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Water Stress Induces Cultivar Dependent Changes in Stomatal Complex, Yield and Osmotic Adjustments in *Glycine max* L.

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Abstract: *Glycine max* (L.) Merril. cultivars TGX 536-02D (cv. A) and TGX 923-2E (cv. B) were subjected to water stress for 7 days at the vegetative stage (T_2), flowering/fruiting stage (T_3), seed development stage (T_4) and the control (T_1) with water potential (ψ_w) values of about -1.5, -2.3, -2.7 and -0.3 MPa, respectively. Stressed T_3 and T_4 plants of both cultivars had very low tolerance (20-46%). Trichomes were present in the abaxial epidermis of T_3 and T_4 plants with higher occurrence in cv.B and stomata were sunken. Generally, abaxial and adaxial stomatal aperture of stressed cultivars decreased in the order $T_2>T_3>T_4$ stages and there was a corresponding increase in stomatal index in stressed plants. The low tolerance of T_3 and T_4 plants coupled with significant reduction in stomatal aperture with increase in stomatal index may have caused the drastic reduction in number of pods and seed yield of these stressed plants. While yield of T_2 plants of cv. B was also significantly reduced ($p<0.05$), 100 seed weight of cv. A was significantly enhanced. Both cultivars are capable of osmotic adjustments as shown by the accumulation of sugars and the reduction of lipids and starch in seeds of stressed plants with greater accumulation of sugar in cv. B. Hence the reproductive stage is critical in the growth of both cultivars but cv. B shows greater osmotic and structural adjustments than cv. A.

Key words: Soybean, water stress, stomatal complex, osmotic adjustments

INTRODUCTION

Plants have evolved various means of tolerating or avoiding adverse effects of unfavourable environmental factors. Such tolerance or resistance may change as a plant grows and develops. A plant may be susceptible to stress induced injury at one stage of development but at another stage, it may be completely resistant. The stage at which water stress imposes very drastic effects on the plant is referred to as the crucial/critical stage (Forbes and Watson, 1992). Generally, water stress at the vegetative stage of growth has little effect on growth and seed yield but deficit at reproductive stage, reduces yield (Ma *et al*., 2006). Severe water stress levels of about -3.3 MPa did not cause desiccation injuries in leaf tissues of several *sorghum* varieties prior to anthesis but at post anthesis stage these plants were badly affected (Sullivan, 1972). Water stress at the vegetative stage even caused a two-fold enhancement in growth and yield in *Spigelia anthelmia* (Umebese and Iloba, 2001).

The stomatal complex which consists of the stoma, together with its bordering guard cells and subsidiary cells controls leaf gas exchange and water loss. Plants respond to water deficits by closing their stomata. Thus, stomatal aperture reduces, stomatal resistance increases and stomatal conductance is decreased (Steudle, 2000). However, the drawback of the stomatal closure for plants is that their carbon gain is lowered and growth is impaired (Kramer and Boyer, 1995).

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Plants adapt to stresses by different mechanisms, including changes in morphological patterns as well as physiological and biochemical processes (Bohnert *et al*., 1995). These include metabolic adjustments that lead to accumulation of organic solutes such as sugars, proline, starches, lipids and proteins (Gill *et al*., 2003).

This study compares the stomatal response, tolerance, adaptive features, yield and osmotic adjustments of two cultivars of *Glycine max* subjected to water stress at various stages of growth.

MATERIALS AND METHODS

Plant Material and Growth Experiment

Seeds of two cultivars of *Glycine max* (TGX536-02D and TGX 923-2E designated cv.A and cv.B, respectively) were collected from the International Institute for Agriculture (IITA), Ibadan, Nigeria.

Plants were raised in a nursery bed for 2 week. Thereafter, plants of the same height were transplanted to plastic pots each filled with 4 kg garden soil. The two cultivars were planted together in each pot to ensure inter-mixing of the root system subjected to similar moisture availability. The first batch of 75 pots was watered daily (control-T_1). Three batches: T_2, T_3 and T_4 were subjected to 7 days water stress at the vegetative (23 days after planting, DAP), flowering/fruit set (65 DAP) and seed development (75 DAP) stages, respectively. The plants were arranged in a randomized complete block design with 5 replications. The pots were kept at the greenhouse of the Botanic garden of the University of Lagos with 12 h photoperiod and a relative humidity of 60% during the day and 68% at night. Temperature range was 29.3±0.3 to 32.3±0.3°C during the day and 21.6±0.5 to 23.4±0.5°C at night.

