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PHYSIOLOGICAL AND BIOCHEMICAL BASIS FOR STAY-GREEN TRAIT IN SORGHUM

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ABSTRACT

Drought is a major cause of sorghum [*Sorghum bicolor* (L.) Moench] yield losses in rain-fed agriculture, especially in the semi-arid and arid agro-ecological zones of Africa and Asia. Stay-green sorghum genotypes are able to maintain grain filling under drought conditions. The trait has been employed in the selection and breeding for post-flowering drought resistance, even though the genes regulating the trait are still being identified. The objective of this study was to assess how leaf area and chlorophyll are maintained in various sources of stay-green; and to determine whether the integrity of the photosynthetic apparatus and enzymes involved in the maintenance of photosynthesis during post-flowering drought stress are regulated differently. A glasshouse experiment was conducted using three stay-green sorghum lines (B35, KS19 and E36-1) and a senescent control, R16, under well-watered (WW) and water-limited (WL) conditions. The size of the canopy at anthesis varied significantly between genotypes, and this profoundly impacted leaf senescence patterns. For example, green leaf area (GLA) at anthesis was highly correlated with the decline in GLA during the first 21 days of grain filling, under both WW ($r = 0.92$) and WL ($r = 0.86$) conditions. These differences in senescence patterns were further exacerbated by the small pot size in this study (10 L). E36-1 is normally designated as a stay-green genotype, but the growth of this 'high leaf area', genotype in a small pot resulted in a senescent phenotype. Green leaf area retention was higher in B35 and KS19, and the loss of GLA started 14 days earlier in the WL E36-1 and R16 plants, compared to B35, with little change in KS19. Chlorophyll levels were higher in B35 and KS19 compared with R16 and E36-1 under WL conditions. Φ_{PSII}, CO_2 assimilation rate, leaf conductance, transpiration rate and leaf water use efficiency were higher in the stay-green genotypes under WL conditions compared to R16. Enzymes involved in leaf nitrogen metabolism and chlorophyll biosynthesis, and photosynthesis were retained at higher levels in the stay-green lines than in R16. Therefore, the stay-green mechanism resulted in reduced destruction

of the photosynthetic apparatus, better nitrogen metabolism and chlorophyll turnover, and maintenance of active enzymes involved in photosynthesis.

Key Words: Green leaf area, photosynthesis, *Sorghum bicolor*

RÉSUMÉ

La sécheresse est une cause majeure des pertes de rendement du sorgho [*Sorghum bicolor* (L.) Moench] dans l'agriculture pluviale, en particulier dans les zones agro-écologiques semi-arides et arides d'Afrique et d'Asie. Les génotypes de sorgho restant verts peuvent maintenir le remplissage du grain dans des conditions de sécheresse. Le trait a été utilisé dans la sélection et la sélection pour la résistance à la sécheresse après la floraison, même si les gènes régulant le trait sont encore en cours d'identification. L'objectif de cette étude était d'évaluer comment la surface des feuilles et la chlorophylle sont maintenues dans diverses sources de persistance; et pour déterminer si l'intégrité de l'appareil photosynthétique et des enzymes impliqués dans le maintien de la photosynthèse pendant un stress de sécheresse après la floraison est régulée différemment. Une expérience en serre a été réalisée en utilisant trois lignées de sorgho vertes (B35, KS19 et E36-1) et un témoin de sénescence, R16, dans des conditions bien arrosées (WW) et limitées en eau (WL). La taille de la canopée à l'anthèse variait considérablement entre les génotypes, ce qui a eu un impact profond sur les profils de sénescence des feuilles. Par exemple, la surface de la feuille verte (GLA) à l'anthèse était fortement corrélée au déclin de la GLA au cours des 21 premiers jours de remplissage du grain, dans les conditions WW (r = 0,92) et WL (r = 0,86). Ces différences de profils de sénescence ont été encore exacerbées par la petite taille du pot dans cette étude (10 L). E36-1 est normalement désigné comme un génotype restant vert, mais la croissance de ce «génotype de grande surface foliaire» dans un petit pot a entraîné un phénotype sénescent. La rétention de la surface des feuilles vertes était plus élevée dans B35 et KS19 et la perte de GLA avait commencé 14 jours plus tôt dans les plantes WL E36-1 et R16, par rapport à B35, avec peu de changement dans KS19. Les niveaux de chlorophylle étaient plus élevés dans B35 et KS19 par rapport à R16 et E36-1 dans des conditions de WL. FPSII, le taux d'assimilation du CO_2, la conductance des feuilles, le taux de transpiration et l'efficacité d'utilisation de l'eau des feuilles étaient plus élevés chez les génotypes restant verts dans des conditions de WL par rapport à R16. Les enzymes impliquées dans le métabolisme de l'azote des feuilles et la biosynthèse de la chlorophylle, ainsi que la photosynthèse, ont été conservées à des niveaux plus élevés dans les lignes vertes restantes que dans R16. Par conséquent, le mécanisme restant vert a entraîné une destruction réduite de l'appareil photosynthétique, un meilleur métabolisme de l'azote et le renouvellement de la chlorophylle, ainsi qu'un maintien des enzymes actives impliquées dans la photosynthèse.

Mots Clés: Zone de la feuille verte, photosynthèse, *Sorgho bicolore*

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth most important global cereal after wheat, rice, maize and barley (FAO, 2014) for food, feed, fibre and fuel; and is particularly well adapted to hot and dry conditions (Paterson *et al*., 2009). A major constraint to sorghum production is inadequate soil water availability. In many parts of Asia and Africa, where sorghum is a staple to millions of people, the crop is cultivated under rain-fed conditions, often utilising stored soil moisture. Rainfall is frequently erratic and patterns are changing due to climate change. Such conditions result in crop losses.

Inadequate rainfall and soil moisture, during and after flowering in sorghum, can result in serious yield losses due to premature plant death, stalk rot, lodging and reduced seed size (Borrell *et al*., 2014a).

Many drought resistant sorghum cultivars stay green until harvest, and the stay-green trait has been used for years by breeders as a measure of post-flowering drought tolerance (Borrell *et al*., 2000b; 2014; Jordan *et al*., 2012). The trait is characterised by the retention of green stems and green upper leaves, even under severe post-flowering drought stress; and is associated with the maintenance of grain fill, reduced lodging, high stem carbohydrate content and resistance to charcoal stem rot under such conditions (Subudhi *et al*., 2000; Tao *et al*., 2000; Burgess *et al*., 2002; Borrell *et al*., 2000ab, 2014ab). Thus, delaying leaf senescence (stay-green) is an effective strategy for increasing cereal production under water-limited conditions (Mahalakshmi and Bidinger, 2002).

A number of different genetic sources of stay-green have been identified and used in breeding programmes around the world (Borrell *et al*., 2000b, 2014ab; Jordan *et al*., 2012). It has been suggested that the origin of stay-green in sorghum is from perennial landraces (Thomas *et al*., 2000), with reduced monocarpic senescence compared with typical annual cereals. In some regions, such as East Africa, these perennial tendencies in locally adapted sorghums are exploited for ratooning to extend the productive period over two or more seasons (Escalada and Plucknett, 1975).

The longevity of leaves (defined as the time in days from initiation to when the leaf area is more than 90% yellow) is significantly reduced for upper leaves at low nitrogen (Thomas and Rogers, 1990; Peng *et al*., 2013). Since the stay-green trait is characterised by delayed-leaf-senescence, it can also be viewed as a consequence of a balance between supply and demand for N during grain-filling (Borrell and Hammer, 2000; Thomas and Ougham, 2014). For example, if N supply from age-related senescence and N uptake during grain filling are matched with grain N demand, the shortfall in N supply for grain filling is greater in senescent than stay-green hybrids, leading to more accelerated leaf senescence in senescent lines (Borrell and Hammer, 2000; Borrell *et al*., 2001).

Genotypic differences in delayed onset and reduced rate of leaf senescence were found to be due to differences in specific leaf N and N uptake during grain filling (Borrell and Hammer, 2000). Reduced CO_2 assimilation caused by reduction in stomatal conductance, reduced concentrations and activities of photosynthetic enzymes, chlorophyll and N loss, among other factors, consequently limit the availability and partitioning of photosynthates into grain filling. Grain yield in sorghum under post-flowering drought stress correlates positively with green leaf area at mid-grain filling (Borrell *et al*., 1999) and green leaf area at maturity (Borrell *et al*., 2000b), confirming that green leaf area duration improves yields under drought stress. The longevity of leaves in stay-green sorghum might be promoted by a combination of several biochemical factors, which interact to regulate N remobilisation and chlorophyll turnover, maintaining the integrity of the photosynthetic apparatus and enzyme activity as well; particularly, those involved in carbon and N assimilation.

