Asian Journal of Agricultural Sciences 3(1): 37-43, 2011
ISSN: 2041-3890

Received: October 13, 2010 Accepted: November 05, 2010 Published: January 10, 2011

Physiological Responses of Bambara Groundnut (*Vigna subterranea* L. Verdc) to Short Periods of Water Stress During Different Developmental Stages

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Abstract: The study was conducted to evaluate the responses of bambara groundnut (*Vigna subterranea* L. Verdc) to short periods of water stress imposed at different growth stages, and the recuperative ability of the species from drought stress. A major problem associated with Bambara groundnut production is its very low yields due to intra-seasonal and inter-seasonal variability in rainfall in semi-arid regions. The response pattern of physiological processes to water stress imposed at different growth stages might provide a basis for development of strategies in order to stabilize yields. Water stress reduced relative water content, chlorophyll fluorescence, stomatal conductance and the reduction was more pronounced in plants stressed during the pod filling stage and less pronounced in plants stressed during the vegetative stage. Chlorophyll content was not affected by water stress at all stages of growth and development. Proline levels were increased by water stress mostly during the pod filling stage. When plants were rewatered after each stress treatment, plants stressed during the pod filling stage failed to fully recover the relative water content and chlorophyll fluorescence. All water stressed plants at different stages of growth and development fully recovered in stomatal conductance and proline concentration. Bambara groundnuts appear to reduce water loss under water stress and have the ability to recover from the effects of water stress after rainfall or irrigation. The nature and extend of damage and the ability of bambara groundnut to recover from water stress depends on the developmental stage at which the plant encounters water deficit.

Key words: Bambara groundnut, chlorophyll, relative water content, proline, stomatal conductance, water stress

INTRODUCTION

Bambara groundnut (*Vigna subterranea* L. Verdc) is an indigenous African leguminous crop grown primarily for its seeds and is increasingly popular as food in rural areas across the African continent. It is the third most important legume after groundnut (*Arachis hypogea*) and cowpea (*Vigna unguiculata*) (Howell *et al.*, 1994). However, despite its importance in the subsistence diet of much of Africa, bambara groundnut is still cultivated from local landraces rather than from varieties bred specifically for particular environments and farm yields are still low. Bambara groundnut is widely regarded as drought tolerant (Linnemann and Azam-ali, 1993). Collinson *et al.* (1997) suggested that drought tolerance of bambara groundnut is a result of osmotic adjustment and low water loss through stomatal closure.

Stomatal closure can result in decreased diffusion and fixation of CO_2 and reduction in photosynthesis. Measurements widely used to measure the effect of water stress include stomatal conductance, chlorophyll fluorescence, chlorophyll content and RWC. Chlorophyll fluorescence measurements may sometimes provide a useful measure of the photosynthetic performance of plants and its real strength lies in its ability to give information that is not readily available in other ways. In particular, fluorescence can give insights into the ability of a plant to tolerate environmental stresses and into the extent to which those stresses have damaged the photosynthetic apparatus (Maxwell and Jonhson, 2000). Assessment of pigment content has also became an effective means of monitoring plant growth and estimating photosynthetic productivity (Chen *et al.*, 2007) and remote estimates of pigment concentration provides an improved evaluation of the spatial and temporal dynamics of vegetation stress (Filella *et al.*, 1995). The accurate measurement of plant water status is also critical in experiments concerned with understanding the effect of differing water supply (Jones, 2007). The measurement of RWC under low soil moisture is of importance since high RWC appears to be a common trait in drought resistant species as species which exhibit restricted changes in

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RWC per unit reduction in water potential are often considered to be relatively drought resistant (Rahaman *et al.*, 2000). Osmotic adjustment is another major physiological mechanism underlying plant resistance to water deficit (Zhu *et al.*, 1997) and over accumulation of osmolytes may help plants to tolerate stress by improving their ability to maintain osmotic balance within the cell (Hasegawa *et al.*, 2000; Apse and Blumwald, 2002). Amino acid proline is one of the osmolytes reported to accumulate intensely in all stressed organs of plants (Aziz and Khan, 2001; Abdalla and El-Khoshiban, 2007).