Measurement of Leaf Water Potential

The degree of stress in the plant was measured by the determination of the leaf water potential (ψ_w), using a pressure bomb chamber (Model 600L, Chas. W. Cook and Sons, England) by the method of Boyer (1967). A pressure as low as −0.3 MPa was regarded as low deficit (high ψ_w) while a pressure as high as −2.0 MPa was regarded as relatively high deficit (low $ø_w$). Measurements were taken in three replicates at the end of each period of water stress, from 4-6 pm daily.

Measurement of Stress Tolerance

After 7 days water stress at different stages of growth the stress was released by re-watering the plants. The plants were observed for recovery. Stress tolerance was measured by calculating the percentage number of plants that recovered from the treatment (Sullivan and Eastin, 1974).

Anatomical Investigations of Changes in the Stomatal Complex

Abaxial and adaxial epidermal strips were prepared by the techniques of Weyers and Travis (1981) and observed under a light microscope (x400, x1000 magnifications and 0.35, 0.26 µ resolutions, respectively). Features observed and studied include, stomatal frequency (observed in 5 replicates and expressed as the number of stomata per mm^2 leaf surface), trichomes, nature of stomata and epidermal cells. The Stomatal Index (SI) which provides a more stable estimate of the stomatal distribution, was calculated from the stomatal frequency (Sfreq) and expressed as: (Salisbury, 1928)

$$\text{Stomatal index} = \frac{\text{S Freq}}{\text{S Freq} + \text{Freq of subsidiary and epidermal cells}} \times 100$$

Measurement of Stomatal Aperture

Whole leaves were immersed in Formalin-Acetic Acid-Alcohol (FAA) for 48 h. Thereafter, they were dehydrated, fixed, impregnated and embedded (Sass, 1958). Transverse sections of 10 μm in thickness were cut through the midrib using a rotating microtome (422 Erma, Tokyo) and these were spread in water at 40°C before placing on microscope slides smeared with egg albumen and the slides were placed on an electric warmer at 55°C for 25 h. Thereafter, slides were placed in xylene for 5 min, hydrated in graded alcohol for 3 min each, rinsed rapidly and then stained with safranin, dehydrated in 90% and absolute alcohol for 1 min before dipping in xylene for 3 min. Sections were mounted in Canada balsam and observed under a light microscope at x400 and x1000 magnifications. Stomatal measurements were made with a micrometer screw guage inserted in the eye piece of the microscope.

Pod and Seed Yield

Final harvest was done at pod maturity, when pods were yellow and rattled when shaken (Gbikpi and Crookson, 1981). Number of pods, mean seed yield (dry weight/plant) and 100 seed weight produced by each plant, were recorded.

Chemical Composition of Harvested Seeds

Harvested seeds were dried at 80°C for 3 days. Powdered samples were analyzed for total ethanol soluble sugar, starch, lipid, protein and crude fibre contents. Ethanol soluble sugar was determined by the phenol sulphuric acid method of Dubois *et al.* (1956). Starch content was determined according to the Anthrone reagent method described by Southgate (1969), while the lipid content was determined as described by AOAC (1980). Crude protein content was determined according to Lowry *et al.* (1951), while the crude fibre content was estimated by the method of Diamond and Denman (1973).

Statistical Analyses

Analyses of Variance (ANOVA) was applied and tests of significance between treatments at $p<0.05$ and $p<0.01$ were performed using Duncan's multiple range test.

