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Influence of Calcium and Magnesium on Growth Rate and Antioxidant Responses of *Glycine Max* Seedlings Grown under Water Deficit and Waterlogged Conditions

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ABSTRACT

Standard procedures were used to determine the influence of calcium and magnesium on growth rate and antioxidant responses of *Glycine max* seedlings grown under water deficit and waterlogged conditions. The results of the 3 – week old seedlings subjected to 14 days growth under water stress conditions revealed significant decrease ($P>0.05$) in growth indices (number of leaf, height difference and relative water content) in water deficit and waterlogged seedlings. However, malonaldehyde and antioxidants (superoxide dismutase, catalase, ascorbic acid and vitamin E) significantly increased ($P>0.05$) in the water stressed samples mostly water deficit seedlings when compared to control group grown under normal condition. In the present study, water supply below and above plant consumptive level for a minimum period of 14 days resulted in generation of reactive oxygen species, peroxidation of lipid and release of defensive mechanism such as endogenous enzymatic and non – enzymatic antioxidants. The macro – nutrients (magnesium and calcium) at the concentration of 40 mg/L mostly magnesium played defensive, compensatory and survival roles in the growth and recovery of soyabean seedlings from water stress effects. Fortification of these macronutrients in agricultural chemicals and adoption in semi arid or arid regions is hereby recommended.

Keywords: water, stress, macro-nutrient, arid, growth.

INTRODUCTION

Water stress is one of the major limitations to the agricultural productivity worldwide, particularly in warm, arid and semi-arid parts of the world; the world population is expanding rapidly and is expected to be around 8 billion by the year 2025. It is a prediction that the increases in world population will occur almost exclusively in developing countries that are suffering from serious nutritional problems and population pressure on the agricultural soils. To feed the increasing world population and sustain well - being of humankind, food production must be increased by up to 100% over the next 25 years. Therefore, for sustaining food security, a high priority should be given to minimizing the detrimental effects of water stress (Waraich *et al*., 2011).

Plants are sessile in nature and, as a result, they do not have the capability to escape from the site of unfavorable environment (Diaz-Vivancos *et al*., 2008). Plants often face the challenges to grow under adverse environmental conditions such as water deficit or excess, high intense light, low or high temperature, salinity, heavy metals, UV rays, insect and pests attack etc. These stresses wield adverse effects on plant growth and development by inducing many metabolic changes, such as the occurrence of an oxidative stress (Hernandez *et al*., 2004).

Some forms of abiotic stresses are experienced throughout the growth and developmental stages of most cultivable crops (Hasanuzzaman *et al*., 2018). Abiotic stress deters the productivity of plants by altering plant growth patterns and physiological responses. Thus, increasing plant resilience in response to abiotic stress is a great challenge in the effort to improve food production by 70% to feed the increasing population by the year 2050 (Wani and Sah, 2014). The combined effects of various stresses that affect crops have become more common such as the occurrence of drought and waterlogging and at the same time, salinity and high temperature stresses in arid and semi-arid regions. Increased production of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) is a common consequences of water-deficient (drought) stress (Clement *et al*., 2008).

Soybean (*Glycine max L.*) is an important dicotyledonous crop due to the high content of oil and protein in its seeds. Soybean has a potential for large-scale production, as such has excelled in the world agricultural economy as the main source of oilseed crop. Singh and Shivakuamar, (2010) stated that at present, soybeans are grown primarily for oil extraction and for use as a high protein meal for animal feed. Consumption of soyabean as cheese and local beverage is common among the Nigerian populace, cheese is consumed in most homes as alternative to meat and beans which are the major sources of protein, since the costs of meat and beans are becoming unaffordable thus making soyabean a staple source of vegetable protein in Nigeria. Soybean production has been increased from about 26 million tons in 1960 to 223 million tons in 2010 due to increases in harvest area and yield (FAO, 2012), one major factor influencing growth and yield of soybean is water stress, however numerous studies indicate that some macronutrients can regulate plant growth, developmental processes and also play crucial role in stress signalling (Hepler, 2005).

Therefore, the objective of this study was to determine the influence of calcium and magnesium on growth rate and antioxidant responses of *Glycine max* seedlings grown under water deficit and waterlogged conditions.

