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Impact of salicylic acid on antioxidants, biomass and osmotic adjustments in *Vigna unguiculata* L. walp. during water deficit stress

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Ameliorative impact of salicylic acid (SA) on *Vigna unguiculata* L. (cowpea) cultivar IT93k-452-1 during water deficit stress was investigated. Plants were subjected to water deficit stress (WD) for 7 days at either the vegetative stage (DVS) or the reproductive stage (DRS) and the leaf water potentials (ψ_w) of -1.9 MPa and -2.01 MPa were obtained, respectively. Stress caused reductions in almost all parameters studied. 3 and 5 mM SA foliar treatment caused increases of 27% in leaf ψ_w, 94% in chlorophyll content, 75% in plant biomass, 7% in nitrate reductase activity and 38% in proline content in DVS plants while the impact was much lower in DRS plants. Stress stimulated almost 3-fold increase in antioxidant vitamin B_{12} in DVS plants and 40% increase in vitamin C in DRS plants and SA (3 mM) further increased the latter by 14%. Overall, SA had a protective influence on the water potential and growth of stressed plants accompanied by an increase in production of osmolyte proline and antioxidant vitamin C.

Key words: Antioxidants, growth, salicylic acid, water stress.

INTRODUCTION

Water deficit stress elicits many different physiological responses in plants. It causes reduction in leaf water potential, stomatal conductance, nitrate reduction and inhibits leaf enlargement while osmolytes such as total soluble sugars and proline are increased (Jafar et al., 2004; Adejare and Umebese, 2007). Prolonged water stress affects virtually all metabolic processes and often results in severe reductions in plant productivity and death of plants. The effect of water deficit varies with the variety and the growth stage of the plants.

All abiotic stresses such as water deficit and salt stress cause increased production of reactive oxygen species leading to oxidative damage. Almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage. Majority of the antioxidant activity is from flavonoids, isoflavone, flavones, anthocyanin, catechin, isocatechine, vitamins B, C, E and β-carotene (Bronzetti et al. 2001; Amic et al., 2003). Others are enzymes such as, catalase, peroxidase and superoxide dismutase (Jaleel et al., 2009). Salicylic acid acts as an endogenous signal molecule responsible for the induction of antioxidant responses that protect plants from damage (Senaratna et al., 2000; Hayat et al., 2007, 2010).

Salicylic acid can stimulate flowering, increase flower life, inhibit seed germination, promote ethylene synthesis (Singh and Usha, 2003), enhance photosynthetic rate and growth rate (Khan et al., 2003). Salicylic acid has

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Abbreviations: SA, Salicylic acid; **NRA,** nitrate reductase activity; **DVS,** vegetative stages; **DRS,** reproductive stages; **HPLC,** high performance liquid chromatography; **ABA,** abscisic acid; **P5CS,** D'-pyroline-5-carboxylate synthetase; **ASC,** ascorbate; **SOD,** superoxide dismutase; **APX,** ascorbate peroxidase; **GR,** glutathione reductase; **WD,** water deficit.

been shown to produce a protective effect in plants under the action of stress factors of different abiotic nature, such as, heat, chilling, osmotic and salt stress (Borsani et al., 2001; Janda et al*.,* 1999; Singh and Usha, 2003; Wang and Li, 2006). Salicylic acid (SA) is an endogenous growth regulator of phenolic nature, which participates in the regulation of physiological processes in plants such as growth, photosynthesis, nitrate metabolism, ethylene production, heat production and flowering (Hayat et al., 2010) and also provides protection against biotic and abiotic stresses such as salinity (Kaya et al*.,* 2002). An efficient N assimilation is said to be favoured by a high rate of CO_2 assimilation (Larsson et al., 1989). SA induced conservation of water in stressed plants also results in the protection of nitrate reductase activity (NRA) in SA treated stressed plants.

The present study is aimed at evaluating the protective effect of salicylic acid on the development of *Vigna unguiculata* L. (cowpea) seedlings during water deficit stress, thereby considering the leaf water potentials, plant biomass, leaf chlorophyll content, nitrate reductase activity, proline concentrations, vitamine B12 and ascorbate contents at both vegetative and reproductive stages.

