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Effect of Polyethylene Glycol Induced Water Stress on Physiological and Biochemical Responses in Pigeonpea (*Cajanus cajan* L. Millsp.)

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Abstract

Water deficit induced by Polyethylene glycol (PEG) affect physiological and biochemical changes in pigeonpea. The plants were subjected to two progressive stresses: moderate (-0.51 MPa) and severe (-1.22 MPa) respectively. The water stress condition was created by irrigating 14 days old grown seedling pot with PEG nutrient solution and decreasing the osmotic potential -0.04 MPa regularly. Relative water content (RWC) content was significantly reduced under water stress condition. Increase in the free proline content during water stress condition suggests that proline is one of the common compatible osmolytes under water stress condition. The genotype exhibited lower accumulation of catalase (CAT) and increased activity of superoxide dismutase (SOD) and Peroxidase (POD) under stressed condition. The present data suggest a relation between proline content and water stress and a well developed antioxidant defense mechanism activated during water stress.

Key Words: Antioxidant enzymes; *Cajanus cajan*; Drought; Polyethylene glycol; Proline

Introduction

Plants are subjected to various abiotic stresses due to unfavorable environmental conditions that affect their growth, metabolism and yield [1] and drought is one of the major abiotic stresses which limit the crop production in arid and semi arid tropics like India.

Pigeonpea (*Cajanus cajan* (L.) Millspaugh) is one of the major grain legume crops of the tropics and subtropics. It is the most important pulse crop which is cultivated in the gross cropped area (3.58 million ha) under pulses providing 20% of the national pulse production (2.51 m tones). This accounts for 90% of the world's Pigeonpea production [2]. Gulbarga region accounts for 70% of total Pigeonpea production of Karnataka state; is a major drought affected area.

Extensive field studies have been conducted for understanding the plant tolerance and oxidative stress in response to water deficit. Osmotic solution such as PEG has been used to impose water stress by exposing the root system of plants can resolve the problem. Addition of PEG to nutrient solution produces osmotic stress over a period of 3-4 weeks. PEG is used successfully to decrease the water potential of plants as it doesn't enter into the root [3]. This neutral polymer is being widely used to impose water stress in plants. Responses of plants to water deficit result in alteration of chlorophyll content, free proline, protein activity and reactive oxygen species. One of the biochemical changes occurring when plants are subjected to stress condition is the accumulation of reactive oxygen species which are inevitable by products of normal cells. Plants have evolved several mechanisms that allow perceiving the stresses and rapidly regulating their physiology and metabolism to cope them. The antioxidant defense mechanism provide an strategy to enhance drought tolerance by increase the rate of reactive oxygen species via enhanced electrolyte leakage in chloroplast and mitochondria. Plants with high levels of antioxidants either constitutive or induced have been reported to have greater resistance to the oxidative damage [4].

SOD is a major scavenger of O_2 and its enzymatic action results in the formation of H_2O_2 and O_2. Catalases and peroxidases are major enzymatic cellular scavenger of CO_2. Removing the highly toxic H_2O_2 produced during dismutation is essential for the cell for the cell to avoid inhibition of the enzymes such as those controlling the calvin cycle in the chloroplast [5]. Catalase, which is present in peroxisome, dismutates H_2O_2 into water and molecular O_2 whereas peroxidase decomposes H_2O_2 by oxidation of substrate such as phenolic compounds and/or antioxidants [6, 7]. Under drought stress condition, plant accumulates osmolytes such as proline and act as osmoprotectant. Genes for enzymes involved in biosynthesis and metabolism of proline indicates that the expression of these genes and accumulation of proline under stress mainly regulated at transcriptional level [8, 9].

The present study was to evaluate the effect PEG induced water stress on biochemical and antioxidant enzymes activities in pigeonpea. Since a little attention has been given by researches to improve the locally cultivated pigeonpea in this area; the present study would help to understand the responses under drought stress condition and its further improvement of present cultivar.

