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Physiological responses of selected African sorghum landraces to progressive water stress and re-watering

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Abbreviations: LWC, leaf water content; c_a, chlorophyll *a*; c_b, chlorophyll *b*; $c_{(x+c)}$, carotenoids; GS II, growth stage II; Mod-RW, moderate re-watered; MS, mild stress; SMC, soil moisture content; SS, severe stress; *Tchl*, total chlorophyll.

Abstract

Sorghum is particularly drought tolerant compared with other cereal crops and is favoured for subsistence farming in water scarce regions of the world. This study was conducted to identify South African sorghum landraces with superior drought tolerance compared with a drought tolerant breeding line (P898012). Seedlings of 14 South African sorghum landrace accessions were initially screened for drought tolerance by assessing percentage leaf water content (LWC) during progressive water deficit. Four landraces (designated LR5, LR6, LR35 and LR36) recorded higher LWC than P898012. These were subsequently evaluated with P898012 during the reproductive growth stage, for their physiological responses to mild (four days) and severe (six days) water stress treatments and a moderate re-watered treatment on day seven. Plant height, soil moisture and LWC were measured during harvests. Chlorophyll, carotenoid and proline contents were quantified. All five genotypes maintained LWC above 80% during mild and severe stress treatments. For LR35 and LR36, LWC were recorded within 8% less in comparison to their well-watered controls following the moderate re-watered treatment. Significantly higher chlorophyll and carotenoid contents were recorded for both LR6 and LR35 in comparison to P898012 during severe stress. When LWC was reduced in LR36 (to 73.68%) and LR35 (to 73.51%), their proline content significantly increased by 14- and 16-fold, respectively. In this study, we have identified four previously uncharacterised sorghum genotypes exhibiting drought tolerance and described their physiological responses during water deficit and moderate re-watering. Aside from their application to breeding, these landraces are valuable resources to elucidate genetic mechanisms that enable drought tolerance in South African sorghum.

Keywords

carotenoid, chlorophyll, drought tolerance, physiology, proline, *Sorghum bicolor*

1. Introduction

Drought is a complex environmental stress and major constraint to crop productivity (reviewed by Mishra and Singh, 2010; Farooq et al., 2012). It is a global problem that may have profound effects on agriculture and food security, especially upon agricultural systems which depend on rain as their primary source of water (Bray et al., 2000; Rosegrant et al., 2002). Subsistence and small-scale farmers, particularly those living in the semi-arid areas of Africa and Asia, are vulnerable to the impacts of drought as they often lack essential resources for additional agricultural inputs and irrigation systems (Glantz, 1987; Leichenko and O'Brien, 2002). Whilst most primary cereal crop species are sensitive to hot and dry climates, sorghum [*Sorghum bicolor* (L.) Moench] is recognized as a remarkably drought tolerant species and is favoured for subsistence farming in water scarce, impoverished regions of the world (House, 1985; McKersie and Leshem, 1994; Wani et al., 2012).

Sorghum, which is indigenous to Africa, is a close relative of sugarcane and cereals such as maize and pearl millet. It is a versatile crop and the utilization of the whole plant is far-reaching; consequently, sorghum is grown for food, animal feed, fibre, fuel and used for some industrial purposes (Wall and Ross, 1970; House, 1985; Paterson et al., 2009). Sorghum is the third most important grain crop cultivated in South Africa after maize and wheat (Sorghum Section 7 Committee, 2007). Worldwide, sorghum is the fifth most

important grain crop with 62 million tonnes produced during 2013 (Wani et al., 2012; FAO, 2015). Although grain sorghum exhibits resilience to the effects of water stress, particular growth stages in its lifecycle are susceptible to drought stress. The early vegetative stage and reproductive stages (pre- and post-flowering) of sorghum are vulnerable to the effects of water deficit (Tuinstra et al., 1997; Kebede et al., 2001; Wani et al., 2012). A drought period during the early seedling stage of sorghum may inhibit establishment of the crop (McKersie and Leshem, 1994). The water demand of sorghum is greatest during the pre-flowering reproductive growth stage (Anon, 2008). Water stress during pre- and post-flowering stages impacts grain development and yield of the crop (McKersie and Leshem, 1994). Therefore, the ability to withstand water deficit at these stages is critical to productivity.

Plants may exhibit various biochemical and physiological mechanisms to ameliorate the effects of drought (Tuinstra et al., 1997; Bray et al., 2000). The process in which plants are able to grow and complete their lifecycle before soil moisture becomes limiting represents the drought escape mechanism. Drought avoidance involves features which aid in decreasing the amount of water loss by the plant whilst drought tolerance encompasses stabilizing mechanisms that protect cellular and metabolic integrity and function at the tissue or cellular level (Tuinstra et al., 1997; Blum, 2011). These mechanisms may work synergistically to bring about successful tolerance during periods of drought.

Water is essential for the myriad of biological processes which contribute to sustaining life. Consequently, periods of water deficit have profound effects on the physiology of all organisms, especially sedentary plants. Aside from a plants' response to continuous water stress, it is important to consider the effect of re-hydration on plant physiology. In field environments water availability is subject to cyclical changes and unpredictable climatic conditions therefore, intermittent rains may follow a drought period (Izanloo et al., 2008; reviewed by Mishra and Singh, 2010). A plants' prompt biochemical response to a re-hydration event is a good indicator of recovery which is dependent on the severity of the preceding water stress. Intensive research has been conducted to understand plant responses to water deficit only however work describing the effects of water stress and re-watering on plants are limited (Takele, 2010; reviewed by Xu et al., 2010, Filippou et al., 2011).

Water stress in plants may manifest as decreased leaf water content and chlorophyll contents. The leaf water content is a measure of plant stress and severe decreases may contribute to structural interruptions of important biological functions in plants leading to injury or tissue death (McKersie and Leshem, 1994). Total chlorophyll content as well as chlorophyll *a* and *b* contents are indicators of overall plant health and directly influence a plants' ability to absorb light for photosynthesis (Malkin and Niyogi, 2000). This is crucial to maintaining vital processes of the plant system.

Some plant protective mechanisms may be activated during abiotic stress, such as increased production of pigments and organic osmolytes. Carotenoids, which include

carotenes and xanthophylls, are pigments closely associated with chlorophylls and play a role in light absorption and photosynthesis (reviewed by Britton, 1995; Malkin and Niyogi, 2000). They also provide photoprotection during abiotic stress. The amino acid, proline, is an important compatible osmolyte which has been found to accumulate in plants during stress (Bray et al., 2000; Ashraf and Foolad, 2007). Proline is suggested to serve an important protective role against abiotic stress in plants due to its distinct biochemical properties which enable this amino acid to have a neutral charge at physiological pH, not affect cellular metabolism and scavenge harmful reactive oxygen species (Van Rensburg et al., 1993; Bray et al., 2000; reviewed by Kavi Kishor et al., 2005).