MATERIALS AND METHODS

Materials

Healthy Soybean seeds were obtained from National Cereals Research Institute (NCRI) Badeggi, Niger State, Nigeria, West - Africa.

Chemicals and Reagents

All chemicals and reagents used were of analytical grade and obtained from BDH Poole, England.

Methods

Growth conditions

Matured glasshouse grown soybean seedlings were used for all the experiments. According to Pattanagul and Madore (1999), ten seeds of soybean were potted in loamy soil and kept in a glasshouse under natural conditions (12 hours day and 12 hours night) and approximately 30ºC and 20ºC day/night temperatures. Planted seeds were watered daily and grown under these conditions for 3 weeks, by which time they had reached the desired height, canopy diameter and have abundant matured source leaves. The experimental design was of seven (7) different groups and these groups were labeled A, B, C, D, E, F and G to indicate the plants watered always, watered once a week, waterlogged, watered once a week with Ca solution (40 mg/l), watered once a week with Mg solution (40 mg/l), waterlogged with Ca solution (40 mg/l) and waterlogged with Mg solution (40 mg/l) respectively. For the water deficit condition, plants were put under stress by light – watering once at the start of the week, and then depriving them of water for the rest of the week. This process was repeated weekly for a 2 – week period, and the described experiments were carried out till the end of week 2. For the waterlogged condition, plants were put under stress by maintaining water above soil level of the plants throughout experimental period (2 weeks). For group A, plants were watered daily throughout 14 – day period. Portable water was used to wet the plants; the volume of water used was at the consumptive level of daily requirements of the plants.

Measurement of Growth Rate

Viability test

Viability test of Oka *et al.,* (2003) for survival rate (i.e. the number of leaves fully germinated and the height differences of the plants before and after treatments and RWC) were considered as the growth rate.

Determination of Relative Water Content (RWC)

For each group, 3 plants were randomly harvested after exposure to water deficit and waterlogged conditions, their roots were quickly rinsed in water and gently blotted dry. Each plant was separately weighed for fresh weight determination. For water – deficit plant, dry weight was determined after 2 – day of incubation in an oven (PHL, E811181, England) at 70ºC (Pattanagul and Madore, 1999). Relative water content was calculated using the equation:

$$\text{Relative Water Content (\%)} = \frac{\text{Fresh Weight} - \text{Dried Weight}}{\text{Fresh Weight}} \times 100$$

Preparation of Plant homogenate

Plant homogenates were prepared according to the method described by Gachotte *et al.,* (1995). All operations were carried out at 4ºC. Whole 35 – day old seedling was grinded, 5 g of grinded sample was homogenized for 10 seconds in 20ml of 0.1M Tris - HCl buffer, pH 7.8, containing 0.5M Sucrose and 1mM EDTA. The homogenate of the mixture was centrifuged at 6000 x g for 15 minutes; the supernatant obtained was kept for further analysis.

Antioxidant Assays

Determination of Malondialdehyde (MDA)

The procedure described by Varshney and Kale (1990) was used to determine malonaldehyde level in experimental seedlings.

About 3mL aliquot of the plant tissue extract was mixed with 2 mL solution containing 0.6 % (m/v) thiobarbituric acid (TBA) and 10 % (m/v) trichloroacetic acid (TCA), reacted for 30 mins. in a boiling water bath *()*, and immediately cooled on ice. The reaction solution was then centrifuged for 5 mins. at 11,600 × *g*. The OD_{532} and OD_{450} values of the resulting supernatant were measured, respectively. The MDA content was estimated using the formula

$$MDA = \frac{[(6.45 \times OD_{532}) - (0.56 \times OD_{450})] \times \text{plant tissue extraction compound (L)}}{\text{Sample weight (g)}}$$

Determination of Superoxide Dismutase (SOD)

The SOD activity was assayed according to procedures described by Tang (1999). To 0.1ml of plant extract, 1.5 mL of PBS at pH 7.4, 0.3 mL of 130 mM methionine, 0.3 mL of 750 µM nitroblue tetrazolium (NTB), 0.3 mL of 110 µM EDTA-Na_2, 0.3 mL of 110 µM riboflavin and 0.5 mL deionized water were added. The solution was allowed to react for 20 mins. at a light intensity of 3000 lux and 25 °C, the OD_{560} value of the solution was assayed for SOD activity and expressed as a specific activity of U mg^{-1} protein.