MATERIALS AND METHODS

Plant material and planting procedure

Seeds of *V. unguiculata* L. cultivar IT93k-452-1 were collected from the International Institute of Tropical Agriculture (IITA) Ibadan, Oyo State. The experiment was set up at the botanic garden of the University of Lagos, Akoka Lagos. Seeds of cowpea were planted 2-3 cm deep in 48 pots of 30.4 cm diameter (4 treatments) each filled with 2 kg garden topsoil mixed with 2 g NPK fertilizer. After three weeks, seedlings were thinned to 4 plants per pot. Plants were subjected to water deficit for 7 days at vegetative (21^{st}-27^{th} days after sowing) and reproductive (45^{th} - 52^{nd} days after sowing) stages (DVS and DRS plants). At the beginning of the 7 day stress period a batch of 12 plants was treated with 3 mM SA as foliar spray, the other batch of 12 plants was treated with 5 mM SA, the third batch which consist of yet another 12 plants was subjected to water deficit only while the fourth batch of 12 plants served as the control (no stress treatment). All plants were watered daily apart from the period of stress treatment. The plants were arranged in a randomized complete block design with 3 replicates. The pots were kept at the green house with 12 h photoperiod and a relative humidity of 60% during the day and 68% at night. Plants were harvested on the last day of stress treatment at each growth stage, dried in an oven at 80°C for 3 days and the biomass taken.

Determination of leaf water potential

After subjecting plants to 7 days water deficit, the degree of stress in the plants was measured using the tissue weight- change method outlined by Hopkins and Hüner (2004). 0.5 g leaf from the different treatments was placed in each of a graded series of 0.2 – 0.8 molal concentration of sucrose in different test tubes. The leaves were removed after 1 h equilibration and dried between filter papers and reweighed. Osmotic potential (ψ_s) of each sucrose solution was calculated using the van't Hoff equation:

$$\psi_s = - C\gamma RT$$

Where, C is the molal concentration, γ is the activity coefficient (a value of 1 for neutral solutes such as sucrose in dilute solution), R is the gas constant (.00831 kg MPa mol^{-1} $^oK^{-1}$), T is the absolute temperature ($^oK = {}^oC + 273$).

The change in weight was calculated as a percentage of the original weight and plotted against ψ_s of sucrose solutions. The leaf water potential (ψ_w) is estimated as equivalent to the ψ_s of the solution in which there is no change in weight (the intercept on the abscissa).

Determination of nitrate reductase activity

Nitrate reductase activity in the leaves was assayed as described by McCashin (2000). 5 ml of the incubation medium (comprising 100 ml 0.1 M phosphate buffer, pH 7.5, 1.5825 g KNO_3 and 1 ml 4% propan-1-ol), was used to incubate 0.5 g sample of leaves in boiling tubes in 3 replicates for a period of 1 h at a room temperature of 30 – 35°C. Then the reaction was stopped by adding 1 ml 1% sulphanilic acid, followed by 1 ml of 0.02 % naphthylethylenediamine dichloride. It was then carefully shaken and left for 20 min for colour development. The blank was prepared by adding 1 ml substrate assay solution, 1 ml 1% sulphanilic acid and 1 ml napthylethylenediamine dichloride together in a boiling tube. After full colour development, the absorbance was measured at 540 nm optical density of the test solution, was determined using a Corning spectrophotometer model 258. A standard curve was prepared using 0-10 µM nitrite ml^{-1}. NRA is proportional to the concentration of nitrite in the reaction medium.

Determination of proline content

Proline content of leaves was determined as described by Bates et al. (1973). 0.5 g of dried ground leaves was homogenized in 10 ml 3% aqueous sulfosalicylic acid and the homogenate filtered through filter paper. 2 ml acid ninhydrin and 2 ml glacial acetic acid were added to 2 ml of the filtrate in a digestion tube and placed in a boiling water bath for 90 min. The reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and agitated vigorously for 30 s. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance read at 520 nm using toluene as the blank. The concentration was determined from a standard curve prepared from 1-10 mg l^{-1} of proline and calculated on a dry weight basis.

Estimation of chlorophyll content

The amount of chlorophyll a and b (mg g^{-1} fresh weight) were calculated using the formula of Maclachlam and Zalik (1963).