The stay-green phenotype can be categorised as "functional" for maintenance of leaf photosynthesis or "non-functional" (cosmetic), where chlorophyll degradation is prevented but photosynthesis competence is lost (Thomas and Howarth, 2000). Senescence, as a whole, may be postponed or slowed down; alternatively, initiation may be on schedule, but pigment metabolism may be compromised by a blockage in a biochemical pathway. Retention of chloroplast proteins, such as light-harvesting complex proteins II (LHCPII), oxygen-evolving complex 33 (OEC33) and Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco), has been observed previously in sorghum containing the KS19 source of stay-green (de Villiers *et al*., 1993), which explains, in part, how stay-green sorghum could maintain photosynthesis for

longer periods than their senescent counterparts. However, these observations were made under non-drought conditions, whereas the trait is expressed under severe drought stress (Srinivas *et al.*, 2008). To maintain photosynthesis, the photosynthetic apparatus and enzymes involved in carbon assimilation, chlorophyll turnover and N assimilation should be actively maintained in green leaves. Since different genetic sources of stay-green may employ different mechanisms and inheritance characteristics (Borrell *et al.*, 2000ab; Vadez *et al.*, 2011; Jordan *et al.*, 2012), assessment of these factors should enhance the understanding of the functioning of the stay-green trait.

The objective of this study was to assess how leaf area and chlorophyll are maintained in various sources of stay-green; and to determine whether the integrity of the photosynthetic apparatus and enzymes involved in the maintenance of photosynthesis during post-flowering drought stress are regulated differently.

MATERIALS AND METHODS

Seed material. Three stay-green sorghum genotypes (B35, E36-1 and KS19) were studied in comparison with a well-known senescent sorghum genotype (R16). Sorghum seeds were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), India, and the Queensland Department of Agriculture and Fisheries' Hermitage Research Facility, Australia. B35 (also known PI534133 or SC35-6 or BTx642) is a BC_1 selection from a converted (dwarf height, early flowering) version of IS12555 durra sorghum, an Ethiopian landrace (Rosenow *et al.*, 1983). It is a stay-green B-line released as BTx642 (Rosenow *et al.*, 2002) and is the parent used in several QTL mapping studies of drought tolerance in sorghum (Walulu *et al.*, 1994; Crasta *et al.*, 1999; Xu *et al.*, 2000).

KS19 is derived from a cross between short Kaura, an important landrace cultivar from Nigeria, and Combine Kafir 60 (Henzell *et al.*, 1984). E36-1 is a stay-green, tall, high-yielding breeding line of the guinea-caudatum hybrid race with Ethiopian origin (van Oosterom *et al.*, 1996). R16 is a high-yielding cultivar from Maharashtra, India, which is adapted to the post-rainy season (grown on stored soil moisture), but has a very rapid rate of leaf senescence (van Oosterom *et al.*, 1996).

Plant culture and treatment. Plants were grown individually in 24.5-cm diameter pots, each containing 10 L of a mixture of soil, peat, grit and perlite in a ratio of 3:3:3:1, in a glasshouse at IBERS, Plas Gogerddan, Aberystwyth, Wales, UK. Glasshouse conditions were 30 °C in the day and 18 °C at night; and natural daylight was supplemented using 400 W high-pressure sodium lamps (Phillips so T Agro) for 12 hr daily. All plants were watered to field capacity, until flowering. Pots were arranged in a randomised block design with 8 replicates. Blocking was intended to address variation in the environment because the room used in the glasshouse was bordered to the East and West by other rooms with plants (*Miscanthus* sp) growing in them. These plants were tall and thus shaded the eastern side in the early morning and the western side in the evening. Hence, there was the need to block out the effect of shading in the morning and evening.

At flowering (when anthers were visible on 50% of main shoot panicles), 4 replicates of each genotype remained well-watered (WW) and 4 replicates were subjected to drought stress by limiting the amount of water supplied. The water-limited (WL) plants were supplied with 250 ml water daily and the soil moisture for the well-watered plants was maintained at field capacity at all times.

All plants were supplied with fertiliser (NPK : 15-30-15) at 14 and 28 days after emergence

and at swelling (growth stage 5 in sorghum). Fertiliser application was at a rate of 2 g pot^{-1} each time. Hence, 6 g of N per pot during the experimental period. This was equivalent to 300 kg of N ha^{-1} (Borrell *et al*., 2000a).

Measurement of green leaf area. Green leaf area (GLA) was measured non-destructively on all leaves of four plants per cultivar per treatment, at anthesis, using a Delta-T Area Meter; and subsequently at weekly intervals by calculation using the length (from collar to tip) and maximal width of the leaf. Preliminary studies using 180 leaves indicated that the relationship between the product of the length and width and the size measured using the area meter was:

$\mathbf{y} = 0.95 + 0.75\mathbf{x}$ with an $R^2 = 94.7\%$ ($P<0.001$),

Where:

y is the area measured using the area meter, and x the product of the measured length and width for each leaf.

This relationship was then used to determine the area according to the method described by Wolfe *et al*. (1988). The area of each leaf was corrected for senescence by subtracting the area of the lamina lost to senescence defined by visible yellowing.

Leaf chlorophyll levels. Non-destructive measurements of chlorophyll content were made at weekly intervals, using a SPAD-502 chlorophyll meter (Minolta, Japan). SPAD values for sorghum have been found to be highly correlated with total leaf chlorophyll, determined by spectrophotometry (Xu *et al*., 2000). Nine readings were taken along each side of the lamina of the fourth leaf from top (flag leaf minus 3; FL-3) in four plants per treatment from 14 to 49 days after flowering (DAA) and the average calculated.

Leaf photosynthesis. Leaf gas exchange was measured on a 5.6cm^2 area of FL-3 using an open portable gas exchange system (CIRAS-1, PP Systems, Hitchin Herts, UK). Carbon dioxide (CO_2) assimilation rates (*A*), leaf conductance (g_L), transpiration rates (*E*) and intercellular CO_2 concentration data were collected by placing each leaf in the cuvette maintained at 30ºC, with an ambient CO_2 concentration of 350 µL L^{-1} and exposed to photosynthetically active radiation (PAR) of 1100 µmol m^{-2} s^{-1}. Measurements were taken on four plants per cultivar per treatment, between 08:00 and 13:00 hr. Photosynthetic water use efficiency (WUE_L) was computed as the ratio of *A*/*E*.

Chlorophyll fluorescence was measured in the same leaves, used for gas exchange. An EARS Plant Photosynthetic Measurement (PPM) System was used to measure the quantum yield of photosynthetic electron transport of photosystem II (Φ_{PSII}), as described by Maxwell and Johnson (2000) in light-adapted leaves. Three readings were taken between the collar and tip of the leaf at equal spacing and averaged.

Leaf proteins. Protein analysis was carried out at 0, 21 and 42 days after flowering (DAA). Protein was extracted from leaf sections cut from the middle of the lamina of each leaf, avoiding the midrib as described by Smart *et al*. (1995). Proteins were separated by SDS gel electrophoresis (Laemmli, 1970), using an equal equivalent fresh weight loading per lane; followed by Western blotting, using the method described in Thomas *et al.* (1999). Individual polypeptides were visualised using a chemiluminescence detection kit (Roche), according to the protocol of the manufacturers, using antibodies (Hilditch *et al*., 1989; Davies *et al*., 1990; Smart *et al*., 1995). Bands were quantified following background correction, using ImageJ (Rasband, W.S., ImageJ, U. S. National

Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016).

Grain yield. Panicle yield was determined after oven drying at 60 ºC for 48 hours, after harvest. Panicles were hand-threshed and seed weight per panicle, and 100 seed weight measured. The 100 seed weight and total seed weight per panicle were used to determine the number of seeds per head as described by Borrell *et al*. (2000a), viz;

$$\text{Number of seeds per panicle (g)} = \frac{\text{Panicle seed weight} \times 100\ (g)}{\text{Weight of 100 seeds}}$$

Harvest index (HI) and the ratio of grain yield to panicle yield (panicle harvest index (PHI)) for each plant were determined (Bidinger *et al*., 1987).

Statistical analysis. Data were analysed using Minitab Release 13 software. Significance of differences between treatment means was determined by using ANOVA and LSD (Fisher's).

RESULTS

Green leaf area (GLA) and chlorophyll retention. Loss of chlorophyll and a consequent decline in green leaf area are the visible expression of leaf senescence. The genotypes studied here varied considerably in their retention of green leaf area (GLA) from flowering to physiological maturity, under both well-watered (WW) and water-limited (WL) conditions (Table 1). Total green leaf area (GLA) per plant at anthesis was highest in E36-1, intermediate in B35 and R16, and lowest in KS19 (Fig.1). Loss of GLA was rapid in E36-1 and R16 plants under both WW and WL conditions between 0 and 28 days after anthesis (DAA). Indeed, by 21 DAA, E36-1 and R16 had lost 72% of GLA under water limitation; while the reductions under WW conditions were 54 and 39% for E36-1 and R16, respectively (Fig. 1).