Materials and Methods

Pigeonpea drought tolerant genotype GRG-295 seeds were germinated in pots containing sand: soil (1:1). Seedlings were grown 22-32^{o}C and ~70% relative humidity under normal day light condition. After 14 days of seedling growth, plants were divided into two groups: 2 control and 2 treatment. The drought stress was induced by irrigating the pot with PEG-6000 nutrient solution. The osmotic potential of the nutrient solution of treatment group was decreased gradually at the rate of -0.04 MPa/day. After 12 days of; the first series of comparative investigations were performed in one control (C1) and treatment (S1) group, when O.P. reached at -0.51 MPa (moderate stress). In second set; Osmotic potential was decreased again at the same rate until stress level reached -1.22 MPa (after 32 days; severe stress). The comparative studies were performed in control (C2) and treatment (S2) group of same age plants. Healthy leaves, free of any disease, from control group and stress induced leaves were used for various physiological and biochemical assays. The concentration of PEG-6000 (g/kg of water) for each water stress was determined using the equation of Michel and Kaufmann [10].

Relative water content

To determine relative water content, 20 leaves from each group were weighed immediately (FW) after harvesting the plant. Leaves were then placed in distilled water for 4 hr and then turgid weight (TW) was measured. Then the leaves were dried in oven at 80^{o}C for 24hr to obtain their dry weight (DW). Relative water content was calculated by the following formula.

RWC= FW-DW/TW-DW x 100

Free proline content

Free proline content was estimated by following the method of Bates et al [11]. Fresh 500 mg of leave samples were homogenized in 5 ml of 3% (w/v) sulphosalicylic acid using mortar and pestle. 2 ml of extract was in test tube and to it 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent was added. The reaction mixture was boiled in water bath at 100^{0}C for 30 min. after cooling the reaction mixture; 4 ml of toluene was added. After thorough mixing, the chromophore containing toluene was separated and absorbance of red color developed was read at 520 nm against toluene blank.

Protein estimation

Proteins were estimated by using Lowry et al. [12] method. 250 mg of fresh leaves were homogenized in 2 ml of 0.1 M phosphate buffer (pH 7.0). The extract was centrifuged at 10000 g for 15 min at 4^{o}C and the supernatant was transferred to the tube containing a mixture of 20 ml acetone and 14ml of β-merceptoehanol for precipitation of proteins. The samples were stored at 0^{o}C for 5 hr and then centrifuged at 10000 g for 20 min. the supernatant was discarded and the pellet was dissolved in 2.5 ml of NaOH solution. 0.2 ml of this sample was used to prepare the reaction mixture. The intensity of blue color developed was recorded at 660 nm on UV visible spectrophotometer.

Activity of Superoxide dismutase

SOD activity was estimated by recording the decrease in absorbance of the enzyme as described by Dhindsa et al [13]. Fresh 500 mg leaves were homogenized in 0.1 ml of phosphate buffer (pH 7.5). The extract was centrifuged at 10000 g for 20 min at 4^{o}C and supernatant was used as enzyme source. 3 ml of reaction mixture containing 0.1 ml of 1.5 M Na_2CO_3, 0.2 ml of 200mM methionine, 0.1 M of 3mM EDTA, 0.1 ml of 2.25 mM NBT, 1.5 ml of 100 mM potassium phosphate buffer (pH 7.5), 1ml of distilled water and 0.05 ml of enzyme samples. The tube without enzyme was taken as control. Reaction was started by adding 0.1 ml 60 µM riboflavin and placing the tubes below a light source of two 15 W fluorescent lamps for 15 min. The reaction was stopped by switching of the light and covering the tubes with black cloth. Absorbance was recorded at 560 nm.

Activity of catalase

250 mg of leaves were homogenized in 3ml of 0.1M phosphate buffer (pH 7). The extract was centrifuged at 10000 g for 20 min at 4^{o}C and supernatant was taken as enzyme source. The catalase activity was determined according to Luck [14]. The assay mixture in total volume of 3 ml contained 0.5 ml of 0.2 M phosphate buffer (pH 7), 0.3 ml of (v/v) H_2O_2 and 0.1 ml of enzyme. The final volume was made 3ml by adding distilled water. The reaction was started by adding enzyme and change in optical density was measured at 240 nm at 0 min and 3 min on UV Vis spectrophotometer.