RESULTS

Water stress was moderate at the T_2 stage with a leaf ψ_w of -1.53 to -1.57 MPa, while it was severe at the reproductive stages (ψ_w: -2.23 to -2.67 MPa). Plants had a high rate of recovery (96.7 and 86.7% for cv.A and cv.B, respectively) when stress was given at the T_2 stage (Table 1). Corresponding to the drastic reduction in ψ_w at the T_3 and T_4 stages, there was a drastic reduction in the stress tolerance of both cultivars. However, at these stages, cv.B had higher percentage recovery (36.7-46.7%) than cv.A (20.0-36.7%).

Stress treatment induced the production of trichomes at the abaxial epidermis of T_3 and T_4 plants with higher occurrence in cv.B (Table 2). T_3 and T_4 plants had sunken stomata. Furthermore, epidermal cells shrank, resulting in increased number of small cells at these stages. In the course of water stress T_3 and T_4 plants of cv.B shed older leaves and produced new leaves with smaller leaf area.

Table 1: Leaf water potential (ψw) and stress tolerance (% No. of plants that recovered and continued growth) of soybean under water stress

Treatment stages	Leaf water potential (ψ_w) (MPa)		Recovery (%)
	Control	Stressed	Stressed
Cultivar A			
Vegetative (T_2)	-0.30±0.00	-1.57±0.03*	96.67±0.00
Flowering/fruiting (T_3)	-0.73±0.03	-2.33±0.03*	36.67±6.00
Seed development (T_4)	-1.00±0.06	-2.67±0.03*	20.00±5.30
Cultivar B			
Vegetative (T_2)	-0.27±0.03	-1.53±0.03*	86.70±4.73
Flowering/fruiting (T_3)	-0.57±0.03	-2.23±0.03*	46.70±3.78
Seed development (T_4)	-1.03±0.09	-2.47±0.07*	36.70±1.70

*Difference between stressed and control ψ_w is significant at $p<0.01$

Table 2: Observed adaptive features of soybean cv. A and cv. B plants subjected to water stress at different stages of growth

	Water stress treatment stage					
	cv. A			cv. B		
Adaptive features	T_2	T_3	T_4	T_2	T_3	T_4
Trichomes	Nil	*	*	Nil	**	**
State of stomata	Normal	Sunken	Sunken	Normal	Sunken	Sunken
Nature of epidermal cells	Normal	Small and numerous	Small and numerous	Normal	Small and numerous	Small and numerous
Leaf shedding	Nil	Nil	Nil	Nil	**	**