Determination of the antioxidant vitamins content

Antioxidant vitamins cyanocobalamin (vitamin B_{12}) and ascorbic acid (vitamin C) contents were determined as described by Van (2006). Ten grams of the fresh leaves from different plants were macerated using a ceramic mortar and pestle. The extract was mixed thoroughly using an electric mixer and centrifuged at 1000 g at 5°C. 1 ml aliquot was made up to 10 ml with distilled water for cyanocobalamin (vitamin B_{12}) content while 1 ml of the extract was made up to 100 ml with distilled water for ascorbic acid (vitamin C). After filtration each was injected into the high performance liquid chromatography (HPLC) at a wavelength of 204 nm. The chromatographic conditions consist of the following: Column-zorbax SB

Table 1. Leaf water potential of cowpea subjected to water deficit (WD) and salicylic acid (SA) treatment at the vegetative and reproductive stages of growth.

Treatment	Water potential (MPa) vegetative stage	Reproductive stage
Control	-0.92[a]	-1.35[a]
WD	-1.97[b]	-2.01[b]
WD + 3 mM SA	-1.88[b]	-1.90[b]
WD + 5 mM SA	-1.44[d]	-1.70[c]

Superscripts with same letters at each growth stage are not significantly different at $p<0.05$ using the Duncan's multiple range tests.

C18 (250 x 4.6 mm, 5 nm), thermostatically regulated at 20°C; the mobile phase was a gradient composition of activities: acetonitrite (A) and 25mM KH_2PO_4 buffer (B) pumped at a flow rate of 0.5 ml min^{-1}. When the gradient elution of A is 8 and 16% and B is 92 and 84% at 0 min and 5 min, respectively, 20 µL of reference standards were injected to determine their respective retention time, and graded concentration of mixed reference standards (vitamin C and B_{12}) were injected. The peak areas (MAU) were acquired and integrated by enhanced integrator for plotting of calibration curve and concentrations given as µg $ml^{-1}$and calculated as mg g^{-1} fresh weight.

All data were analyzed using Statistical Analysis System (SAS) software. When ANOVA showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $p<0.05$.

RESULTS AND DISCUSSION

Leaf water potential of *V. unguiculata* L. cultivar IT93k-452-1 was significantly reduced when plants were subjected to 7 days water deficit stress at the vegetative and reproductive stages (DVS and DRS respectively) of growth, with the reproductive stages (DRS) showing a greater decrease in water potential (Table 1). This stage dependent response to water deficit was shown in an earlier study on soybean leading to a greater increase in stomatal resistance and reduced stomatal capacity at the reproductive stage (Adejare and Umebese, 2007). This resulted in much reduction in growth and yield of plants (Figure 3). Foliar application of salicylic acid (SA) was effective in raising the water potential of stressed plants by 27% though the values were still lower than the control.

Water deficit did not affect the total chlorophyll content of cowpea at both stages of growth (Figure 1). This was also observed by Vasileva and Llieva (2011), when lucerne was subjected to water deficiency stress. However, the application of 5 mM SA to DVS plants significantly ($p<0.05$) enhanced the chlorophyll content over the control. This corroborates the report by Rajasekaran and Blake (1999) that SA has a positive effect on photosynthesis. Nitrate reductase activity (NRA) was significantly reduced by water deficit (Figure 2). 5 mM SA raised the NRA level of vegetative stages (DVS) of plants to that of the control while 3 mM SA increased the NRA of DRS plants though it was still lower than that of the control. There is a strong positive correlation between NRA and relative water content (Umebese and Okeola, 1998). SA increased NRA of stressed plants and this corresponded with the increase in the leaf water potential. This agrees with an earlier report that SA increases the conservation of water in tomato and amaranth during water stress and enhances NRA (Umebese et al., 2009).

Plant biomass was significantly reduced by water deficit, corresponding to the low water potential induced by the 7 days water deficit (Figure 3). Energy requiring processes such as NRA and growth are reduced by water stress since CO_2 fixation is limited, reducing the $NADP^+$ regeneration by the Calvin cycle leading to an over-reduced photosynthetic electron transport chain (Krause, 1994). Though treatment with SA significantly increased ($p<0.05$) plant biomass, values were still lower than the control. In an earlier study, plant biomass was enhanced by SA when tomato and amaranth were subjected to water stress (Umebese et al., 2009). Concentration of SA as low as 0.05 mM increased the level of cell division within the apical meristem of seedling roots which caused an increase in plant growth (Bezrukova et al., 2001).