Since many of bambara groundnut's genotypes are drought tolerant, there is need to establish traits which will provide a basis for breeding. There is hardly any report in literature on its response to short periods of drought stress imposed at different growth stages, or on the recuperative ability of the species from drought stress. The ability to survive and recover rapidly from moisture stress after rainfall or irrigation is critical to the maintenance of growth or production during stress. Thus noting traits for improved recovery may be as economically important as selecting for improved growth during drought. Therefore, the objective of this study was to evaluate the physiological responses of bambara groundnut to short periods of water stress imposed at different growth and developmental stages and their recuperative ability after water stress.

MATERIALS AND METHODS

Experimental site and plant material: The study was conducted between October 2009 to May 2010 in a greenhouse at University of Botswana. The bambara groundnut landrace "Uniswa red" was used and the seeds were obtained from stocks of the EU-funded bambara groundnut research project (BAMLINK) based at the Botswana College of Agriculture, Gaborone, Botswana.

Experimental design and crop management: Two trials were carried out and the experiment was arranged in a completely randomised design with four replications. The treatments were as follows:

Trial 1 - Control (plants were well watered throughout), plants were stressed during the vegetative stage [plants were stressed for 21 days, starting 25 Days After Sowing (DAS)], plants were stressed during the flowering stage (plants were stressed for 21 days, starting 46 DAS) and plants were stressed during the pod filling stage (plants were stressed for 21 days, starting 80 DAS).

Trial 2 - Control (plants were well watered throughout), plants were stressed during the vegetative stage [plants were stressed for 21 days, starting 25 Days After Sowing (DAS)], plants were stressed during the flowering stage (plants were stressed for 21 days, starting 46 DAS) and plants were stressed during the pod filling stage (plants were stressed for 21 days, starting 60 DAS).

Four seeds were sown per pot at 4 cm depth, and seedlings were thinned to one per pot at emergence. The black plastic pots, measuring 225 millimetres in diameter and 450 millimetres in height, were each filled with a 17 kg mixture of normal field soil and sand in 5:3 volume ratios. A basal fertiliser (NPK, 2:3:2) was applied to the soil at a rate equivalent to 265 kg/ha. Plants (pots) were spaced 30cm on benches to preclude competition among treatments. The greenhouse temperature was maintained at 25-28ºC. For each drought treatment, watering was withheld until the pots reached a stress level of 30 % of Plant Available Water (PAW) which was measured according to Rosenthal *et al.* (1987). It took the pots about 10 days to reach 30% of PAW from beginning of stressing and this stress level was maintained for 15 days. During the study period each pot was weighed daily at 09:00 h and water was added if necessary to maintain the stress level. Except for the periods of stress, the watering for all treatments was the same as that for the control plants. During drought recovery, measurements were taken only from leaves existing before rewatering.

Relative Water Content (RWC): RWC was determined using 10 leaf discs (about 13 mm diameter) from leaves of 3 tagged plants per replication. The leaf discs were placed in pre-weighed vials, sealed and reweighed to derive their fresh weight (FW) before being placed in petri dishes lined with two layers of germinating paper saturated with deionised water. These were sealed with tape to prevent evaporation and left overnight under a light source to allow discs to re-hydrate to their turgid weight (TW). Their dry weight (DW) was obtained after overnight drying at 80ºC for 48h. The leaf RWC was then measured and calculated according to Turner and Begg (1981) as:

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

Chlorophyll fluorescence: Chlorophyll fluorescence (estimated from dark adapted F_v/F_m ratio) was measured with a Hansatech Fluorescence Monitoring System (Hansatech instrument limited, Kings Lynn ,England). Measurements were taken on terminal leaflets of three fully expanded, fully sunlit leaves from three tagged plants per treatment per replication. All measurements were taken between 1200 and 1400 h local time. The plants chosen for evaluation were dark adapted for a minimum of 30 min at 25ºC temperature. The dark adapted chlorophyll variable fluorescence/maximal fluorescence (F_v/F_m) ratios and quantum efficiency of photosystem II for each treatment were determined.

