*Indian J. Plant Physiol.*, Vol. 10, No. 4, (N.S.) pp. 344-348 (Oct.-Dec., 2005)

# DIFFERENTIAL RESPONSE OF PEARL MILLET HYBRIDS TO WATER STRESS IN RELATION TO ANTIOXIDANT ENZYMES AND PROLINE

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Received on 31 May, 2005, Revised on 12 Jan., 2006

## SUMMARY

**Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important crop of semi-arid tropics. Due to erratic rainfall, the crop is generally exposed to water stress at different stages, which decreases growth and yield of plants in many ways. In the present study the female parent ICMA 94555 was crossed with eight inbred male lines in summer, 2004. Hybrids were evaluated under terminal drought condition at three different stages, each at 10 days interval (*i.e.* at 60, 70 and 80 DAS) in *Kharif*, 2004 to assess the antioxidant enzymes activity and proline content. Terminal drought condition was created by withdrawing irrigation at 50 days after sowing. Among the eight hybrids tested, four hybrids *viz.*, ICMA 94555 x J 2340, ICMA 94555 x J 2405, ICMA 94555 x J 108 and ICMA 94555 x J 2290 showed increased activities of superoxide dismutase (SOD) and non specific peroxidase (POX) at second and third stage, while catalase (CAT) and ascorbate peroxidase (APOX) activity increased only at second stage. The CAT and APOX activity in these four hybrids recorded 2-3 folds increase as compared to first stage. The results suggested that moisture stress affected the activity of antioxidant enzymes involved in detoxification of activated oxygen species and $H_2O_2$ differently. Proline content increased in all hybrids at second and third stage but these four hybrids showed 2-3 fold higher proline level as compared to first stage. Thus, these four hybrids indicated biochemical adaptability to tolerate water stress condition.**

***Key words*: Antioxidant enzymes, pearl millet, proline, reactive oxygen species, terminal water stress.**

## INTRODUCTION

Pearl millet crop is generally exposed either to terminal or mid season water stress. Water stress triggers varying responses in cellular metabolic activities of plants, which in turn are reflected in growth and yield. At cellular level, water stress induces the production of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\bullet}$), which ultimately cause membrane damage (Tambussi *et al.* 2000). One of the adaptations of the water stress tolerant species / cultivars is the dominance of a defense system of antioxidant enzymes. These enzymes includes superoxide dismutase (SOD), which scavange superoxide radicals and converts them to $O_2$ and $H_2O_2$, $H_2O_2$ is then detoxified by catalase (CAT) and / ascorbate peroxidase (APOX). In addition, non specific-peroxidase plays important role in the antioxidative protection. In higher plants, there is also a strong correlation between increased cellular proline level and the capacity to survive water deficit. The present investigation was, therefore, carried out to study the effect of terminal water stress on activities of some antioxidant enzymes and proline content in pearl millet hybrids in relation to drought tolerance.

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## MATERIALS AND METHODS

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] genotypes were sown at plant breeding farm, B. A. College of Agriculture, Anand, Anand Agril. University, Anand during summer and *Kharif* 2004. The female parent ICMA 94555 was crossed with eight inbred male lines i.e. J 2454, J 2340, J 998, J 2405, J 108, J 2290, J 2440 and J 104 in *summer* 2004 and hybrids were evaluated under terminal drought condition in *Kharif* 2004. Terminal drought condition was created by withdrawing irrigation at 50 days after sowing (DAS) (*i.*e. after boot leaf stage). Antioxidant enzymes activities and proline content were estimated at three different stages, each at 10 days interval (*i.e*. at 60, 70 and 80 DAS) for all above mentioned eight hybrids.

Leaf samples were collected from third upper leaf. Fresh leaves (0.5 g) were ground in a pre-chilled mortar and pestle under ice cold conditions in 5 ml 50 mM sodium phosphate buffer, pH 7.0 with the addition of 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 % (w/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 10,000 g at 4 $^0$C for 20 min. The supernatant was used for the enzyme assays (Krisztina *et al.* 2002).

CAT, APOX and POX activities were determined according to the method of Costa *et al.* (2002). CAT activity was determined in the homogenates by measuring the decrease in absorption at 240 nm in a 3 ml reaction medium containing 50 mM sodium phosphate buffer (pH 7.2), 2 mM $H_2O_2$ and 0.2 ml enzyme extract. APOX activity was measured immediately in fresh extracts and was assayed using 3 ml reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0), 0.1 ml enzyme extract, 0.1 mM $H_2O_2$, 0.5 mM ascorbate and 0.1 mM EDTA. The hydrogen peroxide dependent oxidation of ascorbate was followed by a decrease in the absorption at 290 nm. POX activity was determined in the homogenates by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol in a reaction mixture containing 50 nm sodium phosphate buffer (pH 7.0), 0.1 ml enzyme extract, 0.1 mM EDTA, 10 mM guaiacol and 10 mM $H_2O_2$.The enzymes units were calculated using formula given by Kokkinakis and Brooks (1979).