Determination of Catalase (CAT)

CAT activity was assayed using the procedures described by Cakmak and Horst (1991).

100 µL aliquot of the plant tissue extract was well mixed with 3 mL PBS (pH 7.4) containing 0.1 M H_2O_2, the CAT activity was assayed for at OD_{240}.

Determination of Ascorbic Acid (Vitamin C)

Ascorbic acid content of the samples was determined using 2,6-dichlorophenolindophenol (DCIP) dye - titrimetric method of AOAC (2006).

Extraction solution: 15 g of trichloroacetic acid was dissolved in 40 ml acetic acid and 200 ml distilled water. It was diluted to 500 ml with distilled water and filtered.

Ascorbic acid standard solution: 0.05 g of standard ascorbic acid was dissolved in 60 ml of the extract and made up to 250 ml with distilled water.

DCIP Standard Solution: 0.05 g of 2,6-dichlorophenolindophenol (DCIP) was weighed into a beaker and an equal amount of sodium bicarbonate (Na_2CO_3) was added to facilitate stability of the dye solution. The mixture was dissolved in 100 ml of distilled water and filtered. The 2,6-dichlorophenolindophenol was thereafter standardized by titrating against 10 ml of ascorbic acid stock solution until a faint pink color was obtained. The amount (mg) of ascorbic acid equivalent to 1 ml of dye solution was then calculated.

Ascorbic acid (mg/100g) = C x V x (DF/W)

Where C = mg ascorbic acid/ml dye; V = volume of dye used for titration of 1 ml of diluted sample; DF = Dilution factor and W = weight of sample (g).

Determination of Vitamin E by Ferric-Bipyridine Reducing Capacity (FBRC)

Vitamin E content of the samples was determined using the procedure described by AOAC (2006). 1 ml of plant extract was homogenized with 20ml of ethanol and filtered with Whatman No. 1 filter paper, 1ml of 0.2% Ferric Chloride was added to 1 ml of the filtrate followed by addition of 1ml of 0.5% 2,2-bipyridiyine (Bp) reagent, this mixture was made up of 5ml with distilled water. Absorbance was taken at 535nm using UV- Spectrophotometer (Specord200, Analytikjena, Germany).

STATISTICAL ANALYSIS

Student t-test was used to compare means for significant difference at 5% level of confidence (P>0.05) using SPSS version 19. Mean results were presented using line graph.

RESULTS AND DISCUSSION

The mean number of leaf per experimental seedlings of soyabean grown under water deficit and waterlogged conditions is presented in Figure 1. The untreated seedlings grown under water deficit condition showed the least number of leaf (group B = 7) followed by the waterlogged sample (group C = 12). Treatment with calcium and magnesium significantly increased (at P>0.05) the number of leaf when compared to the untreated seedlings in groups B and C.

The mean values of height of the seedlings between 21 and 35 days are presented in Figure 2. There was significant increase (P>0.05) in heights of normal (A) and treated seedlings (D to G) between 21 and 35 days of growth. However, significant decrease (P>0.05) was observed in water stressed samples (B & C) particularly the water deficit seedlings in group B. waterlogged seedlings treated with magnesium (40mg/L) (group G) compared favourably with group A seedlings grown under normal condition in term of height increase of 9cm.

The mean values of relative water content (RWC) of the experimental seedlings are presented in Figure 3. The mean result of RWC positively correlates with results of growth parameters i.e. number of leaf per seedling and height difference, which followed the same trend with RWC in this present study. The water stressed seedlings mostly water deficit showed marked reduction in water content (B = 21%); treatment with the macronutrients (Ca & Mg) significantly increased (P>0.05) water absorbing and retaining capacities of tissues and cells of the treated seedlings (groups D, E, F & G) to the least percentage increase of 50%.

As shown in Figure 4, malonaldehyde mean value was significantly increased (P>0.05) in untreated water deficit seedlings (group B), calcium and magnesium significantly decreased (P>0.05) MDA levels in treated seedlings (groups D, E, F & G) while group A (control) with the mean value 1.0 mmol/mg protein showed the least MDA value.