The aim of this study was to identify drought tolerant African sorghum genotypes and subsequently evaluate their physiological responses to progressive water stress and subsequent moderate re-watering. The first objective was to screen 14 sorghum landrace accessions for drought tolerance at the seedling stage together with a known drought susceptible (ICSV112) and tolerant breeding line (P898012) during progressive water stress. The second objective was to evaluate the physiological responses of those landraces which compared favourably with P898012 in the seedling stage screen, together with P898012 during progressive water deficit (mild and severe stress) and a moderate re-watered treatment at the drought sensitive, growth stage (GS) II of development. The early seedling and pre-flowering reproductive stages of sorghum are sensitive to water stress. Drought tolerance at these stages are important for plant survival and grain yield.

2. Materials and methods

2.1 Plant material

The seeds of sorghum [*Sorghum bicolor* (L.) Moench] lines and landrace accessions were obtained from the Agricultural Research Council Grain Crops Institute (ARC-GCI) and the National Plant Genetic Resources Centre of the Department of Agriculture, Forestry and Fisheries (DAFF), South Africa, respectively. P898012 is a public genotype that was bred at Purdue University, USA and exhibits pre-flowering and post-flowering drought resistance (Casas et al., 1993; Kumar et al., 2011; Yu et al., 2013). ICSV112 was bred at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Center, India and its cultivation is recommended for rainy seasons or as an irrigated crop during post-rainy seasons (Anon, 1988).

2.1.1 Seedling water stress screen

Three seeds of P898012 (drought tolerant), ICSV112 (drought susceptible) and each landrace accession, were planted in 12 cm diameter plastic plant pots, lined with filter paper in the bottom and filled with 624 g of an autoclaved soil mix consisting of ½ red soil: ½ river sand: 1 vermiculite: 2 compost. The soil was thoroughly wet with water before seeds were sown at a depth of 2 cm and each pot was supplemented with 50 mL Nutrifeed [Starke Ayres (Pty) Ltd., RSA] nutrient solution (1 g/L). There were three replicates per genotype and stress time point. Pots were randomly placed in a controlled growth room facility with a 16 h light / 8 h dark photoperiod at a total energy in the visible region measured by an AccuPAR LP-80 ceptometer (Decagon Devices, Inc., USA) to be 312 $\mu mol.m^{-2}\ s^{-1}$. A HOBO® U10 Temp/RH series data logger (Onset®,

USA) was used to monitor the temperature at 15 min intervals for the duration of the experiment. The temperature was maintained at a range of 26-29^{o}C. Seedlings were grown to the five leaf stage by daily watering with 50 mL water. Before the onset of stress, each pot was watered with 150 mL water and supplemented with 50 mL Nutrifeed (2 g/L). Water was withheld from pots for six, seven, eight and nine days.

2.1.2 Drought simulation at reproductive GS II

One hundred seeds per sorghum genotype (P898012, LR5, LR6, LR35 and LR36) were surface decontaminated by exposure to sodium hypochlorite (1% v/v NaOCl, JIK® Reckitt Benckiser Group plc., RSA) for 10 min followed by a 3X rinse with sterile deionised water. For seed germination, 4 kg of autoclaved soil mix (section 2.1.1) was wet with 500 mL water whereafter it was placed in plastic trays (35 x 25 cm). Surface decontaminated seeds were sown into the soil at a depth of 2 cm and thereafter, the soil was watered with 200 mL water. Trays were watered daily with 400 mL water and maintained at a 16 h light / 8 h dark photoperiod at a total energy in the visible region at 150 µmol.m^{-2} s^{-1}. The temperature was maintained at a range of 24-29^{o}C.

One week old sorghum seedlings were transferred to individual 1L, 15 cm plastic, filter paper-lined plant pots filled with 1 kg autoclaved soil mix (section 2.1.1). The soil was saturated with 250 mL water before planting seedlings and each pot was supplemented with 50 mL Nutrifeed (1 g/L). Appropriately labelled pots were randomly arranged in the growth room. Seedlings were grown for eight weeks after seeds were sown and pots were watered daily with 80 mL water during weeks one to four, 100 mL water during

weeks five to six and 120 mL water from week seven until onset of drought conditions. In addition, pots were supplemented with 50 mL Nutrifeed (2 g/L) fortnightly for eight weeks.

Water stress treatments commenced during GS II, which occurs between 30-60 days after sowing (Du Plessis, 2008). This is a pre-flowering reproductive stage during which the water demand by the plant is greatest (Anon, 2008). There were nine biological replicates per sorghum genotype treatment and control. Before the onset of water stress, each pot was watered with 150 mL water and supplemented with 50 mL Nutrifeed (2 g/L). A progressive water deficit was applied to the drought treatment plants whilst control plants were watered daily with 120 mL water. Three stress treatment time points were investigated: [1] mild stress (MS) after four days of water deficit, [2] severe stress (SS) after six days of water deficit and [3] a moderate re-watered (Mod-RW) treatment during which soil was re-watered with 120 mL water, 5 h prior to harvest on day seven of water deficit.

This study was a controlled pot experiment in which sorghum plants, at the reproductive growth stage (30-60 cm stalk heights), were intensively stressed in 1L pots over several days of water withholding. The water stress time points were chosen after a preliminary screen to determine the degree of stress over a period of nine days (results not shown). Four days after water was withheld, the soil moisture content had decreased to ~50% of well-watered values and this was designated as a mild stress. At six days of water deficit, the soil moisture content had reached its lowest values and remained in this

range until day nine. Therefore, six days of water withholding was labelled as a severe stress condition. A moderate re-watering treatment was conducted on day seven after water was initially withheld. Plants were exposed to water for 5 hours before harvesting to identify prompt biochemical changes in response to water after a period of water deficit.

2.2 Measurements

During water stress at the seedling stage, the percentage leaf water content (LWC) of the third leaf was calculated (Kirkman, 2005; Zeng et al., 2013). The following physiological parameters were measured or quantified at each harvest day during water stress at GS II: (a) plant height was measured from the soil surface to the top of sorghum stalk; (b) percentage soil moisture content (SMC) at a depth of 85 mm was recorded using a soil moisture probe, manufactured by DFM Software Solutions CC. (RSA), and three measurements were recorded for each pot; (c) total chlorophyll, chlorophyll *a* and *b*, as well as carotenoid contents were quantified as outlined by Lichtentaler and Buschman (2001) using a leaf disk from the third leaf of each plant; (d) the third leaf blade was excised for quantification of percentage LWC; and (e) proline was quantified following the protocol described by Bates et al. (1973).