Stomatal conductance: Stomatal conductance (mmols/m^{2}s) was measured with a Decagon Devices Leaf

Table 1: RWC (%) of bambara groundnuts leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatment	Stressed (RWC %)	Recovered (RWC %)	Stressed (RWC%)	Recovered (RWC %)
Control	95.00	96.00	95.00	94.87
Stressed at vegetative stage	86.00	95.00	86.48	94.49
Stressed at flowering stage	85.75	95.00	85.28	94.00
Stressed at pod filling stage	82.75	92.50	83.07	91.00
LSD	1.90	1.39	1.87	0.96

Table 2: Water stress effects on chlorophyll fluorescence ratio F_v/F_m of bambara groundnuts leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatments	Stressed F_v/F_m	Recovered F_v/F_m	Stressed F_v/F_m	Recovered F_v/F_m
Control	0.79	0.80	0.80	0.80
Stressed at vegetative stage	0.67	0.80	0.72	0.80
Stressed at flowering stage	0.66	0.80	0.68	0.79
Stressed at pod filling stage	0.63	0.77	0.65	0.77
LSD	0.11	0.02	0.10	0.02

F_v/F_m= The dark adapted variable fluorescence/maximal flourescence ratio and quantum efficiency of photosytem II

Porometer SC-1. Measurements were taken between 1200 and 1400 h local time on fully expanded leaves of 3 tagged plants per treatment per replication.

Leaf Chlorophyll content: The measurements were taken on fully expanded leaves of 3 tagged plants from each treatment per replication. Two methods were used to determine leaf chlorophyll content.

The non-destructive measurement: This was done using the OPII-SCIENCES CCM-200 chlorophyll content meter to acquire a rapid estimate of leaf chlorophyll content which is expressed as the Chlorophyll Content Index (CCI).

The destructive measurement: Leaf disks of about 0.25 g were used for determination of actual leaf chlorophyll content by photometric methods as described by Porra *et al.* (1989). Chlorophyll was extracted from the leaf tissue using a buffered 80% aqueous acetone solution and absorbance was measured on the supernatant by a UV 160 IPC spectrophotometer. Chlorophyll content was expressed in µg chl.g/Fwt, where Fwt denotes fresh weight.

Leaf proline content: Samples of fresh tissue were weighed (0.5 g) and homogenised in 10 ml of 3 % aqueous sulfosalicyclic acid. The homogenate was then filtered through Whatman filter (grade1) paper. 2 mL of the filtrate was reacted with 2 mL acid-nihydrin and 2 mL glacial acetic acid in a test tube for an hour at 100ºC in a water bath to develop the colours. Soon after removal from the water bath, the test tubes were cooled in an ice bath and proline was extracted with 4ml toluene, mixed vigorously with a test tube stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read in a UV 160 IPC spectrophotometer at 520 nm ,using toluene as a blank. Proline content in fresh tissue was determined by comparing the sample absorbance with the standard proline curve and calculated on a fresh weight basis as µmole proline/g of fresh weight material (Bates *et al.*, 1973).

Statistical analysis: The data collected was subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS). Treatment means were compared using the Least Significance Difference (LSD) at probability level of 0.05.

