WLIS significantly reduced the number of seeds per plant (55.4 and 107.4, respectively) compared to the well-watered treatment (133.1). The same was observed for seed mass per plant where both moderate (18.2 g) and severe (9.0 g) soil WLIS resulted in significant reductions in seed mass compared to the well-watered treatment (23.9 g). Results further revealed that severe (41.2 g) and moderate (56.0 g) soil WLIS both reduced the biomass significantly compared to the well-watered treatment (83.5 g). The effect of soil WLIS on harvest index was inconsistent. Moderate (0.35) soil WLIS resulted in a significant increase in harvest index compared to the well-watered treatment (0.27) and severe soil WLIS (0.29).

Genotypic main effects mean values for agronomic traits for the eight soybean genotypes have been presented in Table 3.4. Since genotypic main effects were only significant for plant height at pod development, seed number and seed mass (g) at harvest in the ANOVA (Table 3.2), these have traits have were considered for further discussion.

The tolerant genotype BL2 (61.76 cm) and the sensitive genotype CV4 (67.72 cm) attained the tallest height. The other genotypes BL1 (56.67 cm), BL3 (56.04 cm), BL4 (58.44 cm), CV2 (58.85 cm) and CV3 (57.72 cm) were moderately tall. The shortest genotype was CV1 (54.03 cm).

In terms of seed numbers produced per plant, it was genotype CV1 (121.9) that ranked the first. Tolerant genotypes BL2 (117.80) and CV2 (109.20) ranked second. The least ranking genotype in terms of seed numbers produced per plant was genotypes BL1 (70.70) and BL4 (83.5). The genotypes mean values suggested that the sensitive genotype CV4 (19.41 g) had the overall highest yielding potential. This was followed by tolerant genotype CV1 (18.2 g) and CV2 (18.14 g). On the contrary, the least genotype in terms of seed mass production was the tolerant genotype BL1 (12.78 g).

Table 3.4 Genotype mean values of agronomical traits measured on eight soybean genotypes treated at three different soil water-limited-induced-stress levels

Genotype	Plant height (cm)			Number of nodes plant ⁻¹			Pods plant ⁻¹		Seeds plant ⁻¹		Biomass (g)	HI
	Ve	Flr	Pd	Ve	Flr	Pd	Number	Mass (g)	Number	Mass (g)		
BL1	36.45	40.23	56.67bc	6.35	12.42	14.83	38.00	27.54	70.70d	12.78e	60.10	0.24
BL2	33.70	37.01	61.67a	5.61	12.47	14.80	66.90	36.59	117.80ab	17.40abcd	69.90	0.26
BL3	32.81	36.47	56.04bc	6.26	12.41	13.94	49.00	31.68	87.80cd	15.26de	60.70	0.26
BL4	33.12	37.68	58.44bc	4.60	11.74	13.34	44.10	34.06	83.50d	16.31bcd	60.90	0.29
CV1	34.19	37.70	54.03c	5.77	12.19	14.02	60.10	35.06	121.90a	18.24ab	57.40	0.34
CV2	31.79	35.58	58.85bc	5.63	11.60	13.61	54.10	35.52	109.20abc	18.14abc	54.10	0.36
CV3*	32.66	35.75	57.72bc	5.72	12.00	13.56	58.60	33.71	99.20bcd	17.78abcd	56.80	0.35
CV4*	38.53	42.45	67.72a	5.74	12.76	14.76	57.40	34.83	99.30abcd	19.41a	62.10	0.31
Mean	34.15	37.86	58.89	5.71	12.20	14.11	53.50	33.62	98.70	16.41	60.30	0.30
LSD _{0.05}	4.75	5.09	6.35	1.12	0.95	1.21	11.15	7.43	22.60	2.85	14.17	0.94

* Drought sensitive genotypes; Ve = vegetative growth stage, Flr = flowering growth stage, Pd = pod development growth stage, HI = harvest index, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

The lack of genotype by WLIS interaction effects suggested that the genotypes did not differ in their response to WLIS treatments. This suggested that genotypes may have different coping mechanisms to deal with drought stress. Since both tolerant and sensitive genotypes were able to produce acceptable seed mass, a different index on seed mass was used to explain tolerance. An exploratory least mean difference in seed mass (seed mass of the well-watered minus seed mass of the severe WLIS level) was calculated for each genotype and used to effectively distinguish genotypes' relative performance under the varying soil WLIS levels. That is, seed mass was used as an index for tolerance to WLIS of the soybean genotypes.

Results of the least mean difference in seed mass per plant (g) and the ranking of genotypes are presented in Table 3.5. Results showed that genotypes BL1, BL3 and CV2 had the least mean mass differences (12.20 g, 12.32 g and 12.76 g, respectively), indicating that their seed mass was least affected by the severe WLIS level. Genotypes BL1, BL3 and CV2 were therefore the most tolerant genotypes and would have the largest yield potential under soil WLIS conditions. The two drought sensitive genotypes CV3* and CV4*, together with BL2, had the largest mean differences, suggesting that these genotypes produced high seed mass under well-watered conditions and least seed mass under severe soil WLIS, and were, therefore, regarded not tolerant. Yielding potential of genotypes CV3*, CV4 and BL2 are, therefore, drastically affected by severe WLIS.

3.3.2 Effects of soil water-limited-induced stress on proline accumulation

Results from the combined ANOVA over three soil WLIS levels and eight genotypes (Table 3.6) showed that both genotype effects and soil WLIS effects were highly significant ($P < 0.001$) for proline accumulation at the flowering and pod development growth stages, respectively. This suggested that proline accumulation varied among genotypes and also varied among the different soil WLIS levels. However, the interaction effects between WLIS level and genotype were not significant for all three growth stages. This suggested that genotypes responded more or less similarly to the different WLIS levels for each treatment respectively.

Table 3.5 Mean difference in seed mass (g plant⁻¹) of eight soybean genotypes exposed to three different soil water-limited-induced-stress levels

Genotype	Seed mass (g plant ⁻¹)			Rank**
	Well-watered	Severe	Mean difference	
BL1	18.28 ^c	6.08 ^c	12.20	1
BL2	24.38 ^b	8.75 ^{ab}	15.63	7
BL3	19.88 ^c	7.56 ^{bc}	12.32	2
BL4	24.00 ^b	9.14 ^{ab}	14.86	5
CV1	25.41 ^{ab}	10.86 ^{ab}	14.55	4
CV2	24.65 ^b	11.89 ^a	12.76	3
CV3*	24.38 ^b	9.36 ^{ab}	15.02	6
CV4*	27.65 ^a	8.17 ^{bc}	19.48	8
<i>Mean</i>	23.58	8.98	14.62	
LSD _{0.05}	2.58	2.58		

* Drought sensitive genotypes; ** Ranking is based on least mean difference. LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

It should be noted that leaf samples at vegetative growth stage were collected before soil WLIS treatment were imposed, which could be the reason that there were no significant effects for both soil WLIS and the genotype on proline accumulation. The large mean square values (ANOVA) observed for WLIS at flowering and pod development growth stages suggested that soil WLIS contributed more to the variation than genotype effects.

Using genotype means for each WLIS level and growth stage, proline accumulation ($\mu\text{mol g}^{-1}$ FW) in response to soil WLIS is graphically represented in Figure 3.1. In general, with an increase in soil WLIS level, proline accumulates at higher concentrations. For severe soil WLIS, proline accumulated sharply between the vegetative and flowering growth stages (2.60-8.69 $\mu\text{mol g}^{-1}$ FW). Proline concentration was the highest (9.23 $\mu\text{mol g}^{-1}$ FW) at pod development.

Table 3.6 Mean square values for proline accumulation ($\mu\text{mol g}^{-1}$ FW) in response to soil water-limited-induced-stress at three different growth stages

Source of variation	Proline ($\mu\text{mol g}^{-1}$ FW)		
	Ve	Flr	Pd
Replication	0.2613	1.7630	0.1567
WLIS	0.1722	156.1608***	197.0782***
Genotype	0.2586	1.8097***	5.4946***
WLIS x genotype	0.3147	0.1270	1.1757
Residual	0.3040	0.1860	0.7454
CV%	20.8000	7.1000	14.0000
LSD _{0.05}	0.9062	0.7088	0.5020

*, **, ***, significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively. FW = fresh weight, Ve = vegetative growth stage, Flr = flowering growth stage, Pd = pod development growth stage, WLIS = water-limited-induced-stress, CV = Coefficient of variation, LSD = least significant difference.

For moderate soil WLIS, proline accumulated at an increasing rate from the vegetative to flowering stage ($2.88\text{--}5.87 \mu\text{mol g}^{-1}$ FW) but then decreased slightly towards the pod development stage ($5.73 \mu\text{mol g}^{-1}$ FW). In well-watered plants, proline concentrations were the lowest at flowering ($3.60 \mu\text{mol g}^{-1}$ FW) and pod development ($3.55 \mu\text{mol g}^{-1}$ FW) growth stages compared to the other two growth stages.

Genotype mean values for proline accumulation across the different soil WLIS levels and for the three growth stages respectively are presented in Table 3.7. Since genotype main effects were significant for flowering and pod development growth stages in the ANOVA (Table 3.6), these stages were considered for further discussion. For the flowering growth stage, tolerant genotype CV2 ($6.5 \mu\text{mol g}^{-1}$ FW) showed the highest genotypic potential for proline accumulation. This was followed by tolerant genotypes BL4 ($6.32 \mu\text{mol g}^{-1}$ FW), BL2 ($6.30 \mu\text{mol g}^{-1}$ FW) and BL1 ($6.22 \mu\text{mol g}^{-1}$ FW). Least ranked were sensitive were the sensitive genotypes CV4 ($5.99 \mu\text{mol g}^{-1}$ FW), and CV3 ($5.56 \mu\text{mol g}^{-1}$ FW) and tolerant genotype CV1 ($5.20 \mu\text{mol g}^{-1}$ FW).

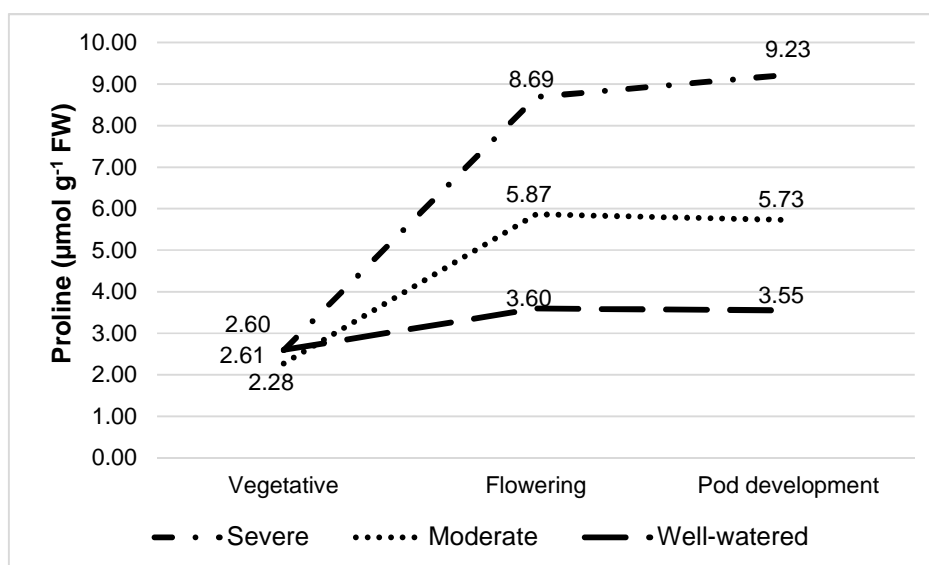


Figure 3.1 Proline accumulation ($\mu\text{mol g}^{-1} \text{FW}$) as related to soil water-limited-induced-stress at different growth stages.

Under pod development, tolerant genotypes BL2 ($7.05 \mu\text{mol g}^{-1} \text{FW}$) and CV2 ($6.99 \mu\text{mol g}^{-1} \text{FW}$). These were followed by another tolerant genotype BL4 ($6.91 \mu\text{mol g}^{-1} \text{FW}$). Least ranking in terms of potential to accumulate proline were the sensitive genotypes CV4 ($5.39 \mu\text{mol g}^{-1} \text{FW}$), CV3 ($5.22 \mu\text{mol g}^{-1} \text{FW}$) and tolerant genotype CV1 ($5.32 \mu\text{mol g}^{-1} \text{FW}$).

3.4 Discussion

Drought stress is a major limitation to yield in rain-fed soybean production areas (Sinclair 2011). Genotypes with increased drought tolerance would improve productivity; however, breeding specifically for drought tolerance is complex, time-consuming and expensive (Pidgeon et al. 2006). In this study, seed mass and related morphological traits, together with proline accumulation, were evaluated under different soil WLIS levels to determine the efficacy of using proline as a physiological marker for soil WLIS among soybean genotypes. This easy, non-destructive and economical method would greatly assist breeding and selection for drought tolerance in South African breeding programmes.

Table 3.7 Mean values for proline accumulation ($\mu\text{mol g}^{-1}$ FW) in soybean genotypes in response to soil water-limited-induced-stress across different growth stages

Genotype	Vegetative	Flowering	Pod development
BL1	2.77	6.22ab	6.36abc
BL2	2.94	6.30ab	7.05a
BL3	2.53	6.34ab	6.13bcd
BL4	2.54	6.32ab	6.91ab
CV1	2.78	5.20d	5.32de
CV2	2.56	6.50a	6.99a
CV3*	2.43	5.56cd	5.22e
CV4*	2.67	5.99b	5.39de
<i>Mean</i>	2.65	6.05	6.17
LSD _{0.05}	0.5232	0.4092	0.819

* Drought sensitive genotypes. LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

Results in the present study have shown that different soil WLIS levels had varying effects on all morphological traits measured. Severe (30%) and moderate (50%) WLIS treatments drastically reduced morphological traits and seed yield components in soybean. Plant height during pod development, number of nodes, number and weight of pods, number and mass of seed and biomass were significantly reduced by moderate and severe soil WLIS, while harvest index increased. In addition, among the three growth stages, pod development was the most critical stage for plant height and number of nodes per plant. Plant height and number of nodes per plant were significantly reduced with both moderate and severe soil WLIS, while no significant reductions in these traits were observed for the vegetative and flowering stages respectively. Akram et al. (2011) found a positive correlation between plant height reduction and number of nodes in response to soil WLIS. Similarly in the present study, such a relation was observed and consequently, less nodes were produced. These morphological traits determines the number of pod bearing nodes and, therefore, have a bearing on the yield performance under soil WLIS (Oz et al. 2009).

In soybean, seed yield potential is mostly driven by pod number and pod weight, which are positively associated with biomass accumulation (Vega et al. 2001). Soybean requires adequate water to achieve maximum yield potential (Westgate and Peterson 1993). The soybean plant at R1 and R2 begins a rapid and steady increase in nutrient and dry weight accumulation (Salvador and Pearce 1995). Water stress at this stage limit the size of the leaves (the photosynthetic factory); in turn affecting the flowering period, number of flowers, number of pods and their weight. Soil WLIS affects active cell division in the young ovules and rapid pod expansion; both processes are sensitive to drought stress (Peterson et al. 1993). Smiciklas et al. (1992) noted that drought stress during flowering and early pod setting increases the probability of pod abortion, and decreased pod setting because of inhibited pod expansion and metabolism. Both moderate and severe soil WLIS were critical for these seed yield components (pod number and pod mass) of soybean plants and significantly reduced values for these traits.

An increase in soil WLIS intensity decreased both the seed number (41.62%) and seed mass (38.08%). Sionit and Kramer (1977) reported that the main reason for reduced seed mass and seed number under soil WLIS is mainly due to the reduction in the assimilate production and

translocation. Since both seed number and seed mass were reduced by the moderate and severe treatments, yield potential also reduced. These results are in agreement to studies conducted by Doss et al. (1974), Eck et al. (1987) and Desclaux et al. (2000). After reducing plant available water to 50% or 30% of the normal conditions, seed yield and components were severely affected (45-88%). The most severe effect of soil WLIS was observed when the soil water deficit was imposed during seed filling (R5-R6) growth stage (Cox and Jolliff 1986; Desclaux et al. 2000; Karam et al. 2005; Dogan and Copur 2007; Shadakshari et al. 2014).

