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EFFECT OF DROUGHT STRESS IN VARIOUS ENZYMES OF *PENNISETUM GLAUCUM*

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Abstract

Introduction: Pearl millet (*Pennisetum glaucum*) is an important cereal of traditional farming systems that has the natural ability to withstand various abiotic stresses such as drought, which is one of the most important manifestations of abiotic stress in plants. These plants have however evolved mechanisms that allow them to adapt and survive prolonged periods of water deficit at some level or form of plant structure, if not at the whole plant level. The hostile conditions augment the formation of reactive oxygen species (ROS) during physiological stresses in plants which are combated by various enzymatic and non-enzymatic mechanisms. The present study aims at examining the role of four important enzymes namely Ascorbic peroxidase (APX), Peroxidase (POX), Catalase (CAT) and Superoxide Dismutase (SOD) expressed during drought stress in pearl millet (*Pennisetum glaucum*). **Method:** 12 and 22 days old seedlings of *Pennisetum* cultivar HHB-68 were subjected to drought stress by treatment of 30% Polyethylene glycol for different time periods 30min (T1), 2hr (T2), 4hr (T3), 8hr (T4), 16hr (T5), 24hr (T6) and 48hr (T7) respectively, monitored by examining RWC of seedlings. The treatment seedlings were then used for investigating the levels of enzymes activity in response to prolonged dehydration periods of 22 days. The enzyme activity of POX, APX, CAT and SOD were assayed. **Result:** Enzymes expression was assayed for each treatment sets at both time intervals. Drought stress was observed to cause remarkable increase in POX, APX and SOD activity while incidence of CAT enzyme decreased with the increasing period of water deficit. **Conclusion:** Prolonged periods of water deficiency causes significant variations in expression of various enzymes in *Pennisetum glaucum*, known to be involved in ROS scavenging and drought stress management. The study provides a sturdy validation of the role of these enzymes as potent mechanisms undertaken by drought resistant plants for successful management of drought stress, which can be used for the development of more efficient and economic drought resistant cultivars.

Keywords: Drought Stress; Enzyme activity; *Pennisetum glaucum*; Relative water content

Introduction

Among various environmental stresses, drought stress has become a critical problem worldwide due to its detrimental effect on plant physiology and performance (Janmohammadi *et al.* 2008). It can be said that it is one of the most devastating environmental stresses. Iran, with an annual rainfall of 240 mm, is classified as one of those dry regions (Jajarmi, 2009). The decrease in rainfall due to world-wide climatic shifts has been predicted to reduce crop yield in semi-arid areas of the world. The reduction of crop yield currently affects approximately 3.6 billion ha (25% of upland in the world) in semi-arid and arid areas (United Nations Environment Program 1991). In these areas, desertification and population growth will exacerbate food shortage.

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] (Known under synonyms: *P. americanum* (L.) Leake or *P. typhoides* (Burm) Stapf and C.E. Hubb.), is an important cereal of traditional farming systems in tropical and subtropical Asia and sub-Saharan Africa. It accounts as the sixth most

important crop after wheat, rice, maize, barley and sorghum in terms of annual global production (FAO 1992). Pearl millet is the staple food grain with a high nutritive value and is also used as a feed, fodder, construction material. Its use as a source of bio fuel is being explored (Wu *et al.*, 2006). It is grown on 29 million ha (FAO 2005) in Africa and Indian sub-continent supporting millions of poor rural families mostly in the drought-prone areas where rain fed agriculture is commonly practiced. Pearl millet is the fourth most important cereal crop in India, after rice, wheat and sorghum, which is widely grown in the states of Rajasthan, Maharashtra, Gujarat and Haryana where the food security of the population depends immensely on pearl millet production.

Extensive field studies have been conducted for understanding the plant tolerance and oxidative stress in response to water deficit. Osmotic solution such as Polyethylene Glycol (PEG) has been used to impose water stress by exposing the root system of plants can resolve the problem. It is used successfully to decrease the water

potential of plants as it doesn't enter into the root and thus cause cytorhysis rather than plasmolysis damages during the growth stages such as germination, seedling or flowering which are the most critical stages susceptible to water stress (Ahmadi *et al.* 2009). It is also a better choice for imposing low water potential than the often used solute because mannitol has been shown to be taken up by plant cell and can cause specific toxic effects on plants growth (Hohl and Schopfer, 1991).