In B35, the decline in GLA was not apparent until 14 DAA; and the rate of loss of GLA was slow thereafter (Fig. 1). Differences between the WW and WL plants began to show from 21 DAA onwards, with reductions of 16 and 29%, respectively, for the WL and WW

TABLE 1. Sorghum green leaf area (cm^2) at various days after flowering in well-watered (WW) and water-limited (WL) plants

Line	Treatment	Days after flowering		
		0	21	42
B35	WW	4635	3908	2977
	WL	4635	3299	2206
E36-1	WW	7003	3238	1477
	WL	7003	1994	921
KS19	WW	1881	1796	1282
	WL	1881	1758	1005
R16	WW	4884	3000	1286
	WL	4884	1392	988
$LSD_{0.05}$		86.8	77.6	75.8

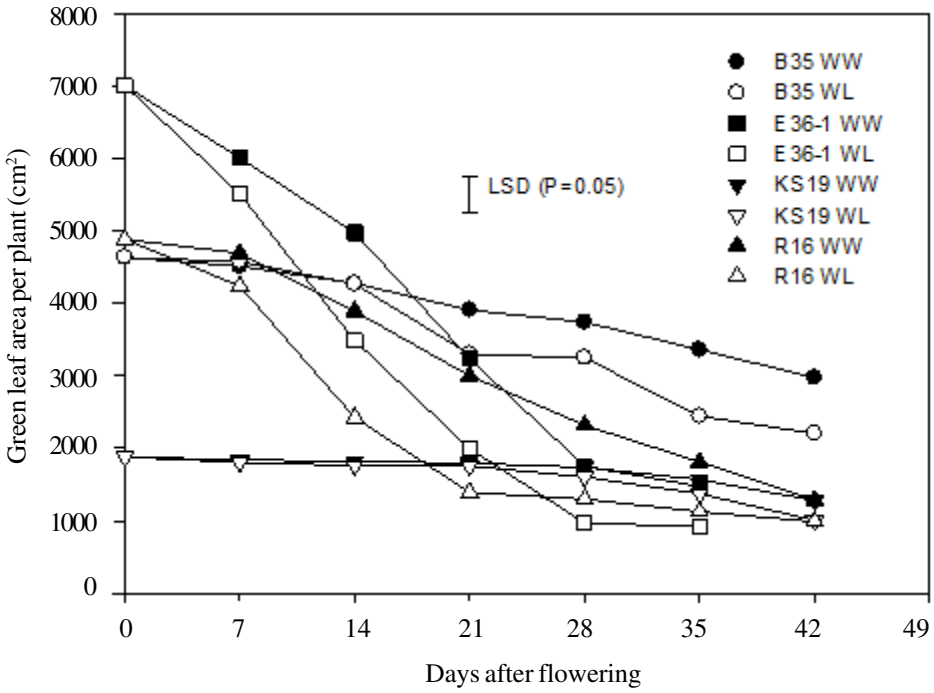

Figure 1. Changes in sorghum green leaf area in sorghum plants. Plants were either well watered (WW) and water-limited (WL) from flowering to physiological maturity. Values are means of four replicates.

plants. In contrast, the loss of GLA in KS19 was slow in both the WL and WW plants, and by 21 DAA they had lost only 7 and 5%, respectively. The loss of GLA increased in KS19 plants, thereafter, and by 42 DAA it had reduced by 32% (WW) and 47% (WL) compared to that at flowering (Table 1). The loss of GLA was generally more rapid in plants with high GLA at anthesis, such as E36-1 (Fig. 2). Nonetheless, B35, which had similar size of GLA as R16, had a slower rate of senescence than R16.

Age-related senescence (WW treatment). GLA at anthesis was positively correlated with percent loss in GLA per day (0-21 DAA; r = 0.92; Fig. 2a). However, R16 (senescent) and B35 (stay-green) exhibited similar canopy sizes at anthesis (~4700 cm^2); yet the decline in GLA during the first 21 DAA was significantly ($P<0.05$) higher in R16 (39%) than B35 (16%); indicating inherent differences in age-related senescence. Age-related senescence continued throughout the experiment, with R16 senescing at twice the rate (1.75% loss in GLA per day) than B35 (0.85% loss in GLA per day). KS19, which had the smallest canopy size at anthesis, did not display senescence until 28 DAA, whereas E36-1, with the largest canopy size at anthesis, was exhibiting significant senescence by 7 DAA.

Stress-induced senescence (WL treatment). The decline in green leaf area (GLA) under water-limited conditions during the first 21 days after anthesis was positively correlated with the loss in GLA per day (0-21 DAA; r = 0.86; Fig. 2b). But again, R16 (senescent) and B35 (stay-green) exhibited similar canopy sizes at anthesis (~4700 cm^2), yet the decline in GLA (Fig. 2) during the first 21 DAA under WL conditions was significantly higher in R16 (72%) than in B35 (29%), indicating inherent differences in stress-

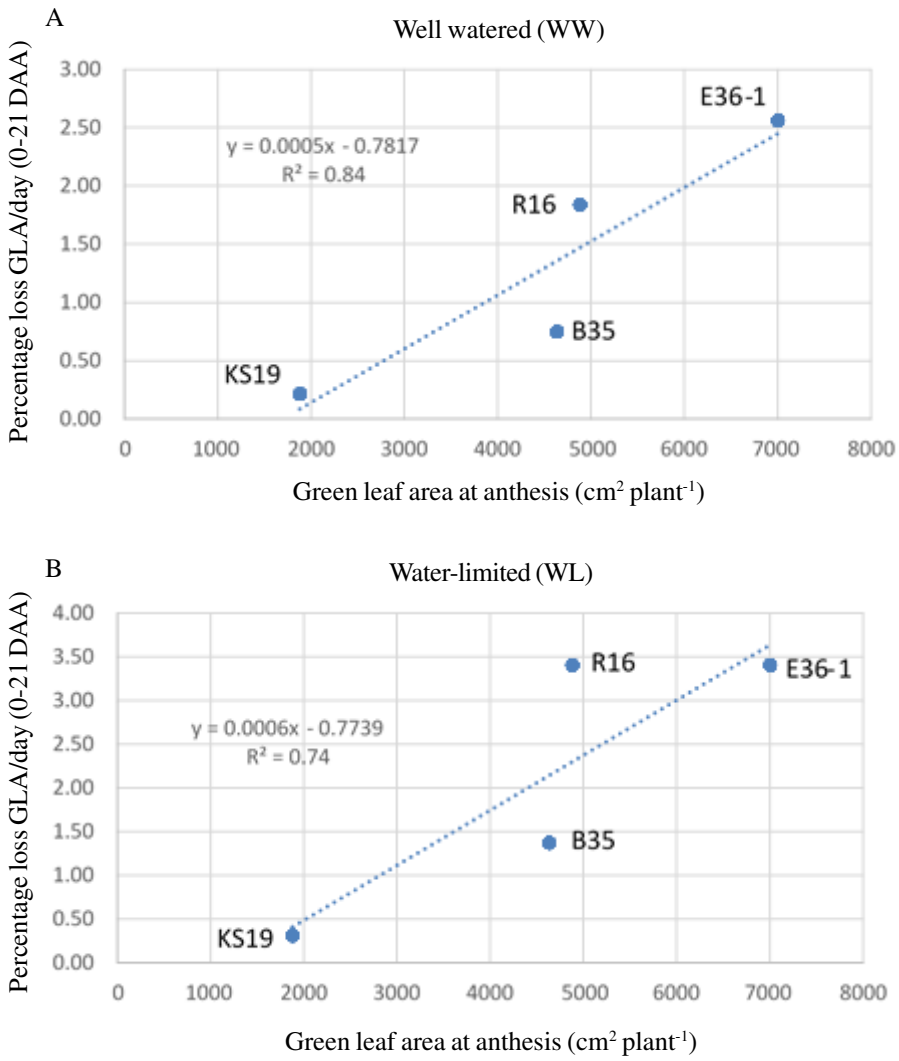

Figure 2. The relationship between sorghum green leaf area at anthesis (cm^2 $plant^{-1}$) and the percentage loss in green leaf area (GLA) per day (between 0 and 21 DAA) for four sorghum genotypes grown under (A) well-watered, and (B) water-limited conditions.

induced senescence. E36-1, which had the largest canopy size at anthesis, senesced at the same rate as R16 (3.4% per day).