Shoot biomass accumulation is considered an important trait to attain high seed yield in grain legumes (Sernaa et al. 2004). Soil WLIS effects were significant for shoot biomass and resulted in reduced values for this trait. Harvest index was significantly affected by soil WLIS. Under severe drought stress conditions HI was reduced in comparison with moderate stress conditions. However, the well-watered treatment showed a lower HI than both the moderate and severe WLIS treatments. On the other hand, Shadakshari et al. (2014) observed a reduction in HI with an increase in soil WLIS. This could be explained by the plasticity of the soybean genotypes during stress and non-stress conditions. Ludlow and Muchow (1990) described genotypic plasticity as a state in which a genotype matches crop phenology to the expected water supply and this is one of the most important traits for adaptation to different types of drought. Since HI is a function of seed yield, there is a possibility that most genotypes were proportionately able to remobilise their sink towards seed under stress conditions.

Significant genotypic responses for plant height (at pod development growth stage) and number and mass of seeds per plant have been observed. Soil WLIS severely reduced plant height among the genotypes. Plant height has a significant bearing on the yield of soybean (Malik et al. 2007) as it determines the number of nodes and pods. Akram et al. (2011) found a significant and positive correlation between higher yielding ability with taller plants including higher number of branches. In the present study under well-watered conditions, moderate and severe soil WLIS, there were no distinct differences between the tolerant and the sensitive genotypes. This could suggest that plant height on its own may not be a good trait for selection for soil WLIS.

Soybean seed yield is a direct function of seed number and mass per plant (Desclaux et al. 2000). Significant genotypic effects for these traits under soil WLIS underlies this fact. It was evident that both of these traits were drastically reduced by soil WLIS, and this was more

pronounced for the severe WLIS treatment. Results are similar to what Desclaux et al. (2000) and Oya et al. (2004) found. The significant genotypic effects observed for seed number and mass suggests that these traits could be potential traits in selection for soil WLIS. There were varied responses among the known tolerant genotypes from the sensitive ones in terms of seed number and mass. Hence a related index, exploratory mean yield difference, was employed to ascertain the most tolerant genotypes.

In this study, a genotype has been considered drought tolerant when its seed mass was relatively high under severe WLIS conditions. This is because the ability of a genotype to perform reasonably well in drought-stressed environments is paramount for stability of production (Raman et al. 2012). According to Sneller and Dombek (1997) and Oya et al. (2004) a tolerant genotype should exhibit less reduction in yield under WLIS conditions compared to sensitive genotypes. In addition, it is speculated that genotypes with the least mean yield difference have a good remobilisation capacity during stress. Oya et al. (2004) have indicated that soybean can effectively remobilise carbon and nitrogen from leaves and stems to seeds. As such, tolerant genotypes often tend to have a steady seed growth rate even when photosynthetic rate is decreased by drought stress (Westgate et al. 1989).

In this regard, genotypes BL1, BL3 and CV2, which showed less seed mass reduction and ranked high as tolerant genotypes, could also be regarded as genotypes with good remobilisation capacity. Sensitive genotypes CV3* and CV4* ranked least (seventh and eighth) from the tolerant, suggesting that these genotypes, although they yielded comparatively under well-watered and moderate soil WLIS, their seed mass under severe stress conditions were minimally affected; hence least mean difference in mass could be a putative drought tolerance indicator for soybeans. Results corroborate studies by Sloane et al. (1990) and Hida et al. (1995) who were able to discriminate drought tolerant and sensitive soybean genotypes based on least mean differences in yield in America and Japan, respectively.

With the irregularity of drought occurrence in a semi-arid country, field selection for drought tolerance using yield performance of the testing genotypes, can be challenging. Therefore, another assay, determination of free proline concentration in leaves (Borgo et al. 2015), has been proposed to evaluate drought tolerance. This method is non-destructive, easy and has

previously shown to effectively distinguish between drought tolerant and sensitive plants (Kapuya et al. 1985; Van Heerden and De Villiers 1996).

In this study, proline accumulated with an increased WLIS level in all soybean genotypes. However, tolerant soybean genotypes showed greater genotypic potential for proline accumulation than the sensitive ones. Results are in agreement with previous studies by Moussa and Abdel-Aziz (2008), Mafakheri et al. (2010) and Masoumi et al. (2011), where proline accumulated significantly with increased drought stress levels and consequently was associated with drought tolerance in maize, chickpea and soybean, respectively. It is suggested that proline acts in membrane and protein protection against the effects of high concentration of inorganic ions and high temperatures, which could be caused by soil WLIS conditions (Verbruggen and Hermans 2008). Proline acts as an osmoprotectant (among other things) by acting as (i) a compatible osmolyte, (ii) a molecular chaperone stabilising the structure of proteins, (iii) a mechanism to store carbon and nitrogen, (iv) a part of stress signal influencing adaptive responses (v) and to balance cell redox status (Ashraf and Foolad 2007; Verbruggen and Hermans 2008). Studies by Peterson et al. (1993) further proved the osmoprotectant role of proline in pods and flowers of tolerant soybeans under soil WLIS conditions. Proline, acting as a compatible solute, helped in maintenance of turgor pressure in the pods and flowers of the tolerant genotypes, thereby avoiding the deleterious effects of low osmotic potential. Solutes such as proline accumulate because they are able to enter the growing regions but metabolism and subsequent water uptake are restricted (Sharp et al. 1990).

In addition, genotype main effects were significant for free proline accumulation. Genotypes BL4, BL2 and CV2 had high potential for proline accumulation, while the sensitive genotypes CV3* and CV4* had the least genetic potential for proline accumulation across the WLIS level. Hence genotypes with potential for high proline accumulation have the capacity to utilise the limited assimilates that are supplied by the plant in the event of low osmotic potential and are able to maintain growth and set seed under drought stress conditions. Peterson et al. (1993) further suggested that genotypes that are able to maintain growth and set seed under water-deficient conditions, also have the potential to accumulate higher osmoprotectant solutes, including proline, in the stressed cells and contribute to maintenance of growth under soil WLIS.

A comparison was made between mean mass difference and proline accumulation results in order to determine if there is a link between tolerance index and proline accumulation for the genotypes tested. This was done to determine if the tolerance observed for genotypes BL1, BL3 and CV2 (which showed least mean mass differences) could be attributed to proline accumulation. It was noted that genotypes BL1 and BL3, depicted as tolerant through least mean mass difference, ranked low in terms of proline accumulation. This could be an indication of a different coping mechanism (other than proline accumulation) of drought tolerance at play in these two genotypes. However, genotype CV2 ranked third for both mean mass difference and genetic potential for proline accumulation. As a result, it is possible that for CV2, proline accumulation was linked to yielding potential. As indicated, drought stress tolerance in plants is a complex trait and different genotypes could show different mechanisms to cope with drought (Manavalan et al. 2009).

3.5 Conclusions and recommendations

This study highlighted the response of soybean to different levels of soil WLIS and the efficacy of using proline as a physiological marker for drought stress. Results showed that soil WLIS reduces seed mass and number per plant, shoot biomass per plant, number and mass of pods per plant, nodes per plant and plant height. Significant differences exist among soybean genotypes for seed mass response to soil WLIS. The seed mass mean difference i.e. the relative performance of a genotype in a soil WLIS against non-stress environment, has also been proved to be a useful index for soil WLIS tolerance. The study has shown that proline accumulated with an increase in soil WLIS level. The genotypes main effects for potential for proline accumulation was different. However, it was observed that less sensitive and tolerant genotypes showed great potential for proline accumulation than sensitive genotypes. Since the premise of the study was to assess the potential of free proline accumulation as a selection criterion for drought tolerance; three drought-tolerant genotypes (BL1, BL4 and CV2) which showed great genotypic potential to accumulate more free proline and once sensitive (CV3) genotype which showed less genotypic potential to accumulate free proline were advanced for further characterisation of their drought stress tolerance mechanisms at different growth stages both under glasshouse and field conditions.

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Chapter 4

Seedling shoot- and root growth responses among soybean genotypes to drought stress

4.1 Introduction

Soybean is the world's leading economic oilseed crop (Tidke et al. 2015). It is the largest source of vegetable oil and protein in the world (Corner et al. 2004) and a primary source of protein in the livestock feed industry in South Africa (Dlamini et al. 2014). In South Africa, soybean is a small but an important growing component of the agricultural economy (De Beer and Prinsloo 2013). This is evident from the exponential growth in area and amount of production in the last two decades (Dlamini et al. 2014). Like in most parts of the world, soybean production in South Africa is limited by inadequate rainfall and soil water-limited-induced-stress (WLIS), which characterises most production regions (Eck et al. 1987; Kobraei et al. 2011; Dlamini et al. 2014), consequently affecting the livestock industry and food security of the country.

Among other measures being employed to address adverse effects of soil WLIS on soybean, selection for morphological and physiological parameters related to soil WLIS tolerance are at the forefront (Manavalan et al. 2009). Drought tolerant mechanisms in soybean have been closely associated with the rooting system and/or rooting pattern (Taylor et al. 1978; Kaspar et al. 1984; Fenta et al. 2014; Nguyen et al. 2014). The response of root traits to the immediate effects of soil WLIS determines the soybean plants' defence to drought (Manavalan et al. 2010; Fenta et al. 2014). This is because the root system is responsible for exploring and acquisition of all the water the plant requires from the soil (Malamy 2005). Plants sense water shortage around their roots and respond instantaneously by sending chemical signals to shoots to initiate various adaptive responses to soil WLIS (Hossain et al. 2014). As such, root characteristics of soybeans have a major impact on survival and productivity under soil WLIS conditions (Manavalan et al. 2010). Root traits respond to soil WLIS through (i) dehydration escape by earliness, matching phenological development with periods of soil availability and (ii) avoidance by the maintenance of high tissue water potential while maintaining water uptake (Turner et al. 2001; Khan et al. 2010).

Genetic variation for consultative ability and phenotypic plasticity in root growth patterns have been reported in soybean (Manavalan et al. 2011; Mastuo et al. 2013). Root traits that have been studied in relation to plant productivity under soil WLIS conditions include: allometry (Hsiao and Xu 2000; Sadras and Calvino 2000; Maseda and Fernandez 2006), root length, biomass, number and branching (Garay and Wilhelm 1983; Manavalan et al. 2010; Fenta et al. 2014), axial root elongation rate, root penetration angle, root tissue density, root system with depth distribution (Mastui and Singhi 2003; Fenta et al. 2014; Nguyen et al. 2014), number of root tips per root system length, aquaporin activity and root hydraulic conductivity (Liu et al. 2014).

Although root traits have shown strong potential in breeding for drought tolerance in soybean (Manavalan et al. 2009), the laborious and difficult procedure involved to phenotype roots without breaking the tap roots is a big setback (Cosmas et al. 2013). However, a “deep-pot” root screening method, which facilitates removal of the root system with minimal damage and evaluation of the soybean root architecture, has been developed (Manavalan et al. 2010). This screening method facilitates efficient root evaluation at seedling stage. The present study was conducted with the aim of discriminating drought tolerant and –sensitive genotypes through root- and shoot morphology. Specific objectives were to determine responses of seedling shoot- and root length, dry matter and root length density among soybean genotypes grown under soil WLIS using the “deep-pot” method.

4.2 Materials and methods

4.2.1 Plant material and trial site

Glasshouse trials were conducted in duplicate (Trial 1 from 06/09/2014 to 07/10/2014 and Trial 2 from 06/11/2014 to 08/12/2014) at the Department of Soil, Crop and Climate Sciences of the University of the Free State, South Africa during the summer season. The plant material used included three drought tolerant genotypes (BL1, BL4 and CV2) and one susceptible genotype (CV3) obtained from the PANNAR SEED® (PTY) LTD breeding programme (Table 4.1). According to their yield performance under field breeding trials by PANNAR SEED® (PTY) LTD, these genotypes were categorised to be drought tolerant and -sensitive, respectively. The four genotypes were chosen based on their differential genotypic potential for free proline accumulation and root- and shoot morphology at seedling growth stage under soil WLIS were characterised.

Table 4.1 Soybean genotypes used in the study and their levels of drought sensitivity

Genotype	Growth habit	Maturity group	Drought sensitivity
BL1	Determinate	6.7	Tolerant
BL4	Indeterminate	7.4	Tolerant
CV2	Indeterminate	7.0	Tolerant
CV3	Determinate	5.7	Sensitive

Glasshouse trials were conducted in deep transparent polythene bags (10 cm diameter and 110 cm length) according to Manavalan et al. (2010) with modifications. Polythene bags were placed in polyvinyl chloride (PVC) tubes (10 cm diameter and 100 cm length) for support and the pots were filled with soil, leaving a 5 cm space at the top (Figure 4.1). Soil used was an aridic ustothents soil (Bainsvlei 2300) with a reddish brown colour and a fine sandy texture, and contained 8-14% clay and 2-4% silt (Soil Classification Working Group 1991). Pots containing the polythene bags were fully saturated with water and left to drain. After seven days, three seeds from each genotype were sown in the pots containing the polythene bags.



Figure 4.1 “Deep-pot” system used for screening shoot- and root morphology of four soybean seedling genotypes grown under water-limited-induced-stress conditions for 21 days from sowing.

4.2.2 Experimental layout and data collection

Pots were laid out in a randomised complete block design (RCBD) with six replications. After germination, seedlings were thinned to one per pot. For the first 12 days after sowing, plants were watered daily with 100 ml of water to maintain the soil at field capacity to ensure strong seedlings; thereafter, soil WLIS was induced by irrigating 100 ml every third day. The soil WLIS was determined using the procedure described in Section 3.2. Twenty-one days after emergence, shoot length (SL) (cm), was measured from the soil contact point to the tip of the plant and number of trifoliate leaves was counted. Shoot dry weight (biomass) (g) was recorded after drying the shoots in the glasshouse at room temperature for 72 hours.

For root measurements, the polythene bags were carefully taken from the PVC tubes and cut longitudinally in order to safely isolate the whole root system from the soil. Tap root length (TRL) was measured on intact roots from the soil contact point to the tip. Total root dry weight (root biomass) (g) was recorded after separating roots from the soil by washing the samples on a 0.5 mm sieve. The root samples were then dried at room temperature, weighed and root biomass determined. After recording tap root length, shoot and root biomass, all roots including tap root and primary roots were cut into 10 cm sections, corresponding to the actual depths from the PVC tubes. For root length density, root samples in each of the 10 cm sections were counted using an infra-red root counter and were converted into root length per soil layer using a standard curve generated from standard samples and actual root length. Mass of the total root per layer was used to calculate the root weight density per layer.

4.2.3 Data analysis

Data were statistically analysed using GenStat Release 18 statistical package (VSN International 2015). A combined analysis of variance (ANOVA) was conducted across the duplicated trials on the data collected in order to partition the different sources of variation. Means were separated using least significant difference (LSD) test.

4.3 Results

4.3.1 Responses of shoot- and root traits

Significant genotype differences were observed for shoot length ($p < 0.01$), number of leaves ($p < 0.01$), tap root length ($p < 0.01$) and root-to-shoot length ratio (TRL/SL) ($p < 0.05$) (Table 4.2). Trial effects were significant for shoot and root dry weight ($p < 0.01$). No significant genotype

by treatment (GxT) interaction effects were observed for any of the traits measured (Table 4.2). This suggested that genotypes responded the same between the two trials for all traits studied. Tolerant genotypes BL1 and BL4 grouped together for shoot length (both large), while the tolerant genotype, CV2, grouped with the sensitive genotype CV3. This, together with shoot dry weight (non-significant genotype effects), indicated that the sensitive genotype CV3 generally showed a good shoot biomass and was not different from the tolerant genotypes, especially BL4 and CV2 (Table 4.3).