The activity of SOD and CAT in experimental seedlings is presented in Figures 5 and 6 respectively. Activity of SOD and CAT significantly increased (P>0.05) in untreated water deficit seedlings (group B) than untreated waterlogged seedlings (group C). CAT significantly decreased (P>0.05) in activity in calcium and magnesium treated groups (D to G) while the macronutrients' effects were not significant at P<0.05 on SOD activity.

As shown in Figures 7 and 8, the concentrations of the non – enzymatic antioxidants (ascorbic acid & E) mostly vitamin E significantly increased (P>0.05) in untreated water stressed seedlings (B = 41.0 mg/100g; C = 40.0 mg/100g) when compared to the seedlings grown under normal condition with vit. C and E mean values as 9.0 mg/100g; and 17 mg/100g respectively. Treatment with calcium and magnesium solutions significantly decreased (P>0.05) the antioxidants concentration thus ameliorating the effects of stress.

Water stress including drought and flooding is considered as a major threat, limiting growth and yield of plants (Thomas *et al*., 2004). Drought is caused by insufficient rainfall or irrigation which results in soil drying, whereas, in flooding, water exists in soil solution causing water logging and submergence. In response to drought and flooding stress, 40–60% yield losses have been reported (Ahmed *et al*., 2013). This accounts for the poor growth rate in water stressed seedlings. Calcium and magnesium ameliorate the effects of water deficit and waterlogging on relative water content of the soybean seedlings.

According to Hassanuzzaman *et al*., (2020), drought provokes stomatal closure, reduced carbon (IV) oxide (CO_2) entry, impaired photosynthetic rate and altered photochemistry in chloroplasts and imbalance in the light harvest and utilization thus causing ROS overproduction. The increased level of malonaldehyde, an index for determining peroxidation of lipid mostly in water deficit seedlings may be attributed to ROS overproduction. Waterlogging induced hypoxic or anoxic conditions generate toxic compounds that impair plant metabolism resulting in ROS over generation and oxidative damages (Loreti *et al*., 2016). Also, increased level of MDA in waterlogged seedlings may be attributed to oxidative damage resulting from ROS overproduction.

Plants primarily deal with oxidative stress via an endogenous defensive mechanism consisting of different enzymatic and non – enzymatic systems such as superoxide dismutase (SOD), catalase (CAT) ascorbate peroxidase (APx), glutathione reductase (GR) and non-enzymatic systems like ascorbic acid, glutathione, phenolic acids; alkaloids; flavonoids; carotenoids; α-tocopherol and non-protein amino acids (Gill and Tuteja, 2010; Kaur *et al*., 2019). The increase in activity and concentration of enzymatic and non – enzymatic antioxidants in water stressed seedlings can be regarded as expression and proliferation of signaled antioxidant systems upon oxidative stress and ROS over generation in the intracellular tissues of water stressed seedlings, this endogenous defensive mechanism is a natural compensation in balancing between oxidation and antioxidation.

Epstein and Bloom (2004) reported that Mg is exceptional in activating more enzymes than any other mineral nutrient. Mg-activated enzymes are ATPases, ribulose-1, 5-bisphosphate (RuBP) carboxylase, RNA polymerase and protein kinases (Shaul, 2002). Mg role as the central atom of the Chlorophyll molecule is perhaps the best-known function of Mg in plants which it is associated with the development of leaf chlorosis, typically interveinal, under Mg deficiency stress. Calcium is considered to play a role in mediating stress response during injury, recovery from injury, and acclimation to stress (Palta, 2000). It has been suggested that Ca is necessary for recovery from drought by activating the plasma membrane enzyme ATPase which is required to pump back the nutrients that were lost in cell damage (Palta, 2000). Since dehydration is the common denominator, Ca also has a role to play in freeze injury tolerance. Calcium also plays a role as calmodulin which controls the plant metabolic activities and enhances the plant growth under drought condition.

In the present study, water supply below and above plant consumptive level for a minimum period of 14 days in a controlled experiment results in peroxidation of intracellular lipid, generation of reactive oxygen species and release of defensive mechanism such as endogenous enzymatic and non – enzymatic antioxidants. The macro – nutrients (magnesium and calcium) at the concentration of 40 mg/L mostly magnesium played defensive, compensatory and survival roles in the growth and recovery of soyabean seedlings from water stress effects. Fortification of magnesium and calcium in agricultural chemicals is hereby recommended.