RESULTS

Relative Water Content (RWC): Stressing bambara groundnuts for 21 days significantly reduced ($p<0.05$) RWC compared to the control plants in both trial 1 and 2 at all stages of growth and development (Table 1). Depending on the stage of bambara plant development, water stress reduced the RWC of plants by 9-12.25% in trial 1 and 8.97-12.56% in trial 2. However, stressing bambara groundnut plants at the pod filling stage had the highest reduction of the leaf RWC (Table 1). Only the RWC of the plants stressed at the vegetative and flowering stages recovered fully after rewatering attaining values near or close to those observed in control plants (Table 1). However, recovery of RWC for the pod filling stage failed to reach the values for control and had the lowest recovery of 3.65% in trial 1 and 4.08% in trial 2 as compared to control plants (Table 1).

Chlorophyll fluorescence: There was a 15.2-20.3% and 10-18.75% significant reduction of chlorophyll fluorescence (dark adapted F_v/F_m ratio) in trial 1 and 2, respectively, in water stressed bambara groundnut plants at different stages of growth and development compared to non-stressed plants (Table 2). Rewatering bambara groundnut plants after water stress during the vegetative and flowering stages significantly ($p<0.05$) increased the dark adapted F_v/F_m ratio to the same level of that of non-stressed plants (Table 2). However, the bambara plants which were stressed during the pod filling stage had the lowest recovery of 3% in trial 1 and 4% in trial 2 and they failed to significantly ($p<0.05$) recover from water stress as compared to non-stressed plants (Table 2).

Stomatal conductance: Stomatal conductance was significantly reduced ($p<0.05$) by water stress and was about 10 times lower in stressed treatments compared to non-stressed plants (Table 3). The plants which were stressed during the pod filling stage had the highest stomatal conductance reduction of 90% in trial 1 and 91% in trial 2 and those stressed during the vegetative stage had the smallest reduction of as compared to the non stressed control plants also in both trial 1 and 2. There was no significant difference in stomatal conductance among the stressed bambara groundnut plants (Table 3). Rewatering bambara groundnuts after water stress resulted in a significant ($p<0.05$) recovery of stomatal conductance in plants which were stressed during the vegetative and flowering stages only. There was however, a none significant increase or recovery of stomatal conductance in plants which were stressed during the pod filling stage as compared to non stressed plants (Table 3).

Chlorophyll content (destructive method): There was no significant reduction ($p<0.05$) of chlorophyll content of bambara groundnut plants after water stress was experienced during the vegetative, flowering and pod filling stages (Table 4). Significantly lower chlorophyll content was observed in both the control and stressed treatments at pod filling stage compared to plants water stressed at vegetative and flowering stages (Table 4).

Chlorophyll content (non-destructive method): There was no significant effect ($p<0.05$) of water stress on chlorophyll content using the Chlorophyll Content Index (CCI) measurements (Table 5). The CCI values for chlorophyll during the pod filling stage for both control and stressed plants were lower than those for plants water stressed at vegetative and flowering stages of growth and development. These results are consistent with those of destructive method of chlorophyll analysis (Table 5).

Proline content: Water stress significantly increased ($p<0.05$) proline concentration in bambara groundnut plants water stressed at the vegetative, flowering and pod filling stages compared to the non-stressed plants (Table 6). Depending on the stage of development, water stressed plants produced about four fold increases in the amount of proline compared to non-stressed plants (Table 6). Plants which were water stressed during the pod filling stage had the lowest increase in proline content (368%) and (285%) (Table 6) while plants which were water stressed during the vegetative stage had the highest increase in proline content (402%) and (301%) (Table 6). Rewatering significantly ($p<0.05$) reduced proline concentrations in all the previously water stressed plants at different stages of growth and development (Table 6).