Table 4.2 Combined analysis of variance showing mean square values for shoot- and root traits of soybean seedlings under water-limited-induced stress at 21 days after sowing

Source of variation	Shoot length (cm)	No of trifoliate leaves	Shoot dry weight (g)	Tap root length (cm)	Root dry weight (g)	TRL/SL	RDW/SDW
Replication	2.38	1.02	0.07	70.70	0.03	0.39	0.01
Trial (T)	1.02	1.02	1.56**	78.70	0.51**	0.22	0.01
Genotype (G)	40.34**	7.74**	0.11	1552.60**	0.02	4.76*	0.02
GxT	12.56	1.74	0.05	53.50	0.04	1.53	0.02
Residual	10.80	1.07	0.13	170.50	0.02	1.28	0.01
CV%	18.80	26.80	23.70	16.30	23.60	24.00	24.70

*, **, significant at $P < 0.05$, $P < 0.01$, respectively. CV = coefficient of variation, TRL/SL = tap root length to shoot length ratio, RDW/SDW = root dry weight to shoot dry weight ratio.

Table 4.3 Combined mean values for shoot- and root traits of soybean seedlings under water-limited-induced stress conditions at 21 days after sowing

Source of variation	Shoot length (cm)	No of trifoliate leaves	Shoot dry weight (g)	Tap root length (cm)	Root dry weight (g)	TRL/SL	RDW/SDW
BL1	19.58 ^a	4.42 ^a	1.55	80.10 ^b	0.60	4.22b ^c	0.40
BL4	18.12 ^{ab}	3.08 ^b	1.59	95.50 ^a	0.69	5.37 ^a	0.45
CV2	15.25 ^c	3.25 ^b	1.37	75.30 ^{bc}	0.67	5.13 ^{ab}	0.49
CV3*	16.96 ^{bc}	4.67 ^a	1.50	68.80 ^c	0.67	4.13 ^c	0.45
Mean	17.33	3.85	1.50	79.90	0.66	4.71	0.45
LSD _{0.05}	2.72	0.86	0.29	10.82	0.13	0.94	0.09

* Drought sensitive genotype. TRL/SL = tap root length to shoot length ratio, RDW/SDW = root dry weight to shoot dry weight ratio. Means followed by the same letter of the alphabet in the column are not significantly different.

The three tolerant genotypes (BL1, BL4 and CV2) ranked in the top three positions for both tap root length and TRL/SL ratio, while the drought sensitive genotype (CV3) ranked last. This suggested that the drought sensitive genotype developed a shorter tap root (as indicated in Figure 4.2) or had a reduced root elongation rate compared to the tolerant genotypes due to the soil WLIS. In Figure 4.2 it is evident that the three tolerant genotypes had longer root systems than the sensitive genotype (CV3).



Figure 4.2 Root lengths of four soybean seedling genotypes with CV3 as the sensitive genotype under soil water-limited-induced-stress conditions at 21 days after sowing.

4.3.2 Response in root architecture

The root architecture system under soil WLIS was investigated by determining the distribution of the root length density (cm cm^{-3}) and dry matter (root weight density) (mg cm^{-3}) through the soil profile (Figures 4.3 and 4.4). Results showed that the root systems of all four cultivars were mostly contained in the top 0-40 cm soil profile. There were no marked differences in root distribution among the tolerant genotypes (BL1, BL4 and CV2). The tolerant genotypes' roots explored the whole soil profile to the deepest part.

On the other hand, it was evident that the sensitive genotype (CV3) had the same root distribution (root length density and dry matter) like the tolerant genotypes in the top 0-90 cm soil profile. However, the sensitive genotype's roots could not explore the deeper profile -

beyond the 90 cm depth mark. This suggested that the tolerant genotypes, unlike the sensitive one, could endure the soil WLIS by exploring moisture trapped in the deep soil profiles.

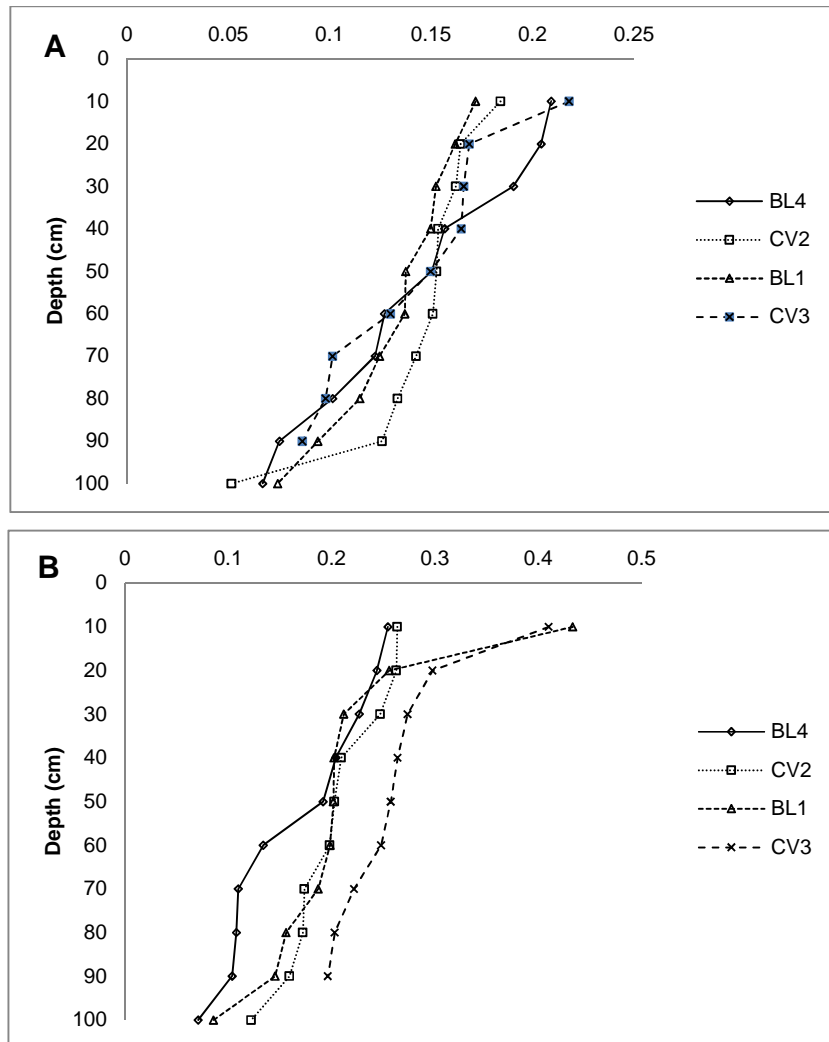


Figure 4.3 Root length density (cm cm⁻³) distribution with depth for four soybean seedlings grown under soil water-limited-induced stress conditions for duplicate trials (A) Trial 1 and (B) Trial 2.

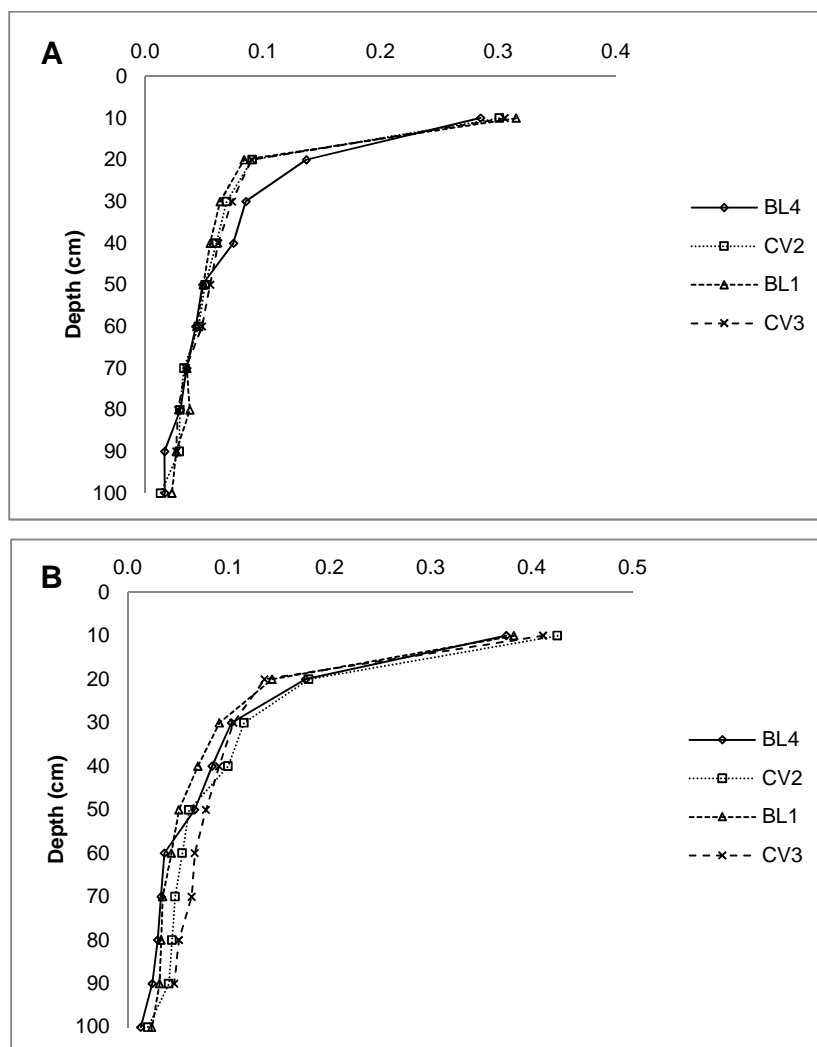


Figure 4.4 Root weight density (mg cm^{-3}) distribution with depth for four soybean seedlings grown under soil water-limited-induced stress conditions for duplicate trials (A) Trial 1 and (B) Trial 2.

4.4 Discussion

Drought stress tended to increase biomass partitioning to the roots. This was demonstrated by the increase in the tap root length to shoot length ratio (TRL/SL) especially among the tolerant soybean genotypes. Similar trends were reported by Manavalan et al. (2009) who suggested that TRL/SL determines the effective proportion of the roots supporting the above ground shoots. This is significant for survival under soil WLIS conditions. The large tap root length to shoot length ratio observed for the drought tolerant genotypes might have translated to the ability of the tolerant cultivars to access and get more edaphic resources per unit of the

above ground shoot (Beebe et al. 2013; Okogbenin et al. 2013). A large tap root length to shoot length ratio suggests a large root surface area per unit of shoot length and this significantly increases the capacity of the tolerant genotypes to efficiently absorb and utilise soil water per unit dry matter (Hossain et al. 2014). In addition, the drought sensitive soybean genotype indicated to have a lower ability to penetrate the deeper soil.

The seedling genotypic variation observed between drought tolerant and –sensitive genotypes for root and shoot traits, in response to soil WLIS, was in agreement with results of Creelman et al. (1990), Hsiao and Xu (2000), Fenta et al. (2014) and Hossain et al. (2014). Drought tolerant soybean genotypes were associated with a deep-rooting phenotype and a large root-to-shoot ratio (length and/or mass), unlike the drought sensitive soybean genotypes. Thus, drought tolerant genotypes tend to demonstrate a phenomenon called “balanced growth”, whereby plants respond to drought by stimulating or maintaining root growth while effectively reducing shoot growth (Bloom et al. 1985). Increases in root versus shoot growth under drought conditions enhance the genotype’s drought coping ability due to increased root-to-leaf surface ratio, continued production of new root tips, and enhancement of plant capacity for acquiring water to support existing shoots (Cosmas et al. 2013). The observed shift in allometry under drought stress and the ability of the tolerant genotypes to grow roots according to the distribution of available soil water, thus, increase the productivity of tolerant genotypes under soil WLIS conditions. This has been attributed to the action of ethylene and abscisic acid in the tolerant soybean genotypes (Sharp and LeNoble 2002; Tseng et al. 2013) and is triggered by soil WLIS conditions.

4.5 Conclusions and recommendations

Soybean genotypes showed varying seedling shoot- and root morphology in response to soil WLIS conditions. The tolerant and sensitive genotypes did not differ much for shoot- and root biomass but significant differences were observed for shoot length, tap root length and tap root length to shoot length ratio. Drought tolerant genotypes showed deep rooting ability and larger root-to-shoot ratios compared to the drought sensitive genotype. Consequently, the drought tolerant genotypes increased biomass partitioning to the roots as a coping mechanism to soil WLIS. The “deep-pot” system was effectively used to phenotype roots of soybean seedlings in a non-destructive manner. The system showed potential to differentiate between drought tolerant and -sensitive genotypes at seedling growth stage. However, in future studies

more genotypes and more root traits need to be analysed in order to efficiently classify genotypes as drought tolerant or drought sensitive.

4.6 References

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Chapter 5

Associations among agronomical traits, drought tolerance indices and proline accumulation in selection for drought tolerance in soybean

5.1 Introduction

Soil water-limited-induced-stress (WLIS) is one of the most damaging abiotic stresses that affects agriculture (Farshadfar et al. 2013). It has an impact on yield and yield stability of soybeans and acts simultaneously on many traits leading to a drastic decrease in yield (Ludlow and Muchow 1990; Dogan et al. 2007). The effects of soil WLIS on growth and yield of plants are dependent upon the species and variety (Çakir 2004). Doorenbos and Kassam (1979) reported that depending on varietal characteristics, soybeans use about 450-700 mm of water throughout the growing period.

The sensitivity of a plant to drought stress also varies by developmental stage (Doorenbos and Kassam 1979). Researchers have reported different critical periods for soil WLIS in soybeans. The most critical developmental growth stage for soil WLIS in soybean has been reported to be the reproductive stage (Doorenbos and Kassam 1979; Dogan et al. 2007). Foroud et al. (1993) found that soybeans are more susceptible to soil WLIS at R1 (beginning of flowering) and R5 (beginning of seed setting) stages. Soil WLIS imposed throughout the plant's growth cycle reduces vegetative growth and affects flowering and seed yield (Hodges and Heatherly 1983). Dogan et al. (2007) suggested that soybean is most sensitive to soil WLIS during seed-filling, followed by flowering and the vegetative stage. On the other hand, Meckel et al. (1984) indicated that soil WLIS during seed-fill stage, shortens the seed-filling stage and consequently lowers the yield.

Despite numerous studies being conducted on the crop's response to soil WLIS, the use of different definitions of soil water deficit stress makes replications and validation of such studies within a crop difficult, more so in different crops (Mastrorilli et al. 1995). As such, there is a need to properly characterise drought stress and the growth stage, at which the plant's response to stress is being investigated. Katerji et al. (1993) suggested that an accurate determination of a sensitive growth stage is possible only if the method used causes a water deficit of the same intensity at all the analysed growth stages.

Breeding for drought stress tolerance is therefore complicated by the lack of reproducible screening techniques and the inability to routinely create defined and repeatable soil WLIS conditions (Manavalan et al. 2009; Talebi et al. 2009), especially under field conditions. Loss of yield is the main concern of plant breeders; hence, the emphasis is on direct selection for yield performance under soil WLIS conditions (Manavalan et al. 2009). Achieving a genetic increase in yield under drought stressed conditions has been a difficult challenge for plant breeders as better progress has been achieved in favourable environments (Richards et al. 2002). This is attributed to the low heritability of seed yield and the complexity of genotype by environment (GxE) interaction, which limit the development of cultivars tolerant to soil WLIS stress.

Several yield-based tolerance and/or susceptibility indices, which are based on mathematical relationships between yield under non-WLIS conditions and WLIS conditions, have been used to characterise the behaviour of genotypes in stress and non-stress conditions, and to screen for soil WLIS tolerant genotypes (Mitra 2001). These indices provide a measure of soil WLIS based on yield loss under stress conditions in comparison to non-stress conditions (Mitra 2001). In addition to the yield-based tolerance indices, proline accumulation in genotypes under stress conditions has also been proposed as a potential selection criterion for soil WLIS tolerance in soybean (Manavalan et al. 2009). However, to better understand the data structure and relations of multiple traits, like the different drought stress indices and yield potential under soil WLIS conditions, the use of multivariate statistical analysis, such as principal component analysis (PCA), factor analysis and correlations, is encouraged (Janmohammadi et al. 2014). This would help bring selection efficiency in soybean drought stress tolerance breeding programmes.