The retention of GLA (%GLA) was greater in the B35 (84%) and KS19 (98%) than in E36-1 (62%) and R16 (46%) under WL, compared to WW plants at 21 DAA. In all genotypes, water limitation either brought forward the onset of leaf senescence and/or enhanced the rate of leaf senescence. GLA declined rapidly from flowering in the water-limited E36-1 and R16 plants; whereas in the WL B35 and KS19 plants, the decline occurred after 7 and 28 DAA, respectively. Once senescence had started, KS19 senesced at a greater rate (2.0% per day) than B 35 (1.6% per day). There was a significant difference in GLA between WL and WW R16 plants at 14 DAA; whereas this was not apparent between WL and WW B35 plants until 35 DAA.

Among the WW plants, chlorophyll levels of FL-3 were highest in B35 and KS19

throughout the sampling period, with no significant differences between them or between flowering and physiological maturity (Fig. 3). R16 also had higher levels of chlorophyll than E36-1 when well-watered, but both genotypes displayed a steady reduction in chlorophyll levels during the course of the experiment.

Chlorophyll levels were reduced in all the WL plants compared to the WW plants, but the timing of the onset of this reduction differed between genotypes. In E36-1 and R16, chlorophyll levels were significantly ($P<0.05$) reduced by water limitation compared to the WW plants, but the reduction was immediate in E36-1 and delayed by one week in R16. However, the reduction was more rapid in R16, particularly, in the second and third weeks after flowering. In B35 and KS19, the differences in chlorophyll levels between WW and WL plants were not significant until 22 and 35 DAA, respectively.

Leaf photosynthesis and water use efficiency. Quantum yield of PSII electron transport (Φ_{PSII}) declined slowly in the well-watered plants (Fig. 4). There was no significant difference between cultivars, except during the last two weeks of sampling, when R16 and E36-1 plants had significantly lower Φ_{PSII} (Fig. 4). For B35 and KS19 plants, there was no difference in Φ_{PSII} between WW and WL treatments throughout the sampling period, except at the last sampling date when

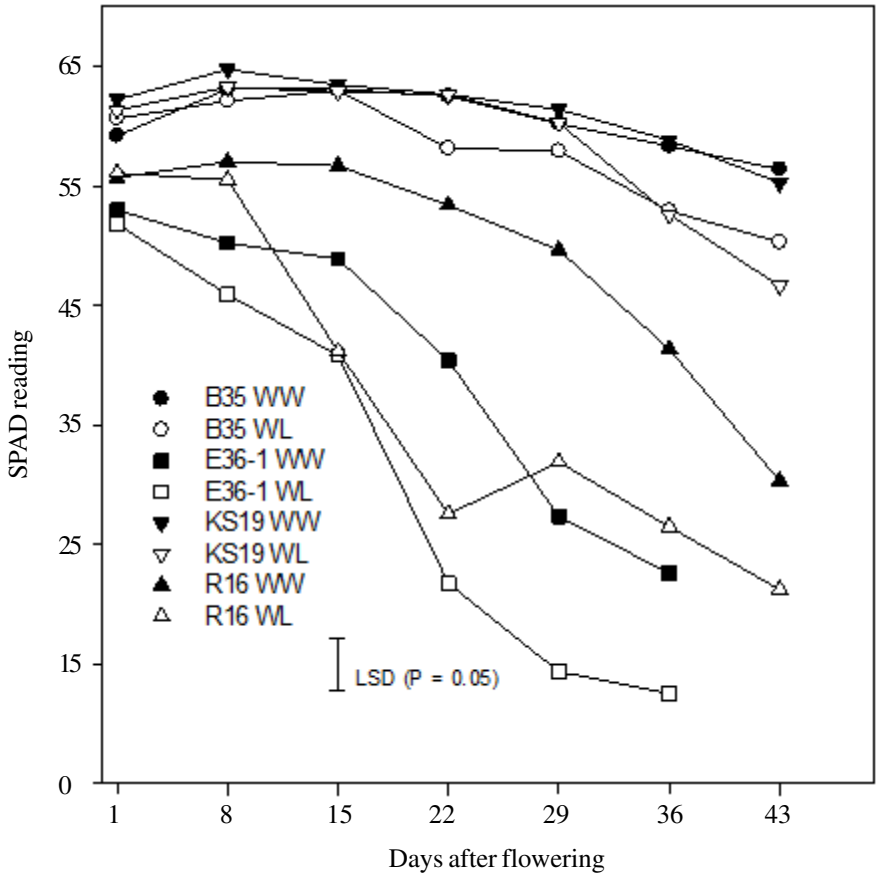

Figure 3. SPAD readings showing levels of chlorophyll retained in well-watered (WW) sorghum plants and water-limited (WL) from 14 to 49 DAA. Values are means of 4 replicates.

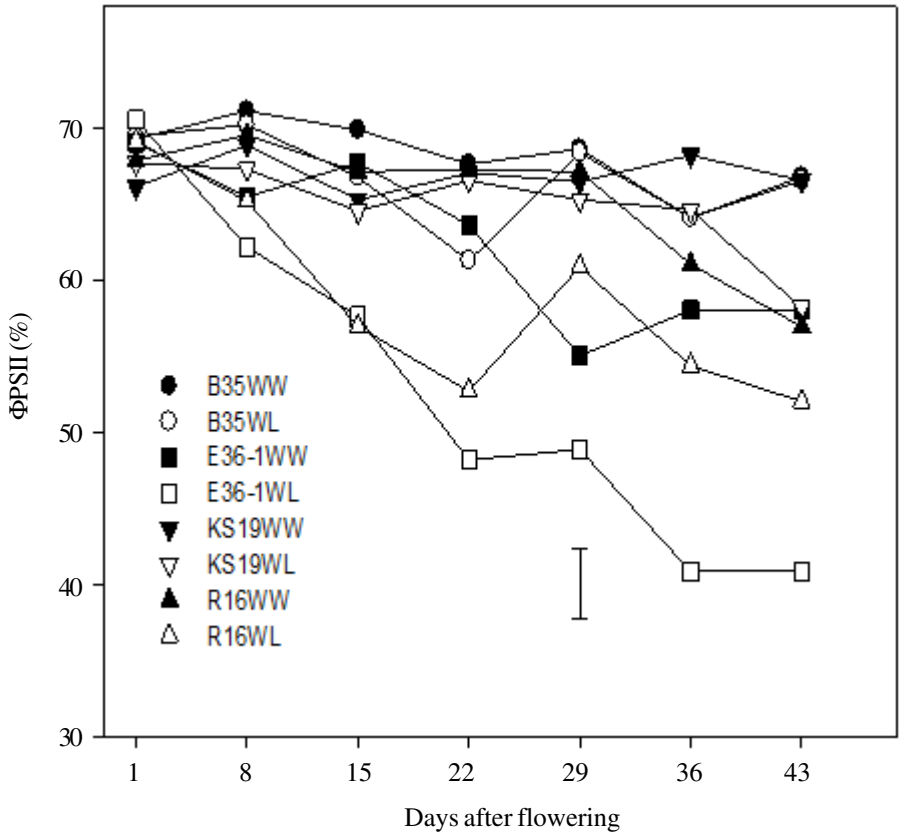

Figure 4. Changes in quantum efficiency of photosystem II ($Ö_{PSII}$) in well-watered (WW) and water-limited (WL) sorghum plants from flowering to physiological maturity. Values are means of 4 replicates.

it was significantly (P<0.05) lower in WL for KS19 plants. Water limitation significantly (P<0.05) reduced Φ_{PSII} in E36-1 and R16 compared to the WW treatment from 15 to 43 DAA, in parallel to the loss in chlorophyll (Fig. 4).

Carbon dioxide assimilation rate (*A*) and leaf conductance (g_L) were higher in R16 than any of the other lines at flowering; but declined sharply thereafter in both the WW and WL plants (Fig. 5). By the end of the third week, *A* was reduced significantly (P<0.05) in E36-1 and R16 compared with the respective WW plants. The reductions were 68 and 59% in in R16 and E36-1, respectively, compared to only 20 and 14% reductions in B35 and KS19, respectively. Furthermore, in the water-limited treatment, carbon dioxide assimilation rate was significantly (P<0.05) higher in B35 and KS19 than in E36-1 and R16 at the end of week 3. At physiological maturity, under both WW and WL treatments, *A* was significantly (P<0.05) higher in B35 and KS19 than in E36-1 and R16.

Leaf conductance was significantly (P<0.05) reduced by water limitation in B35, E36-1 and R16 at the end of the third week (Fig. 5); whereas in KS19 there was no difference between the WW and WL plants. However, the absolute values of g_L were higher in B35 and KS19 than in E36-1 and R16 under WL conditions. Hence, under WL conditions, B35 and KS19 were able to reduce water losses *via* transpiration better than E36-1 and R16.

