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Biosciences,

Council for Industrial and Scientific Research (CSIR),

PO Box 395,

Pretoria,

0001,

South Africa

Physiological responses of selected African sorghum landraces to progressive water stress

N. Devnarain[1,2,*], B. G. Crampton[2], J. V. W. Becker[3], M. M. O'Kennedy[1,*]

[1] *Council for Scientific and Industrial Research (CSIR) Biosciences, Pretoria, South Africa*

[2] *Department of Plant Science, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa*

[3] *African Centre for Gene Technologies (ACGT), Pretoria, South Africa*

Corresponding author:

Martha M. O'Kennedy

CSIR Biosciences,

PO Box 395,

Pretoria,

0001,

South Africa

Tel: +27 12 841 3773

Fax: +27 12 841 2185

e-mail address: mokennedy@csir.co.za

* These authors contributed equally to this work

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Abstract

This study was conducted to identify African sorghum genotypes with superior drought tolerance compared with a drought tolerant breeding line (P898012). Seedlings of 14 sorghum landrace accessions were initially screened for drought tolerance by assessing absolute leaf water content (% AWC) during progressive water deficit. Four landraces (designated LR5, LR6, LR35 and LR36) recorded higher % AWC than P898012. These were subsequently evaluated together with P898012, for their physiological responses to water stress during the reproductive growth stage. Plants were subjected to mild (4 days) and severe (6 days) water stress treatments and a moderate re-watered treatment on day seven. Plant height, soil moisture and % AWC were measured during harvests. Chlorophyll, carotenoid and proline contents were quantified. All five genotypes maintained % AWC above 80 % during mild and severe stress treatments. For LR35 and LR36, % AWC was recorded within 8 % or less in comparison to their controls during 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 % AWC was reduced during the re-watered treatment in LR36 (to 73.68 %) and LR35 (to 73.51 %), their proline contents significantly increased by 14- and 16-fold, respectively. These four landraces would be valuable for breeding and further, to elucidate genetic mechanisms that enable drought tolerance in African sorghum.

Keywords

Running Title: Drought tolerant African sorghum landraces

Introduction

Drought is a complex environmental stress and major constraint to crop productivity (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 are dependent 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 is 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 57 million tonnes produced during 2012 (Wani et al. 2012, FAO 2014). 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; whilst water deficit 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 mechanisms that stabilize and protect cellular and metabolic integrity and functions at the tissue or cellular level (Tuinstra et al. 1997). 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. Water stress in plants may manifest as decreased leaf water content and chlorophyll contents. The absolute 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. 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 as an important protective role against abiotic stress in plants due to its distinct biochemical properties which enables this amino acid to have a neutral charge at physiological pH, not affect cellular metabolism and capable of scavenging 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. 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 during the drought sensitive, growth stage (GS) II of development. P898012 is a public genotype which exhibits pre-flowering and post-flowering drought resistance (Yu et al. 2013).

Materials and methods

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.

Seedling drought stress

Three seeds of P898012, ICSV112 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 tap water before planting and each pot was supplemented with 50 ml Nutrifeed nutrient solution (2 g/l). Sorghum seedlings were randomly placed and grown 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 to be 312 μmol m^{-2} s^{-1}. A Hobo Pro RH/Temp series data logger 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 three leaf stage, approximately 14 days from planting by daily watering with 50 ml tap water. Before the onset of stress, each pot was watered with 150 ml tap water and supplemented with 50 ml Nutrifeed nutrient solution (4 g/l). Water was withheld from pots for 6, 7, 8 and 9 days with three replicates per sorghum genotype and stress time point.