A lysimeter glasshouse experiment was, therefore, conducted with the aim of evaluating and identifying efficient selection criteria for tolerance to soil WLIS in soybean. Specific objectives of this study were: (i) to determine the effects of soil WLIS, subjected at three growth stages on morphological traits of soybean genotypes, (ii) to determine the effects of soil WLIS, subjected at three growth stages on proline content of soybean genotypes, (iii) compare the capacity of the different tolerance indices to identify genotypes having high yield potential under both WLIS and non-stress conditions, and (iv) to determine associations among yield

potential, the tolerance indices and proline accumulation under WLIS and non-stress conditions.

5.2. Materials and methods

5.2.1 Plant material and trial design

A replicated glasshouse experiment was conducted in the weighing lysimeter unit of the Department of Soil, Crop and Climate Sciences, at the University of the Free State (UFS) during 2014 and 2015. The lysimeters were filled with known masses of air-dried, A- and B-horizon of Bainsvlei Amalia 2300 soil. Filter sand was placed at the bottom of cylinders before soil was packed. A mulch of Styrofoam was placed on the soil surface to prevent evaporation (Figure 5.1).

Plant material used included three drought tolerant genotypes (BL1, BL4 and CV2) and one susceptible genotype (CV3) obtained from the PANNAR SEED® (PTY) LTD breeding programme (Table 5.1). According to their yield performance under field breeding trials by PANNAR SEED® (PTY) LTD, these genotypes were categorised to be drought tolerant and -sensitive, respectively. The four genotypes were chosen (out of the eight) based on their differential genotypic potential for free proline accumulation (Chapter 3), had different modes of soil WLIS coping mechanisms and the number of genotypes to be used were limited by the number of lysimeters available.

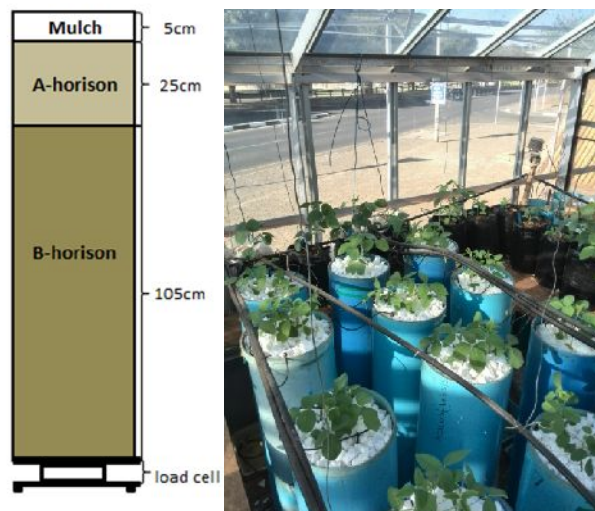


Figure 5.1 Schematic diagram and actual weighing lysimeter at University of the Free State.

Table 5.1 Soybean genotypes used in the study and their drought sensitivity levels

Genotype	Growth habit	Maturity group	Drought sensitivity
BL1	Determinate	6.7	Tolerant
BL4	Indeterminate	7.4	Tolerant
CV2	Indeterminate	7.0	Tolerant
CV3	Determinate	5.7	Sensitive

The trial was a factorial experiment laid out in a randomized complete block design (RCBD) with two factors and three replications. Factor one comprised of four water treatments; plants subjected to soil WLIS respectively at (i) vegetative, (ii) flowering and (iii) pod-development stage, and (iv) a non-WLIS control treatment (well-watered throughout crop growth). WLIS was initiated by terminating irrigation for ± 26 days, depending on the length of the growth period under consideration. Factor two included four soybean genotypes with known drought sensitivities from the previous screening experiment (Chapter 3). Two seeds of each of the genotypes were sown directly in a lysimeter. Soil WLIS treatments in the lysimeters were initiated when 50% of the experimental units had reached the respective growth stages of vegetative, flowering and pod-development as described by Fehr et al. (1971).

5.2.2 Agronomical trait measurements

Agronomical traits were measured at harvest (on mature plants) and included; top pod height (cm), bottom pod height (cm), number of primary branches per plant, number of nodes per plant, number of pods per plant, number of aborted pods per plant, number seeds per plant, seed mass per plant (g), 100-seed mass (g), biomass per plant (g) and harvest index (HI) per plant. Harvest index (HI) was determined as dry seed mass (g) divided by the total above-ground biomass (g) (seed plus shoot).

5.2.3 Extraction and determination of free proline content

Proline content was determined using leaves sampled from plants subjected to all treatments. A ninhydrin-based extraction method, as described by Gibon et al. (2000) and modified by Carillo et al. (2011), was followed. The method used was described in Chapter 3, section 3.2.3.

5.2.4 Calculation of drought tolerance indices

Seven drought tolerance indices with relative change in proline content (Table 5.2) were used to compare genotypes based on their tolerance to soil WLIS. The drought tolerance indices were calculated using relationships described in Table 5.2; where Y_p = seed mass under non-WLIS conditions, Y_s = seed mass under soil WLIS (during pod development), \bar{p} and \bar{s} = mean mass under non-WLIS and soil WLIS conditions, respectively, Prl_p = proline content under non-WLIS conditions, Prl_s = proline content under soil WLIS conditions.

Table 5.2 Yield-based drought selection indices and proline accumulation used

Selection criteria	Formula	Reference
Mean productivity (MP)	$MP = (Y_p + Y_s)/2$	Hossain et al. (1990)
Stress susceptibility index (SSI)	$SSI = [1 - (Y_s/Y_p)]/[1 - (\bar{s}/\bar{p})]$	Fischer and Maurer (1978)
Stress tolerance (TOL)	$TOL = Y_p - Y_s$	Hossain et al. (1990)
Stress tolerance index (STI)	$STI = (Y_p \times Y_s)/(\bar{p})^2$	Fernandez (1992)
Geometric mean productivity (GMP)	$GMP = (Y_p \times Y_s)^{0.5}$	Fernandez (1992)
Yield index (YI)	$YI = Y_s/\bar{s}$	Lin et al. (1986); Gavuzzi et al. (1997)
Yield stability index (YSI)	$YSI = Y_s/Y_p$	Bousslama and Schapaugh (1984)
Relative change in proline (ΔPrl)	$Prl = Prl_p - Prl_s$	

Y_p = seed mass under non-WLIS conditions, Y_s = seed mass under WLIS, \bar{p} and \bar{s} = mean mass under non-WLIS and WLIS conditions, respectively, Prl_p = proline content under non-WLIS conditions, Prl_s = proline content under WLIS conditions.

5.2.5 Associations among seed yield, proline and drought tolerance indices

Correlation coefficients between Y_p , Y_s , Prl_p , Prl_s and the other quantitative indices of drought tolerance were calculated. Principal component analysis (PCA), based on the correlation matrix, was used to further understand the relationships among the tolerance indices and selection criteria.

5.2.6 Data analysis

Data on morphological traits and free proline concentrations were subjected to analysis of variance (ANOVA) in order to partition the various sources in the data structure. Means were separated by least significant difference (LSD). Correlation analysis and PCA were also

performed to delineate the pattern of the association and relations existing among seed mass, proline accumulation and the different drought tolerant indices. It has been demonstrated by Farshadfar et al. (2013) that correlation analysis between yield and the tolerance indices can be a good criterion for screening the best genotypes and indices to be used. Data were analysed using GenStat Release 18 statistical package (VSN International 2015).

5.3 Results

5.3.1 Effects of soil water-limited-induced-stress on agronomical traits

Responses of soybean genotypes to soil WLIS at different growth stages are presented in Table 5.3. Results suggested significant trial effects for height of the top ($P<0.001$) and bottom pods (cm) ($P<0.05$), number of branches per plant ($P<0.001$), number of nodes per plant ($P<0.01$), number of pods per plant ($P<0.01$), number of aborted pods per plant ($P<0.001$), seed mass (g) per plant ($P<0.001$) and plant biomass (g) ($P<0.01$). Significant growth stage effects were observed for number of nodes per plant ($P<0.01$), number of pods per plant ($P<0.001$), number of aborted pods per plant ($P<0.05$), seed mass (g) per plant ($P<0.001$), 100-seed mass (g) ($P<0.001$), plant biomass (g) ($P<0.05$) and harvest index (%) ($P<0.01$). Genotype (G) effects were significant for height of the top pod (cm) ($P<0.001$), number of aborted pods per plant ($P<0.001$) and seed mass (g) per plant ($P<0.001$). There were significant trial by growth stage interaction (TxGS) effects for number of aborted pods per plant ($P<0.01$) and 100-seed mass (g) ($P<0.05$) as well as significant trial by growth stage by genotype interaction (TxGSxG) effects for height of the bottom pod (cm) ($P<0.05$).

The mean values for agronomical traits' responses to soil WLIS at various growth stages are presented in Table 5.4. Results indicated that pod development and flowering growth stages were the most sensitive to soil WLIS, as the yield component (number of seeds per plant, seed mass per plant, 100-seed mass and biomass) mean values were lower for these two growth stages. Soil WLIS imposed at both flowering and the vegetative growth stage significantly reduced number of pods per plant, seed mass per plant and harvest index, respectively compared to the control.

Table 5.3 Mean square values for agronomical traits of four soybean genotypes subjected to soil water-limited-induced-stress at three different growth stages

Source of variation	Pod height		Branches plant ⁻¹	Nodes plant ⁻¹	Pods plant ⁻¹	Aborted pods plant ⁻¹	Seeds plant ⁻¹		100- seed mass	Biomass (g)	HI
	Top (cm)	Bottom (cm)	Number	Number	Number	Number	Number	Mass (g)	(g)		
Replication	993.4	24.8	3.5	1489.0	1489.0	64.4	13802.0	1009.6	14.1	1635.0	1.2
Trial (T)	35766.8***	96.0*	42.0***	23830.0**	23830.0**	2860.2***	159021.0	7234.1***	9.4	45034.0**	0.4
Growth stage (GS)	903.4	3.7	3.0	18683.0**	18683.0***	868.5*	118668.0	5453.7***	53.5***	11751.0*	5.9**
Genotype (G)	5387.2***	15.7	6.9	6125.0	6125.0	1892.2***	58566.0	2632.0***	21.6	8229.0	2.9
TxGS	301.4	42.3	5.7	3858.0	3858.0	701.5**	13423.0	95.8	35.2*	349.0	1.1
TxG	378.3	2.2	7.0	3125.0	3125.0	226.3	8565.0	375.4	15.8	1873.0	1.4
GSxG	786.2	23.2	1.3	1819.0	1819.0	253.3	11194.0	395.5	10.7	1233.0	0.9
TxGSxG	214.5	10.2*	2.2	1817.0	1817.0	125.9	8395.0	393.0	7.1	1664.0	0.5
Residual	684.5	15.1	3.1	2690.0	2690.0	215.4	10041.0	421.2	8.7	4114.0	1.1
CV%	23.2	26.4	21.1	34.9	34.9.0	89.4	39.4	42.0	15.9	41.1	34.1

*, **, ***, significant at P<0.05, P<0.01, P<0.001 respectively. HI = harvest index, CV% = coefficient of variation.

Table 5.4 Growth stage mean values for agronomical traits measured on four soybean genotypes subjected to soil water-limited-induced-stress

Growth stage	Pod height		Branches plant ⁻¹	Nodes plant ⁻¹	Pods plant ⁻¹	Aborted pods plant ⁻¹	Seeds plant ⁻¹		100-seed mass (g)	Biomass (g)	HI
	Top (cm)	Bottom (cm)	Number	Number	Number	Number	Number	Mass (g)			
Pod development	129.5	16.08	8.1	23.29	146.7b	16.9a	183.0	40.8c	16.50c	142.2b	0.29c
Flowering	120.1	14.4	9.1	22.8	140.3b	11.2b	223.0	45.0c	18.7ab	189.0a	0.25c
Vegetative	139.0	16.8	8.8	23.0	169.2b	12.8ab	225.0	61.5b	20.0a	180.0a	0.33b
Well-watered	144.8	15.6	10.0	23.6	204.2a	8.8b	348.0	99.9a	19.2ab	207.6a	0.50a
<i>Mean</i>	<i>133.3</i>	<i>15.7</i>	<i>9.0</i>	<i>23.2</i>	<i>165.1</i>	<i>12.5</i>	<i>244.0</i>	<i>61.8</i>	<i>18.6</i>	<i>179.7</i>	<i>0.34</i>
LSD _{0.05}	7.6	2.2	1.0	1.9	29.9	8.5	57.8	11.8	1.7	37.0	0.03

* Drought sensitive genotype. HI = harvest index, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

Significant genotype effects of soil WLIS imposed at the different growth stages are shown in Table 5.5. Significant differences between genotypes only occurred for top pod height, the number of aborted pods per plant and seed mass per plant. The tolerant genotype CV2 ranked in the top position with the highest mean values respectively for top pod height (135.2 cm and it was significantly higher than the rest of the genotypes), number of nodes per plant (26.8), number of pods per plant (169.5), number of seeds per plant (326), seed mass per plant (63.1 g, and it was significantly higher than the rest of the genotypes) plant biomass (181.9 g) and harvest index (0.36). Tolerant genotype (CV2) had the lowest number of aborted pods per plant (9.4) under soil WLIS conditions. CV2, thus, showed the best yield potential under soil WLIS conditions compared to BL2 and BL4 (Table 5.5).

The sensitive genotype CV3 ranked in the bottom position with the lowest mean values, respectively for top pod height (103.0 cm), number of nodes per plant (20.6), number of seeds per plant (217), seed mass per plant (38.0 g), 100-seed mass (17.5 g), plant biomass (138.2 g) and harvest index (0.28) (Table 5.5). CV3, therefore, showed the poorest yield potential under soil WLIS conditions compared to the tolerant genotypes. However, this genotype had the highest number of branches per plant (8.9). CV3 was not significantly different from BL2 and BL4 for most traits (except for number of aborted pods per plant). In addition, there was no clear distinction between the two tolerant genotypes, BL2 and BL4, for all traits (except for number of aborted pods per plant). Genotype BL2 had the highest number of aborted pods per plant (29.3) and was significantly higher than all other genotypes. This showed that, as a consequence to soil WLIS, BL2 resorted to pod abortion as a coping mechanism.

The combined interaction effects of soil WLIS on seed yield components at the different growth stages are presented in Table 5.6. Results indicated that pod development and flowering growth stages were the most sensitive to soil WLIS, as the yield component mean values were the lowest for these two growth stages. It was evident that 100-seed mass, seed mass per plant and seed number per plant had the lowest mean values for soil WLIS at pod development growth stage (16.5 g, 32.3 g and 183, respectively). This was followed by the flowering growth stage with mean values of 18.68 g, 41.7 g and 223 for 100-seed mass, seed mass per plant and seed number per plant, respectively. However, the growth stage by genotype (GSxG) interaction effect was not significant; thus, genotypes responded the same across the different growth stages.