Activity of peroxidase

Peroxidase activity was assayed as described by Putter [15]. 250 mg of leaves were homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.0). The extract was centrifuged at 10000 g for 20 min at 4^{o}C and supernatant was taken as enzyme source. The assay mixture of 3 ml contained 1.5 ml of).1 M phosphate buffer (pH 7.0), 1 ml freshly prepared 10mM guaicol, 0.1 ml enzyme extract and 0.1 ml of 12.3 mM H_2O_2. Initial absorbance was read at 436 nm and then increase in the absorbance was noted at the interval of 30 sec on UV-Vis spectrophotometer.

Results

Effect of water stress on physiological parameters

The plants were subjected to extensive level of water stress of -0.51 MPa and -1.22 MPa induced by polyethylene glycol-6000. It was observed that severe stress (-1.22 MPa) clearly affect the relative water content as compare to the control of same age group plant.

The significant differences in RWC was observed as compare to control (C1) and stressed (S1) of young leaves (26 days old). The sharp decrease in RWC was noted in old leaves (46 days old) of stressed plants (S2) plants as compare to control (C2). In both C1 and C2 more than 90% of relative water content was observed. A decrease of 25% RWC was noted in S1 as compare to the C1 while ~56% of decrease in RWC was observed in S2 plants as compare to C2 of same age group plants.

It is reported that PEG induced significant water stress in plants and not having any toxic effects [16]. A progressive moderate stress (-0.51 MPa) cause a significant decrease in RWC whereas drastic decrease in RWC seen at progressive severe stress (-1.22 MPa) level.

Effect on total chlorophyll content

PEG induced drought stress imposed to plants, significantly decreased chlorophyll a, chlorophyll b and total

chlorophyll content both at the stress level. The highest content of chlorophyll a was observed in C2 leaves while both progressive stresses of -0.45 MPa and -1.22 MPa caused significant in the leaves chlorophyll contents (Table.1). Unlike chlorophyll a, it is clear that a progressive stress adversely affect chlorophyll b content. A decrease of total chlorophyll with drought stress implies a lowered capacity for light harvesting. Since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments.

Table 1. Relative water content, chlorophyll content and activities of antioxidant enzymes in Pigeonpea leaves under normal and PEG induced water stressed condition. The values are mean ± SEM of three replications.

Group	Relative Water Content (in %)	Chlorophyll content (mg gm^{-1} F.W.)				Antioxidant enzyme activity		
		Chl a	Chl b	a/b	Total Chl	SOD (U µg prot^{-1})	POD (ΔA_{436} min^{-1} mg prot^{-1})	CAT U mg^{-1} prot^{-1}
C1	92.67±0.67^{a}	1.25±0.03a	1.14±0.04^{a}	1.10	2.35±0.05	5.70±0.21	11.14±0.03	2.20±0.86
S1	69.33±1.33^{b}	0.78±0.03	0.58±0.04^{b}	1.34	1.43±0.05	7.67±0.12	16.68±0.11	1.33±0.42
C2	90.33±1.45^{a}	1.79±0.04	1.10±0.07^{a}	1.63	2.68±0.07	6.37±0.09	12.21±0.07	2.76±0.67
S2	40.00±2.08^{c}	1.48±0.03	0.74±0.04^{ab}	2.00	2.19±0.09	9.03±0.24	27.58±0.08	1.67±0.48

Effect on free proline and protein content

The change in the free proline content was measured in both control and stressed induced plant leaves. The results depicted on Fig. 1 show that a high increase in the proline accumulation in the leaves during progressive stresses in pigeonpea. In young (C1) and old (C2) plant leaves, free proline accumulation was 1.47 and 2.07 µMgm^{-1} F.W. respectively. Under progressive mild stress (S1) increased free proline content increased up to 12.17 µMgm^{-1} F.W. and 54.47 µMgm^{-1} F.W. accumulation of proline was observed in old leaves (S2) under severe progressive stress condition. The results shown in Fig. 2 indicates increased level of protein concentration at both moderate and progressive stress as compare to control of same age group.