2.2.1 Leaf water content

The third leaf blade of each plant was excised at the leaf collar for quantification of LWC. The fresh weight (FW) was immediately measured after excision at harvest. Individual leaves were placed in (5 x 8 cm) brown paper bags and oven-dried at 70^{o}C

for 48 h. The dry weight (DW) was then recorded and LWC was calculated on a fresh weight basis using the following equation (Kirkman, 2005; Zeng et al., 2013):

$$LWC\ (\%) = [(FW - DW) / FW] \times 100$$

The LWC of seedling leaves harvested following six, seven, eight and nine days of water stress were calculated with three biological replicates per treatment. Leaves from sorghum plants stressed during GS II were harvested at four days and six days of water withholding and 5 h after re-watering on day seven with nine biological replicates per treatment and control.

2.2.2 Quantification of chlorophylls and carotenoids

Prior to excision of the third leaf blade for LWC (section 2.2.1), circular leaf disks (9 mm in diameter) were punched using a cork borer for quantification of chlorophylls and carotenoids. Leaf disks were placed on ice and maintained in the dark until extraction of leaf pigments. Individual leaf disks were homogenized in 1 mL of 80% acetone [Merck (Pty) Ltd, Germany] using a mortar and pestle. A further 1 mL of 80% acetone was added to the homogenized sample and centrifuged at 10 000 rpm for 1 min. The optical density of the supernatant was measured using a quartz cuvette and 80% acetone as a blank with a Beckman Coulter™, Inc. (USA), DU®800 spectrophotometer at three wavelengths: 470 nm for carotenoids, 646.8 nm for chlorophyll *b* and 663.2 nm for chlorophyll *a* quantification.

The following equations were used to quantify the chlorophyll and carotenoid contents according to Lichtentaler and Buschman (2001):

(1) c_a (µg/mL) = 12.25 $A_{663.2}$ – 2.79 $A_{646.8}$

(2) c_b (µg/mL) = 21.50 $A_{646.8}$ – 5.10 $A_{663.2}$

(3) *Tchl* (µg/mL) = $c_a + c_b$

(4) $c_{(x+c)}$ (µg/mL) = (1000 A_{470} – 1.82c_a – 85.02c_b) / 198

(1) Chlorophyll *a* quantification, (2) Chlorophyll *b* quantification, (3) Total chlorophyll and (4) equation for the quantification of carotenoids, where (x + c) = xanthophylls and carotenes.

The concentration of leaf pigments was represented on a fresh weight basis as mg.g^{-1} FW.

2.2.3 Proline quantification

The protocol used for the quantification of proline was adapted from Bates et al. (1973). Chemicals were purchased from Sigma-Aldrich® Co. LLC. (USA). Acid-ninhydrin reagent was prepared by dissolving 1.25 g ninhydrin in 30 mL glacial acetic acid and 20 ml 6 N orthophosphoric acid by warming and gentle swirling. The reagent was kept cool on ice and used within 24 h. Flash frozen leaf material from the fifth leaf (0.5 g FW) was ground to a fine powder in liquid nitrogen and homogenized in 5 mL 3% (w/v) sulphosalicylic acid. The homogenate was centrifuged at 10 000 rpm for 5 min. Equal parts (2 mL) of the supernatant, acid-ninhydrin reagent and glacial acetic acid were combined in a test-tube and placed in a water bath at 100^{o}C for 1 h. The reaction, which produced a red colour, was terminated by being placed on ice. Toluene (4 mL) was added to the reaction mixture, vigorously mixed and left at ambient room temperature

(25-28^{o}C) for 15 min until separation of layers was observed. The optical density of the chromophore-containing toluene (upper phase) was measured at 520 nm using a quartz cuvette and toluene as a blank with a Beckman Coulter^{TM}, Inc. (USA), DU$^{®}$800 spectrophotometer. Proline concentration was determined from a standard curve and calculated on a fresh weight basis using the following formula (Bates et al., 1973):

Proline (mg.g^{-1} FW) = [(µg proline/ml x 4 ml toluene) / (0.5 g sample/2.5)] / 1000

2.2.4 Data analysis

The LWC data recorded for sorghum seedlings during water stress were analysed using STATISTICA version 6.0 (Statsoft® Inc., USA) and each treatment was analysed with three replicates. The statistical program, GenStat® version 12 (VSN International, UK) was used to evaluate significance of data obtained from sorghum plants stressed during the reproductive, GS II and each treatment/control consisted of nine replicates. Data were initially tested for normality using the Kolmogorov-Smirnov ($P < 0.05$) test. Details of the specific analyses are presented in the appropriate sections. Microsoft© (2010) Excel was used to generate graphs, calculate means and Standard Error (SE) values.

3. Results and Discussion

Two independent water stress investigations were conducted to: (1) screen 14 South African, sorghum landrace accessions for drought tolerance during the vegetative, seedling stage together with drought susceptible ICSV112 and drought tolerant P898012 breeding lines and (2) assess the physiological responses of landraces selected from the

water stressed seedling screen, during the drought sensitive, reproductive GS II of sorghum development at eight weeks after emergence. Four landraces recorded higher LWC during water deficit at the seedling stage compared with P898012. Water stress at GS II, demonstrated the physiological drought responsive mechanisms activated in the four South African landraces and results indicate that the selected landraces compared favourably with P898012 with respect to the physiological parameters measured.

3.1 Progressive water deficit at seedling stage

The performance of 14 sorghum landrace accessions was evaluated during progressive water stress at the seedling stage with a drought sensitive line, ICSV112 and drought tolerant breeding line, P898012. This screen was conducted to select sorghum landraces with superior drought tolerance compared with P898012. The LWC was measured using excised leaf blades harvested after withholding water from pots for six, seven, eight and nine days. There were three replicates for each sorghum genotype and stress time point. The calculated LWC values for all investigated genotypes were statistically analysed by ANOVA ($P < 0.05$, df = 63, F statistic = 17.72, n = 3) and a significant difference was found amongst the sorghum genotypes and stress treatments. A graphical representation of the change in percentage LWC compared with P898012 is presented in Figure 1 to highlight the landraces which responded better (positive values on y-axis) than the drought tolerant breedling line by maintaining higher LWC during progressive water deficit.

[Figure 1]

It is essential for young seedlings to tolerate water stress as this may impact the establishment of the crop following germination (McKersie and Leshem, 1994). As expected, the drought sensitive line, ICSV112, performed poorly in comparison to P898012 during all stress treatments. During the most severe stress (after nine days of water withholding), the lowest LWC was recorded for ICSV112 which was 33% lower in comparison to P898012 (Fig. 1). LR5, LR6, LR35 and LR36 were the only landraces with superior performance compared with drought tolerant P898012 during the imposed water deficits. At nine days of water stress, LR5, LR6, LR35 and LR36 recorded higher LWC in comparison to P898012 (Fig. 1, 8.4%, 12.5%, 11.9% and 14.3%, respectively). The LWC recorded for these four landraces during nine days of water withholding ranged between 76.5% and 82.4%. Therefore, these four potentially drought tolerant landraces were subjected to further treatments at the reproductive, pre-flowering GS II to assess their physiological responses to water deficit and moderate re-watering.