Leaf conductance correlated with CO_2 assimilation rate, with the coefficients of 0.77 (P<0.001), 0.73 (P<0.001), 0.52 (P <0.01) and 0.74 (P<0.001) for B35, E36-1, KS19 and R16,

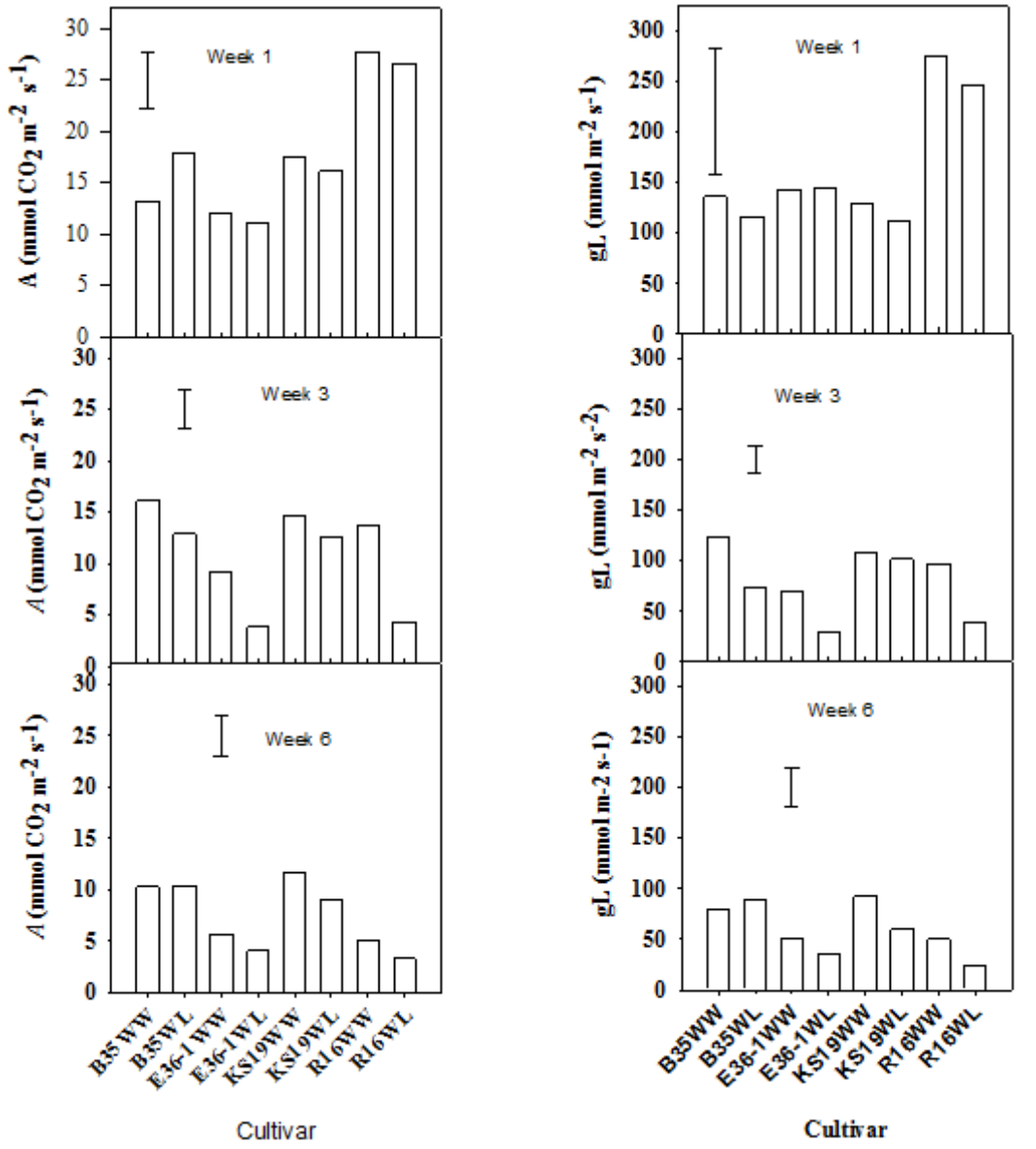

Figure 5. Effect of water limitation on CO_2 assimilation rate (A) and leaf conductance (g_L) at the end of 1, 3 and 6 weeks after sorghum flowering. Plants were either well-watered (WW) or water-limited (WL). Means are values of 4 replicates. Vertical lines above are the LSD values at P = 0.05 for the corresponding sampling date.

respectively; under well-watered conditions. The corresponding coefficients for the water-limited plants were 0.65 (P<0.001), 0.82 (P<0.001), 0.82 (P<0.001) and 0.81 (P<0.001), respectively, for B35, E36-1, KS 19 and R16.

Transpiration rate (*E*) was also significantly (P<0.05) higher in R16, compared to the stay-green lines at flowering (Fig. 6). Generally, *E* was lower in the WL plants and corresponded with $g_{L,}$ indicating that stomatal conductance mainly regulated transpiration. The R-square

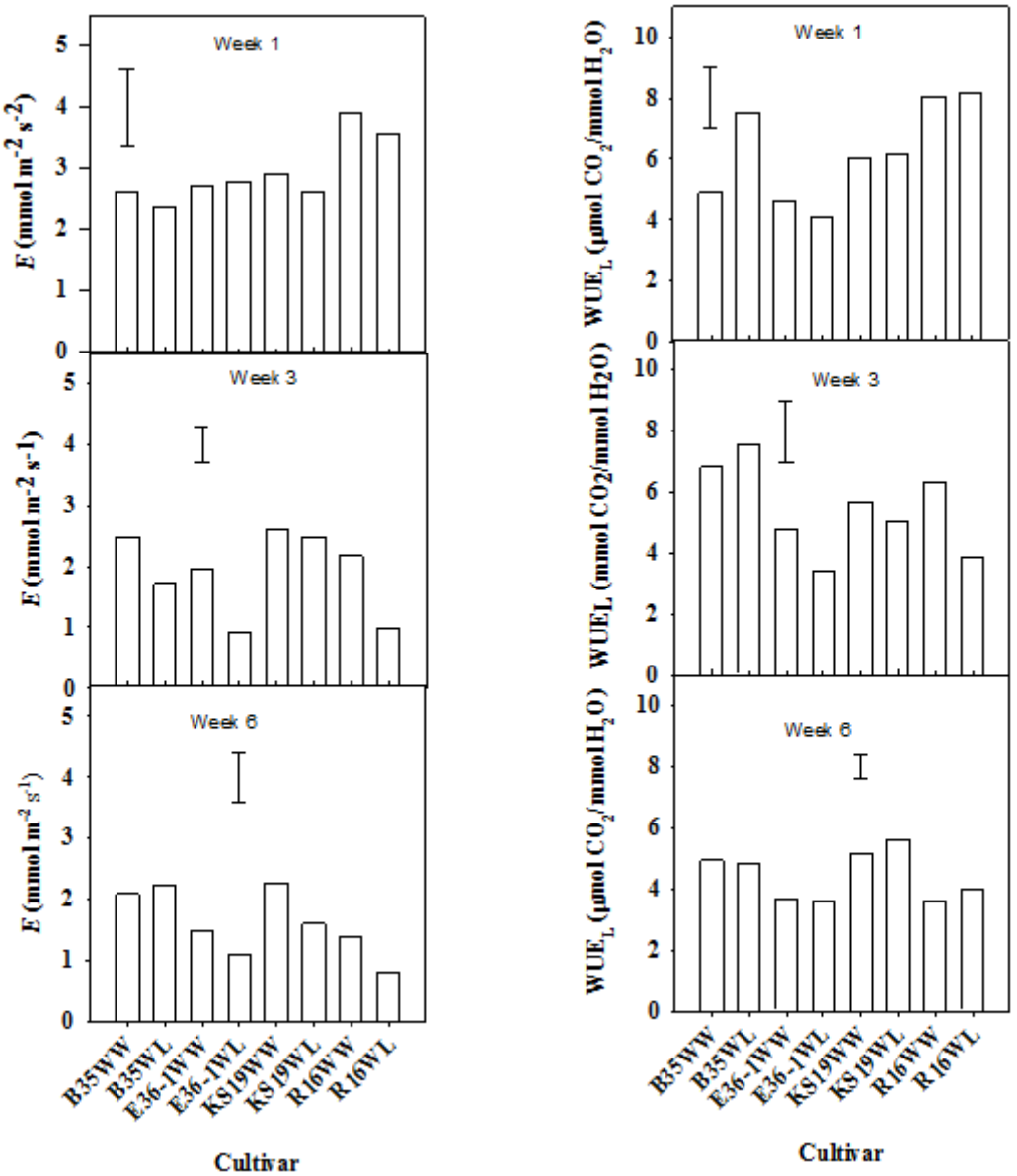

Figure 6. Effect of water limitation on transpiration rate (*E*) and photosynthetic water use efficiency (WUE_L) at the end of 1, 3 and 6 weeks after sorghum flowering. Plants were either well-watered (WW) or water-limited (WL). Means are values of 4 replicates. Vertical lines above are the LSD values at P = 0.05 for the corresponding sampling date.

values were 90.9% for B35 and E36-1, 81.6% for KS19 and 86.1% for R16, under well-watered conditions. The corresponding values for B35, E36-1, KS19 and R16 water-limited plants were 93.7, 95.5, 89.7 and 86.5%, respectively. For R16, transpiration rate was significantly ($P<0.05$) reduced at the end of the third week in WL plants (by 72%), compared with WW plants (by 44%). Similarly, in E36-1, *E* was significantly ($P<0.05$) reduced (67%) by water limitation, compared to the WW (28%) from flowering at the end of the third week. For B35 and KS19, water limitation did not have a significant ($P>0.05$) effect on *E* at any sampling dates.