Proline was significantly decreased in the DVS plants while it was significantly increased in DRS plants. Both concentrations of SA enhanced proline production during water stress at both stages of growth (Figure 4). The accumulation of the osmolyte proline, in plants is a mechanism by which plants resist water stress and develop anti-stress reaction. Water deficit induces accumulation of proline in seedlings and also results in a decline in metabolic activity of plants cells, and this is inevitably reflected in inhibition of their growth (Bezrukova et al., 2001; Umebese et al., 2009). SA induced abscisic acid (ABA)-mediated protective reactions in plants involves increased production of proline by the expression of the gene encoding D'-pyroline-5-carboxylate synthetase (P5CS) in proline biosynthesis. When plants constantly produce high levels of proline, they are capable of surviving drought (Delauney and Verma, 1993). Thus, the increased production of proline by SA in plants subjected to water deficit at the two growth stages may have

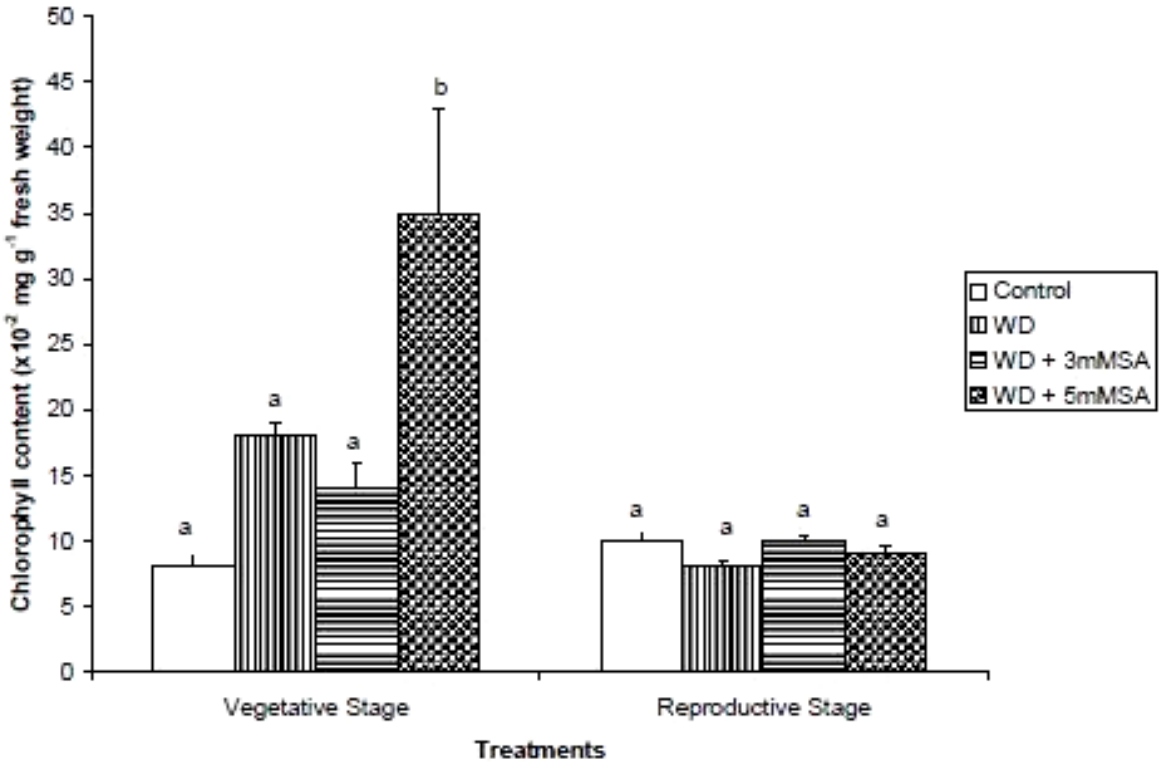

Figure 1. Chlorophyll content of cowpea subjected to water deficit (WD) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