Drought simulation at reproductive GS II

One hundred seeds per sorghum genotype (P898012, LR5, LR6, LR35, LR36) were surface decontaminated by exposure to sodium hypochlorite (1 % v/v NaOCl) for 10 min followed by a 3X rinse with sterile deionised water. For seed germination, 4 kg of soil mix (as per seedling experiment) was wet with 500 ml tap water and autoclaved three times, whereafter it was placed in plastic trays (35 x 25 cm). Surface sterilized seeds were sown into the soil at a depth of 2 cm and thereafter, the soil was watered with 200 ml tap 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 1-l, 15 cm plastic, filter paper-lined plant pots filled with 1 kg autoclaved soil mix. The soil was prepared by being saturated with 250 ml tap water before planting seedlings and each pot was supplemented with 50 ml half-strength Nutrifeed nutrient solution (1 g/l). Appropriately labelled pots were randomly arranged in the growth room. Seedlings were grown for

eight weeks after seeds were sown by daily watering 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 full-strength 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). There were nine biological replicates per sorghum genotype for each treatment and control. Before the onset of water stress, each pot was watered with 150 ml water and supplemented with 50 ml full-strength 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 water stress time points were investigated: [1] mild stress (MS) after 4 days of water deficit, [2] severe stress (SS) after 6 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.

At each harvest, the plant height measurements from the soil surface to the growing point were recorded. A soil moisture probe, manufactured by DFM Software Solutions CC., was used to record the percentage soil moisture content (% SMC) and three measurements were recorded for each pot during harvest. A leaf disk was punched from the third leaf of each plant at each stress time point and used to determine total chlorophyll, chlorophyll *a* and *b*, as well as carotenoid content as outlined by Lichtentaler and Buschman (2001). Thereafter, the third leaf was excised for

determination of absolute leaf water content (% AWC). A 0.5 g sample of the fifth leaf was excised and flash frozen for quantification of proline, following the protocol described by Bates et al. (1973).

Percentage absolute leaf water content (% AWC)

The third leaf of each plant was excised for quantification of percentage absolute leaf water content (% AWC). 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 $^{\circ}$C for 48 h. The dry weight (DW) was then recorded and % AWC was calculated on a fresh weight basis using the following equation (Hall and MacHardy 1981):

$$\% \text{ AWC} = [(FW - DW) / FW] \quad x \quad 100$$

The % AWC of seedling leaves harvested following 6, 7, 8 and 9 days of water stress were calculated with three biological replicates per genotype and treatment. Leaves from sorghum plants stressed during GS II were harvested at 4 days and 6 days of water withholding and 5 h after re-watering on day seven.

Quantification of chlorophylls and carotenoids

Circular leaf disks (9 mm diameter) were punched from the third leaf of sorghum plants using a cork borer. These 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 DU®800 spectrophotometer at 3 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) $Tchl = c_a + c_b$

(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 concentrations of leaf pigments were represented on a fresh weight basis as mg g^{-1} FW.

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 %
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 $^{\circ}$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
$^{\circ}$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 DU$^{®}$800 spectrophotometer.
The 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

Data analysis

The leaf water content (% AWC) data recorded for water stressed sorghum seedlings
were analysed using STATISTICA version 6.0 (Statsoft$^{®}$ Inc., USA) and each
treatment/control was analysed with three replicates. The statistical program, GenStat$^{®}$
version 12 (VSN International, UK) was used to analyse data obtained from sorghum
plants stressed during the reproductive, GS II and each treatment/control was analysed
with 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.

Results and Discussion

Two independent water stress investigations were conducted to: (1) screen 14 southern African, sorghum landrace accessions for drought tolerance during the vegetative, seedling stage together with drought susceptible ICSV112 and drought tolerant P898012 and (2) assess the physiological responses of landraces selected from the initial screen to water stress, during the drought sensitive, reproductive GS II of sorghum development at eight weeks after emergence. Four African landraces recorded higher leaf water contents during water deficit at the seedling stage compared with P898012. Water stress at GS II, demonstrates the physiological drought responsive mechanisms activated in the four African landraces and results suggest that selected landraces compared favourably to the drought tolerant P898012 with respect to the physiological parameters measured.