Leaf water use efficiency (WUE_L), computed as *A*/*E,* generally declined from flowering and the effect of water limitation was most apparent at the end of the third week from flowering (Fig. 6). In B35, there was no difference between the WW and WL treatments; while in KS19, E36-1 and R16, WUE_L was reduced by 11, 28 and 39%, respectively, by water limitation at the end of the third week; indicating a genotype by treatment interaction. However, water treatment had no impact on WUE_L at week 6. These findings indicate that WUE_L was greater in the stay-green lines under WL conditions.

Specific proteins. Trends in band intensity of a range of photosynthetic, nitrogen metabolism and other leaf proteins assessed at 21 DAA and at physiological maturity (42 DAA) in relation to that at flowering are presented in Table 2. In both B35 and KS19, these proteins were not affected by water limitation. For E36-1, only D1 proteins, OEC33, GS1 and Glu-t-RNAL were reduced in the water-limited, compared to the well-watered plants. For the senescent R16 plants, D1, cytochrome *f*, LHCPII, PEPCK, PEPC, Glu-t-RNAL and GSAAT were reduced by water limitation.

Grain yield. Seed number per plant, grain dry mass per plant (mean seed weight) and HI did not differ significantly ($P>0.05$) between the well-watered (WW) and water-limited (WL) plants in all lines (Table 3). However, the values of these parameters were higher in all the stay-green lines than in the senescent cultivar. The harvest index (HI) in all three stay-green lines was significantly ($P<0.05$) higher than in R16. B35 and E36-1 also had significantly ($P<0.05$) higher number of seeds and grain dry mass than R16. Grain dry mass was not reduced in KS19, with minimal reductions in E36-1 (8%) and B35 (13%) due to water limitation, compared to the significant ($P < 0.05$) reduction of 35% in R16. However, grain dry mass was still low in KS19 under WW conditions, similar to R16.

Panicle harvest index (PHI) was also higher in the stay-green lines than in R16, where it was significantly ($P<0.05$) reduced in WL compared to WW. PHI did not differ between B35 and E36-1, but significantly ($P<0.05$) lower in KS19. These results indicate that water deficit reduced seed set in B35 and R16 by 13 and 21 %, respectively; and reduced seed size in E36-1 and R16 by 6 and 18 %, respectively.

DISCUSSION

Findings from the present study indicate that stay-greenness in the three stay-green sorghum genotypes may have different physiological and biochemical basis and can be influenced by the pot size for glasshouse studies. All WL plants were given the same limited amount of water per day, and pot size was constant. The E36-1 plants had much greater leaf area per plant at anthesis (Fig. 1), thus would use more water, and would be more stressed. Hence, in these plants, WL induced rapid senescence which allowed limited expression of stay-green. However, the yields under WL had some degree of protection as there was only non-significant difference in yield (62.5 SEM 7.9 and 57.2 SEM 3.7 g $plant^{-1}$ in WW and WL, respectively). By a similar argument, the smallest plants at anthesis (KS19) used the least amount of water and were not very stressed, and had similar

TABLE 2. Band intensity (%) relative to that at sorghum flowering time. Plants were either well-watered (WW) or water-limited (WL). Band were quantified following background correction using ImageJ

	21 DAA		42 DAA	
	WW	WL	WW	WL
D1 protein				
B35	89.3	88.7	91.7	86.9
KS19	103.1	94.4	97.4	94.2
E36-1	94.9	66.1	68.4	59.7
R16	99.3	82.6	55.8	44.0
OEC33				
B35	100.1	98.8	100.3	97.2
KS19	99.2	97.7	102.6	99.3
E36-1	107.6	96.9	95.8	56.8
R16	85.5	79.5	66.7	65.5
Cytochrome f				
B35	105.6	97.5	98.6	100.3
KS19	106.0	97.4	103.8	97.3
E36-1	66.9	63.2	22.8	25.2
R16	105.1	71.8	48.9	30.7
LHCP11				
B35	100.0	100.6	100.4	111.0
KS19	110.6	134.1	103.8	127.2
E36-1	100.3	78.5	36.8	34.6
R16	96.9	75.9	82.6	35.7
PEPCK				
B35	112.5	90.1	29.7	31.7
KS19	145.3	156.4	102.3	98.3
E36-1	36.6	17.0	6.7	6.4
R16	145.6	103.0	88.6	51.2
PEPC				
B35	116.1	100.7	103.4	105.6
KS19	92.3	93.1	82.0	79.3
E36-1	84.2	80.9	40.0	43.6
R16	114.9	107.0	72.8	28.9
Rubisco (large sub-unit)				
B35	102.1	101.0	104.5	101.4
KS19	101.2	101.1	99.2	101.9
E36-1	101.5	104.4	103.4	99.1
R16	101.2	99.1	98.2	103.3

TABLE 2. Contd.

	21 DAA		42 DAA	
	WW	WL	WW	WL
GS1				
B35	101.8	102.2	101.8	104.9
KS19	103.7	103.7	101.9	102.2
E36-1	419.2	277.1	499.4	277.0
R16	113.6	167.9	206.6	187.8
GS2				
B35	99.2	101.8	104.4	103.7
KS19	104.7	103.7	99.5	101.4
E36-1	98.0	93.0	90.5	92.8
R16	88.6	90.6	83.1	76.5
Glu-t-RNAL				
B35	104.9	98.4	103.7	104.5
KS19	101.6	104.5	103.7	102.8
E36-1	80.6	61.0	73.5	78.2
R16	92.0	103.6	93.7	73.7
GSAAT				
B35	91.3	97.1	117.9	116.1
KS19	101.6	99.5	102.2	102.1
E36-1	101.1	83.9	92.8	84.2
R16	98.9	119.0	102.7	86.5
PORB				
B35	69.7	55.3	53.1	44.4
KS19	108.0	131.5	90.3	92.7
E36-1	n.d.	n.d.	n.d.	n.d.
R16	104.6	53.9	26.1	27.9

D1 protein = the reaction centre polypeptide of photosystem II; OEC33 = oxygen evolving complex; GS1 = cytoplasmic glutamine synthetase; Glu-t-RNAL = Glutamyl-tRNA ligase: EC 6.1.1.17; LHCPII = light-harvesting complex proteins II; PEPCK = phospho*enol*pyruvate carboxykinase; PEPC = phospho*enol*pyruvate carboxylase; GSAAT = glutamic semi-aldehyde aminotransferase

yields in WW and WL. By comparison, the stay green had a much higher leaf area at the end of the experiment under WL (2206 and 988 cm^2 for B35 and R16, respectively), than the two genotypes (B35, stay-green, and R16 not stay green) with intermediate leaf area at anthesis. Furthermore, there was only a slight reduction in yield by WL in the stay green B35 (yield 44.9 SEM 2.8 and 38.9 SEM 4.1 g $plant^{-1}$ in WW and WL respectively) whilst there was a much larger proportional reduction in yield by WL in the genotype without stay green, R16 (yield 10.2 SEM 0.8 and 6.6 SEM 0.7 g $plant^{-1}$ in WW and WL respectively. Thus overall this reinforces the value of the stay green character in maintaining yield under water limitation and shows it has no negative impact under lower stress levels.