Table 5.5 Genotype mean values for agronomical traits measured on four soybean genotypes subjected to soil water-limited-induced-stress during three growth stages

Genotype	Pod height		Branches plant ⁻¹	Nodes plant ⁻¹	Pods plant ⁻¹	Aborted pods plant ⁻¹	Seeds plant ⁻¹		100-seed mass (g)	Biomass (g)	HI
	Top (cm)	Bottom (cm)	Number	Number	Number	Number	Number	Mass (g)			
BL2	108.3b	14.2	7.94	20.71	152.9	29.3a	235	48.2b	19.2	149.8	0.32
BL4	105.4b	14.8	7.83	22.17	134.6	12.0b	257	46.0b	19.6	154.1	0.30
CV2	135.2a	14.1	8.67	26.77	169.5	9.4b	326	63.1a	18.1	181.9	0.36
CV3*	103.0b	15.6	8.92	20.60	137.9	15.1b	217	38.0b	17.5	138.2	0.28
<i>Mean</i>	<i>113.0</i>	<i>14.7</i>	<i>8.34</i>	<i>22.56</i>	<i>148.7</i>	<i>16.4</i>	<i>25.8</i>	<i>48.8</i>	<i>18.6</i>	<i>156.0</i>	<i>0.31</i>
LSD _{0.05}	15.10	2.240	1.016	1.914	29.93	8.47	57.8	11.84	1.71	18.51	0.62

* Drought sensitive cultivar. HI% = harvest index, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

Table 5.6 Combined interaction effects of soil water-limited-induces stress at different growth stages on seed yield components

Growth stage	100-seed mass (g)					Seed mass plant ⁻¹ (g)					Number of seeds plant ⁻¹				
	BL2	BL4	CV2	CV3*	Mean	BL2	BL4	CV2	CV3*	Mean	BL2	BL4	CV2	CV3*	Mean
Pod development	16.83	17.42	16.21	15.54	16.50	31.7	27.1	43.2	27.2	32.3	198	153	207	175	183
Flowering	20.67	19.75	17.29	17.00	18.68	50.5	35.7	48.5	32.2	41.7	224	189	291	189	223
Vegetative	21.96	20.79	19.25	17.96	19.99	52.1	61.4	67.2	36.0	54.2	208	300	321	192	225
Well-watered	17.17	20.33	19.79	19.46	19.19	58.4	60.0	93.4	56.3	67.0	310	387	484	312	348
Mean	19.16	19.57	18.14	17.49		48.2	46.0	63.1	38.0		235	257	326	217	

* Drought sensitive cultivar.

5.3.2 Effects of soil water-limited induced stress imposed at different growth stages on proline content

Results of the combined ANOVA (Table 5.7) suggested that growth stage effects (GS) were highly significant ($P = 0.001$) for proline accumulation. This indicated that proline content was significantly different between the different growth stages. Furthermore, genotype (G) effects were significant ($P = 0.01$) for proline content as well as trial by genotype (TxG) effects ($P < 0.05$). This suggested that genotypes were significantly different in proline content and that they responded differently between the two trials. This was probably due to differences in day length and temperature as the trials were planted in glasshouses during winter and summer, although these were controlled as far as possible.

Results further indicated that the mean values for proline content increased with the growth stage in which soil WLIS imposed (Table 5.8). The mean proline content for the well-watered treatment was $1.47 \mu\text{mol g}^{-1} \text{FW}$ and a gradual increase in proline content was observed for soil WLIS imposed at the vegetative ($4.48 \mu\text{mol g}^{-1} \text{FW}$), flowering ($6.03 \mu\text{mol g}^{-1} \text{FW}$) and pod development ($6.20 \mu\text{mol g}^{-1} \text{FW}$) growth stages, respectively.

Genotype CV2 had the highest genotypic mean value for proline content of all genotypes across the soil WLIS imposed at all three (vegetative, flowering and pod development) growth stages with a mean of $5.15 \mu\text{mol g}^{-1} \text{FW}$. This was followed by genotype BL4 that ranked in the second position for proline content across the three growth stages and it had a mean of $4.68 \mu\text{mol g}^{-1} \text{FW}$. Genotype BL2 and the sensitive genotype CV3 ranked in the bottom two positions for genetic potential for proline content across all three growth stages and they had the lowest means of $4.17 \mu\text{mol g}^{-1} \text{FW}$ and $4.19 \mu\text{mol g}^{-1} \text{FW}$, respectively.

Table 5.7 Combined mean square values for proline content ($\mu\text{mol g}^{-1}$ FW) analysed for four soybean genotypes subjected to soil water-limited-induced stress imposed at three growth stages

Source of variation	Proline content ($\mu\text{mol g}^{-1}$ FW)
Replication	2.7858
Trial (T)	0.0469
Growth stage (GS)	115.0449***
Genotype (G)	5.2013**
TxGS	2.7535
TxG	0.3927*
GSxG	1.4621
TxGSxG	0.2025
Residual	0.9793
CV%	21.8

*, **, ***, significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively. CV = coefficient of variation.

Table 5.8 Mean values for proline accumulation ($\mu\text{mol g}^{-1}$ FW) in soybean genotypes in response to soil water-limited-induced-stress at different growth stages

Genotype	Growth stage				Mean
	Well-watered	Vegetative	Flowering	Pod development	
BL2	1.61	4.13	5.31	5.62	4.17
BL4	1.74	4.62	6.21	6.14	4.68
CV2	1.15	5.04	6.84	7.56	5.15
CV3*	1.39	4.14	5.77	5.46	4.19
Mean	1.47	4.48	6.03	6.20	
LSD _{0.05} (Genotype)	0.571				
LSD _{0.05} (Growth stage)	0.571				

* Drought sensitive cultivar. LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

5.3.3 Comparing genotypes' response to soil water-limited-induced-stress based on yield potential, proline content and the tolerance indices combined

To compare the use of different stress tolerance/susceptibility indices in screening for soybean genotypes under soil WLIS conditions, seed mass of the soybean genotypes under both non-stress (well-watered) (Yp) and soil WLIS conditions (imposed during pod development growth stage) (Ys) were used to calculate the different indices as indicated in Table 5.2. Results on the mean values for seed mass under WLIS (Yp) and non-WLIS conditions (Ys) suggested that genotype CV2 (93.4 g and 43.2 g, respectively) had the highest yield potential and it also had the highest mean productivity (MP = 68.3 g) and geometric mean productivity (GMP = 63.5 g) compared to rest of the genotypes (Table 5.9).

Genotype CV2 showed the largest tolerance index (TOL) value (50.2 g) indicating that this genotype showed the largest reduction in seed mass of all genotypes and it had a low yield stability index (YSI = 0.46). On the other hand, CV2 showed the largest increase in proline content ($6.4 \mu\text{mol g}^{-1} \text{FW}$) in response to soil WLIS and therefore this genotype was considered the most tolerant to soil WLIS conditions during pod development of the four genotypes analysed.

Genotype BL2 had a lower yield potential (58.4 g and 31.7 g for Yp and Ys respectively) and showed lower mean values for mean productivity (MP = 45.1 g) and geometric mean productivity (GMP = 43.0 g) compared to CV2. BL2 was sensitive to soil WLIS during pod development since it had a stress susceptibility index value of less than one (SSI = 0.9) and it also showed the smallest increase in proline content ($4.0 \mu\text{mol g}^{-1} \text{FW}$). However, it had the lowest tolerance index value (TOL = 26.7 g) and the highest yield stability index value (YSI = 0.54) of all four genotypes. This suggested the genotype will be more productive under soil WLIS conditions.

Genotype BL4 showed an acceptable yield potential under well-watered conditions (Yp value of 60.0 g); however, it showed a large reduction in seed mass under WLIS (Ys value of 27.1 g), thus, a high tolerance index value (TOL = 32.9 g). BL4 had a stress susceptibility index of above 1 (SSI = 1.1) and low yield index value (YI = 0.8) indicating that this genotype will be more productive under non-WLIS conditions. Genotypes BL4 and CV3 performed more or less the same for the parameters shown in Table 5.9.

Table 5.9 Mean values for seed mass per plant (g) (Yp and Ys), drought tolerance indices and proline accumulation obtained for plants subjected to soil water-limited-induced-stress during pod development

Genotype	Yp (g)	Ys (g)	MP	SSI	TOL	STI	GMP	YI	YSI	PrIp ($\mu\text{mol g}^{-1}$ FW)	PrIs ($\mu\text{mol g}^{-1}$ FW)	PrI ($\mu\text{mol g}^{-1}$ FW)
BL2	58.4	31.7	45.1	0.9	26.7	0.02	43.0	1.0	0.54	1.6	5.6	4.0
BL4	60.0	27.1	43.6	1.1	32.9	0.02	40.3	0.8	0.45	1.7	6.1	4.4
CV2	93.4	43.2	68.3	1.0	50.2	0.03	63.5	1.3	0.46	1.2	7.6	6.4
CV3*	56.3	27.2	41.6	1.0	28.8	0.02	39.0	0.8	0.49	1.2	5.5	4.3
Mean	67.0	32.3	49.6	1.0	34.7	0.02	46.5	1.0	0.49	1.4	6.2	4.9

* Drought sensitive cultivar, Yp = seed mass under non-WLIS conditions, Ys = seed mass under WLIS conditions (during pod development), MP = mean productivity, SSI = stress susceptibility index, TOL = tolerance index, STI = stress tolerance index, GMP = geometric mean productivity, YI = yield index, YSI = yield stability index, PrIp = proline under non-WLIS conditions, PrIs = proline under WLIS conditions, PrI = relative change in proline.

The sensitive genotype CV3 generally exhibited a low yield potential but at the same time it was sensitive to WLIS conditions because of its low values for mean productivity (MP = 41.6 g), geometric mean productivity (GMP = 39.0 g), low yield index (YI = 0.8) and low proline content values under non-WLIS (1.2 $\mu\text{mol g}^{-1}$ FW) and WLIS (5.5 $\mu\text{mol g}^{-1}$ FW) conditions, respectively (Table 5.9).

5.3.4 Associations among yield potential, proline accumulation and tolerance indices

The correlation coefficients between Yp, Ys, proline content and the other tolerance indices for soil WLIS were determined in order to identify the most desirable and efficient drought stress tolerant selection criteria, based on associations among indices. Seed mass under both well-watered (Yp) and soil WLIS (Ys) conditions at pod development were positively correlated with mean productivity (MP) ($r = 0.99$; $r = 0.97$, respectively), relative change in proline content (Prl) ($r = 0.98$; $r = 0.90$, respectively), proline content under WLIS (PrIs) ($r = 0.97$; $r = 0.88$, respectively), stress tolerance index (STI) ($r = 0.99$; $r = 0.97$, respectively) and yield index (YI) ($r = 0.95$; $r = 1.00$, respectively) (Table 5.10). However, Yp was significantly and positively correlated with geometric mean productivity (GMP) ($r = 0.99$) and tolerance index (TOL) ($r = 0.97$), while Ys was not significantly correlated with these two parameters. The significant positive relationships between Yp and Ys with the stress tolerance and stability indices, respectively suggested that these criteria were more effective in characterising and identifying the responses of genotypes to soil WLIS; more especially the identification of a tolerant and stable genotype. In addition, the positive relationships between the tolerance indices (STI, TOL and YI) with Ys and PrIs suggested that proline content and the change thereof could be used as potential indices for identifying drought sensitive soybean genotypes under soil WLIS conditions.

In order to identify more clearly, which combinations of the drought tolerance indices can be effectively utilised together as selection tools for genotypes under soil WLIS conditions, a principal component analysis (PCA) was done. Results indicated that the first two principal components (PCs) explained cumulatively 99.98% of the variation (Table 5.11), which corresponds to the whole percentage variance in the data set. PC1 accounted for 98.38% of the variation, PC2 for 1.60% and PC3 for 0.01%. Tolerance indices, which contributed more positively to PC1 together with Yp, were GMP, MP and TOL.

Table 5.10 Simple correlation coefficients between yield (Yp and Ys), drought tolerance indices and proline content

	GMP	MP	Prl	PrIp	PrIs	SSI	STI	TOL	YI	YSI	Yp
GMP	-										
MP	0.99**										
Prl	0.95**	0.97**									
PrIp	-0.49	-0.49	-0.57								
PrIs	0.94*	0.95*	0.96**	-0.33							
SSI	0.27	0.31	0.51	-0.16	0.53						
STI	0.99**	1.00**	0.97**	-0.49	0.95*	0.31					
TOL	0.94*	0.95*	0.99**	-0.47	0.98**	0.57	0.95*				
YI	0.98**	0.97**	0.90*	-0.47	0.88*	0.11	0.97**	0.87			
YSI	-0.27	-0.31	-0.51	0.16	-0.53	-1.00***	-0.31	-0.57	-0.11		
Yp	0.99**	0.99**	0.98**	-0.49	0.97**	0.40	0.99**	0.97**	0.95*	-0.40	-
Ys	0.98	0.97**	0.90*	-0.47	0.88*	0.11	0.97**	0.87	1.00**	-0.11	0.95*

*, **, significant at $P < 0.05$, $P < 0.01$ respectively. GMP = geometric mean productivity, MP = mean productivity, PrI = relative change in proline, PrIp = proline under non-WLIS conditions, PrIs = proline under WLIS conditions, SSI = stress susceptibility index, STI = stress tolerance index, TOL = tolerance index, YI = yield index, YSI = yield stability index, Yp = seed mass under non-WLIS conditions, Ys = seed mass under WLIS conditions (during pod development).

Table 5.11 Loadings of seed mass, the tolerance indices and proline content on the first four principal components

Tolerance indices	PC1	PC2	PC3	PC4
Yp	0.64049	-0.16037	0.01287	0.16312
Ys	0.26489	0.55383	0.02149	0.215569
MP	0.45269	0.19673	0.01718	0.18942
SSI	0.00105	-0.02067	0.00144	-0.76547
TOL	0.3756	-0.71419	-0.00862	-0.05251
STI	0.0002	0.00009	-0.00008	0.00009
GMP	0.41337	0.33574	-0.03769	-0.54894
YI	0.0082	0.01714	0.00065	0.00668
YSI	-0.00055	0.0107	-0.00068	0.00241
PrIp	-0.00548	-0.00112	0.81328	-0.02442
PrIs	0.03348	-0.05166	0.45883	-0.01448
PrI	0.03897	-0.05054	-0.35444	0.00994
Eigenvalues	2291.3	37.3	0.3	0
Percentage variation	98.38	1.60	0.01	0
Cumulative %	98.38	99.98	99.99	99.99

PC = principal component, Yp = seed mass under non-WLIS conditions (during pod development), Ys = seed mass under WLIS conditions (during pod development), MP = mean productivity, SSI = stress susceptibility index, TOL = tolerance index, STI = stress tolerance index, GMP = geometric mean productivity, YI = yield index, YSI = yield stability index, PrIp = proline under non-WLIS conditions, PrIs = proline under WLIS conditions, PrI = relative change in proline content.

This suggested that selecting for these tolerance indices together would bring efficiency in a breeding programme in coming up with a stable and high yielding genotype under both WLIS and non-WLIS conditions. In addition, indices which contributed significantly to PC2 were GMP, Ys and TOL and for PC3 they were PrI, PrIp and PrIs.

The first two principal components were plotted on a biplot to further observe associations between all parameters analysed (Figure 5.2). Yan and Rajcan (2002) and Dehghani et al. (2008) suggested that the association between two parameters is approximated by the cosine of the angle between their vectors. Yan and Holland (2010) expounded that an acute angle represents more or less positive association, an obtuse angle shows a negative association, whilst a right angle represents a no association.