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 performances to the drought tolerant breeding line. The percentage absolute leaf water content (% AWC) was measured using excised leaves harvested following withholding water for 6, 7, 8 and 9 days. There were three replicates for each sorghum genotype and stress time point. The calculated % AWC 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 % AWC

compared with P898012 is presented in Figure 1 to highlight the landraces which responded better (positive values on y-axis) than the known drought tolerant breedling line by maintaining higher % AWC 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 9 days of water withholding), the largest negative change in % AWC (-33 % difference) compared with P898012 (68.1 %) was recorded by ICSV112 (35 %). LR5, LR6, LR35 and LR36 were the only landraces with superior performance compared with drought tolerant P898012 during the imposed water deficits. At 9 days of water stress, LR5, LR6, LR35 and LR36 recorded higher % AWC in comparison to P898012 (8.4 %, 12.5 %, 11.9 % and 14.3 %, respectively). The recorded % AWC for these four landraces during 9 days of water withholding ranged between 76.5 % and 82.4 %. Therefore, these four potentially drought tolerant landraces were subjected to further drought treatments to assess their physiological responses to water stress at the reproductive pre-flowering, GS II of sorghum development.

Physiological responses of sorghum to water deficit at the reproductive GS II stage

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

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breeding line (P898012) and four potentially drought tolerant landraces (LR5, LR6, LR35 and LR36) selected from the seedling stress treatment in order to compare their physiological responses to simulated drought conditions at GS II. The percentage 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.

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. Following commencement of water stress, the lower leaves of sorghum plants began to show the first signs of stress and appeared yellow and wilted. This correlated with a common observation that older leaves dessicate 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 been more physiologically stressed than the other genotypes and responded by activating a drought avoidance mechanism.

The 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).

[Table 1]

During the water stress treatments, % SMC ranged between 45-55 % during mild stress, 32-37 % during severe stress and 44-61 % during the moderate re-watered treatment. The % SMC recorded for the well-watered, control pots across all stress time points were statistically similar (Table 1). The % SMC between treatment pots and control pots were significantly different for all sorghum genotypes therefore some degree of water deficit was inflicted on treatment plants.

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

[Figure 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 previously been 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.

Percentage absolute leaf water content (% AWC)

At each harvest, the percentage absolute leaf water content (% AWC) of the third leaf was determined using an equation incorporating the leaf fresh and dry weights. Overall, there was a significant difference in % AWC amongst sorghum genotypes (Fig. 3, ANOVA analysis, $P < 0.05$, df = 29, F statistic = 11.63, n = 9). The % AWC values

across stress treatments ranged: 85-88 % during mild stress, 82-86 % during severe stress and 74-85 % during re-watered treatments.

[Figure 3]

A significant difference was found only during the moderate re-watered treatment when % AWC recorded for LR35 and LR36 were statistically lower compared with % AWC recorded for the other genotypes (Fig. 3). In comparison to their controls, there was a reduction in % AWC for LR35 and LR36 of 6.1 % and 7.8 %, respectively. Overall, the % AWC 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). According to Bray et al. (2000), relative leaf water contents typically range between 85-95 % and a critical reduction to less than 50 % may lead to tissue death. These sorghum genotypes were able to maintain % AWC above 80 % during 4 and 6 days of water deficit. After the moderate re-watered treatment at day seven of water deficit, a < 8 % reduction in % AWC 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).

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 on a spectrophotometer at 663.2 nm and 646.8 nm, respectively. Statistical analysis of c_a data revealed that there was a significant difference amongst sorghum genotypes during treatments and controls (ANOVA analysis, $P < 0.05$, df = 29, F statistic = 13.38, n = 9). Data obtained during mild stress 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 water stressed plants and between 2.83 mg g^{-1} FW (P898012 and LR36) and 3.29 mg g^{-1} FW (LR6) for well-watered plants.