TABLE 3. Sorghum grain yield parameters of well-watered (WW) and water-limited (WL) plants

Line	Treatment	Seeds/plant	Mean seed weight (g)	PHI (%)	HI (%)	Mean 100 seed weight (g)
B35	WW	1185.5 ± 69.7	44.91 ±2.82	84.96 ± 0.41	36.36 ± 1.54	3.79 ± 0.11
	WL	1027.0 ±119.9	38.92 ± 4.13	83.87 ± 1.73	33.33 ± 3.02	3.81 ± 0.06
E36-1	WW	1593.0 ±231.0	62.45 ± 7.93	85.98 ± 0.50	23.44 ± 2.13	3.95 ± 0.09
	WL	1543.0 ± 98.4	57.21 ± 3.69	85.90 ± 0.11	25.15 ± 1.43	3.71 ± 0.03
KS19	WW	421.1 ± 96.2	10.06 ± 2.42	75.07 ± 2.65	26.59 ± 3.66	2.38 ± 0.06
	WL	447.0 ±115.0	10.32 ± 2.38	72.98 ± 2.71	26.29 ± 3.42	2.38 ± 0.11
R16	WW	269.9 ± 22.5	10.21 ± 0.82	50.31 ± 1.36	7.52 ± 0.73	3.79 ± 0.10
	WL	214.4 ± 26.5	6.59 ± 0.74	40.33 ± 3.05	5.56 ± 0.66	3.09 ± 0.08
$LSD_{0.05}$		335.22	11.04	5.54	6.85	0.24

Values are Means ± SEM of 4 replicates; PHI = Panicle harvest index, HI = Harvest index

Biochemical data relating to leaf senescence can only be interpreted with understanding of whole plant physiology. Hence, three key limitations to the current study, likely to confounded leaf senescence patterns include: (i) the pot size was small, (ii) there were large genotypic variations in leaf size at anthesis, and (iii) the four genotypes evaluated were not from a common genetic background.

In an experiment to determine the impact of pot size on root and shoot growth (Yang *et al*., 2010), small pots (less than 28 litres in volume) significantly inhibited tillering and shoot growth for both sorghum and maize plants. Although the same total amount of slow release fertiliser was applied to large and small pots during pot filling in the study of Yang *et al.* (2010), visible signs of nitrogen deficiency were obvious in lower leaves of plants growing in small pots. This suggests that the small pot size in our experiment (10 L) affected tillering, shoot growth, root:shoot ratio and N uptake.

In our study, fertiliser was supplied at intervals during the study to augment the effect of nitrogen deficiency that would have resulted from limiting root expansion in the small-volume growth pots. However, different nutrient requirements due to differences in plant sizes could have impacted senescence differently among the genotypes. All of these factors could have impacted on senescence patterns and grain yield. Indeed, a reduction in N uptake and assimilation has been reported in previous studies on different plant species with restricted soil volume for root growth (Ronchi *et al*., 2006; Zhu *et al*., 2006; Yang *et al*., 2007). Hence, the results of our study need to be considered in the context of this limitation.

Canopy size at anthesis. The size of the canopy at anthesis varied greatly between genotypes, and this profoundly impacted leaf senescence patterns (Fig. 1). For example at anthesis, the GLA of E36-1 (7003 cm^2) was almost four times that of KS19 (1881 cm^2) and since the WL plants were supplied with only 250 ml water daily after anthesis, regardless of genotype, those genotypes with a larger leaf area would have used more water than those with a smaller leaf area, thereby potentially affecting leaf senescence patterns. In fact, the percentage decline in leaf area during the first 21 days after anthesis was in the following order: E36-1 (72%), R16 (71%), B35 (29%) and KS19 (7%).

This decline in leaf area highly correlated (r = 0.87) with the initial canopy size at anthesis: E36-1 (7003 cm^2), R16 (4884 cm^2), B35 (4635 cm^2) and KS19 (1881 cm^2).

Therefore, to a large extent, senescence patterns were determined by the initial size of the canopy at anthesis; hence, leaf senescence patterns need to be interpreted in light of canopy size at anthesis. Indeed, Borrell *et al.* (2014a) found that *Stg* loci reduce canopy size at flowering by modifying tillering, leaf number and leaf size; and that smaller canopy size at flowering reduces pre-anthesis water use, which under post-flowering water stress increases water availability during grain filling and, consequently, grain yield. Thus, the designation of E36-1, which has been identified as one of the best sources of stay-green (Badigannavar *et al.*, 2018), was not confirmed in this study.

Maintenance of photosynthesis during grain filling is likely to be highly associated with GLA at anthesis in this study, since this factor was the key driver of leaf senescence. Indeed, photosynthesis is dependent on available green leaf area, which can be reduced by senescence, thus reducing canopy phosynthesis (Badigannavar *et al.*, 2018).

Changes in other parameters during grain filling, including chlorophyll proteins, quantum yield, and CO_2 assimilation, were largely a consequence of differences in GLA at anthesis. Therefore, biochemical data needs to be interpreted in relation to whole plant physiology.

Genetic background. The four genotypes evaluated in this study (B35, KS19, E36-1 and R16) were selected to represent different sources of stay-green. However, they differed significantly in other traits such as maturity and plant height. Understanding how genetic and biochemical mechanisms contribute to phenotypic responses of crop plants should provide opportunities for breeding and selecting genotypes for various environmental conditions (Boyles *et al.*, 2019). Therefore, any comparisons in the physiological or biochemical mechanisms underpinning drought adaptation in these lines, will likely be confounded by variation in other traits e.g. maturity and height (this is why near-isogenic lines are commonly used in physiological studies to remove such confounding factors). Nonetheless, the current findings confirm B35 and KS as stay-green genotypes with different physiological mechanisms for staying green after anthesis under drought stress.

Green leaf area and chlorophyll retention. The results presented here indicate that the drought treatment used was sufficient to prevent premature plant death and enable the trait to be expressed. Two types of senescence were assessed in this study: age-related (WW) and stress-induced (WL). Clearly, B35 and KS19 demonstrated the stay-green trait by maintaining green canopy, even at maturity (Badigannavar *et al.*, 2018), but through different mechanisms. E36-1 behaved as the senescent genotype, R16, maybe due to the factors already discussed under the section for small pot.

Photosynthesis. Photosynthesis, measured as quantum yield of photosystem II and carbon dioxide assimilation (Fig. 2) indicated that the stay-green sorghum genotypes maintained photosynthesis for longer periods compared to the senescent R16. Stay-green sorghum genotypes retain green leaf area during grain filling for longer periods than *go-browns* (Borrell *et al.*, 2000ab, 2014; Harris *et al.*, 2007), presumably maintaining photosynthetic capacity for longer in stay-green lines. The retention of chloroplast proteins, such as LHCPII, OEC33 and Rubisco, until late in senescence observed in the KS19 source of stay-green indicates that photosynthesis may be maintained for longer periods during senescence in these genotypes (de Villers *et al.*, 1993).

Findings in our study have confirmed that photosynthesis is maintained for longer periods in stay-greens under water limitation. Quantum yield (Φ_{PSII}), a measure of quantum efficiency of CO_2 assimilation (number of quanta absorbed per mole of CO_2 reduced), is a good

guide to photosynthetic functionality of leaves (Maxwell and Johnson, 2002). Φ_{PSII} was reduced by water limitation and declined 14 days earlier in R16 compared to B35 and KS19. The comparison between R16 and B35 is particularly insightful since the confounding effect of leaf size at anthesis was largely removed.

B35 and KS19 plants, which had higher Φ_{PSII}, also had higher CO_2 assimilation rates (Fig. 5). The reduction CO_2 assimilation rates in B35 and KS19 by water limitation was not significant ($P>0.05$). Though the reduction in E36-1 was significant ($P<0.05$), the decline was also more gentle compared to R16. However, as explained earlier, E36-1 exhibited a large GLA at anthesis, and combined with the small pot size, resulted in leaf senescence. Nonetheless, our findings confirm that photosynthesis is maintained for longer periods in stay-green genotypes, when water is limiting. The maintenance of photosynthesis could also be explained, in part, as a result of chlorophyll retention, since chlorophyll is a major chloroplast component for photosynthesis (Anjum *et al*., 2011). However, the retention of chlorophyll post-anthesis was likely a consequence of GLA at anthesis.

Leaf conductance (g_L) and transpiration rates (E) were also higher in the stay-green plants compared to the senescent R16 cultivar (Fig. 5). Stomatal conductance is a measure of transpiration rate and photosynthetic potential of plants under drought stress (Badigannavar *et al*., 2018). In R16 plants, the reduction in leaf conductance and transpiration rates resulted in a corresponding reduction in CO_2 assimilation rate (A), which is in agreement with reports by Anjum *et al*. (2011) that reduction in CO_2 assimilation is a consequence of reduced stomatal conductance by drought stress. B35, which had similar GLA as R16 at anthesis, maintained photosynthesis for longer periods under both WW and WL conditions. Furthermore, whereas transpirational water use efficiency (WUE_L) declined with water limitation in R16 and E36-1, WUE_L was increased in B35 and KS19 plants. These findings confirm that B35 and KS19 plants were drought tolerant since drought tolerance in sorghum is associated with the ability to maintain high stomatal conductance, transpiration rate and photosynthesis under drought stress (Tsuji *et al*., 2003).