Table 3: Stomatal conductance (mmol/m^{2}s) of bambara groundnuts leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatment	Stressed	Recovered	Stressed	Recovered
Control	300.8	306	345	347
Stressed at vegetative stage	29.8	304	30	345
Stressed at flowering stage	28.9	300	28.8	344
Stressed at pod filling stage	27.5	26.7	27.5	295
LSD	14.2	10.8	13.9	11.3

Table 4: Water stress effects on chlorophyll content (µg chl.g/Fwt) of bambara groundnuts leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatments	Stressed	Recovered	Stressed	Recovered
Control a	302	302	298	297
Stressed at vegetative stage	298	301	292	298
Control b	303	302	303	302
Stressed at flowering stage	301	299	300	299
Control c	256	248	248	236
Stressed at pod filling stage	254	250	246	236
LSD	5.34	4.62	4.39	4.06

control a, control b and control c are chlorophyll contents of the control plants at vegetative, flowering and pod filling stages, respectively

Table 5: Chlorophyll content index values of bambara groundnut leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatments	Stressed	Recovered	Stressed	Recovered
Control a	47	47	45	47
Stressed at vegetative stage	46	46.7	43	46.7
Control b	47	46.6	46	48
Stressed at flowering stage	46.8	46.4	44.8	47
Control c	38.2	36.7	34	30
Stressed at pod filling stage	37.2	36	33	29
LSD	3.5	3.3	3.7	3.5

Where: control a, control b and control c are chlorophyll contents of the control plants at vegetative, flowering and pod filling stages respectively.

Table 6: Proline content (µmole proline/g of fresh weight material) of bambara groundnuts leaves after water stress and after recovery from water stress in trial 1 and 2

	Trial 1		Trial 2	
Treatment	Stressed	Recovered	Stressed	Recovered
Control	1.05	1.41	1.5	1.6
Stressed at vegetative stage	5.45	1.42	6.01	1.65
Stressed at flowering stage	5.13	1.43	5.89	1.7
Stressed at pod filling stage	5.08	1.5	5.78	1.8
LSD	0.27	0.14	0.31	0.36

DISCUSSION

Water stress during the vegetative, flowering and pod filling stages of growth of bambara groundnuts decreased RWC (Table 1). Plants stressed during the pod filling period had the lowest RWC amongst the stressed treatments and did not fully recover after rewatering. This may be because the plants were on their last stage of growth hence they were aged compared to those at the vegetative and flowering growth stages and the plant's ability to recover is a function of plant age. As there were RWC reductions of between 9-12.3%, this showed that bambara groundnut plants can maintain relatively high RWC values despite the development of moisture stress (Collinson *et al.*, 1997). Similar results have been reported in rice (*Oryza Sativa*) (Lafitte, 2002), groundnuts (*Arachis hypogea*) (Pimratch *et al.* 2008) and maize (*Zea mays*) (Efeoglu *et al.* 2009). This is a very important trait which indicates drought resistance as species which exhibit restricted changes in RWC per unit reduction of water potential are often considered to be relatively drought resistant.

In all water stressed bambara groundnut plants, there was a decline in chlorophyll fluorescence (estimated from dark adapted F_v/F_m ratio) (Table 2). This decrease in F_v/F_m can be attributed to the down regulation of photosystem II activity and/or impairment of photochemical activity which indicates damage in the functionality of the photosynthetic apparatus. This is because water stress reduces photosynthesis directly because dehydrated protoplasm has a lowered photosynthetic capacity (Bjorkman and Demming, 1987). The F_v/F_m ratio completely recovered after rewatering in plants which were stressed during the vegetative and flowering stages but failed to recover during the pod filling stage. Similar results of plants lowering their F_v/F_m under water stress and then recovering after rewatering were also obtained in kidney beans (Miyashita *et al.*, 2005) and tobacco (Galle *et al.*, 2009). The increase in F_v/F_m usually results in increase in dry matter production because of return to normal photosynthetic rate. Failure of plants stressed during the pod filling stage to recover may indicate damage to the photosynthetic apparatus, and full photosynthetic recovery may have been limited by irreversible damage of photosystem II activity during stress (Andrade *et al.* 1999).