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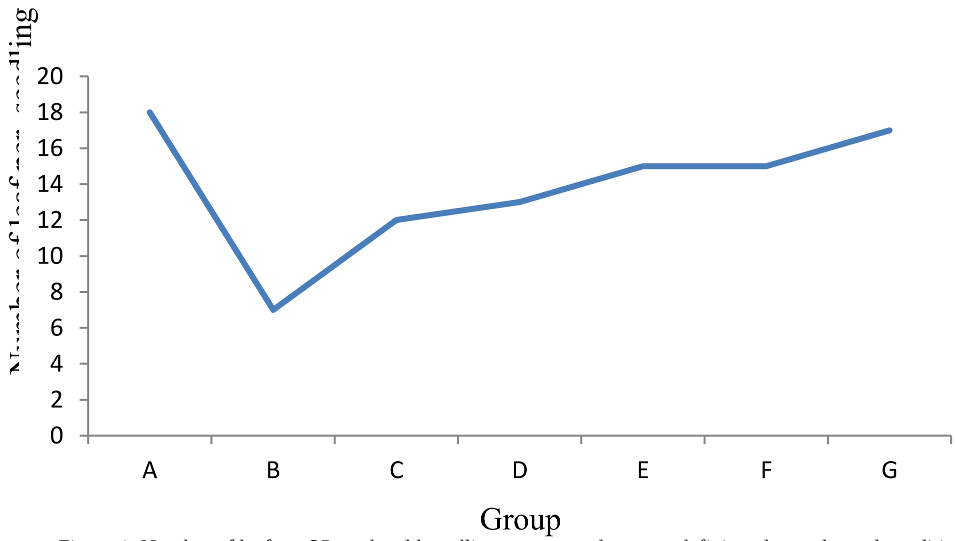

Figure 1: Number of leaf per 35 weeks old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

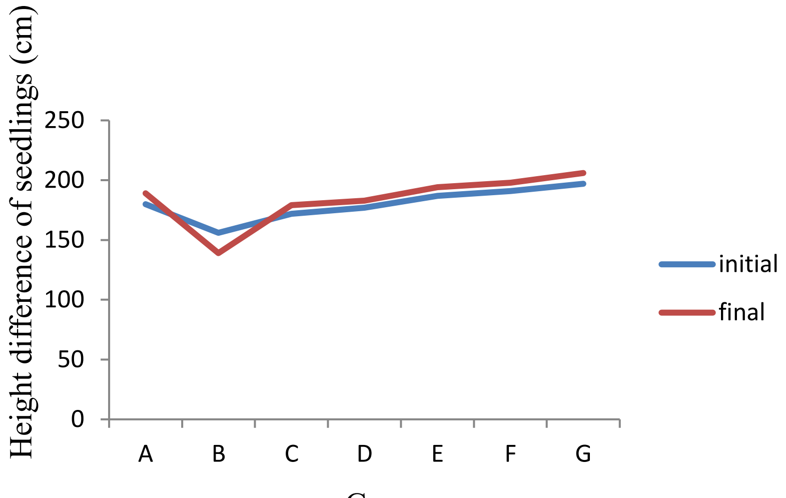

Figure 2: Height difference between 21days and 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