Chlorophyll biosynthetic pathway. The abundance of both Glu-tRNAL and GSAAT proteins did not change much between flowering and physiological maturity in any of the stay-green genotypes studied under both WW and WL conditions (Table 2). This indicates that chlorophyll biosynthesis and turnover were maintained in these genotypes, unlike in R16, in which both proteins were reduced by water-limitation at 42 DAA (Table 2). Glutamate-tRNA ligase (Glu-tRNAL) and GSAAT are important in the biosynthesis of chlorophylls (Czarnecki and Grimm, 2012). Therefore, the maintenance of these proteins for longer periods partially accounted for prolonged photosynthesis in the stay-green genotypes.

POR B is the constitutively expressed and the only remaining POR in light-grown plants (Vavilin and Vermaas, 2002). It persisted in KS19 and was not detectable in leaves of E36-1 from flowering to physiological maturity under both WW and WL conditions. The higher amounts of POR B in B35 compared with R16 at 42 DAA, despite equivalent GLAA, supports the involvement of this enzyme in the maintenance of chlorophyll turnover in stay-green genotypes, particularly under water limitation.

Glutamine synthetase (GS) isoenzymes. Abundance of GS isoenzymes in the cytosol (GSI) and chloroplast (GSII) did not change much from flowering to physiological maturity (Table 2). The exception was the reduction in GSII in R16. Glutamine synthetase (GS) catalyses the synthesis of glutamine from ammonium and glutamate (Németh *et al*., 2018). These authors argue that GS plays a

central role as a regulator between the nitrogen and carbon cycles by maintaining the glutamine-glutamate pool in the chloroplast on the level of substrates, in addition to its ammonia assimilation function. We found no differences in GSI proteins between the well-watered and water-limited plants, except that GSI was reduced in E36-1 under water limitation.

Re-assimilation of ammonium by GSII is crucial to plants as levels of ammonium released during photorespiration could be more than the primary N assimilation (Igamberdiev *et al*., 2014)). For instance, barley (*Hordeum vulgare*) mutants defective in GSII and containing low amounts of GSI grown in air, died because they were unable to re-assimilate ammonium released from photorespiration (Oliveira *et al*., 2002). Hence, the loss of chlorophyll in R16 under water-limitation might have resulted from reduction in the abundance of GSII. The high levels of GSII in the stay-green sorghums might have enhanced the re-assimilation of N from photorespiration, even though photorespiration is supposed to be low in C4 plants (Makino *et al*., 2003).

Chloroplast proteins. Abundance of LHCPII differed very little between the WW and WL plants of the stay-green genotypes (B35 and KS19) (Table 2); but was reduced by water-limitation in E36-1 and R16 plants. The reduction in LHCPII between anthesis and maturity was also higher in E36-1 (56%) and R16 (53%) compared with B35 (10.3%) and KS19 (5.1%). LHCPII is a major contributor to the overall loss of protein during leaf senescence (Matile, 1992). In stay-green genotypes, LHCPII remains stabilised and proteolytic cleavage is restricted due to a small N-terminal that protrudes into the stoma (Thomas and Donnison, 2000).

The 33 kDa oxygen-evolving complex protein (OEC33) is known to stabilise the catalytic Mn cluster, which is essential for water oxidation (Zhang *et al*., 1998). A reduction in or degradation of OEC33, as a result of drought, therefore, would lead to a decline in CO_2 assimilation since electron transport would be adversely affected. Since the abundance of OEC33 did not change much between the well-watered and water-limited stay-green plants (B35 and KS19 (Table 2) it could partially account for the higher and prolonged photosynthetic rates. R16 and E36-1 displayed reductions in OEC33 of 18 and 41%, respectively, under WL conditions.

The stay-greens (B35 and KS19) maintained a greater proportion of D1 proteins between flowering and physiological maturity, compared to the senescent R16 under water limitation (Table 2). Reactive oxygen species degrade D1 protein and inactivate PSII (Miyake *et al*., 2005). Damaged D1 protein is removed and degraded, and the PSII complex repaired by prompt insertion of newly synthesised D1 protein (Henmi *et al*., 2003). This implies that the integrity of PSII was maintained better in the stay-green plants under water-limited conditions compared to R16 plants. Since chlorophyll is required for the integration of newly synthesised D1 protein (Mullet *et al*., 1990), the loss of chlorophyll in the WL R16 plants might have prevented the integration of newly synthesised D1 protein (if any was synthesised).

Cyt *f* is involved in transferring electrons from plastoquinone (PQ) to Plastocyanin (PC); while Fd transfers electrons from PSI to NADP. Cyt *f* and Fd (results not shown) were also retained throughout in the stay-green genotypes (B35 and KS19) under water limitation, unlike in the E36-1 and R16 genotypes. Davies *et al.* (1990) have reported that Cyt *f* is more stable in stay-greens than in senescent genotypes. The reductions of Cyt *f* and Fd proteins in the WL E36-1 and R16 genotypes could also result in reduced electron transport and overall photosynthesis rates. Indeed, in antisense lines with reduced Fd, Holtgrefe *et al*. (2003) found that cyclic electron transport, determined by quantum yields of PSI and PSII, was enhanced,

whereas CO_2 assimilation rate in some lines showed photoinhibition.

Photosynthetic enzymes. Phosphoenol pyruvate carboxylase (PEPC) was also maintained at higher levels in the stay-green plants (B35 and KS19) compared to E36-1 and R16 under water limitation (Table 2). The availability of functional PEPCK in the WW and WL stay-green plants could have resulted in better maintenance of photosynthesis than in E36-1 and R16 under water-limiting conditions. Photosynthesis in C_4 plants, including sorghum, involves the enzymes PEPCK, PEPC and Rubisco, among others. PEPC occupies a key position as the initial CO_2-fixing enzyme of the C_4 pathway, and is a major control point in this pathway (Liu *et al*., 2017).

The activity of PEPC is regulated at many levels, including the phosphorylation catalysed by phospho*enol*pyruvate carboxylase kinase (PEPCK) (Monreal *et al*., 2013). The phosphorylation of PEPC catalysed by PEPCK enables PEPC to perform its catalytic activities. Therefore, under WL conditions, the 42% reduction in PEPCK (Jeanneau *et al*., 2002) activity could have resulted in the deactivation of PEPC in R16 (60% reduction). Blocking of PEPCK synthesis in the leaf of maize and sorghum leads to marked inhibition of CO_2 assimilation, not due to stomatal closure, but as a consequence of a decrease in other photo-activated C_4 cycle enzymes and perturbation of the Benson-Calvin cycle (Jeanneau *et al*., 2002). Therefore, current findings support the reports that phosphorylation of PEPC by PEPCK is fundamental for CO_2 assimilation in the C_4 photosynthetic pathway (Jeanneau *et al*., 2002). The reduction in the amounts of both PEPC and PEPCK, could partially cause reduced CO_2 assimilation rate in the WL R16 plants. In the same vein, the high CO_2 assimilation rates observed in the stay-green plants, particularly B35, were likely associated with the retention of high amounts and activities of PEPC and PEPCK under water limitation.

The reduction in the abundance of PEPC and PEPCK in the WL R16 plants could have impaired the production of malate needed for CO_2 production in the bundle sheath cells and, consequently, CO_2 assimilation rate in these plants.

The abundance of rubisco in leaves is controlled by the rate of its synthesis and degradation (Parry *et al*., 2002). The abundance of the large subunit (LSU) of rubisco in all the genotypes was not affected by water limitation throughout the sampling period. Observations on the effect of drought on rubisco (LSU) are varied (Zasheva *et al.,* 2009). In some studies, it has been found that drought has no effect on rubisco (Pellox *et al*., 2001) whereas in other studies drought resulted in reduction of the large subunit of the enzyme (Inmaculada *et al*., 2006; Zasheva *et al.*, 2009). Therefore, our findings are similar to those reported Pelloux *et al*. (2001).

Grain yield. The present study has shown that maintenance of GLA does not result in lower grain yields due to poor sink strength. This is in agreement with findings obtained from field studies (Borrell *et al*., 2000b; Borrell *et al*., 2014ab; Jordan *et al*., 2012). Panicle harvest index (PHI) was maintained in B35, E36-1 and KS19 under water limitation; whereas in R16 it was significantly ($P<0.05$) reduced,. These differences in PHI are due, in part, to the lower seed set in WL R16 compared with the other genotypes. Similarly, seed size, measured as mean 100 seed weight, was maintained in B35 and KS19, but reduced in WL E36-1 and R16 plants. Even in E36-1, it was apparent that seed size was less reduced compared to R16 by water limitation.

The stay-green sorghum genotypes, particularly B35 and KS19, exhibit delayed leaf senescence and maintain photosynthesis for longer periods as a result of maintenance of the integrity of the photosynthetic apparatus, chlorophyll biosynthesis and turn over, as well as photosynthetic enzymes under drought stress. Furthermore, the onset of senescence

is delayed and/or the rate of senescence is reduced in stay-green (B35 and KS19) compared with senescent genotype. E36-1 does not behave as B35 or KS19, confirming sorghum plants may stay green differently.

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