3.2 Physiological responses of sorghum to water deficit at reproductive GS II

Two progressive water stress treatments (MS and SS) and a moderate re-watered treatment (Mod-RW) were applied to five sorghum genotypes: a drought tolerant breeding line (P898012) and four potentially drought tolerant landraces (LR5, LR6, LR35 and LR36) selected from the seedling water stress screen in order to compare their physiological responses to simulated drought conditions at GS II. Germination amongst these genotypes ranged from 63% recorded for P898012 to 77% for LR35. LR36, LR5 and LR6 recorded germination values of 76%, 74% and 72%, respectively.

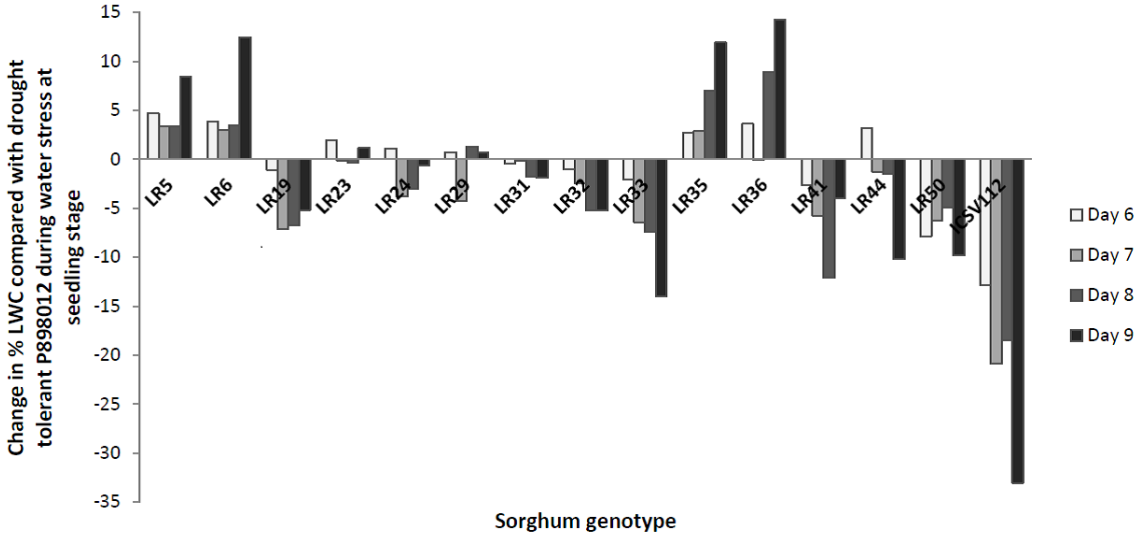

Figure 1: A representation of the change in leaf water content (% LWC) of sorghum seedlings compared with drought tolerant P898012 during progressive water deficit. Water was withheld from seedling pots for six, seven, eight and nine days. Bars on the positive y-axis represent sorghum landraces which performed better than P898012 whilst bars on the negative y-axis represent genotypes which performed poorer than P898012 in terms of their ability to maintain % LWC during water deficit.

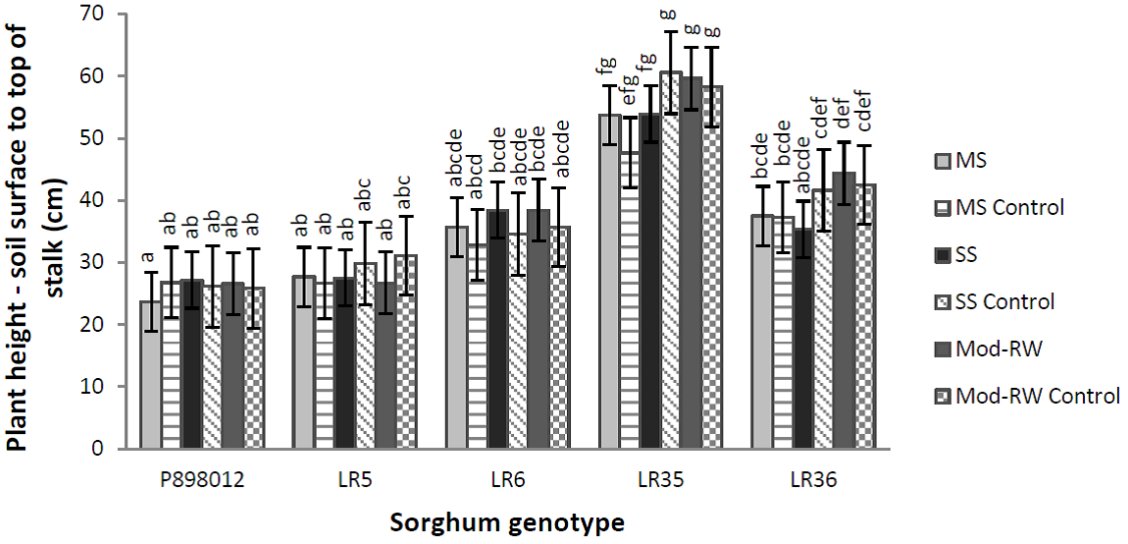

Figure 2: Plant height profiles of sorghum genotypes (P898012, LR5, LR6, LR35 and LR36) during water stress treatments imposed at GS II. Plant heights were measured from the soil surface to the top of sorghum stalk. Dissimilar alphabet characters assigned using the Holm Sidak (5%) post-hoc test denote a statistical significance (data were analysed using an ANOVA analysis, $P < 0.05$, df = 29, F statistic = 21.90, mean ± SE, n = 9).

Water was withheld from treatment pots (progressive water stress) at eight weeks after seeds were sown. This was approximately 53 days after seedling emergence, at which time the sorghum plants were approaching the boot stage of development (Du Plessis, 2008). Following commencement of water stress, chlorosis and wilting was observed in the lower leaves of sorghum plants. This correlated with a common observation that older leaves desiccate and die first during water deficit as a mechanism to reduce leaf area and plant water use (Blum, 2011). Leaf rolling and erect leaves were observed in LR35 plants. Morphological changes to the leaf structure by rolling or folding have been reported as a response to drought stress in cereal species (O'Tool and Cruz, 1980; Fernandez and Castrillo, 1999; Kusaka et al., 2005). Kusaka et al. (2005) reported leaf folding in drought tolerant pearl millet during stress and suggested that this morphological change was an adaptive response to severe drought stress by reducing the surface area exposed to evaporation. Therefore, LR35 may have responded to the water deficit by activating a drought avoidance mechanism.