The hostile conditions augments the formation of reactive oxygen species (ROS) such as H₂O₂ (hydrogen peroxide), O₂⁻ (superoxide) and OH (hydroxyl) radicals, through enhanced seepage of electrons to molecular oxygen (Arora *et al.* 2002). ROS may act as secondary messenger involved in stress signal transduction pathway (Chamnongpol *et al.*, 1998) but inconsistent ROS production damages plants by oxidizing photosynthetic pigments, membrane lipids, proteins and nucleic acids (Yordanov *et al.*, 2000). There are various non-enzymatic and enzymatic anti-oxidant systems Possessed by the plants to protect themselves from oxidative damages (Mittler, 2001). Non-enzymatic antioxidants including β-carotenes, ascorbic acid (AA), α-tocopherol (α-toc), reduced glutathione (GSH) and enzymes including: superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR) (Xu *et al.* 2008). Superoxide dismutases (SODs), a group of metalloenzymes, are considered as the first defence against ROS, being responsible for the dismutation of O₂⁻ to H₂O₂ and O₂. CAT, APX, POD are enzymes that catalyze the conversion of H₂O₂ to water and O₂ (Gratao *et al.* 2005). The capability of scavenging ROS and reducing their damaging effects may correlate with the drought tolerance of plants (Tsugane *et al.* 1999). The concentrations of these enzymes indicate their level of activities which is a direct significance of the level of ROS in the system (Michel and Kaufmann, 1973). With these objectives the present study was conducted to study the effects of drought on *Pennisetum glaucum* seedlings by scrutinizing the RWC and enzyme activities of the treated seedlings.

Materials and Methods

Seed Material and Stress Treatment

Seeds of the Pearl millet cultivar HHB-68 drought tolerant were obtained from the Pearl Millet, CCS Haryana Agricultural University, Hisar. Seeds washed thoroughly with distilled water and germinated in triplicates in autoclaved pots (15 cm diameter and 8 inch depth) containing autoclaved Soilrite™ at 33°C with 16h light/8h dark photoperiod at the National Phytotron in IARI India.

Drought stress was induced in the first two sets of seedlings on 12th and 22nd days of germination by exposing them to 30% PEG 6000 (Polyethylene glycol) and 1mM MES for

different time periods 30 min (T1), 2 hr (T2), 4 hr (T3), 8 hr (T4), 16 hr (T5), 24 hr (T6) and 48hr (T7) respectively to stimulate drought stress at -1.25Mpa (osmotic potential). The other set of seedlings was maintained as control. Osmotic potentials of PEG 6000 were calculated as described by Michael and Kaufman. The first of the two treatment sets were used to calculate RWC, while the other treatment set was used for enzyme assays.

Relative water content (RWC)

Relative water content of 12 and 22 days old seedlings subjected to drought stress was measured by the procedure described by Barrs and Weatherley (1962). The seedling samples were weighed to obtain fresh weight (FW). The samples were immediately hydrated by soaking in double distilled water in a closed Petri dish for 4 h under normal room light and temperature for turgidity. Thereafter the samples were taken out, wiped with tissue paper and immediately weighed to obtain turgid weight (TW). Samples were then packed in butter paper and dried at 80°C for 24 hr and weighed evaluate dry weight (DW). Relative water content of the samples was calculated as per the following formula:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Enzyme Assays

The second set of treatment seedlings were used for investigating the levels of enzymes activity in response to prolonged dehydration periods of 22 days. 1 g of plant tissue from control and treated plants was homogenized on ice in 4 ml extraction buffer (50mM phosphate buffer pH 7.0, containing 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 1% polyvinylpyrrolidone). The homogenate was centrifuged for 25 min at 15 000 × g and 4°C. The supernatant was used for enzyme activity assays. The means ± SD were calculated from the data of at least 3 independent measurements. The activities of different enzymes such as APX, POX, CAT and SOD were determined spectrophotometrically.

Ascorbate Peroxidase (APX) Assay

APX activity was determined as described by Nakano and Asada (1981). The reaction mixture for the peroxidase contained 50 mM potassium phosphate, pH 7.0, 0.5 crude plant extract, 0.1 mM hydrogen peroxide and 0.1 mM EDTA in a total volume of 1 ml. The absorbance at 290 nm was recorded 10 to 30 sec after the addition of all the components in the reaction mix.

Catalase (CAT) and Superoxide dismutase (SOD) Assay

The activity of catalase as well as superoxide dismutase was assayed after the method of Chance and Maehly (1955) with the slight alterations. The reaction mixture included the crude plant extract in 300 μmoles of phosphate buffer, pH 6.8, 100 g moles of H₂O₂. After incubation at 25 C for 1 min, the reaction was stopped by adding 10 ml of 2% (v/v)

H₂SO₄. A control was run at the same time in which the enzyme activity was stopped at "zero" time. One unit of catalase activity is defined as that amount of enzyme which breaks down 1 μmol of H₂O₂ min under the assay conditions described.