Key: Blue and wine colours indicate 21 and 35 days old seedlings respectively

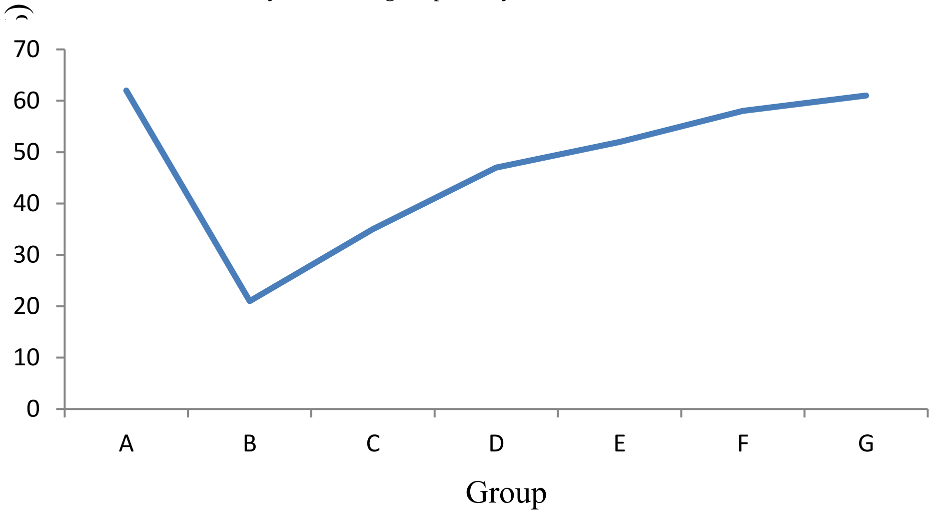

Figure 3: Relative Water Content (RWC) of 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

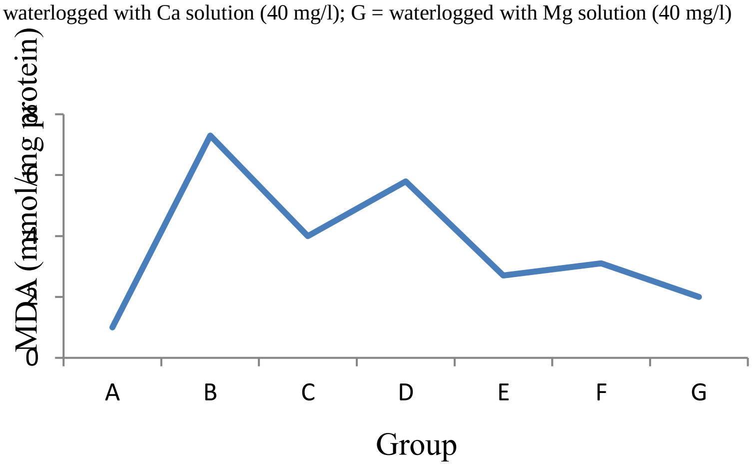

Figure 4: Malonaldehyde (MDA) status of 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

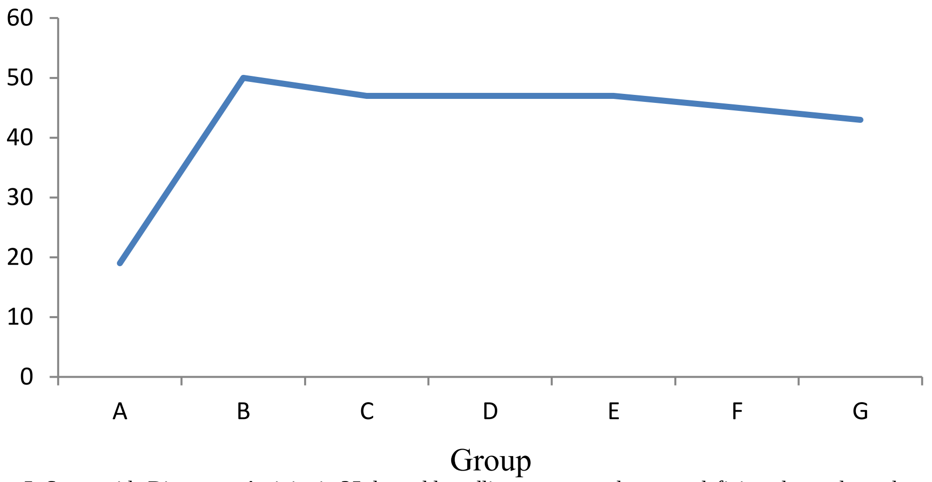

Figure 5: Superoxide Dismutase Activity in 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