The percentage soil moisture content (SMC) of individual pots was measured on each harvest day using a soil moisture probe in order to compare the uniformity of the water stress treatments. There was a significant difference in SMC between treatments (Table 1, ANOVA analysis, $P < 0.05$, $df = 29$, F statistic $= 72.23$, $n = 9$).

During water stress, SMC ranged between 45-55% during MS, 32-37% during SS and 44-61% during Mod-RW treatments. For individual sorghum genoytpes, the SMC of

Table 1: The mean soil moisture content (%) recorded during harvests for treatment and control pots following water stress treatments. Measurements were recorded during mild and severe water stress treatments and a moderate re-watered treatment. Dissimilar alphabet characters assigned using the Holm Sidak (5%) post-hoc test denote a statistical significance (data were analysed using ANOVA, $P < 0.05$, df = 29, F statistic = 72.23, mean percentage, n = 9).

Stress	Genotype	Mean soil moisture content (%)	
		Treatment	Control
Mild stress	P898012	54.58^{de}	78.42^{gh}
	LR5	53.10^{de}	80.45^{h}
	LR6	48.41^{d}	76.37^{gh}
	LR35	48.76^{d}	74.53^{gh}
	LR36	44.57^{cd}	75.94^{gh}
Severe stress	P898012	32.22^{a}	80.04^{h}
	LR5	35.30^{abc}	75.30^{gh}
	LR6	35.32^{abc}	67.39^{fg}
	LR35	33.23^{ab}	78.41^{gh}
	LR36	36.76^{abc}	75.12^{gh}
Moderate re-watered	P898012	47.93^{d}	71.11^{fgh}
	LR5	60.70^{ef}	75.44^{gh}
	LR6	48.97^{d}	75.11^{gh}
	LR35	43.98^{bcd}	78.30^{gh}
	LR36	45.03^{cd}	75.71^{gh}

well-watered, control pots were statistically similar as seen by shared statistical alphabets (Table 1). SMC for P898012, LR5 and LR6 control pots ranged between 71-80%, 75-80% and 67-76 %, respectively. For LR35 and LR36, SMC of control pots were measured at 74-78% and 75%, respectively. As all other experimental conditions (e.g. room temp, humidity, pot size, watering volume) were consistent and there were nine biological replicates with each replicate being a mean of three individual measurements to minimise error, inherent genotypic variation may have influenced SMC of control plants. The SMC between treatment pots and its corresponding control pots were significantly different therefore a degree of water deficit was inflicted on the treatment plants.

The plant height profiles of the sorghum genotypes were noted during harvest when plants were measured from the soil surface to the top of sorghum stalk. Statistical analysis of height data showed a significant difference in the plant height profiles amongst the sorghum genotypes investigated (ANOVA analysis, $P < 0.05$, $df = 29$, F statistic = 21.90, $n = 9$). Data for this measurement revealed that phenotypically LR35 was significantly taller when compared with P898012, LR5, LR6 and LR36 (Fig. 2).

Generally, taller sorghum genotypes are favoured for cultivation, except in areas in which mechanical harvesting methods are employed (Quinby and Schertz, 1970). Tall sorghum plants which are primarily grown by small-scale farmers in Africa and Asia

may be used as fuel and building material after grain harvest (Maiti et al., 2012). Jordan et al. (2003) investigated the performance of sorghum hybrids and found a strong correlation between increased plant height and grain yield. This correlation has been previously observed in sorghum (Graham and Lessman, 1966; Liang et al., 1969). George-Jaeggli et al. (2011) reported that increased biomass of tall sorghum plants was important for increased grain yield after investigating the direct effects of a major dwarfing gene on sorghum shoot biomass, grain yield and yield components. According to Blum (2011), total leaf area is the most dominant factor influencing whole plant transpiration and when grown in a pot of equal volume, a larger plant would require more frequent watering than a smaller plant. Therefore, under uniform water stress conditions imposed on the five sorghum genotypes investigated, the phenotypically different plant heights exhibited by these genotypes likely influenced their water demand and the severity of water deficit experienced.

3.2.1 Leaf water content measured during GS II

At each harvest, the LWC of the third leaf was determined using an equation incorporating the leaf fresh and dry weights. Overall, there was a significant difference in LWC amongst sorghum genotypes (Fig. 3, ANOVA analysis, $P < 0.05$, $df = 29$, F statistic = 11.63, $n = 9$). The LWC values across stress treatments ranged: 85-88% during MS, 82-86% during SS and 74-85% during Mod-RW treatments.

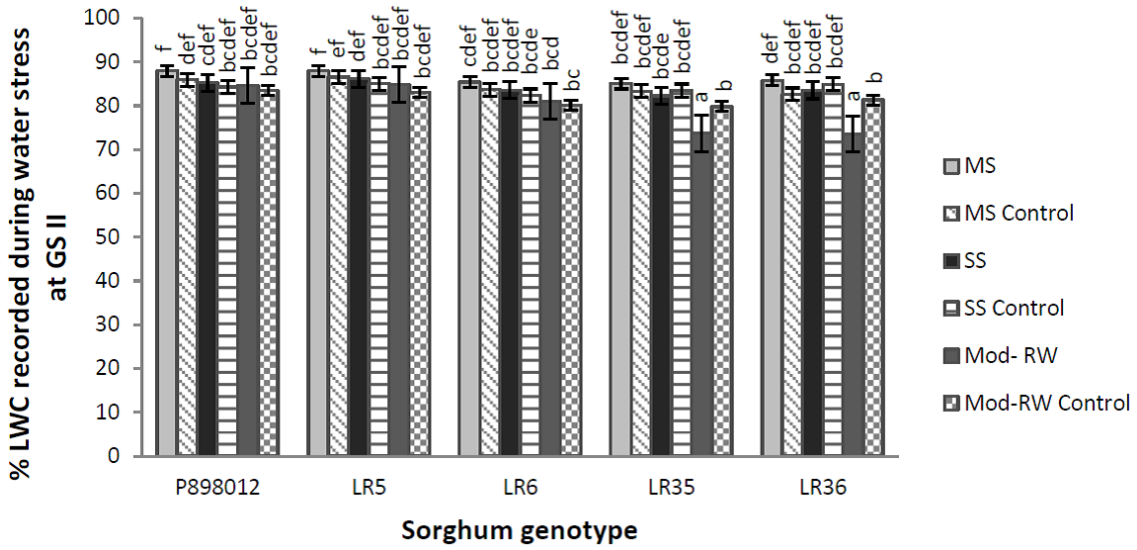

Figure 3: The leaf water content (% LWC) amongst five sorghum genotypes (P898012, LR5, LR6, LR35 and LR36) following mild and severe water stress treatments and a moderate re-watered treatment. Dissimilar alphabet characters assigned using the Holm Sidak (5%) post-hoc test denote a statistical significance (data analysed by an ANOVA analysis, $P < 0.05$, df = 29, F statistic = 11.63, percentage ± SE, n = 9).