Fig. 1. Effect of different level of PEG induced water stress on proline content in pigeonpea leaves. The datas are mean ± SEM of three replications. The means with the same letter donot differ statistically by Turkey's one way ANOVA test (P≤ 0.05).

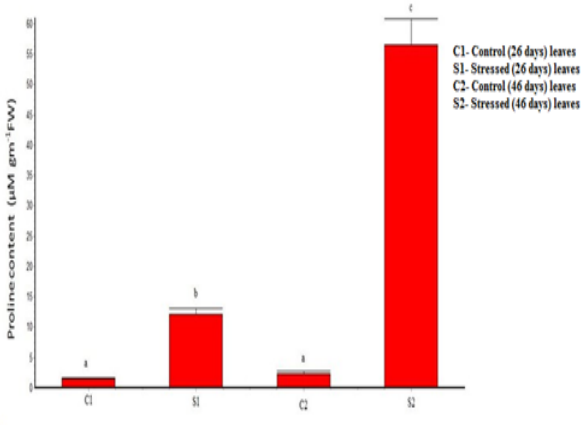
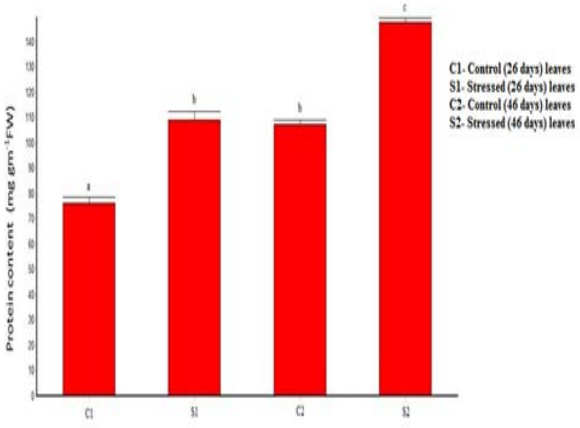


Fig. 2. Effect of different level of PEG induced water stress on protein concentration in pigeonpea leaves. The datas are mean ± SEM of three replications. The means with the same letter donot differ statistically by Turkey's one way ANOVA test (P≤ 0.05).



Effect on Antioxidant enzymes activity

The results pertaining to the effect of PEG induced water stress on SOD, POD and CAT is summarized in Table. 1. The SOD activity of leaves increases at both mild (S1) and severe (S2) stress condition as compare to the control plant leaves. A decrease in CAT activity was observed under both the stress condition while POD activity was significantly higher. The enhancement of SOD and POD activities in pigeonpea genotype under stress condition shows a well organized defense system against ROS under stress condition.

Discussion

In the present investigation, pigeonpea responses were studied to progressive induced different level of osmotic stress by using PEG-6000 in the medium. Several methods which range from withdrawal of water to plants to the use of chemicals such as polyethylene glycol, mannitol etc., have been employed to create water stress in plants. It has been reasonably well established that polyethylene glycol induced water stress mimics that caused by withdrawal of water from plants. Plant exposes their root system to this solution and no other toxicities were observed at plant level following the

addition of PEG-6000 [16]. A decrease in the RWC observed in both progressive mild and severe water stress. Relatively higher RWC was noted in progressive mild stress than severe stress indicating that plants have the ability to sustain their water content under mild stress, whereas this ability lost under severe stress treatment. Decrease in the RWC in PEG induced water stress was reported in rice leaves [17] and in Tomatos [3]. According to results of Bayoumi [18] RWC may be attributed to differences in the ability of the variation to absorb more water from the soil and/or the ability to control water loss through stomata and RWC parameter can be used to select high yielding genotypes that maintain cell turgor under water stress environment to give relative high yield.