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Van Rossun *et al.* (1997). The 3 ml reaction mixture contained 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 0.1 ml enzyme extract, 75µM NBT and 2 µM riboflavin, which was added last and tubes were shaken and placed 30 cm below a light bank consisting two fluorescent tube light. The reaction was initiated by switching on the light and was allowed to run for 10 minutes while switching off the light stopped the reaction. The absorbance of the reaction mixture was read at 560 nm. A non-irradiated reaction mixture did not develop colour and served as control. The reaction mixture-lacking enzyme developed the maximum colour and this decreased with increasing volume of enzyme extract. One unit of SOD activity was taken as the quantity of enzyme that reduced the absorbance reading to 50 per cent in comparison with those lacking enzymes.

For estimation of proline content fresh leaves (0.5 gm) were ground in mortar and pestle with 10 ml of 3 % sulphosalicylic acid and the homogenate was centrifuged at 5000 g. Two ml of the filtrate was reacted with 2 ml acid ninhydrin (containing 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml of 6M phosphoric acid) and 2 ml glacial acetic acid in test tubes and kept for one hour at 100 $^0$C in water bath. The test tubes were transferred to an ice bath. The reaction mixture was vortexed with 4 ml of toluene. Toluene layer was separated and absorbance was read at 520 nm. A standard curve of proline was used for calibration. (Bates *et al.* 1973). Samples were analysed in triplicate and data were analysed using simple CRD (Panse and Sukhatme 1967).

## RESULTS AND DISCUSSION

In all the hybrids 10 days of water stress did not follow any clear-cut pattern of antioxidant enzymes activity in relation to consecutive stress. The hybrid nos. 2, 4, 5 and 6 showed about 2-3 fold increase in APOX activity at 20 days of water stress over 10 days of water stress (Table 1). The activity of APOX in above four hybrids was more or less maintained at 30 days of water stress and showed higher rate of detoxification. The hybrid nos. 1, 3, 7 and 8 showed more or less similar

**Table 1.** Activities of ascorbate peroxidase (APOX) and catalase (CAT) in eight hybrids of pearl millet at three different stages of terminal drought stress.

| Hybrids | APOX (units min $^{-1}g^{-1}$ fw) | | | CAT (units min $^{-1}g^{-1}$ fw) | | |
|---|---|---|---|---|---|---|
| (Number) | I | II | III | I | II | III |
| ICMA 94555 x J 2454 (1) | 8.70 | 5.90 | 7.30 | 3.45 | 2.80 | 3.00 |
| ICMA 94555 x J 2340 (2) | 5.15 | 17.50 | 16.85 | 2.50 | 7.95 | 3.00 |
| ICMA 94555 x J 998 (3) | 6.05 | 6.70 | 8.90 | 3.75 | 4.10 | 4.85 |
| ICMA 94555 x J 2405 (4) | 5.10 | 13.20 | 11.30 | 4.95 | 10.95 | 3.25 |
| ICMA 94555 x J 108 (5) | 5.30 | 13.35 | 8.20 | 2.30 | 7.75 | 3.60 |
| ICMA 94555 x J 2290 (6) | 8.60 | 14.40 | 10.15 | 3.45 | 6.25 | 3.55 |
| ICMA 94555 x J 2440 (7) | 9.65 | 8.20 | 9.25 | 3.15 | 3.20 | 2.15 |
| ICMA 94555 x J 104 (8) | 7.10 | 7.60 | 8.60 | 3.85 | 3.90 | 4.15 |
| SE m± | 0.192 | 0.599 | 0.595 | 0.195 | 0.453 | 0.114 |
| CD 0.05% | 0.575 | 1.796 | 1.784 | 0.575 | 1.357 | 0.342 |
| CV % | 4.72 | 9.56 | 10.23 | 9.71 | 13.37 | 5.74 |

APOX activity at all three stage. APOX is the important enzyme in $H_2O_2$ detoxification (Noctor and Foyer 1998), catalyses the reduction of $H_2O_2$ to water by ascorbate.

CAT activity (Table 1) at 20 days stress increased 3.10, 2.21, 3.37 and 1.01 fold in hybrid nos. 2, 4, 5 and 6 compared to 10 days stress. The CAT activity was also 2-3 fold higher in above hybrids compared to hybrid nos. 1, 3, 7 and 8 at 20 days stress. Catalase scavenges $H_2O_2$ by breaking down directly to form water and oxygen and an increase in its activity is related with increase in stress tolerance (Kraus *et al.* 1995). CAT is indispensable for ROS detoxification during stress (Willekens *et al.* 1997).