A significant difference was found only during the Mod-RW treatment when LWC recorded for LR35 and LR36 were statistically lower when compared with the other genotypes (Fig. 3). In comparison to their controls, there was a reduction in LWC for LR35 and LR36 of 6.1% and 7.8%, respectively. Overall, the LWC for these five sorghum genotypes was maintained above 73% during all water stress treatments at GS II.

Water potential gradients are critical for uptake of water by plants. The soil water potential should be higher than the water potential of root tissues to enable this process (Bray et al., 2000). The regulation of cellular water content and solute potentials by osmotic adjustment are important for plant tolerance to water deficit. Water loss from cells may lead to mechanical disruptions within cells (McKersie and Leshem, 1994). In addition, re-watering affects the severity of ion leakage and membrane disruption due to the irreversible changes in cell membranes as a consequence of water deficit. Giles et al. (1976) investigated water stress and re-watering on the ultrastructure of *Sorghum bicolor* leaf cells. These researchers found that in mature sorghum plants within 3 hours of re-watering after 7 days water stress, leaf tissue showed signs of recovery by starch accumulation in bundle sheath cells, opening of stomata and a reduction in abscisic acid levels (Giles et al., 1976). Therefore, maintenance of the membrane structure during water stress is important for drought tolerance (Ristic et al., 1996; Triparthy et al., 2000).

According to Lambers et al. (2008), the water content of tissues such as leaves and roots typically range between 70-95%. In this study, sorghum genotypes were able to maintain LWC compared with their well-watered controls and above 80% during four and six days of water deficit. After the moderate re-watered treatment at day seven of water deficit, a less than 8% reduction in LWC was recorded for LR35 (73.68%) and LR36 (73.51%) compared with their well-watered, controls; despite an increase in SMC during this treatment by 10.7% and 8.3% from the severe stress for LR35 and LR36, respectively (Table 1). The water demand of these landraces may have increased as the progressive water stress treatments were imposed and they were potentially more physiologically stressed at harvest on day seven. The severity of water deficit experienced by LR35 in particular may have been a consequence of its height, being significantly taller than that of the other genotypes thereby influencing total leaf area and whole plant transpiration (Fig. 2).

3.2.2 Drought induced changes to chlorophyll, carotenoid and proline contents

At each harvest, a leaf disk was used to calculate chlorophyll and carotenoid contents after spectrophotometer measurements. The chlorophyll *a* (c_a) and *b* (c_b) contents were measured at 663.2 nm and 646.8 nm, respectively. Statistical analysis of c_a data revealed there was a significant difference amongst sorghum genotypes during treatments and controls (Table 2, ANOVA analysis, $P < 0.05$, df = 29, F statistic = 13.38, n = 9). Data obtained during MS were statistically similar between sorghum genotypes and ranged between 2.41 $mg.g^{-1}$ FW (LR5) and 3.01 $mg.g^{-1}$ FW (LR6) for stressed plants and

Table 2: A comparison of mean chlorophyll, carotenoid and proline contents between treatments and controls amongst five sorghum genotypes (P898012, LR5, LR6, LR35 and LR36) during two water stress treatments and a moderate re-watered treatment. Dissimilar alphabet characters for each column was assigned using the Holm Sidak (5%) post-hoc test and denote a statistical significance (data were analysed using ANOVA analyses, $P < 0.05$, df = 29, F statistics: c_a = 13.38, c_b = 9.62, *TChl* = 13.28, carotenoid = 16.14, proline ($\log_{10}$) = 9.94, mean, n = 9).

Stress	Genotype	*Chlorophyll a* (mg.g^{-1} FW)		*Chlorophyll b* (mg.g^{-1} FW)		Total chlorophyll (mg.g^{-1} FW)		Carotenoid (mg.g^{-1} FW)		Proline (mg.g^{-1} FW)	
		Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control
Mild stress	P898012	2.57^{abc}	2.83^{abcd}	0.82^{abcdef}	0.69^{abcd}	3.40^{abc}	3.52^{abcde}	0.47^{abc}	0.55^{abcd}	1.00^{abcd}	0.49^{ab}
	LR5	2.41^{ab}	3.00^{abcde}	0.60^{a}	0.74^{abcde}	3.01^{ab}	$3.74^{abcdefgh}$	0.46^{abc}	0.59^{abcde}	0.68^{abcd}	0.42^{a}
	LR6	3.01^{abcde}	$3.29^{abcdefg}$	0.71^{abcd}	0.68^{abcd}	$3.72^{abcdefg}$	$3.97^{abcdefgh}$	0.58^{abcde}	0.68^{cdefg}	1.06^{abcd}	0.76^{abcd}
	LR35	2.95^{abcde}	3.14^{abcde}	0.69^{abcd}	0.80^{abcdef}	$3.64^{abcdefg}$	$3.94^{abcdefgh}$	0.61^{abcde}	0.65^{abcd}	0.92^{abcd}	0.63^{abcd}
	LR36	2.87^{abcd}	2.83^{abcd}	0.70^{abcd}	0.66^{abc}	3.57^{abcdef}	3.49^{abcd}	0.55^{abcd}	0.56^{abcd}	1.79^{de}	1.01^{abcd}
Severe stress	P898012	**2.24^{a}**	**$3.46^{bcdefgh}$**	**0.66^{abc}**	$0.92^{abcdefg}$	**2.90^{a}**	**$4.38^{bcdefghij}$**	**0.41^{a}**	**0.64^{bcde}**	0.88^{abcd}	0.80^{abcd}
	LR5	**2.40^{ab}**	3.00^{abcde}	**0.64^{ab}**	$0.89^{abcdefg}$	**3.04^{ab}**	$3.89^{abcdefgh}$	**0.45^{ab}**	0.59^{abcde}	0.70^{abcd}	0.52^{abc}
	LR6	**$3.82^{defghij}$**	$3.93^{defghij}$	0.99^{bcdefg}	1.04^{defgh}	**$4.82^{defghijkl}$**	$4.97^{fghijkl}$	**0.74^{defgh}**	0.73^{defgh}	1.27^{bcd}	0.65^{abcd}
	LR35	**$3.85^{defghij}$**	4.04^{efghij}	**1.14^{fgh}**	1.11^{efgh}	**4.99^{ghijkl}**	5.15^{hijkl}	**0.73^{defgh}**	0.79^{efghi}	2.79^{bcde}	0.58^{abc}
	LR36	3.09^{abcde}	$3.25^{abcdefg}$	$0.89^{abcdefg}$	$0.91^{abcdefg}$	$3.98^{abcdefgh}$	$4.16^{abcdefghi}$	0.61^{abcde}	0.66^{bcdef}	3.86^{de}	0.65^{abcd}
Moderate re-watered	P898012	$3.60^{cdefghi}$	3.19^{abcdef}	$0.89^{abcdefg}$	$0.88^{abcdefg}$	$4.49^{cdefghijk}$	$4.07^{abcdefghi}$	0.72^{defgh}	0.64^{bcde}	1.66^{cde}	1.56^{cde}
	LR5	$3.85^{defghij}$	4.31^{ghij}	1.03^{cdefgh}	1.12^{efgh}	$4.88^{defghijkl}$	5.43^{ijkl}	0.74^{defgh}	0.87^{fghi}	0.71^{abcd}	0.74^{abcd}
	LR6	4.63^{ij}	4.82^{j}	1.21^{gh}	1.40^{h}	5.84^{kl}	6.22^{l}	**0.92^{hi}**	0.89^{ghi}	1.66^{bcd}	0.76^{abcd}
	LR35	4.29^{fghij}	4.50^{hij}	1.14^{fgh}	1.22^{gh}	5.43^{ijkl}	5.71^{jkl}	**0.92^{hi}**	0.91^{hi}	**11.18^{ef}**	**0.70^{abcd}**
	LR36	$3.88^{defghij}$	4.68^{ij}	1.05^{defgh}	1.22^{gh}	$4.92^{efghijkl}$	5.90^{kl}	**0.99^{i}**	0.89^{ghi}	**16.52^{f}**	**1.21^{bcd}**