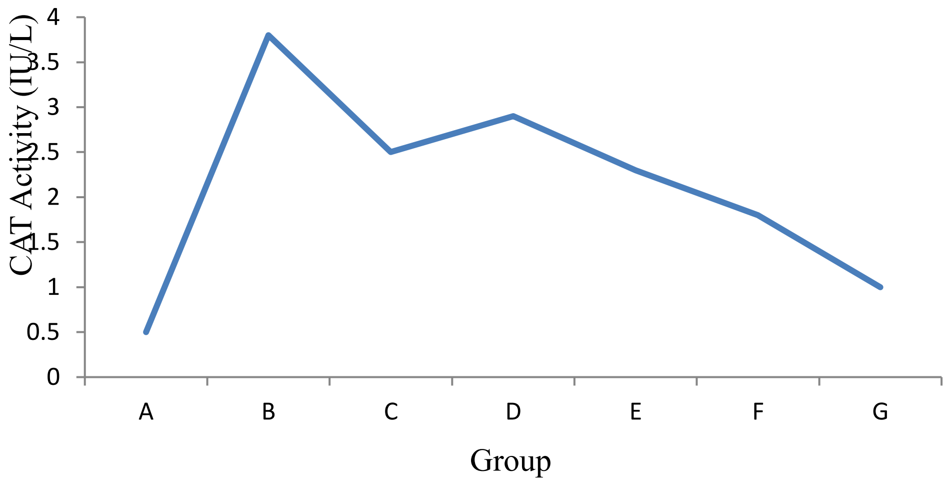

Figure 6: Catalase activity in 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

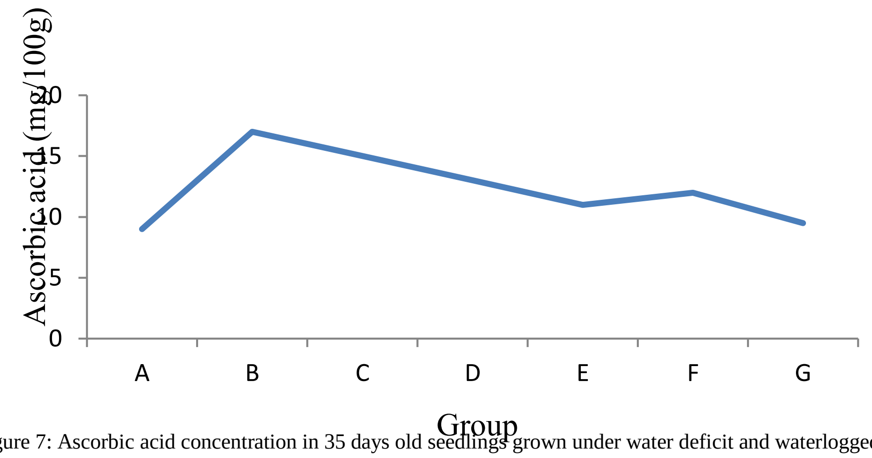

Figure 7: Ascorbic acid concentration in 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)

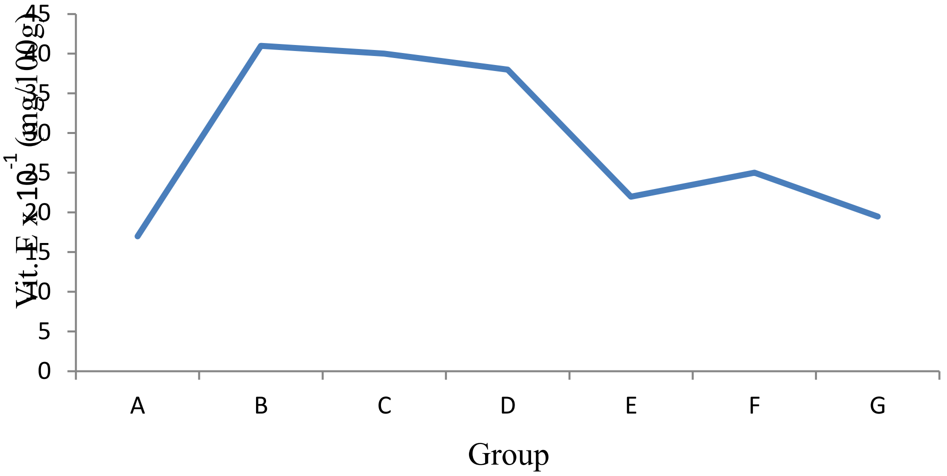

Figure 8: Vitamin E concentration in 35 days old seedlings grown under water deficit and waterlogged conditions

A = watered always; B = watered once a week; C = waterlogged; D = watered once a week with Ca solution (40 mg/l); E = watered once a week with Mg solution (40 mg/l); F = waterlogged with Ca solution (40 mg/l); G = waterlogged with Mg solution (40 mg/l)