*Present, **Abundant

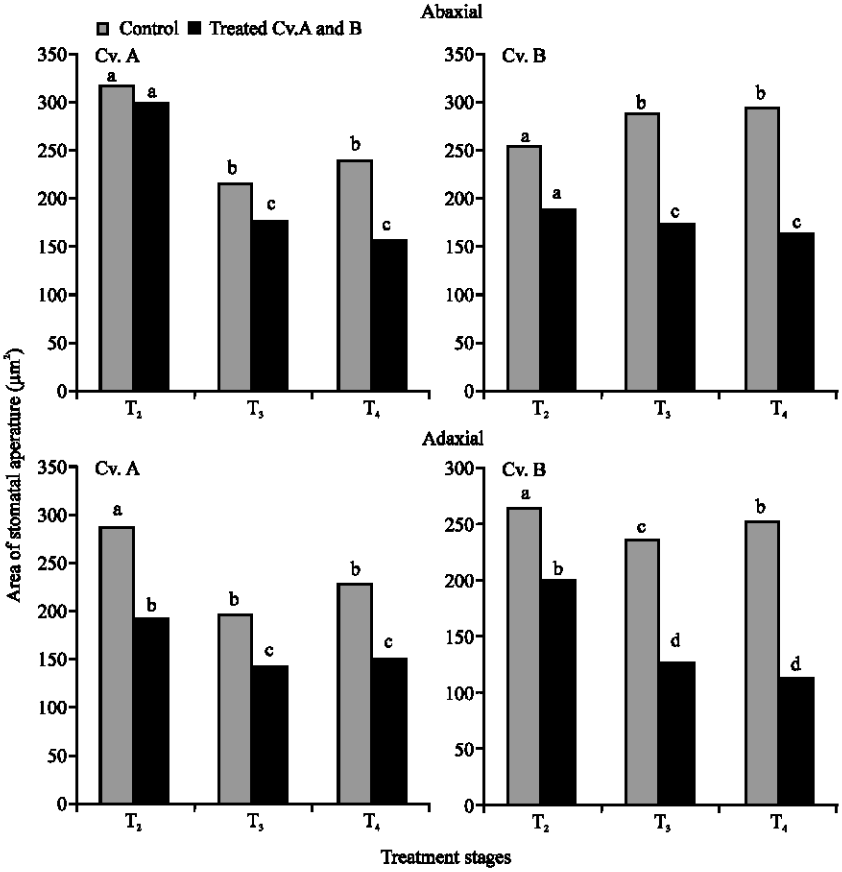

Fig. 1: Abaxial and adaxial area of stomatal aperture of soybean cv.A and cv.B subjected to water stress at different stages of growth. Vertical bars with similar letters are not significantly different ($p<0.05$)

Generally, both cultivars had higher area of stomatal aperture in the abaxial than the adaxial epidermis (Fig. 1). Almost all stress treatments caused significant reductions ($p<0.05$) in the area of stomatal aperture of both abaxial and adaxial epidermis. Reduction in aperture in both abaxial and adaxial epidermis was more marked in T_3 and T_4 plants than the T_2 plants. Stomatal index, (expressed from stomatal frequency) increased significantly ($p<0.05$) in the abaxial epidermis of all stressed plants and in the adaxial epidermis of T_4 and T_3 plants of cv.A and cv.B respectively (Fig. 2). The reduction in aperture size corresponded mostly with the increase in stomatal index.

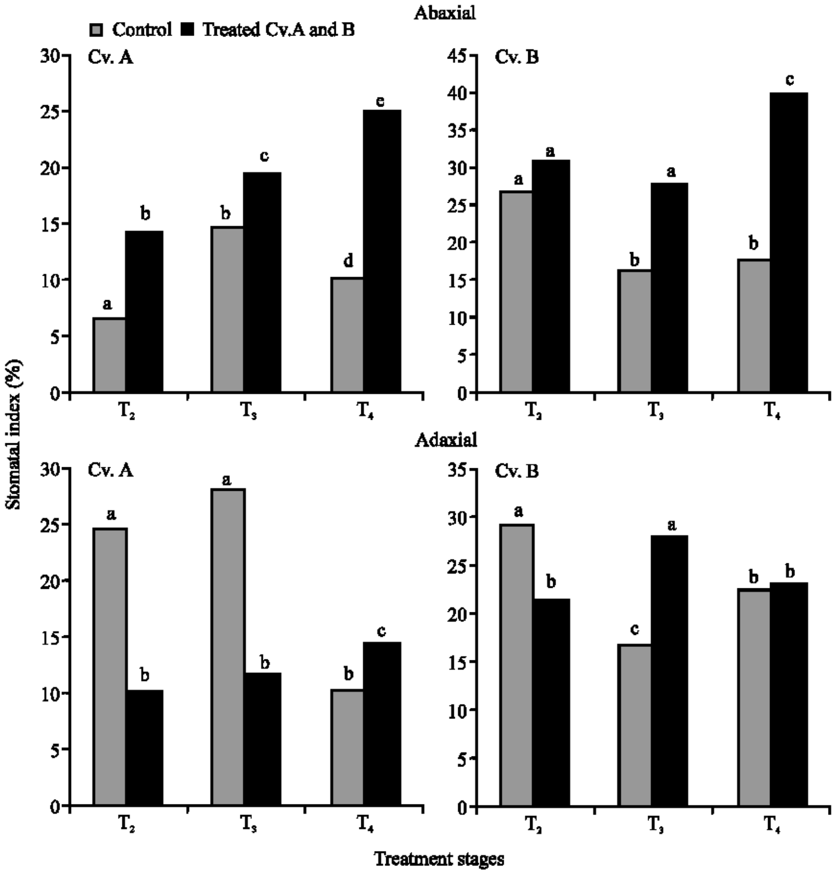

Fig. 2: Abaxial and adaxial stomatal index of soybean cv.A and cv.B subjected to water stress at different stages of growth. Vertical bars with similar letters are not significantly different ($p<0.05$)