[Table 2]

During the severe stress treatment, 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 to values obtained for LR35 (3.85 mg g^{-1} FW) and LR6 (3.82 mg g^{-1} FW). The c_a content of LR36 and the four other sorghum genotypes during severe stress were statistically similar. The c_a content ranged between 3.60 mg g^{-1} FW (P898012) and 4.63 mg g^{-1} FW

(LR6) during the moderate re-watered treatment. Values obtained from treatment and control plants were statistically similar during the moderate re-watered 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 mild stress treatments, 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 water stressed plants and between 0.66 mg g^{-1} FW (LR36) to 0.80 mg g^{-1} (LR35) for the control plants. During the severe stress treatment, 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 severe stress. The c_b content between sorghum genotypes were statistically similar during the moderate re-watered 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 mild stress and ranged between 3.01 mg g^{-1} FW (LR5) and 3.72 mg g^{-1} FW (LR6) for water stressed plants and between 3.49 mg g^{-1} FW (LR36) and 3.97 mg g^{-1} FW (LR6) for control plants. During severe stress, *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 severe stress. During the moderate re-watered 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 severe stress, c_a and *Tchl* values obtained for 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 stress and non-stressed plants; whilst a 75 % reduction was found in the non-"stay green" genotype (Xu et al. 2000). In this study, *Tchl* recorded for LR6, LR35 and LR36 during severe stress 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 severe stress (Table 2).

The carotenoid 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 mild stress, carotenoid contents 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 severe stress were observed for LR6 and LR35 at 0.74 mg g^{-1} FW and 0.73 mg g^{-1} FW, respectively. The carotenoid contents recorded for LR6 and LR35 were significantly different to values obtained from P898012 and LR5 plants. During the moderate re-watered treatment, LR6, LR35 and LR36 obtained statistically similar carotenoid 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, the carotenoid contents were significantly maintained between stressed and non-stressed plants for all treatments, with the only exception of P898012 during severe water stress which recorded a significant reduction in carotenoid content of 35.9 % when stressed. Whilst during severe stress, the four selected landraces statistically maintained carotenoid contents 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 minor 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. The ability of these sorghum landraces to maintain both chlorophyll and carotenoid levels during water stress may indicate that their photosynthetic apparatus 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 that there was a significant difference in free 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 mild and severe stress, proline content between treatment and control plants were statistically similar. The proline content during mild stress 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 severe stress, 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 moderate re-watered 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 moderate re-watered treatment for

LR36 and LR35. The highest proline content was recorded for LR36 (16.52 mg g^{-1} FW) which was significantly different to its control value (1.21 mg g^{-1} FW) and to values obtained for the other genotypes during this treatment (Table 2). LR35 recorded the second highest proline content of 11.18 mg g^{-1} FW which was significantly different to its control (0.70 mg g^{-1} FW) and to values obtained for LR5 and LR6.

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 abiotic 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). Further, 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 have 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

these landraces some degree of osmoprotection and antioxidant action thereby inhibiting tissue or plant death.

Conclusions

Screening of southern African sorghum landraces for drought tolerance at the seedling stage revealed four genotypes which exhibited superior drought tolerance in comparison to a recognized drought tolerant breeding line, P898012. Further investigations of these landraces together with P898012, at the drought sensitive GS II, revealed their physiological responses to water deficit. All five sorghum genotypes maintained their % AWC compared with their controls during mild and severe water stress at the reproductive growth stage. During the moderate re-watered treatment after seven days of water deficit, the % AWC recorded for LR35 and LR36 were within 8 % or less 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 when their % AWC 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. Collectively, results from these physiological measurements demonstrate some of the response mechanisms activated by four putatively drought tolerant sorghum landraces and a drought tolerant breeding line during water deficit. All four African landraces exhibit 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 are warranted and subsequent gene

expression analyses of selected sorghum genotypes, using custom-designed microarrays, are in progress.

Acknowledgements

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