Peroxidase (POX) Assay

The reaction mixture for peroxidase assay included the crude plant extract in 125 moles of phosphate buffer, pH 6.8, 50 moles of pyrogallol, 50 M moles of H₂O₂. This was incubated for 5 min at 25 C after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H₂SO₄. The amount of purpurogallin formed was determined by taking the absorbance at 420 nm (Shannon *et al.*, 1966).

Results

Relative Water Content

The relative water content (RWC) of drought treated 22 days old seedlings decreased with the increased duration of drought stress. The relative water content (RWC) of drought treated seedlings decreased with the increased duration of drought stress. The RWC of 22 days old seedlings decreased from 92.8% in control to 76.43% after 4 hrs of PEG treatment. However it increased to 85.62% in 8hrs, and further decreased to 56.20% after 48hrs (Table 1).

Table 1: Variation in Relative Water Content of 12 and 22 days *Pennisetum glaucum* (HHB-68) Seedlings under Normal and Various Drought Stress Conditions induced by PEG6000 (Osmotic Potential= -1.25)

PEG 6000 Treatment	Relative Water Content (%±S.E)	
	22 days seedlings	12 days seedlings
Control	92.80±0.010	98.81±1.031
30 Min	87.45±0.721	93.30±0.051
2 hrs	83.23±1.161	86.21±4.131
4 hrs	76.43±0.873	75.57±0.278
8 hrs	85.62±0.239	69.68± 0.571
16 hrs	65.74±0.313	68.75±5.421
24 hrs	62.25±0.772	58.55±0.421
48 hrs	56.20±0.283	44.75±0.327

Result are shown as mean + standard error (p<0.05) from three replicate

On the other hand, in the 12 days old seedlings, a constant degradation in the RWC level was observed where it decreased from 98.81% in control to 44.75% after 48 hrs of stress treatment.

Enzyme Activity

On 22nd day of germination, four different enzymes namely APX, POX, CAT and SOD were tested in the fresh seedling extracts for ascertaining their possible roles in drought stress for different time periods of stress induction. The level of catalase in the fresh extract of PEG treated plants

decreased from 56.5 U/g FW in control to 35.4 U/g FW in the seedling subjected to 48 h water deficiency. All other enzymes were found to have enhanced activity after prolonged treatment of drought. Level of SOD increased up to 2.2 fold in 48 h drought induced seedlings. Similarly APX and POX levels also accelerated up to 3.5 fold and 4.2 fold, respectively at 48 h of treatment (Fig. 1).

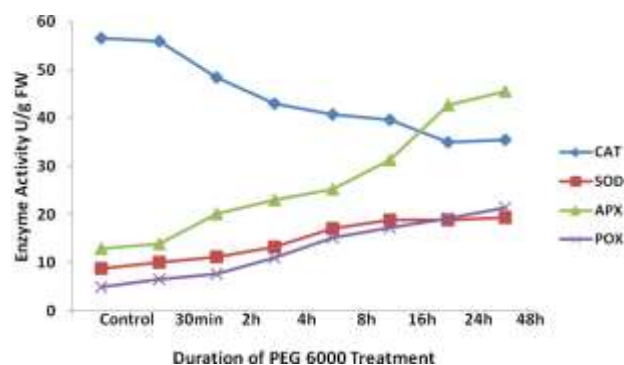


Fig. 1: Variation in Enzyme (CAT, SOD, APX, POX) Activity of 22days *Pennisetum glaucum* (HHB-68) seedlings under Normal and Various Drought Stress Conditions induced by PEG6000 (Osmotic Potential= -1.25)

Discussion

Drought is one of the most important manifestations of abiotic stress in plants. It is the major yield-limiting factor of crop plants and it actively and continuously determines the natural distribution of plant species (Carvalho, 2008). As sessile organisms, plants have to cope with drought stress at least at some point in their life cycle. They have however evolved mechanisms that allow them to adapt and survive periods of water deficit, if not at the whole plant level, at some level or form of plant structure.

Our study shows that pearl millet seedling had highest RWC (92.80%) in control (no water stress condition) and gradually with increasing duration of low water stress with conditions, RWC decreased with lowest (56.2%) at 48 hr of drought induction. However it was observed that at 8 hr of low water stress condition, RWC of seedlings dropped to as low 85.62%. It is quite possible that plant system tried to rebound back to water deficit stress by adapting various mechanisms to escape, avoid or tolerate drought stress (Levitt *et al.*, 1972), although these are not mutually exclusive. The plant drought response will depend on the species inherent "strategy" but also on the duration and severity of the drought period. If prolonged over to a certain extent drought stress will inevitably result in oxidative damage due to the over production of reactive oxygen species (Mittler, 2002). Reactive oxygen species (ROS), also called active oxygen species (AOS) or reactive oxygen intermediates (ROI) are the result of the partial reduction of atmospheric O₂. There are basically four forms of cellular ROS, singlet oxygen (O₂), superoxide radical (O₂⁻),

hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO^\cdot), each with a characteristic half-life and an oxidizing potential. ROS can be extremely reactive, especially singlet oxygen and the hydroxyl radical and, unlike atmospheric oxygen, they can oxidize multiple cellular components like proteins and lipids, DNA and RNA. Unrestricted oxidation of the cellular components will ultimately cause cell death (Levine *et al.*, 1994).