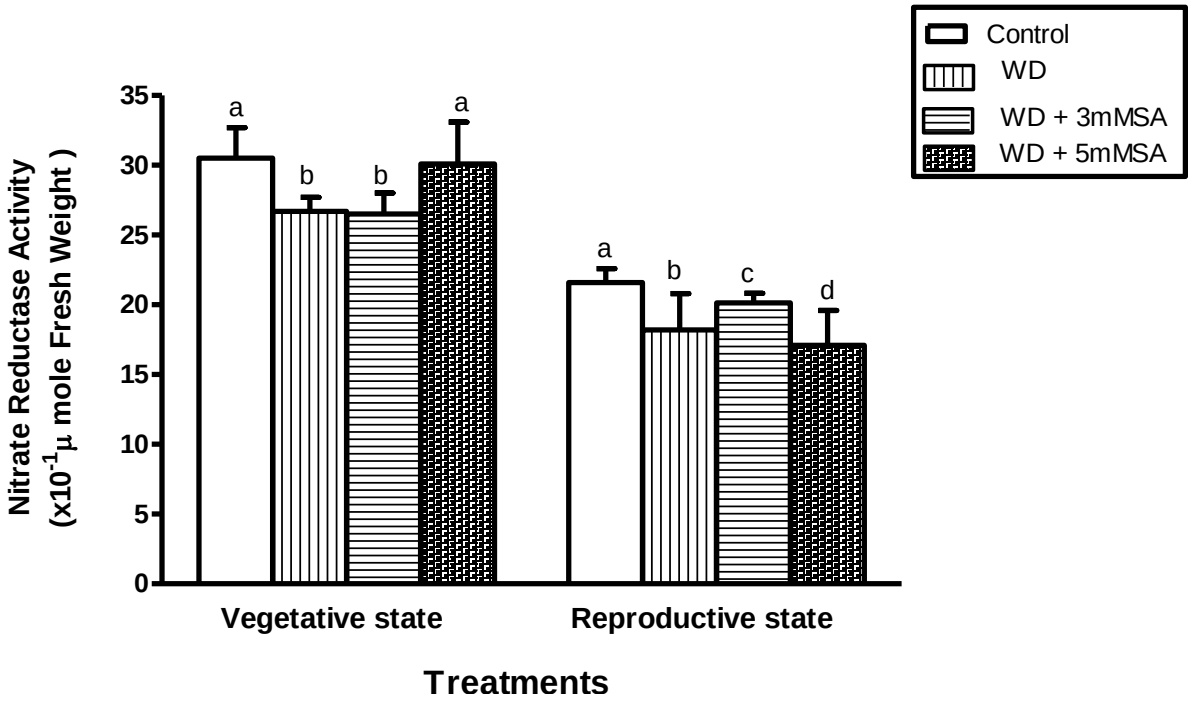

Figure 2. Nitrate reductase activity of cowpea subjected to water deficit (D) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