Table 3: Yield of soybean cultivars subjected to water stress at different growth stages

Growth parameters (control)	Water stress treatment stages			
	T_1	T_2	T_3	T_4
Cultivar A				
No. of pods	15.17a	17.70a	4.10b	2.37b
Seed yield (g plant^{-1})	2.23a	2.47a	0.64b	0.68c
Seed weight (g)	7.90a	12.66b	7.80a	6.94a
Cultivar B				
No. of pods	21.17a	12.13b	6.37c	4.45c
Seed yield (g plant^{-1})	3.18a	1.82b	0.95c	0.33c
Seed weight (g)	7.88a	7.84a	7.90a	8.04a

Treatment means with similar letter(s) on the horizontal axis are not significantly different at $p<0.05$

Water stress caused significant reductions ($p<0.05$) in pod number, seed yield and 100 seed weight of both cultivars at the T_3 and T_4 stages (Table 3). Cv.B was also affected at the T_2 stage but cv.A showed significant enhancement in 100 seed weight. The marked reduction in aperture size of T_3 and T_4 plants of both cultivars and increased stomatal index corresponded with the drastic reduction in yield.

Table 4: Chemical composition of harvested seeds of soybean plants subjected to water stress at different growth stages

Chemical composition (%) (control)	Water stress treatment stages			
	T_1	T_2	T_3	T_4
Cultivar A				
Sugars	15.40a	17.90b	19.00b	18.00b
Starch	16.50a	15.40b	14.00c	13.50c
Crude protein	36.07a	36.10a	40.67b	43.30c
Lipids	20.03a	17.83b	15.00c	15.00c
Crude fibre	4.40a	4.50a	3.50b	3.33b
Cultivar B				
Sugars	16.00a	16.07a	21.07b	21.40b
Starch	15.20a	17.60b	18.23b	19.50^b
Crude protein	41.00a	37.00b	36.00bc	35.07c
Lipids	21.00a	17.00b	15.00b	15.00b
Crude fibre	4.33a	4.20a	4.00a	3.80a

Treatment means with similar letter(s) on the horizontal axis are not significantly different at $p<0.05$

Stress treatment induced various changes in the chemical composition of the seeds (Table 4). Sugar content was significantly increased in stressed plants ($p<0.05$). However, starch and protein contents followed different patterns in both cultivars. In cv.A, starch was significantly reduced from early vegetative to seed development (T_2-T_4 stages) and protein was significantly increased while in cv.B, there was a significant increase in starch content and a significant decrease in protein content from T_2-T_4. stages. Lipid content and crude fibre were reduced in both cultivars from T_2-T_4 and all but the crude fibre of cv.B plants were significantly different ($p<0.05$) from the control (T_1).

DISCUSSION

Severe water deficit affects many of the physiological processes associated with growth and death of plants may result (Belaygue, 1996). A leaf water potential of -2.23 to -2.65 MPa at the reproductive stage of soybean, cultivars TGX 536-02D: cv.A and TGX 923: cv.B, is severe since it caused very low recovery rate in both cultivars. Death of plants may result when there is uncontrolled destruction of the photosynthetic apparatus due to the accumulation of Reactive Oxygen Species (ROS). These are toxic forms of oxygen formed in plants under environmental stress conditions like drought, which limit CO_2 fixation (Krause, 1994; Hopkins and Hüner, 2004). The effects of water deficit vary with the degree, duration of water deficit, variety and growth stage of the plant (Belaygue, 1996; Adejare and Umebese, 1998). Recovery rate of cv.A was higher than cv.B when stress was given at the vegetative stage but lower with stress at the reproductive stage.