In our study, there was observed decrease in the CAT activity with the increase in the duration of drought stress. On the other hand, the levels of SOD and POX increase maximally up to 3.5 and 4.2 folds respectively from the control to maximum period of drought induction (48 h). Superoxide Dismutase, Catalyses and peroxidase (SOD, CAT and POX) play an essential role in scavenging for H_2O_2 toxicity. The combined action of CAT and SOD converts the toxic superoxide radical (O_2^\cdot) and hydrogen peroxide (H_2O_2) to water and molecular oxygen (O_2), thus averting the cellular damage under unfavorable conditions like drought stress (Noctor *et al.*, 2000; Reddy *et al.*, 2000; Chaitanya *et al.*, 2002). The decline in CAT activity is regarded as a general response to many stresses (Abedi and Pakniyat, 2010). CAT catalyses the dismutation of hydrogen peroxide into water and oxygen whereas peroxidase decompose H_2O_2 by oxidation of substrates (McKersie and Leshem, 1994). In the present study, the CAT activity was found decreasing with the increasing duration of drought induction. The level of catalase in the fresh extract of PEG treated plants decreased from 56.5 U/g FW in control to 35.4 U/g FW in the seedling subjected to 48 h water deficiency. The decrease in CAT activity indicates its inactivation by the accumulated H_2O_2 induced by water shortage and can be explained partly by photo inactivation of the enzyme (Zhang and Kirkham, 1990). The increased levels of POX and SOD justifies the reduced activity Catalase (CAT) in enhanced drought stress by corroborating that in Pearl Millet these might be the active scavenging system.

The major scavenging mechanisms include superoxide dismutase (SOD), enzymes and metabolites from the ascorbate-glutathione cycle, and catalase (CAT) (Noctor and Foyer, 1998, Willekens *et al.*, 1997). They are located throughout the different compartments of the plant cell, with the exception of catalase that is exclusively located in peroxisomes. SOD is the front-line enzyme in ROS attack since it rapidly scavenges superoxide, one of the first ROS to be produced, dismutating it to oxygen and H_2O_2 (Bowler *et al.*, 1992). However, this reaction only converts one ROS to another, and H_2O_2 also needs to be destroyed since it promptly attacks thiol proteins. The major enzymatic cellular scavengers of H_2O_2 are catalase and ascorbate peroxidase (APX) (Noctor and Foyer, 1998, Willekens *et al.*, 1997).). They have however different affinities or this ROS and seem to have different cellular roles in H_2O_2

scavenging. In fact CAT does not need a reductant to scavenge H_2O_2 making it reducing power-free, whereas APX needs a reductant, ascorbate. On the other hand, CAT has a lower affinity for H_2O_2 (mM range) than APX (mM range) (Mittler, 2002). It has been shown that ascorbic acid is an important plant metabolite has been implicated in the regulation of different processes associated with plant growth and development and maintains the osmotic status of the stressed tissue (Prabha and Bharti, 1980).

Moreover, in the chloroplast, the Mehler reaction occurring during photosynthesis is an important alternative sink for electrons, but it produces superoxide as side effect. This active oxygen species is however rapidly dismutated by a membrane bound superoxide dismutase (SOD), producing H_2O_2 . H_2O_2 is then locally converted to water by ascorbate peroxidase (APX). This may be possible explanation of the elevated levels APX in the extreme drought conditions obtained in our results.

Conclusion

Drought is the major yield-limiting physiological factors affecting the crop harvest worldwide. The major drought resistant plant cultivars possess various enzymatic and non-enzymatic machineries to combat the negative effects of drought. The present paper summarizes the significant effects of SOD, CAT, APX, POX in drought stress induced by PEG 6000 in *Pennisetum glaucum* and identifies the study of enzymatic modifications as an important parameter to scrutinize the mechanisms involved in drought stress which could be useful to the plant breeders to cultivate effective drought resistant cultivars. However, the unraveling of the enzymatic systems and their functioning in other important crops remains an area to explore further.

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Conflict Of Interest Statement

We declare that we have no conflict of interest.

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