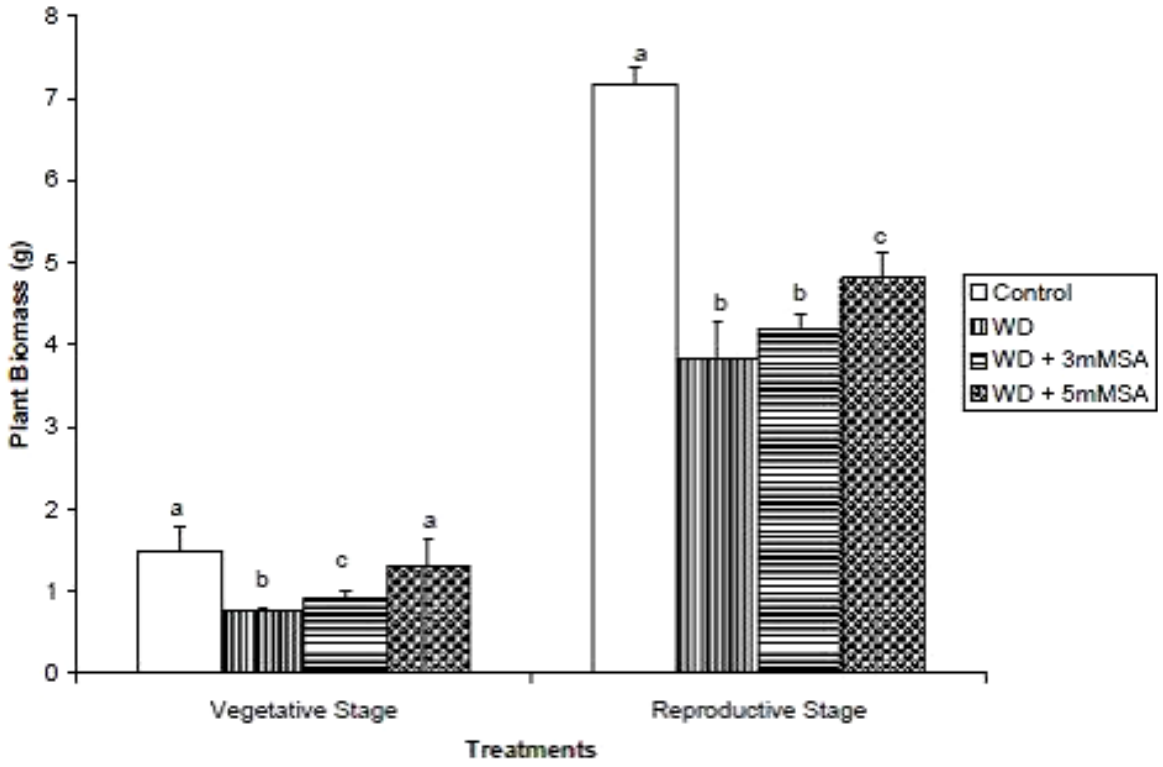

Figure 3. Plant biomass of cowpea subjected to water deficit (D) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

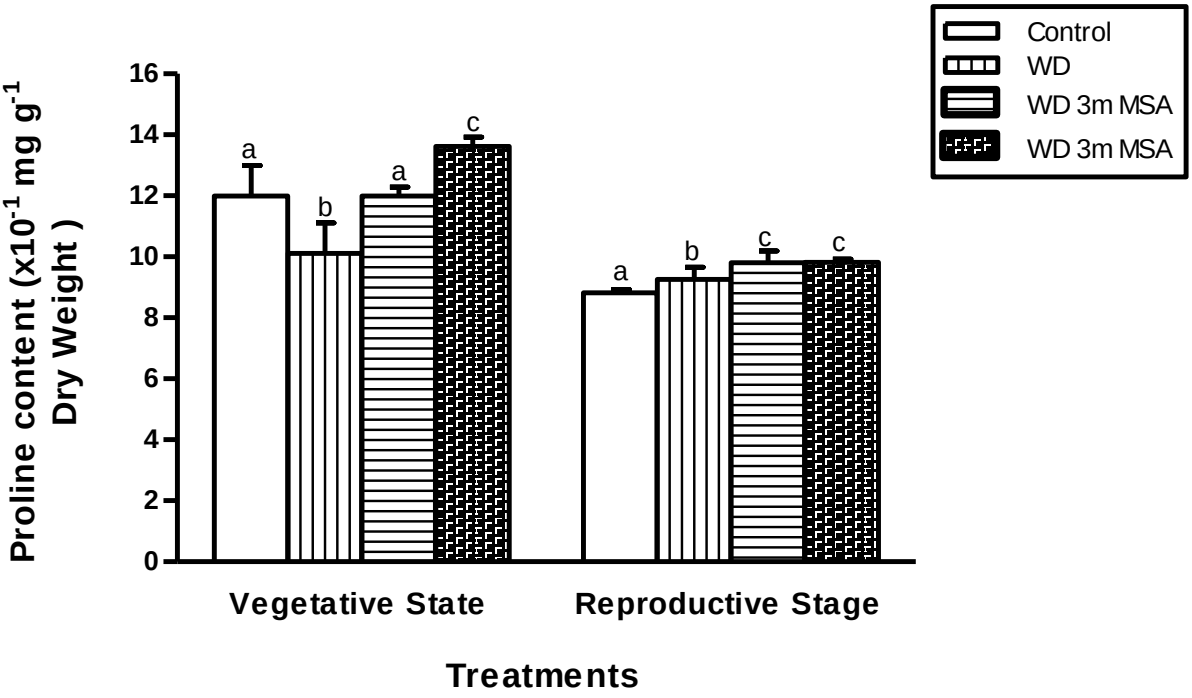

Figure 4. Proline content of cowpea subjected to water deficit (D) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