Under water stress, the anatomy of root tissue changes because stress induces the development of apoplastic barriers to water and ion flow (Steudle, 2000). Similarly, the leaf tissues of soybean showed remarkable changes in anatomy by the presence of trichomes in T_3 and T_4 plants which hinders the outward loss of water by transpiration and contributes to the reduction of incident radiation on the plant while sunken stomata give a low stomatal conductance. The shedding of older leaves and production of new leaves with smaller leaf area in cv.B is referred to as leaf area adjustment; another mechanism for reducing transpiration during limited water availability (Hopkins and Hüner, 2004). Similar decreases in leaf area expansion during water deficit were observed in cassava and this arrested growth assists plants in attaining high productivity in environments with cycles of intermittent drought followed by re-watering (Alves and Setter, 2004a, b). Cv.B developed greater changes in anatomy and is thus, more adapted to water stress than cv.A at the reproductive stages (T_3 and T_4).

A method of altering the water status of the plant is to decrease stomatal conductance (Steudle, 2000). When water status in a leaf falls below a threshold value, stomata respond by closing which buffers the effects of water stress (Bradford and Hsiao, 1982). Water stress reduced the stomatal aperture of the abaxial and adaxial epidermis of both soybean cultivars with greater reduction in T_3 and T_4 plants of cv.B than those of cv.A. There was also a general increase in stomatal index in the abaxial and adaxial epidermis. The reduction of stomatal aperture reduces leaf transpiration and prevents the development of excessive water deficits in the tissues (Kramer and Boyer, 1995). Stomatal closure during water stress is also associated with the maintenance of xylem integrity controlling the risk of embolism (Jones and Sutherland, 1991). It has been shown to maintain the water pressure in the leaf rachis xylem preventing extensive development of cavitation (Cochard *et al*., 2002). Cavitation renders xylem conduits non-conductive (Pickard, 1981). Cavitation avoidance is a physiological function associated with stomatal regulation during water stress (Cochard *et al*., 2002).

Stomatal closure may prevent excessive water loss in plants but it reduces carbon gain and at certain stages of plant development, this closure reduces growth and yield. When carbon fixation is limited there is a reduction in $NADP^+$ regeneration by the Calvin cycle. This leads to severe reduction in photosynthetic electron transport chain, forming ROS in the chloroplasts, which is destructive (Krause, 1994). In many plants growth and yield are not affected when stress is given at the vegetative stage (Ma *et al*., 2006) and some plants even show enhancement in growth and yield (Umebese and Iloba, 2001). Cv.A showed slight enhancement in yield when it was subjected to stress at the vegetative stage. Water deficit reduces biomass production in these soybean cultivars and the effect is more pronounced when water deficit is given at the reproductive stage (Adejare and Umebese, 1998). This result from the observed significant reduction in net assimilation rate and relative growth rate of the two cultivars stressed at the reproductive stage. Severe reduction in yield can occur when plants are subjected to water stress at the reproductive stage. A single drought event at the juvenile stage had little effect on seed yield while stress at anthesis or seed-fill stage caused reduction in seed yield (Ma *et al*., 2006). This is evident in the significant reduction in pod number in both cultivars stressed at the reproductive stage.

The chemical composition of seeds of stressed plants showed that the seeds of both cultivars have large capacities for osmotic adjustments when subjected to water stress. This is indicated by their ability to accumulate solutes under stress. Seeds of cv.B accumulated more sugars than cv.A at the reproductive stage. The increased accumulation of sugars is due to the increase in hydrolytic enzymes such as ribonuclease and amylase under water stress (Todd, 1972). Accumulation of sugars, a characteristic of mature seeds appears to be very important for the development of desiccation tolerance (Hoekstra *et al*., 2001). Enzymes produced under stress conditions also convert the oils and crude fibres of oil seeds to carbohydrates, which can be used for metabolic activities (Cholan, 1978). This was evident in the decrease of lipids and crude fibres of seeds of both cultivars under stress at all stages of growth.

The reproductive stage (T_3 and T_4) of soybean cvs. A and B is the critical stage in which water stress affects yield drastically. Application of water at this stage will greatly benefit the plant. Cv. A tolerates water stress better than cv. B at the vegetative stage of growth but at the reproductive stage cv. B is more resistant to water stress. The adaptive features of cv. B include the prompt response of stomata to loss of turgor, shedding of old leaves and the production of small leaves with numerous trichomes. Cv.B is also more osmotically adjusted to water stress than cv.A in having higher sugar and starch contents under stress.

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