Water stress experienced during the vegetative, flowering and pod filling stages of growth of bambara groundnuts significantly reduced stomatal conductance (Table 3). This tendency of reduction of stomatal conductance under water stress is consistent with observations made by Collinson *et al.* (1997) and Cornellisen (2005) in bambara groundnuts. Decreased stomatal conductance results in lower net carbon dioxide assimilation rate, lower intercellular carbon dioxide and lower chloroplastic carbon dioxide tension. The carbon dioxide insufficiency will reduce photosynthetic efficiency and dry matter production and may have negative impact on plant growth and yield. Plants stressed during the vegetative stage and flowering stage completely recovered their stomatal conductance after rewatering. Recovery of stomatal conductance may result in increased carbon dioxide diffusion into the leaves to attain higher photosynthetic rates which favours higher biomass and higher crop yield. However, the plants which were stressed during the pod filling period did not fully regain stomatal conductance after rewatering. This may be because the plants were in their final stage of development: the older the plant the lower the regenerative capacity (Jones *et al.*, 1989).

Results from measuring chlorophyll showed that water stress did not significantly reduce ($p<0.05$) bambara groundnut leaf chlorophyll content (Table 4, 5). Cornellisen (2005) reported similar results on bambara groundnut. This showed that bambara groundnut plants maintain high amounts of chlorophyll content despite the development of moisture deficit stress and this trait can be considered to be a line of defence against drought which can result in drought resistance. Lower amounts of chlorophyll content were observed in the control plants during the grain filling stage as compared to other control plants for other stages of growth. This may be because chlorophyll tends to decline rapidly during leaf senescence (Merzlyak *et al.*, 1999) and at this stage some leaves were senescing.

In the current study the concentration of free proline accumulation in stressed bambara groundnut leaves were higher by up to 4 times than in non stressed plant leaves (Table 6). This increase in proline concentration under water stress has been observed in other crops like maize (Mohammadkhan and Heidari, 2008), Wheat (Johari-Pireivatlou *et al.*, 2010) and cowpeas (Patil, 2010). Proline is reported to result in drought tolerance and changes in proline content in several crops have been correlated with their capacity to tolerate and adapt to arid environments (Kishor *et al.*, 1995). Although proline's role in plant osmotolerance remains controversial it is however, thought to contribute to osmotic adjustment, detoxification of reactive oxygen species and protection of membrane integrity during water stress (Hare and Cress, 1997). Plants stressed during the vegetative stage accumulated more proline than those stressed during the flowering and pod filling stages. This was attributed to plant age, as plants were still young during the vegetative stage and therefore, more actively growing resulting in more proline accumulation than bambara plants at later stages of growth and development (Andreas, 1995). Rewatering the plants completely eliminated the effects of water stress on proline accumulation and proline levels dropped and equalled those of non-stressed plants. The decrease in proline levels due to rewatering may be a result of the rapid catabolism of proline during recovery to provide nitrogen for recovering tissues, reducing equivalents that support mitochondrial oxidative phosphorylation and the generation of ATP for recovery from stress and repair of stress-induced damage (Hare and Cress, 1997; Hare *at al.*, 1998).

CONCLUSION

The current study showed that water stress resulted in the reduction in RWC, stomatal conductance and chlorophyll fluorescence and in the increase in proline content. Leaf chlorophyll content was however, not reduced by water stress at all stages of growth and development. The nature and extend of damage and the ability of bambara groundnut to recover from water stress depends on the developmental stage at which the plant encounters water deficit. Bambara groundnut plants are most sensitive to water stress during the pod filling stage, followed by the flowering stage and less sensitive in the vegetative stage. It was recommended that, future research should be done on water stress under field conditions and also using various bambara groundnut landraces for comparison.

ACKNOWLEDGMENT

The authors would like to acknowledge the financial support of Moses Vurayai. The authors also thank Professor Sesay for his help and also the Botswana College of Agriculture and University of Botswana for allowing them to use their facilities and technical support.

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