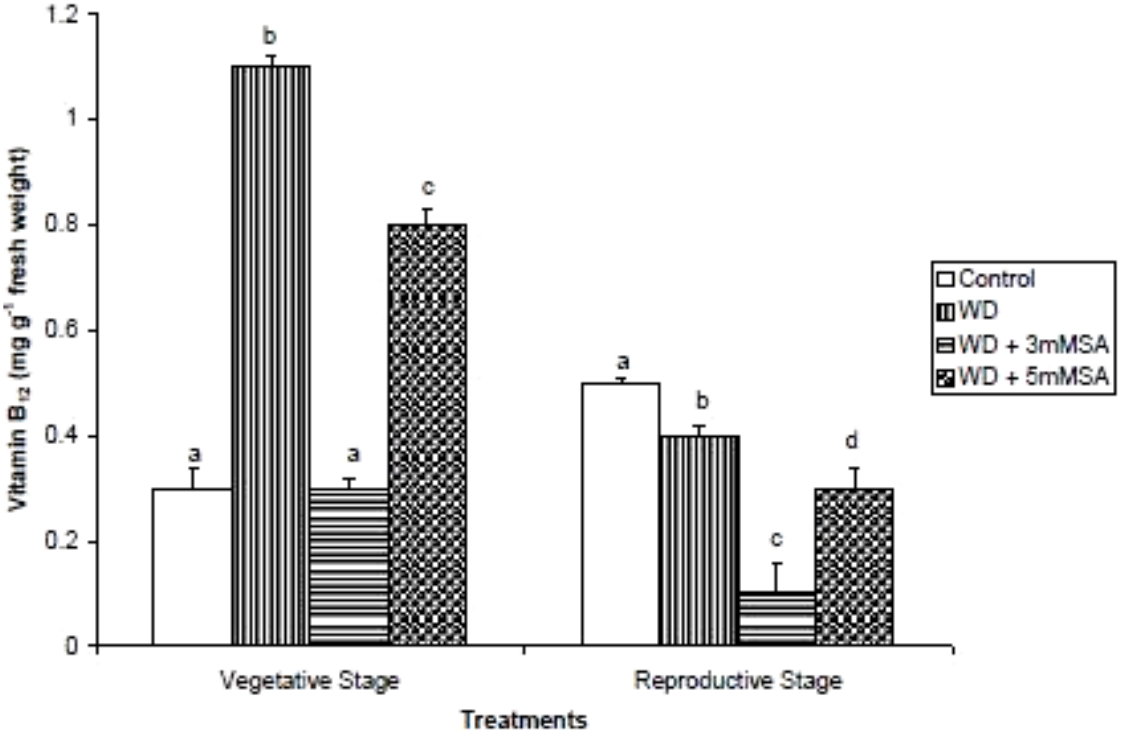

Figure 5. Antioxidant vitamin B_{12} of cowpea subjected to water deficit (D) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

resulted in the observed enhancement of plant biomass.

The antioxidant vitamin B_{12} was significantly enhanced ($p<0.05$) by water stress in DVS plants but significantly reduced in DRS plants (Figure 5). Vitamin C was also stimulated by water deficit but this was in DRS plants (Figure 6). Thus, the stage at which plants were subjected to water deficit affected the type of antioxidant induced; vitamin B_{12} at the vegetative stage and vitamin C at the reproductive stage. An increase in the level of antioxidants is believed to reflect an increase in the formation of reactive oxygen species (Foyer et al., 1997). All abiotic stresses such as water deficit stimulate the production of significant quantities of reactive oxygen species in sub-cellular compartments or organelles which are highly destructive to their proteins, membrane lipids and nucleic acids (Matsumura et al., 2002). The control of these oxyradicals is achieved by antioxidant systems within the cells. Experiments show that vitamin B is a potent quencher of singlet oxygen with quenching rates comparable to or greater than those of vitamin C and E, two of the most efficient biological antioxidants known (Bilski et al., 2000). Treatment with 3 mM SA resulted in significant reduction in vitamin B content at both stages of growth of stressed plants while 5 mM SA caused a reduction only at the reproductive stage. Thus, SA had a retarding effect on vitamin B_{12} in stressed plants. Both concentrations of SA caused significant reduction in vitamin C content in DVS plants but in DRS plants, 3 mM SA stimulated further increase in this vitamin. This enhancement of vitamin C by 3 mM SA corresponds with the increase in NRA observed in stressed plants subjected to this dose of SA. Vitamin C supplement has been shown to increase NRA in white headed cabbage (Heród-Leszczyńska and Miedzobrodzka, 1993). This shows the ameliorative action of SA when cowpea is subjected to water stress at the reproductive stage of development. The sensitivity of vitamin C to reversible oxidation suggests its role in cellular oxidation-reduction reactions (Verma and Verma, 2007).