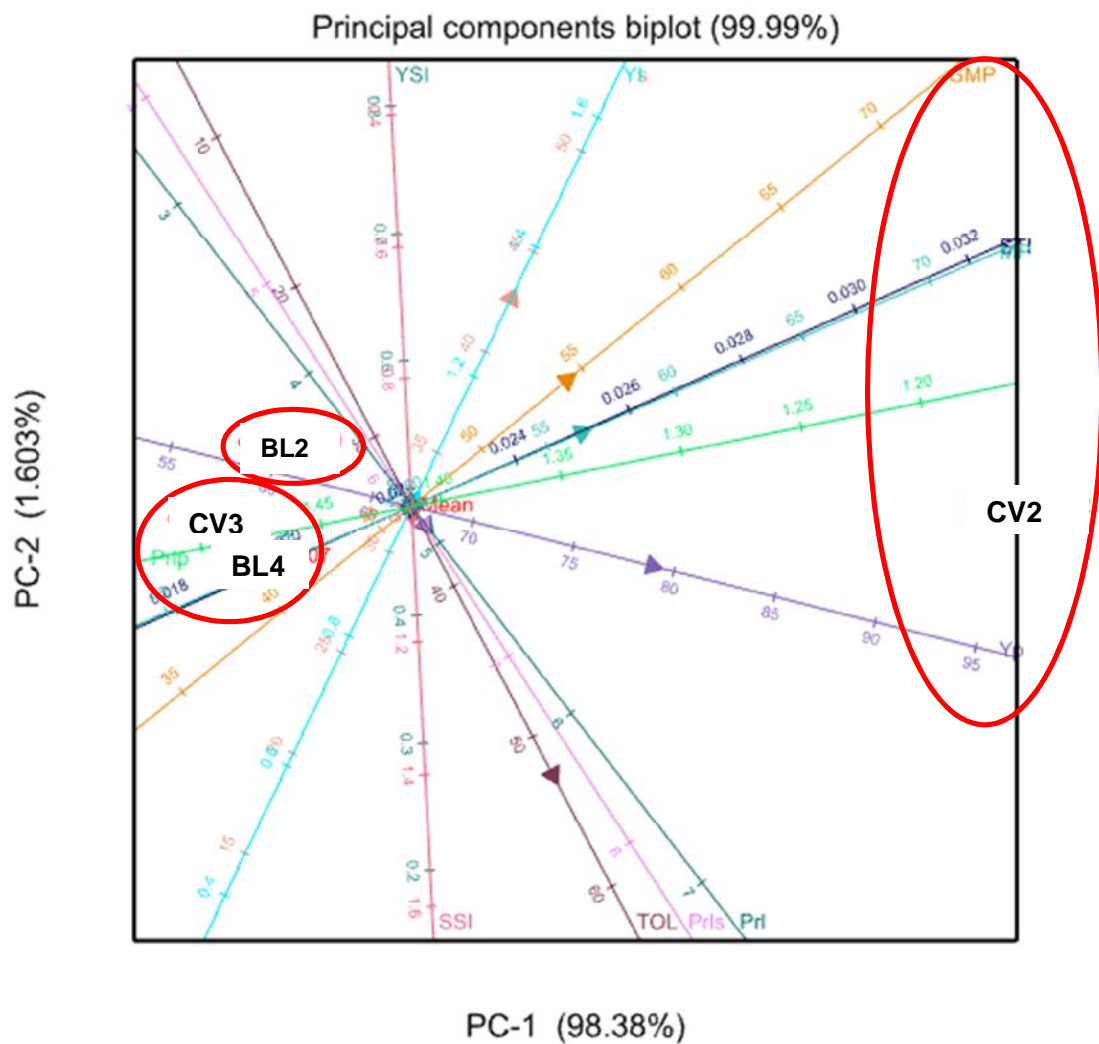


Figure 5.2 Biplot showing the first two principal components (PC1 and PC2) indicating the relations between yield, proline and various stress and susceptibility indices.

Strong positive associations were observed between YI and Ys, MP and STI; GMP with MP and STI, respectively; and among TOL, PrIs and PrIp because they had acute angles and were in the same direction (Figure 5.2). Strong negative associations were also observed between YSI and SSI, and between PrIp with MP and STI, respectively because they had obtuse angles. The biplot also indicated that genotypes BL2, CV3 and BL4 clustered together; whilst CV2 significantly differed from that cluster. Genotype CV2 associated closely with Yp, GMP, MP and STI, and less with PrIp, PrIs and TOL. Therefore, CV2 could be regarded as a more stable genotype and will be high yielding under both non-WLIS and WLIS conditions.

The biplot further showed that the second dimension of the PCA, known as the stress tolerant dimension, separated the stress-tolerant and the sensitive genotypes based on their associations with the indices on the plot. In this case, BL2 will be more suitable for non-WLIS conditions, whilst the sensitive genotype CV3 and tolerant BL4 will be more suitable for WLIS conditions.

5.4 Discussion

The most critical period for soybean genotypes to have adequate soil water resources has been demonstrated to be the pod development and seed-filling growth stages. Results have shown that soybean yield potential components [100-seed mass (g), seed mass per plant (g) number of seeds per plant and biomass (g)] were significantly reduced by soil WLIS imposed during pod development and flowering growth stages, respectively. This collaborates studies by Kranz et al. (1998) whereby soybean yield was significantly reduced by limited soil water resources both under pod development, as well as beginning of full bloom. According to Ouda et al. (2008) soybean plants are most vulnerable to soil WLIS especially during flowering and seed-filling stages. Soil WLIS during these growth stages leads to a significant decrease in soybean seed yield. This is because drought stress at these reproductive growth stages disrupts photosynthesis and remobilisation of sink in soybean plants (Madani et al. 2010; Moghaddam et al. 2014), and causes reduction in the number and weight of seed (Richards et al. 2001). Furthermore, the sensitivity of these growth stages is also exacerbated by the fact that soybean plant ages from R1 (beginning bloom) through to R5 (seed enlargement); hence, its ability to compensate under stressful conditions decreases and yield loss increase (Foroud et al. 1993).

Similarly, the morphological traits [number of pods per plant, number of aborted pods per plant, number of branches per plant, plant height (cm), height of top and bottom pods (cm)] are also drastically affected by soil WLIS at flowering and pod development stages. Kobraei et al. (2011) also observed positive and significant relationships between seed yield losses and reductions in morphological traits, arising from soil WLIS at flowering and pod development growth stages.

The observed genotypic differences among cultivars in response to soil WLIS have also been reported in soybean (Van Heerden and Kruger 2002). This was attributed to the different mechanisms which genotypes utilise to overcome the effects of soil WLIS (Lobato et al. 2008). Such genotypic variations in coping mechanisms to soil WLIS can be utilised as an opportunity for germplasm improvement by breeders. Despite the sensitivity of the genotypes being known, the responses to drought stress observed in terms of yield attributes were significantly different. This suggested that the tolerant (BL2, BL4 and CV2) and sensitive (CV3) genotypes utilised different mechanisms to avert the adverse effects of drought stress.

Apart from changes in yield attributes, one mechanism utilised by plants to overcome limited-water stress effects is biochemical; through changes in proline levels (Vendruscolo et al. 2007). Other than being an osmoprotectant, proline act as a potent non-enzymatic antioxidant; hence, prevents oxidative damage in plant tissues that may be caused by reactive oxygen species (ROS), stabilise deoxyribonucleic acid (DNA), membranes and protein complexes, and provide a source of carbon and nitrogen for growth after stress relief (Szabados and Savoure 2010; Rejeb et al. 2014). In the present study, it was found that under non-WLIS conditions proline content in the leaves were similar in both the tolerant and susceptible genotypes. However, proline content increased with progressive soil WLIS in all genotypes. The tolerant genotype CV2 accumulated more proline than the rest of the genotypes. Hossain et al. (2014) reported that leaf proline levels of both tolerant and susceptible genotypes were similar until day six of the stress cycle, but after day 12 the proline content of the tolerant genotypes peaked. Other studies also reported higher proline levels in tolerant genotypes of crops and trees such as tomato (Claussen 2005), beans (Turkan et al. 2005) and poplar (Guo et al. 2010). Furthermore, like in maize (Moussa and Abdel-Aziz 2008) and chick pea (Mafakheri et al. 2010), it was possible to discriminate the sensitive genotype CV3 from the tolerant genotype CV2 using proline accumulation under WLIS conditions.

The sharp increase in proline content under WLIS might theoretically be attributed to genes for synthesis and degradation of proline, which are up-regulated strongly under soil WLIS. This is an adaptation strategy of soybean plants to prevailing stress; the purpose of which is to overcome the adverse effects of the stressful condition (de Ronde et al. 2001; Masoumi et al. 2011; Hayat et al. 2012). The increased proline level, therefore, supplies energy for growth and survival during stress and during the stress relief period, and thereby helps the plant to tolerate the water-limited stress conditions (Sankar et al. 2007).

Seed mass (both under WLIS and non-WLIS conditions) correlated positively and significantly with proline accumulation. Similar results were observed by Shivkumar et al. (1998), Silverira et al. (2003) and Bayoumi et al. (2008) where proline accumulation was closely associated to high yielding potential under soil WLIS conditions. This suggested that the accumulated proline might have been acting as a compatible solute regulating and reducing water loss from the cell during episodes of water deficit; hence, reducing the effects of WLIS on plant growth and seed mass loss in the tolerant genotype.

Proline accumulation under soil WLIS was also found to be positively and significantly correlated with the drought tolerance indices. Mitra (2001) suggested that an efficient index must have significant correlations with both the seed yield and drought tolerance indices. Seed mass under non-WLIS conditions (Y_p) was significantly and positively correlated, respectively with proline content (in plants exposed to WLIS), GMP, MP, STI, TOL and YI. The multivariate analysis (PCA) of the stress tolerance indices indicated the relationships between the indices and the different genotypes. This helped to characterise the genotypes yielding potential in both non-WLIS and WLIS conditions.

Improvement in seed yield potential of crops grown in both normal and drought stress conditions is paramount in any breeding programme. Therefore, selecting cultivars based on their strong yielding ability under both WLIS and non-WLIS conditions, and the tolerance stress indices (as identified by the PCA) would improve the selection efficiency for stable genotypes. The correlation analysis provided the relationships between the yield potential and tolerance indices, while the PCA indicated which tolerance indices were closely associated with which genotype. Hailegiorgis et al. (2011) and Janmohammadi et al. (2014) used the factor analysis method and the PCA to better understand the data structure and trait relations under varied soil WLIS conditions. The two methods were found to give complementary

information that helped in constructing selection indices for their breeding programmes. The multivariate analysis method has strongly corroborated the fact that proline accumulation can be used as a drought tolerance index to discriminate between tolerant and sensitive soybean genotypes. The strong relationships between yielding ability under both the stress conditions and the tolerance indices suggested the suitability of using proline accumulation as a drought tolerance index. Similarly, using multiple statistical procedures, Esack et al. (2015) could predict F1 progenies with a high YSI in tea through selection of breeding materials which accumulate high proline concentrations under WLIS conditions. Proline accumulation was further found to be a highly heritable trait, which plays a significant role in screening for drought tolerance in tea.

5.5 Conclusions and recommendations

Soybean yield potential is adversely affected by soil WLIS. Results have shown that effects of soil WLIS are more severe when plants are exposed to drought stress during pod development growth stage, followed by flowering. Genotypic differences existed for seed mass, 100-seed mass, number of aborted pods, and height of the top and bottom pods, among soybean plants exposed to soil WLIS. Plants utilise different mechanisms to avert the effects of soil WLIS. One such mechanism is through biochemical compounds, more specifically proline accumulation. It has been demonstrated that proline content is higher in plants exposed to soil WLIS. However, genotypic differences exist in rate of proline accumulation with prolonged soil WLIS. This study has shown that tolerant genotypes tend to accumulate more proline than sensitive genotypes. In tolerant plants proline plays an osmoprotectant role. It is possible to discriminate sensitive genotypes from tolerant genotypes using proline accumulation in stressed plants. Proline content under WLIS conditions correlated positively with yield potential (Y_p and Y_s) as well as the tolerance indices GMP, MP and TOL. Hence it can be used as a drought stress tolerance indicator in soybean. Use of multivariate analysis gave a good dimensional view of how proline associates with other drought tolerance indices and consequently, yielding potential which is key in any breeding programme. Use of multivariate statistical procedures is, therefore, recommended for breeding programmes that use data with multiple variables.

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Chapter 6

Field evaluation of soybean for seed yield and proline accumulation under varying soil water-limited-induced-stress conditions

6.1 Introduction

Soybean is the sixth most grown agricultural crop in the world (FAOSTAT 2016). It is grown in many parts of the world; mostly as a primary source of vegetable oil and protein for use in food, feed and industrial applications (Corner et al. 2004). The soybean crop requires a high amount of water to obtain a high yield and good quality seed (Sweeney et al. 2003). Soil water-limited-induced-stress (WLIS) during growth and development of soybean is a major constraint (Tefera 2011). In addition, global warming has worsened the situation in most agricultural regions (Xonocostle-Cazares et al. 2010). Conventional breeding of soybean continues to deliver improved varieties to farmers across the world (Richards 2006). Development of extra-early and drought tolerant varieties have become the focal breeding objectives for most soybean breeding programmes especially in the sub-Saharan region, to avert the effects of soil WLIS (Tefera 2011). However, breeding of improved soybean genotypes for water-limited environments through selection on seed yield only is difficult. This is because of the variability in the amount of rainfall and soil water, as well as the temporal distribution thereof from year to year at test environments (Ludlow and Muchow 1990). It has been reported that genotypic variance for seed yield is low under soil WLIS and that plant characteristics, which influence productivity under water-limited environments, are differently expressed in diverse years (Blum 1988; Ludlow and Muchow 1990; Zangi 2005) making selection under these conditions challenging.

Soil WLIS induces complex physical-chemical processes in plants. These involve many biological macro-molecules and micro-molecules such as nucleic acids [deoxyribonucleic acid (DNA), ribonucleic acid (RNA), microRNA], proteins, carbohydrates, lipids, hormones, ions, free radicals and mineral elements (Bayoumi et al. 2008). Proline, a compatible solute, accumulates in the cytosol as one of the earliest biochemical responses of plants to soil WLIS (Hanson and Hitz 1982; Bandurska 2000). A number of studies have found that soil WLIS adapted varieties have higher proline levels, suggesting a correlation between proline

accumulation and soil WLIS tolerance (Ben et al. 2008; Parida et al. 2008; Verslues and Juenger 2011).

Results have demonstrated that proline has a protective effect on nitrate reductase activity and cell membranes under soil WLIS (Bandurska 1998). According to Schwab and Gaff (1990) soil WLIS tolerance in plants, among others, is attributed to the composition of the cell membrane surroundings, with special reference to compatible solutes. Cell membrane stability is considered to be one of the best physiological indicators of soil WLIS tolerance (Blum and Ebercon 1981). Free proline accumulation in stressed plants reduce stress-induced cellular acidification or prime oxidative respiration to provide energy needed for recovery (Hare and Cress 1997). Whereas a large body of data indicated that proline may act as a protective compatible substance (Ashraf and Foolad 2007; Mafakheri et al. 2010; Moustakas et al. 2011), there are no conclusive data that verifies whether proline accumulation in wilting plant tissues provides a protective, membrane stabilizing effect, and more specifically in field studies (Ashraf and Foolad 2007).

Breeding efforts to improve soil WLIS tolerance in soybean could be aided by the identification of biochemical markers associated with improved field performance under soil WLIS conditions (Bayoumi et al. 2008). The strategy of indirect selection using secondary traits such as free proline has succeeded only in soybean, maize, canola, and wheat (Delauney and Verma 1993; Van Heerden and Kruger 2002; Ashraf and Foolad 2007; Manavalan et al. 2009), due to problems with repeatability and lack of phenotyping strategies (Manavalan et al. 2009). The aim of this study was to evaluate the effect of soil WLIS on soybean genotypes grown under field conditions. The objectives were to evaluate the effects of soil WLIS on (i) morphological traits and seed yield and (ii) free proline accumulation in soybean genotypes grown under field conditions.

6.2 Materials and methods

6.2.1 Trial design, growing conditions and plant material

A field experiment was conducted in a rain-out shelter at the farm Barendspan (29°25'19.1"S 25°31'44.5"E), Petrusburg District, southern Free State, South Africa, during the summer season of 2014/2015. Petrusburg is characterised by hot and dry summers. It received a summer rainfall of 452 mm during the months of November 2014-March 2015 and the average

maximum temperature during the same period was 31°C (data obtained from ARC–ISCW 2015), making it an ideal site for soil WLIS studies. The trial was a factorial experiment laid out in a randomized completely block design (RCBD) with two factors and three replications. Plots consisted of four rows, spaced 0.45 m apart and 3 m in length. Factor one comprised of four water treatments where plants were subjected to different soil WLIS levels, respectively throughout the growth cycle: (i) 75% deficit irrigation, (ii) 50% deficit irrigation, (iii) 30% deficit irrigation (severely stressed) and (iv) a well-watered control treatment. Drip-irrigation was used with dripper nozzles supplying different amounts of water to achieve the four different soil WLIS treatments. Seedlings were subjected to normal irrigation conditions until the third trifoliate leaf stage was reached to ensure good plant establishment. After this stage, the different soil WLIS treatments followed. The trial was irrigated once a week.