Chlorophyll content was also affected during the present investigation which shows that long progressive stress along with some other environmental factor may affect photosynthetic ability of the plant system. In our present report it was observed that Chla is more sensitive than Chlb to PEG induced water stress. Hsu and Kao [17] also demonstrated that PEG induced water stress cause decrease in total chlorophyll content in rice leaves. Hassanzadeh et al. [19] observed decrease in Chla but increase in Chlb content under drought stress in seasame. A reason for decrease in chlorophyll content as affected by water deficit is that drought or heat stress by producing reactive oxygen species (ROS) such as O_2 and H_2O_2, can lead to lipid peroxidation and consequently, chlorophyll destruction also, with decreasing chlorophyll content due to the changing green color of the leaf into yellow, the reflectance of the incident radiation is increased.

A variety of organic solutes accumulate in osmotically stressed plants in which proline appears to be widely distributed osmolytes under stress condition not only in plants but in bacteria also [8, 9]. In the present investigation, it was observed that a severe progressive stress in pigeonpea leads to about 25 fold more accumulation of proline as compare to control of same age group while a 6 fold increase was observed in progressive mild stress. Zgalli et al. [3] reported 10 fold increase in proline accumulation under PEG induced water stress condition in tomato plants that shows that a high proline accumulation observed under PEG stress may produce an adoptive response for pigeonpea under water stress condition. The accumulation of proline under drought stress condition is well established in other plants like in Ragi [20] and Bhendi [21]. It was observed that in Sugarcane, proline has little or no any significant role under water as well as salinity stress condition [22]. Increased level of proline in PEG induced water stressed plants may be an adaptation the purpose of which is to overcome the stress conditions. Proline accumulates under stress conditions supplies energy for survival and growth and thereby helps the plant to tolerate stress [8, 9, 20, 21, 25].

A slight but significant increase in protein level was also observed under both the progressive stresses. Our results depicting increased protein level are in agreement with Kandpal et al. [20] where they also found an increase in soluble proteins during water stress and the dramatic increase in the proline levels. These results clearly suggest that the contribution of proteolysis may not be the major factor responsible for this phenomenon.

Contradictory results have been reported for activities of antioxidant enzymes in number of different plant species. These variations in antioxidant enzymes induced by stress not only depend on severity and duration of the stress treatment and also depend on species and age of the plant [5]. In our report, we observed that the activities of SOD and POD enhances by increasing the duration of stress, whereas declined CAT activities were observed in both progressive stresses induced by PEG as compare to the control. An increase in SOD and POD activity and decrease in CAT activity was also reported during drought stress in Liquorice [6]. It was reported that SOD, POD as well as CAT activities increases in response to PEG induced drought stress in gerbera and Sesame [23, 24]. Aktas et al [25] also reported an increase in all three antioxidant enzyme activities during drought tolerance induced by abscisic acid in Laural seedlings. Sharp decreased activity of SOD and an increased activity of POD were reported in Doritaenopsis [26]. Deceased activity of SOD and CAT was reported in wheat subjected to long term field drought as well as PEG induced water deficit in wheat [7, 27]. It was observed that SOD and CAT activities increases in pigeonpea under oxidative stress induced by waterlogging [28].

Plants are well endowed with antioxidant molecules and scavenging systems which establish a link between tolerance to water stress and rise in antioxidant enzyme concentration in photosynthetic plants. SOD is thought to provide the first line of defense against the toxic effects of reactive oxygen intermediates by converting H_2O_2 to H_2O and O_2. SOD catalyses conversion of O_2^- radicals to H_2O_2 and O_2. Peroxidases have a higher capacity for the decomposition of H_2O_2 and the capacity of catalases significantly reduced under all stress treatments. Catalases may not be the most important during scavenging ROS since little or no catalases in mitochondria and chloroplast where much O_2 free radical generated [29].

Conclusions

Our present results indicate that a progressive water stress induced PEG-6000 cause significant physiological and biochemical changes in pigeonpea. RWC parameter can be used to select high yielding genotypes that maintain cell turgor under water stress environment. Enhanced proline accumulation during stress indicates that proline is thought to play a cardinal role as an osmo-regulatory solute in plants. The increased activities of antioxidant enzymes including SOD and POD indicates that an effective antioxidant defense mechanism possess by pigeonpea for scavenging reactive oxygen species and protect them from destructive oxidative reactions.

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