between 2.83 mg.g^{-1} FW (P898012 and LR36) and 3.29 mg.g^{-1} FW (LR6) for well-watered plants.

During SS, c_a content for P898012 (2.24 mg.g^{-1} FW) and LR5 (2.40 mg.g^{-1} FW) were statistically similar to each other but were significantly lower compared with values obtained for LR35 (3.85 mg.g^{-1} FW) and LR6 (3.82 mg.g^{-1} FW). The c_a content of LR36 during SS was statistically similar to the four other sorghum genotypes. The c_a content ranged between 3.60 mg.g^{-1} FW (P898012) and 4.63 mg.g^{-1} FW (LR6) during the Mod-RW treatment. Values obtained from treatment and control plants were statistically similar during this treatment.

Statistical analysis of c_b data revealed that there was a significant difference amongst sorghum genotypes for treatment and control plants (ANOVA analysis, $P < 0.05$, df = 29, F statistic = 9.62, n = 9). During MS, c_b contents were statistically similar and ranged between 0.60 mg.g^{-1} FW (LR5) and 0.82 mg.g^{-1} FW (P898012) for stressed plants and between 0.66 mg.g^{-1} FW (LR36) to 0.80 mg.g^{-1} FW (LR35) for the control plants. During SS, c_b recorded for LR35 (1.14 mg.g^{-1} FW) was significantly higher to values obtained for P898012 (0.66 mg.g^{-1} FW) and LR5 (0.64 mg.g^{-1} FW). LR6 (0.99 mg.g^{-1} FW) and LR36 (0.89 mg.g^{-1} FW) recorded c_b values that were statistically similar to the c_b value obtained by LR35 during SS. The c_b content between sorghum genotypes were statistically similar during the Mod-RW treatment and ranged between 0.89 mg.g^{-1}

FW (P898012) and 1.21 mg.g^{-1} FW (LR6) for treatment plants and between 0.88 mg.g^{-1} FW (P898012) to 1.40 mg.g^{-1} FW (LR6) for well-watered, control plants.

There was a significant difference in total chlorophyll (*Tchl*) content between sorghum genotypes for treatment and control plants (ANOVA analysis, $P < 0.05$, df = 29, F statistic = 13.28, n = 9). The *Tchl* content amongst sorghum genotypes were statistically similar during MS and ranged between 3.01 mg.g^{-1} FW (LR5) and 3.72 mg.g^{-1} FW (LR6) for stressed plants and between 3.49 mg.g^{-1} FW (LR36) and 3.97 mg.g^{-1} FW (LR6) for control plants. During SS, *Tchl* contents for P898012 (2.90 mg.g^{-1} FW) and LR5 (3.04 mg.g^{-1} FW) were significantly lower to *Tchl* of LR35 (4.99 mg.g^{-1} FW) and LR6 (4.82 mg.g^{-1} FW). *Tchl* ranged between 3.89 mg.g^{-1} FW (LR5) and 5.15 mg.g^{-1} FW (LR35) for control plants during SS. During the Mod-RW treatment, *Tchl* values were statistically similar and ranged between 4.49 mg.g^{-1} FW (P898012) and 5.84 mg.g^{-1} FW (LR6) for treatment plants and between 4.07 mg.g^{-1} FW (P898012) and 6.22 mg.g^{-1} FW (LR6) for control plants (Table 2).

Overall, the four sorghum landraces significantly maintained chlorophyll contents compared with their controls during all stress treatments. During SS, c_a and *Tchl* values obtained for the drought tolerant line, P898012 were significantly reduced in comparison to its control. Xu et al. (2000) investigated water stress of sorghum genotypes with and without post-flowering drought tolerance (i.e. “stay green”). In the drought tolerant genotype, these researchers reported a 23% reduction in total chlorophyll content between stressed and non-stressed plants; whilst a 75% reduction

was found in the non-"stay green" genotype (Xu et al., 2000). In our study, *Tchl* recorded for LR6, LR35 and LR36 during SS were statistically similar (< 4.33% reduction in *Tchl* between stressed and non-stressed plants). Total chlorophyll content was significantly reduced in stressed P898012 by 33.8% when compared with its control during SS (Table 2).

The carotenoid [$c_{(x+c)}$] content was calculated using spectrophotometer readings at 470 nm. There was a significant difference in the carotenoid content amongst sorghum genotypes during water stressed treatments and well-watered controls (ANOVA analysis, $P < 0.05$, df = 29, F statistic = 16.14, n = 9). During MS, $c_{(x+c)}$ were statistically similar between sorghum genotypes for treatment and control plants and ranged between 0.46 mg.g^{-1} FW (LR5) and 0.61 mg.g^{-1} FW (LR35). The highest carotenoid values during SS were observed for LR6 and LR35 at 0.74 mg.g^{-1} FW and 0.73 mg.g^{-1} FW, respectively. The $c_{(x+c)}$ recorded for LR6 and LR35 were significantly different to values obtained from P898012 and LR5 plants. During the Mod-RW treatment, LR6, LR35 and LR36 obtained statistically similar values (0.92-0.99 mg.g^{-1} FW) which were higher than values recorded for P898012 (0.72 mg.g^{-1} FW) and LR5 (0.74 mg.g^{-1} FW) during this stress time point (Table 2).