Factor two included four soybean genotypes. Plant material used were obtained from the PANNAR® SEED (PTY) LTD breeding programme and included three drought tolerant genotypes (BL1, BL4 and CV2) and one sensitive genotype (CV3) (Table 6.1). According to their yield performance under field breeding trials by PANNAR SEED® (PTY) LTD, these genotypes were categorised to be drought tolerant and -sensitive, respectively. The four genotypes were chosen based on their differential genotypic potential for free proline accumulation (Chapter 3), and were used for this study. Seeds were sown by hand in furrows, and inoculated with inoculated with *Bradyrhizobium japonicum* strain WB74 at planting. Seed were sown in order to achieve a plant population of 350000 plants per ha. Plants were fertilised using Omnia NPK 2:3:4 (31) + 0.5% Zn, as a top dressing twice during the season. Weed and pest control practises were followed when needed and as recommended for soybean (DAFF 2010).

Table 6.1 Soybean genotypes used in the study and their levels of drought sensitivity

Genotype	Growth habit	Maturity group	Drought sensitivity
BL1	Determinate	6.7	Tolerant
BL4	Indeterminate	7.4	Tolerant
CV2	Indeterminate	7.0	Tolerant
CV3	Determinate	5.7	Sensitive

6.2.2 Agronomical trait measurements

Data on agronomical traits and seed yield attributes were collected at harvest. Single plant measurements were done on 10 randomly selected plants per plot. These included top and bottom pod height (cm), number of primary branches per plant, total number of pods per plant, number of aborted pods per plant, number of seeds per plant, total seed mass per plant (g). Seed yield per plot (kg ha^{-1}), 100-seed mass (g) (the mass of 100 seed) and total biomass (g) (total above ground material) were determined on a per plot basis. Harvest index (HI) was determined as dry seed mass (g) divided by the total above-ground biomass (g) (seed plus shoot).

6.2.3 Extraction and determination of free proline content

Proline content was determined and measured using leaves sampled from plants subjected to all treatments following a ninhydrin based extraction method as described by Gibon et al. (2000) and modified by Carillo et al. (2011). The method used was described in Chapter 3, section 3.2.3. Leaf samples were collected at three growth stages (vegetative, flowering and pod development), respectively for proline analysis in order to capture the interaction of soil WLIS and developmental stage effects on proline accumulation.

6.2.4 Data analysis

Data on agronomical traits, seed yield attributes and free proline content were subjected to analysis of variance (ANOVA) in order to partition the various sources of variation in the data structure. Means were separated by least significant difference (LSD). Data were analysed using GenStat Release 18 statistical package (VSN International 2015).

6.3 Results

6.3.1 Effects of soil water-limited-induced-stress on agronomical traits and yield attributes

Responses of soybean genotypes to soil WLIS are presented in Table 6.2. Soil WLIS effects were significant for top pod height and bottom pod height ($P < 0.05$, respectively), number of branches per plant ($P < 0.01$), number of pods per plant ($P < 0.05$), seed number and mass per plant ($P < 0.001$, respectively), 100-seed mass ($P < 0.05$), seed yield ($P < 0.001$), biomass ($P < 0.001$) and harvest index ($P < 0.05$).

Table 6.2 Analysis of variance showing mean square values for agronomical traits of four soybean genotypes subjected to soil water-limited-induced-stress

Source of variation	Pod height (cm)		Branches plant ⁻¹	Number of pods plant ⁻¹		Seed plant ⁻¹		100-seed mass (g)	Seed yield (kg ha ⁻¹)	Biomass (g)	HI
	Top	Bottom	Number	Total	Aborted	Number	Mass (g)				
Replication	92.5	35.44	11.083	942.0	16.15	501	44.9	0.244	137116	141.9	0.016115
WLIS	1415.6*	160.81*	45.833**	2408.9*	12.19	29198***	874.7***	6.991*	5371403***	5365.4***	0.019661*
Genotype (G)	3091.0***	208.36**	29.944*	1067.0	71.19**	4234	112.9	8.732**	346348**	561.5	0.000453
WLISxG	114.1	42.64	4.556	382.7	12.00	1708	67.2	2.658	95588	598.6	0.004807
Residual	333.0	36.50	9.839	737.2	13.83	3605	103.7	1.829	754474	346.3	0.005200
CV%	21.7	27.9	51.6	56.1	142.8	52.0	44.1	6.8	15.9	38.6	14.5

*, **, ***, significant at P<0.05, P<0.01, P<0.001 respectively. HI = harvest index, WLIS = water-limited-induced-stress, G = genotype, CV = coefficient of variation.

Genotype effects were significant for top pod height ($P<0.001$), bottom pod height ($P<0.01$), number of branches per plant ($P<0.01$), number of aborted pods per plant ($P<0.01$), 100-seed mass ($P<0.01$) and seed yield ($P<0.001$). There were no significant genotype x WLIS interaction effects for any the traits evaluated. This indicated that genotypes responded similarly across the different WLIS treatments. Agronomical traits and seed yield attributes, which were significant in the ANOVA will be further discussed.

In general, the agronomical traits and yield attributes were negatively affected by the different soil WLIS treatments (Table 6.3). Significant differences were observed across all four soil WLIS treatments for top pod height (ranging from 72.4 cm for the severe to 97.6 cm for the well-watered treatment) and seed yield (ranging from 962.0 kg ha⁻¹ for the severe to 2576.0 kg ha⁻¹ for the well-watered treatment). This indicated that plant height and seed yield were significantly reduced with an increase in the severity of the WLIS treatment. The well-watered and low WLIS (75%) treatments ranked in the top two positions, respectively for top pod height, number of branches per plant, number of pods per plant, seed number per plant, seed mass per plant, seed yield and biomass. However, significant differences were observed between the well-watered and the low WLIS (75%) treatments for top pod height, number of pods per plant, number of seeds per plant, seed mass per plant, seed yield and biomass.

The moderate (50%) and severe (30%) WLIS treatments ranked in the bottom two positions for top pod height, bottom pod height, number of branches per plant, number of seeds per plant, seed yield and biomass and their values were significantly lower than the mean (Table 6.3). However, no significant differences were observed between the low and the severe WLIS treatments for these traits, except for seed yield. The severe WLIS treatment resulted in the lowest values for top pod height (72.4 cm), bottom pod height (18.2 cm), number of seeds per plant (70.2), seed mass per plant (14.9 g), seed yield (962.0 kg ha⁻¹) and biomass (31.9 g). However, 100-seed mass (g) and the harvest index (%) showed different responses with the highest values for the moderate (50%) WLIS treatments at 21.0 g and 55.5%, respectively. Thus, the effects of soil WLIS on 100-seed mass and the harvest index were not linear as with the other agronomical traits and yield attributes.

Table 6.3 Mean values of agronomical traits measured on four soybean genotypes subjected to soil water-limited-induced-stress

WLIS	Pod height (cm)		Branches plant ⁻¹	Number of pods plant ⁻¹		Seed plant ⁻¹		100-seed mass (g)	Seed yield (kg ha ⁻¹)	Biomass (g)	HI (%)
	Top	Bottom	Number	Total	Aborted	Number	Mass (g)				
Well-watered	97.6a	21.3b	8.6a	68.1a	3.8	177.9a	34.2a	19.8b	2576.0a	77.2a	0.46c
Low (75%)	87.6b	26.7a	6.5ab	46.8b	2.8	131.4b	25.1b	19.4b	1814.0b	50.5b	0.49b
Moderate (50%)	79.2c	20.2bc	4.0b	34.4c	1.4	82.3c	18.2c	21.0a	1555.0c	33.0c	0.56a
Severe (30%)	72.4c	18.2c	5.2b	44.3bc	2.3	70.2c	14.9c	19.5b	962.0d	31.9c	0.49b
<i>Mean</i>	<i>84.2</i>	<i>21.6</i>	<i>6.1</i>	<i>48.4</i>	<i>2.6</i>	<i>115.5</i>	<i>23.1</i>	<i>19.9</i>	<i>1727.0</i>	<i>48.2</i>	<i>0.50</i>
LSD _{0.05}	7.45	2.5	2.6	11.08	1.5	24.51	4.2	0.5	112.2	15.5	0.029

HI = harvest index, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

In Table 6.4, genotype BL1 ranked in the top position, respectively for number of branches per plant (7.7), number of pods per plant (60.8), number of seeds per plant (135.4), seed mass per plant (26.5 g), biomass (54.2 g) and harvest index (0.50), indicating that this genotype showed the highest yield potential. However, BL1 ranked third in terms of seed yield (1754.0 kg ha⁻¹) and its value was significantly lower than the 1888.0 kg ha⁻¹ observed for BL4, which ranked first for seed yield. The lower yield observed for BL1 could be attributed to the high number of pods (6.2) aborted by this genotype.

Genotype BL4 (Table 6.4) ranked in the top position for 100-seed mass (20.7 g) and seed yield (1888.0 kg ha⁻¹) and second for top pod height (94.9 cm) and number of branches per plant (6.7). This genotype generally grew taller and had a high number of branches; however, it performed the poorest for seed number (93.6) and mass (19.5 g) per plant among all genotypes. The sensitive genotype, CV3, were the shortest with a top pod height of 65.1 cm and it ranked in the bottom position for 100-seed mass (18.9 g) and seed yield (1488.0 kg ha⁻¹), respectively. On the other hand, CV3 performed well and above average for the yield attributes number of pods per plant (49.8), seed number (125.9) and mass (24.5 g) per plant, biomass (53.2 g) and harvest index (0.50). In this case the smaller seeds (seed-mass of 18.9 g) might have contributed to the lower seed yield.

6.3.2 Effects of soil water-limited-induced-stress on proline content under field conditions

Results of the combined ANOVA (Table 6.5) suggested highly significant genotype and WLIS ($P < 0.001$) effects, respectively for proline content. Significant genotype x WLIS interaction (GxWLIS) effects ($P < 0.001$) were observed which indicated that the ranking of genotypes changed across the different WLIS treatments. Significant WLIS x growth stage (WLISxGS) interaction effects ($P < 0.01$) suggested that the proline content, at the different growth stages, varied among the different WLIS treatments.

Table 6.4 Genotype mean values for agronomical traits measured on four soybean genotypes subjected to soil water-limited-induced-stress

Genotype	Pod height (cm)		Branches plant ⁻¹	Number of pods plant ⁻¹		Seed plant ⁻¹		100-seed mass (g)	Seed yield (kg ha ⁻¹)	Biomass (g)	HI
	Top	Bottom	Number	Total	Aborted	Number	Mass (g)				
BL1	77.1b	17.8c	7.7a	60.8	6.2a	135.4	26.5	20.5a	1754.0b	54.2	0.50
BL4	94.9a	26.3a	6.7a	44.2	1.9b	93.6	19.5	20.7a	1888.0a	39.7	0.49
CV2	99.8a	23.9b	4.0b	38.8	0.7c	107.0	21.9	19.6b	1778.0ab	45.6	0.49
CV3*	65.1c	18.4c	5.9ab	49.8	1.7bc	125.9	24.5	18.9c	1488.0c	53.2	0.50
<i>Mean</i>	<i>84.2</i>	<i>21.6</i>	<i>6.1</i>	<i>48.4</i>	<i>2.6</i>	<i>115.5</i>	<i>23.1</i>	<i>19.9</i>	<i>1727.0</i>	<i>48.2</i>	<i>0.49</i>
LSD _{0.05}	7.5	2.4	2.6	11.1	1.5	24.5	4.2	0.5	112.2	15.5	0.29

* Drought sensitive cultivar. HI = harvest index. LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

Table 6.5 Combined analysis of variance showing mean square values for proline content ($\mu\text{mol g}^{-1}$ FW) determined at three growth stages in four soybean genotypes subjected to soil water-limited-induced stress

Source of variation	Proline content ($\mu\text{mol g}^{-1}$ FW)
Replication	0.8920
Genotype (G)	4.8171***
WLIS	137.8639***
Growth stage (GS)	0.6086
GxWLIS	3.0787***
GxGS	0.7374
WLISxGS	1.8761**
GxWLISxGS	0.4084
Residual	0.5678
CV%	24.3

*, **, ***, significant at $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively. FW = fresh weight, G = genotype, GS = growth stage, WLIS = water-limited-induced-stress, CV = coefficient of variation.

Results in Table 6.6 suggested that under well-watered conditions, the mean proline content was the lowest ($1.365 \mu\text{mol g}^{-1}$ FW) compared to the different WLIS treatments and genotypes did not differ significantly for proline content. However, with an increase in soil WLIS intensity, proline content increased significantly from $1.541 \mu\text{mol g}^{-1}$ FW (low WLIS at 75%) to $5.296 \mu\text{mol g}^{-1}$ FW (severe WLIS at 30%) and significant differences were observed between genotypes for proline content. Under well-watered and low WLIS (75%) conditions the sensitive genotype, CV3, had the highest proline contents (1.497 and $1.802 \mu\text{mol g}^{-1}$ FW, respectively), while genotype BL2 had the lowest proline contents for these two treatments (1.248 and $1.394 \mu\text{mol g}^{-1}$ FW), respectively.

Table 6.6 Mean values for proline content ($\mu\text{mol g}^{-1}$ FW) at different growth stages in soybean genotypes in response to soil water-limited-induced-stress

Genotype	Proline ($\mu\text{mol g}^{-1}$ FW)				
	Well-watered	Low (75%)	Moderate (50%)	Severe (30%)	Mean
BL2	1.248a	1.394b	4.127b	5.420b	3.047
BL4	1.460a	1.457ab	4.055b	4.491c	2.866
CV2	1.254a	1.511ab	4.965a	6.811a	3.635
CV3*	1.497a	1.802a	3.684c	4.460c	2.861
Mean	1.365	1.541	4.208	5.296	
LSD _{0.05} (Genotype)	0.3526				
LSD _{0.05} (Soil WLIS)	0.3526				

* Drought sensitive cultivar. FW = fresh weight, LSD = least significant difference. Means followed by the same letter of the alphabet in the column are not significantly different.

This situation changed under moderate (50%) and severe (30%) WLIS conditions where the sensitive genotype CV3 had the lowest proline contents (3.684 and 4.460 $\mu\text{mol g}^{-1}$ FW), respectively and genotype CV2 had the highest proline contents (4.965 and 6.811 $\mu\text{mol g}^{-1}$ FW), respectively.

Results on mean proline content at the different growth stages and across the different soil WLIS levels are presented in Table 6.7. Results suggest that there was a significant interaction between the WLIS levels across growth stages. Proline content was least for the well-watered (1.365 $\mu\text{mol g}^{-1}$ FW) and low (75%) (1.541 $\mu\text{mol g}^{-1}$ FW) WLIS levels across all the growth stages. The proline content started to pick up at moderate (50%) WLIS level across all three growth stages. Proline content was highest at the severe (30%) WLIS level.

Table 6.7 Combined mean values for proline content ($\mu\text{mol g}^{-1}$ FW) at different growth stages in soybean genotypes in response to soil water-limited-induced-stress

Water-limited-induced-stress	Growth stage			Mean
	Vegetative	Flowering	Pod development	
Well-watered	1.389a	1.083a	1.623a	1.365
Low (75%)	1.391a	1.204a	2.029a	1.541
Moderate (50%)	3.962b	4.590b	4.071b	4.208
Severe (30%)	5.152c	5.730c	5.005c	5.296
<i>Mean</i>	2.973	3.152	3.182	
LSD _{0.05} (Soil WLISxGS)	0.6108			

FW = fresh weight, LSD = least significant difference.

6.4 Discussion

Seed yield is a quantitative trait and is, therefore, affected by genetic as well as environmental effects such as the soil moisture status (Turner et al. 2014). Soil WLIS has a negative effect on yield as many yield determining physiological processes depend on moisture (Farooq et al. 2009). Under soil WLIS plants lose their water status, which in turn adversely affects growth and development. The damaging effect of soil WLIS depends not only its severity but also on the development stage at which it occurs (Jongdee et al. 2002).