Plants must possess efficient antioxidant system to enable them to endure oxidative damage under unfavorable conditions such as high/low temperatures, water deficit and salinity (Sreenivasulu et al., 2000). Various associations between water stress and endogenous levels of water-soluble antioxidants have been described (Zaman and Das, 1991), in which ascorbate (ASC) is also required for the re-conversion of SA to SA yielding monodehydroascorbate, since ASC is highly reactive against phenoxyl radicals generated by peroxidases during oxidative stress (Kawano and Muto, 2000). It can be suggested that the exogenous application of SA is accompanied by increased rates of ROS, which leads to an increased load on the ascorbate-glutathione cycle leading to a depletion of tASC. The results are supported

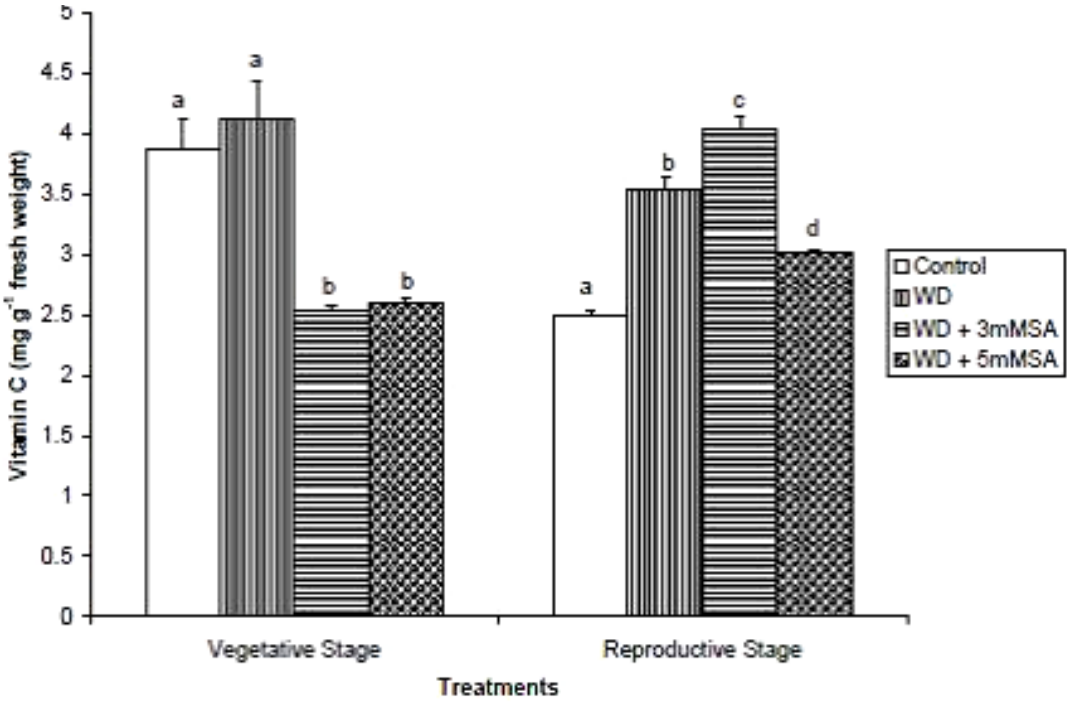

Figure 6. Antioxidant Vitamin C of cowpea subjected to water deficit (D) and salicylic acid (SA) treatment at vegetative and reproductive stages of growth (Bars with same letters at each growth stage are not significantly different at P<0.05 using the Duncan's multiple range test).

by the observation of Shi and Zhu (2008), where SA-treatment enhanced the activities of dehydroascorbate reductase. Mwanamwenge et al. (1999) explained that when ROS increases, chain reactions start, in which superoxide dismutase (SOD) catalyzes the dismutation of O^2 radicals to molecular O_2 and H_2O_2. The H_2O_2 is then detoxified in the ascorbate-glutathione cycle which involves the oxidation and re-reduction of ascorbate and glutathione through the ascorbate peroxidase (APX) and glutathione reductase (GR) action (Ouchi, et al., 1990; Pasternak et al., 2005).

In conclusion, cowpea is more sensitive to water deficit at the reproductive stage than the vegetative stage resulting in reductions in plant biomass and nitrate reductase activity. Treatment of stressed plants with salicylic acid caused significant improvement of those parameters and enhanced proline accumulation, an anti-stress reaction. Plants subjected to water deficit at different stages of growth produced different types of antioxidants: vitamin B_{12} in plants stressed at the vegetative stage and vitamin C in those stressed at the reproductive stage. Salicylic acid enhanced vitamin C content in the latter which may have caused the enhancement of nitrate reductase activity.

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