Overall, carotenoid [$c_{(x+c)}$] content was significantly maintained between stressed and non-stressed plants for all treatments, with the exception of P898012 during SS which recorded a significant reduction of 35.9% when stressed. In contrast, the four selected landraces statistically maintained $c_{(x+c)}$ during SS in comparison to their well-watered

controls. Carotenoids encompass carotenes and xanthophylls which are derivatives of carotenes. Carotenoids in chloroplasts are important components for photosynthesis as they play a role in light harvesting (Santabarbara et al., 2013). The main role of carotenoids during photosynthesis involves their ability to sequester damaging oxygen radicals and triplet chlorophyll which are readily produced by photosynthetic complexes during light harvesting (McKersie and Leshem, 1994; Malkin and Niyogi, 2000; Santabarbara et al., 2013). A review by Demmig-Adams and Adams (1996) highlighted the role of xanthophyll cycle carotenoids in photoprotective energy dissipation during photosynthesis, without which the process of photosynthesis may be severely inhibited.

Takele (2010) found that both chlorophyll and carotenoid contents in pre- and post-flowering drought tolerant sorghum were reduced during dehydration. Chlorophyll and carotenoid contents in the water stressed pre-flowering sorghum had recovered following re-watering whilst no recovery was observed on post-flowering sorghum (Takele, 2010). Rehydration may be as harmful to the photosynthetic apparatus and cell membranes as dehydration. Cell membrane damage is exacerbated upon re-watering contributing to tissue death, particularly when the preceding water stress was severe (Alpert and Oliver, 2002; Takele, 2010; reviewed by Xu et al., 2010). In the present study, the ability to maintain both chlorophyll and carotenoid levels during water stress and moderate re-watering may indicate that the photosynthetic apparatus of the sorghum landraces was not functionally damaged as a result of the imposed water deficits.

At each harvest time point, the fifth leaf was excised for quantification of proline and represented as milligrams per gram of fresh weight ($mg.g^{-1}$ FW). Statistical analysis revealed there was a significant difference in proline content amongst sorghum genotypes between treatment and control plants (Table 2). Data were log_{10} transformed and analysed using ANOVA ($P < 0.05$, df = 29, F statistic = 9.94, n = 9). During MS and SS, proline content between treatment and control plants were statistically similar. The proline content during MS ranged between 0.68 $mg.g^{-1}$ FW (LR5) and 1.79 $mg.g^{-1}$ FW (LR36) for treatment plants and between 0.42 $mg.g^{-1}$ FW (LR5) and 1.01 $mg.g^{-1}$ FW (LR36) for control plants. During SS, proline content for treatment plants ranged between 0.70 $mg.g^{-1}$ FW (LR5) to 3.86 $mg.g^{-1}$ FW (LR36) whilst well-watered plants recorded values between 0.52 $mg.g^{-1}$ FW (LR5) to 0.80 $mg.g^{-1}$ FW (P898012).

Proline values obtained from the Mod-RW treatment ranged between 0.71 $mg.g^{-1}$ FW (LR5) and 16.52 $mg.g^{-1}$ FW (LR36); whilst values for well-watered plants ranged between 0.70 $mg.g^{-1}$ FW (LR5) and 1.56 $mg.g^{-1}$ FW (P898012). An evident increase in proline content was observed during the Mod-RW treatment for LR35 and LR36. The highest proline content was recorded for LR36 (16.52 $mg.g^{-1}$ FW) which was a 14-fold increase compared with its control value of 1.21 $mg.g^{-1}$ FW and significantly different to values obtained for the other genotypes. LR35 recorded the second highest proline content of 11.18 $mg.g^{-1}$ FW which was a 16-fold increase compared with its control (0.70 $mg.g^{-1}$ FW) and significantly different to values obtained for LR5 and LR6 during this treatment (Table 2).

The accumulation of proline in plants may be due to increased synthesis or decreased degradation during abiotic stress conditions (Hare et al., 1998). An increase of proline during stress may be a mechanism which ameliorates the effects of the stress (Bray et al., 2000; Coruzzi and Last, 2000). During osmotic stress in plants, proline as a compatible solute may prevent disruption of proteins, protein complexes and membranes (Van Rensburg et al., 1993; Bray et al., 2000). Furthermore, compatible solutes may function as antioxidants by minimizing the effects of oxygen radical ions during stress (Bohnert and Shen, 1999; reviewed by Matysik et al., 2002). Van Rensburg et al. (1993) found a positive correlation between proline accumulation and the level of drought tolerance in tobacco (*Nicotiana tabacum* L.) which correlated with the cultivars' ability to maintain membrane integrity during stress. Sivaramakrishnan et al. (1988) reported that drought resistant sorghum lines accumulated higher levels of proline compared with drought susceptible lines during water stress. Increased proline production through transgenics has also been linked to increased abiotic stress tolerance (Kavi Kishor et al., 1995; Sawahel and Hassan, 2002).

Therefore, the accumulation of proline in LR35 and LR36 as water stress progressed may have offered the sorghum landraces some degree of osmoprotection and antioxidant action thereby inhibiting plant death.

4. Conclusions

Screening of South African sorghum landraces for drought tolerance at the seedling stage revealed four previously uncharacterised drought tolerant genotypes which

maintained superior LWC in comparison to a recognized drought tolerant breeding line, P898012. Further investigations of these landraces with P898012 at the drought sensitive GS II, revealed their physiological responses to water deficit and re-watering. All five sorghum genotypes maintained LWC compared with the well-watered controls during mild and severe water stress at GS II. During the moderate re-watered treatment after seven days of water deficit, P898012, LR5 and LR6 significantly maintained LWC compared with their controls; whilst LWC recorded for LR35 and LR36 were less than 8% lower in comparison to their controls. Data recorded for proline, which is recognized as a protective compound during abiotic stress, revealed a significant 14-16 fold increase during the moderate re-watered treatment in LR36 and LR35, respectively, when their LWC had decreased to 73%. Overall, LR35 and LR6 maintained significantly higher chlorophyll and carotenoid contents during water stress compared with P898012 and the other landraces. The physiological parameters evaluated under controlled growth room conditions in this study indicated that these selected landraces may be drought tolerant. Collectively, these results demonstrate some of the response mechanisms activated by a drought tolerant breeding line and four putatively drought tolerant sorghum landraces during water deficit and re-watering. All four South African landraces investigated exhibited important drought tolerance characteristics which will be valuable for incorporation into breeding programmes. Molecular investigations to elucidate a full profile of possible drought responsive mechanisms in selected sorghum genotypes are in progress.

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