In this study soil WLIS significantly affected most morphological traits and seed yield attributes evaluated in soybean. Seed yield was reduced by as much as 63% (976 kg ha^{-1} under severe WLIS compared to 2576 kg ha^{-1} under well-watered conditions). The decrease in soybean seed yield due to soil WLIS could be attributed to the reduction in seed size (100-seed mass), number of seeds produced per plant, number of aborted pods and reduced canopy (number of branches and plant height) and these findings are in agreement with previous studies on soybean (Brevadan and Egli 2003; Angra et al. 2010). Yield reduction of this magnitude has also been reported in other crops such as sorghum, maize, rice and wheat (Mastrorilli et al. 1995; Cakir 2004; Kamoshita et al. 2008; Candogan et al. 2012; Wang et al. 2013; Mwadzigeni et al. 2016a). Frederick et al. (2001) reported a reduction in both total seed yield

and branch seed yield due to drought stress in soybean. An economic seed yield reduction in the range of 46-71%, due to soil WLIS, has also been reported (Samarah et al. 2006). This was attributed mainly to soil WLIS at flowering and seed-filling developmental stages. Apparently, during flowering, seed yield is reduced as a result of massive flower abortion caused by soil WLIS (Farooq et al. 2006). Yield reduction at seed-filling occurs due to a reduction in the assimilate partitioning to the developing pod, below the threshold level necessary to sustain optimal seed growth (Yadav et al. 2004); and activities of sucrose and starch biosynthesis enzymes which become inactive due to soil WLIS (Farooq et al. 2006).

A decrease in the number of branches, as well as the height of pods on plants, and number of pods per plant are related to the reduction in plant canopy as a result of soil WLIS. Pandey et al. (1984) have suggested that reductions in canopy size resulted in reduced leaf area which in turn is responsible for a drought induced reduction in seed yield. On the other hand, Manavalan et al. (2009) have suggested that reduction in canopy (leaf area) may also represent an important drought avoidance mechanism, particularly the increased leaf senescence which results in less water use. The tolerant soybean genotypes BL1, BL4 and CV2 have demonstrated low seed yield reduction with soil WLIS under field conditions. In contrast, seed yield of the sensitive genotype, CV3, was drastically reduced. The tolerant genotype CV2 demonstrated reduced canopy under soil WLIS; though its seed yield was not drastically reduced. This could be a form of drought avoidance mechanism employed by the genotype to optimise use of the limited soil water available and reduce its loss (Manavalan et al. 2009).

Recently, it is important to find affordable and reliable physiological indicators, which can help in the selection of drought-adaptive genotypes (Hura et al. 2007). This is in consideration of the unreliability of field selection of soil WLIS; since severity of natural drought stress is uncontrollable in most field conditions (Chen et al. 2016). Genotypic differences in response to soil WLIS have been identified in proline accumulation in many crop species (Efeoglu et al. 2009). As such proline accumulation, has been proposed to be useful as a complementary strategy for selection of drought tolerant genotypes in plant breeding (Abebe et al. 2003; Mwadzingeni et al. 2016b). However, this approach still requires validation for its usefulness in screening soybean germplasm for improved yield under drought stressed conditions. The

synthesis of free proline is widely used by plants to stabilize membranes and maintain the conformation of proteins when experiencing soil WLIS (Verbruggen and Hermans 2008). An increase in proline content under soil WLIS conditions was evident in the present study and this is in accordance with earlier observations made on soybean (Masoumi et al. 2011), maize (Chandrasekar et al. 2000; Efeoglu et al. 2009) and wheat (Geravandi et al. 2011).

It was evident that proline accumulated gradually from the well-watered treatment to the low (75%) treatment but then it rose sharply to moderate (50%) and severe (30%) WLIS treatments. This observation provided convincing evidence of a cause-and-effect relationship between proline content and soil WLIS. That is, proline accumulated under severe soil WLIS to serve as a defence against soil WLIS by acting as a compatible solute (Hare and Cress 1997). Genotypic differences in proline accumulation or interactions between genotype and WLIS treatment were observed in the present study. It was evident that tolerant genotypes BL1, BL4 and CV2 accumulated higher proline contents as opposed to the sensitive genotype CV3. The tolerant genotype (CV2) had very high levels of proline consistently across all the soil WLIS levels. Genotypic differences were evident in wheat cultivars for proline content when exposed to soil WLIS (Mwadzingeni et al. 2016a). In a study by Mwadzingeni et al. (2016a) a positive correlation between grain yield and proline content under drought stressed conditions was also observed but this was more prominent in tolerant wheat genotypes.

Through such genotypic differences in proline accumulation under soil WLIS, several researchers have proposed that proline could be a good indicator for soil WLIS tolerance in wheat (Van Heerden and De Villiers 1996; Kumar et al. 2015), which could be useful during genotype selection. In the past, many methods have been used to evaluate soil WLIS tolerance of crops (Zu et al. 2017). Proline, though it is only an indirect indicator of soil WLIS tolerance (Manavalan et al. 2009), has demonstrated to be simple, rapid and accurate to operate in order to distinguish drought tolerant genotypes in soybean. Genotypic variations were observed from known tolerant and sensitive genotypes under field conditions. However, to quantify the usefulness of proline as a WLIS tolerance indicator, there is a need to have well-characterised checks (known tolerant and susceptible genotypes) when testing genotypes, that could guide as a cut-off point for tolerance limits.

6.5 Conclusions and recommendations

Soil WLIS is a major constraint in soybean production. Results have demonstrated a seed yield loss of over 60% due to the deleterious effects of soil WLIS. The yield loss is mainly a result of reduction in seed number, seed size, number of pods per plant, pod abortion and plant canopy reduction, which is directly responsible for the generation of assimilates. Yield reduction was more pronounced in the sensitive genotype than in the tolerant ones. For purposes of breeding for soil WLIS tolerance, it is desirable to develop a screening method that is simple, rapid and accurate. By using proline accumulation under soil WLIS it was possible to distinguish between tolerant and sensitive soybean genotypes. The tolerant genotypes tended to accumulate higher levels of proline under soil WLIS than the sensitive one. The calorimetric-based proline assay was easy to perform and is quantifiable and reproducible. However, it is recommended that in addition to use of a proline-based method other phenotyping based methods such as yield determination and the tolerance indices should be carried out to better understand the mechanisms of drought tolerance, which the different genotypes are displaying.

6.6 References

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Chapter 7

General conclusions and recommendations

Soybean is an important source of high quality vegetable oil and protein. Despite the fact that production area for the grain legume has doubled over the last decade; South Africa remains a net importer of soybean and soybean products. South Africa imports soybean cake and soybean oil, mainly for the livestock feed industry. Among the many production limitations, soil water-limited-induced-stress (WLIS) is the major factor. This is exacerbated due to the fact that as a country South Africa is a water resource scarce nation. Hence production of soybean and other major cereal and legume crops has been pushed to the arid and semi-arid environments of the country; and mostly depends on supplemental irrigation.

Breeding for soil WLIS tolerant soybean genotypes is one of the sustainable way of dealing with drought. Direct selection for yield and soil WLIS tolerance in multiple environments is often employed as a means of selecting tolerant soybean genotypes by breeders. However this conventional direction selection is inefficient due to among other reasons; (i) associated costs, (ii) unpredictability of the climate in test environments and (iii) implications of the GxE on heritability of the trait. Hence the study was carried out with the aim of investigating other possible indicators of screening for soil WLIS tolerance for soybean.

Soybean germplasm (BL1, BL2, BL3, BL4, CV1, CV2, CV3 and CV4) obtained from the PANNAR SEED® (PTY) LTD breeding programme, South Africa were screened for soil WLIS tolerance. The conventional agronomical traits and yield based soil WLIS indices, as well as the seedling root traits and the biochemical, free proline concentration were used in the screening process. Agronomical traits that were measured included plant height, number of nodes, number and mass of pods per plant, number and mass of seeds per plant, total biomass and harvest index. Results showed that soil WLIS had a significant effect on the agronomical traits. Soil WLIS reduced the seed mass and number per plant, 100-seed mass, shoot biomass per plant, number and mass of pods per plant, number of nodes and plant height. It was evident that soil WLIS effects and main genotype effects were significant for the

soybean genotypes exposed to stress at the different growth stages. Lack of significant WLIS x genotype interaction suggested that the genotypes did not respond differentially to drought stress. Using the agronomical traits alone, it was difficult to discriminate tolerant from sensitive soybean genotypes.

Seed mass mean difference i.e. the relative performance of a genotype in a soil WLIS against non-stress environment was used to discriminate tolerant and sensitive genotypes. It was observed that tolerant genotypes tended to have least mean difference; suggesting better remobilisation capacity under soil WLIS than the sensitive genotypes. The effects of soil WLIS was significant among the genotypes agronomical traits. Increase in soil WLIS resulted in increased proline accumulation in the soybean genotypes. Unlike in other studies, the concentrations of proline accumulation under soil WLIS did not differ significantly among the soybean genotypes. However significant genotypic main effects for free proline accumulation suggested that less sensitive and tolerant genotypes tended to accumulate more proline than sensitive genotypes. Three tolerant (BL1, BL4 and CV2) and sensitive (CV3) genotypes were therefore advanced based on their genotypic potential for higher proline accumulation to further characterise the mode of drought tolerance at different growth stages.

Root characteristics have been closely associated with plants ability to tolerate soil WLIS. This is because the root system is responsible for the exploration and acquisition of soil water that the plant needs. In Chapter Four, the seedling shoot- and root traits among the three tolerant (BL1, BL4 and CV2) and the sensitive soybean genotype (CV3) were investigated. Results showed seedling genotypic variation among the drought tolerant and –sensitive genotypes for root and shoot traits. Soil WLIS tended to enhance partitioning of the biomass towards the roots. Tolerant soybean genotypes tended to have an increased root to shoot length ratio. This phenomenon enhances the ability of the tolerant genotypes access and acquisition of more edaphic resources per unit of the above ground shoot. Furthermore tolerant genotypes also demonstrated deep rooting ability as a coping mechanism to soil WLIS. Using these seedling root traits, tolerant genotypes were ably discriminated from the sensitive one. It is very difficult to phenotype root system. The modified ‘deep-pot’ method used to phenotype these seedling root traits in a non-destructive manner proved very effective for the purpose, and is recommended for similar kind of work.

In Chapter five, a glasshouse lysimeter trial was carried out to further characterise the mode of stress responses among the tolerant and sensitive soybean genotypes. Genotypes were subjected to soil WLIS at different growth stages (control, deficit irrigation during vegetative, flowering and pod-development growth stages). Results collaborated earlier findings that yield potential is adversely affected by soil WLIS. Results indicated that yield potential reduce much more when the plants are exposed to soil WLIS during the pod development growth stage and then during flowering. During these reproductive growth stages soil WLIS affects the availability of water which are critically needed for the active cell divisions that takes place in the developing ovules. Soil WLIS at these growth stage also leads to flower and pod abortion. Genotypic variation was observed for 100-seed mass, seed mass and number per plant, number of aborted pods and height of highest and lowest pods amongst soybean genotypes exposed to soil WLIS.

Soil WLIS also showed significant effects on proline concentration in stressed soybean plants. Proline concentration increased with an increase in soil WLIS. It was observed that proline accumulated most during the pod-development growth stage. Furthermore tolerant soybean genotypes tended to accumulate more proline concentration with an increase in soil WLIS than sensitive genotypes. In tolerant genotypes proline plays an osmoprotectant role. This suggested that proline accumulation could be a potential soil WLIS indices between tolerant and sensitive genotypes.

Yield-based tolerance and/or susceptibility indices were used to further characterise the sensitivity of genotypes to soil WLIS. These indices include mean productivity (MP), stress susceptibility index (SSI), stress tolerance (TOL), stress tolerance index (STI), geometric mean productivity (GMP), yield index (YI), yield stability index (YSI), seed mass under stress (Yp), Seed mass under non-WLIS, and seed mass under WLIS (Ys). The efficiency of the yield based indices in discriminating tolerant and drought genotypes was investigated alongside the free proline accumulation. Proline content under soil WLIS conditions correlated positively with yield potential (Yp and Ys). Results further showed that proline content under soil WLIS also correlated with the tolerance indices GMP, MP and TOL.

These association of proline accumulation under soil WLIS and Yp, Ys, GMP, MP and TOL were also collaborated with results from the principal component analysis (PCA). The PCA gave a good dimensional view of how proline associated with the drought tolerant indices and consequently, yielding potential which is key in any breeding programme. Strong and positive associations were observed between YI and Ys, MP, STI; GMP with MP, and STI, and TOL, Prls and Prlp. The tolerant genotype CV2 clustered separate from the rest of the genotypes (BL1, BL4 and CV3) and was closely associated with Yp, GMP, MP and STI, and less with Prlp, Prls and TOL and could be regarded as a more stable genotype which will be high yielding under both non-stress and WLIS conditions.

The second dimension of the PCA separated the stress-tolerant and the sensitive genotypes. Based on the PCA, BL2 will be more suitable for non-stress conditions, whilst CV3 and BL4 will be more suitable for WLIS conditions. Use of multivariate procedures added value to the interpretation of the data structure; it is therefore recommended that where investigations involve multiple variables like what most breeding programmes are involved in, it should be used.

A field experiment was conducted in a rain-out shelter to validate results on proline accumulation obtained from the two glasshouse experiments (Chapter three and five). The four soybean genotypes (BL1, BL4, CV2 and CV3) were subjected to different levels of soil WLIS [well-watered, low (75% deficit irrigation), moderate (50% deficit irrigation) and severe (30% deficit irrigation)] through-out the growing season in a rain-out shelter. Results showed that soil WLIS and the genotype effects were significant. Bottom and top pod height, number of branches, number of aborted pods, number and mass of seeds per plant, 100-seed mass, total seed yield and biomass were reduced by increased soil WLIS. The severe soil WLIS treatments reduced the agronomical and yield attributes drastically. Significant differences were observed among the genotype due to effects of soil WLIS for pod and bottom pod height, number of branches, number of aborted pods, 100-seed mass and total seed yield. Seed yield was reduced by as much as 63%. Yield reduction was more pronounced in the sensitive genotype than in the tolerant ones.

The soil WLIS effects on proline content on the soybean genotypes under field conditions collaborated the premise that free proline accumulation can be used as a selection indices for drought tolerance. Results suggested significant effects for soil WLIS treatments and the main genotypic effects as well as the interaction of the two. This suggested that the genotypes responded differentially in free proline accumulation due to increased soil WLIS. It was evident that proline accumulated with increased soil WLIS. Furthermore, tolerant genotypes (CV2, BL1 and BL4) tended to accumulate proline concentration much more than the sensitive genotype (CV3) under soil WLIS.

Soil WLIS reduces agronomical traits that affects yielding ability of the soybean genotypes. Consequently seed yield of soybean is reduced by soil WLIS. The effect of soil WLIS is more pronounced when soil WLIS is imposed at pod development growth stage. Agronomical traits alone cannot discriminate sensitive from tolerant soybean genotypes. However seed mass mean difference has potential to act as a drought stress indices in soybean. It has also been demonstrated that soybean accumulate proline under soil WLIS. The accumulation increases with an increase in soil WLIS. Genotypic variation exists among soybean genotypes for proline accumulation under soil WLIS stress. Using proline accumulation under soil WLIS sensitive genotype (CV3) was identified from the tolerant genotypes (CV2, BL1 and BL4). Hence proline accumulation can be used as a biochemical drought stress indices for soybean. As a drought stress indicator, proline accumulation associates significantly with yield based drought stress indices. Seedling root traits, traits such as tap root length and root to shoot length ratio have potential to discriminate sensitive from tolerant genotypes. These drought stress indices can be very useful for breeders dealing with large germplasm, since these essays can be done at an early stage and associated costs can be served.