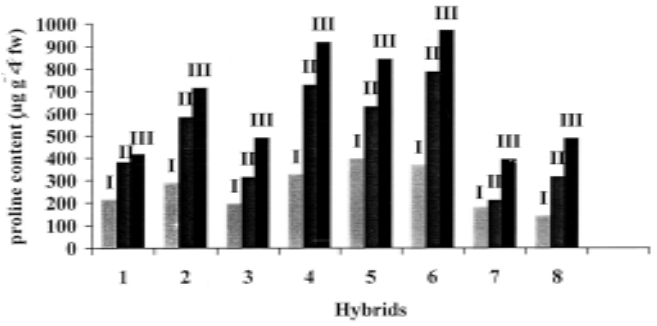
Peroxidase (POX) activity increased in hybrid nos. 2, 4, 5 and 6 at second and third stages (20 days and 30 days of water stress) while, decreased in hybrid nos. 1, 3, 7 and 8 compared to I$^{st}$ stage (10 days water stress). The activity of POX in hybrid 2 increased by 52.31 % at second stage followed by hybrids no. 4, 5 and 6 where the increase was 44.47 %, 4.44% and 7.18 % compared to first stage (Table 2). The study, therefore, showed an increase in activity of enzymes having protective role in hybrid nos. 2, 4, 5 and 6. Similar results were obtained in upland rice (Srivalli *et al.* 2003) and in drought tolerant varieties of black papper (Thankamani *et al.* 2003).

The activity of SOD showed an increase at 20 days and 30 days water stress compared to 10 days water stress in hybrid nos. 2, 4, 5, 6 and 8 (Table 2). The increase in SOD activity was highest at 30 days of water stress in hybrid 4, which was 2.34 fold more compared to 10 days water stress. Hybrid nos. 2, 5, 6 and 8 showed 36.95, 94.05, 43.28 and 45.07 % increase in activity compared to 10 days stress, respectively. Water stress induced increase in SOD activity has been reported in maize (Jhagtap and Bhargava 1995). Superoxide dismutase being one of the protective enzymes during water stress (Blum and Eberun 1981) an increase in its activity under stress situation is expected, especially in the stress tolerant varieties. The declining activity of SOD and POX in hybrids 1, 3 and 7 results in accumulation of $H_2O_2$ and consequently greater cell injury. The perusal of results reflects an increased activity of the scavenging enzymes *viz.* SOD, POX, APOX and CAT in response to water stress. Similarly, higher activities of these enzymes were also reported by Zhang and Kirkham (1996) in sorghum and Srivalli *et al.* (2003) in rice cultivars. However, Sairam *et al.* (1998) found higher activities of only APOX and CAT in drought tolerant wheat genotypes.

**Table 2.** Activities of superoxide dismutase (SOD) and peroxidase (POX) in eight hybrids of pearl millet at three different stages of terminal drought stress

| Hybrids | SOD (units min $^{-1}g^{-1}$ fw) | | | POX (units min $^{-1}g^{-1}$ fw) | | |
|---|---|---|---|---|---|---|
| (Number) | I | II | III | I | II | III |
| ICMA 94555 x J 2454 (1) | 17.31 | 15.30 | 14.70 | 66.40 | 57.33 | 65.17 |
| ICMA 94555 x J 2340 (2) | 16.23 | 13.83 | 22.2 | 48.80 | 53.22 | 74.33 |
| ICMA 94555 x J 998 (3) | 24.71 | 12.43 | 11.53 | 92.00 | 51.60 | 71.60 |
| ICMA 94555 x J 2405 (4) | 16.30 | 21.53 | 38.27 | 45.20 | 54.73 | 65.30 |
| ICMA 94555 x J 108 (5) | 25.23 | 29.55 | 48.97 | 57.60 | 57.17 | 60.17 |
| ICMA 94555 x J 2290 (6) | 27.33 | 31.61 | 39.17 | 57.80 | 60.47 | 61.95 |
| ICMA 94555 x J 2440 (7) | 33.43 | 18.90 | 19.50 | 64.80 | 45.12 | 52.45 |
| ICMA 94555 x J 104 (8) | 13.30 | 15.30 | 19.17 | 84.20 | 69.85 | 74.25 |
| SE m± | 0.560 | 0.466 | 0.482 | 1.884 | 0.740 | 0.634 |
| CD 0.05% | 1.707 | 1.397 | 1.447 | 5.648 | 2.218 | 1.901 |
| CV % | 4.54 | 4.08 | 3.13 | 5.05 | 2.28 | 1.67 |

High proline content was observed in leaves and roots of *Lathyrus sativus* under water stress that can withstand drought (Tyagi *et al.* 1999). The proline content was found to increase both at 20 and 30 days of water stress in all the hybrids. Maximum accumulation was observed in hybrid nos. 2, 4, 5 and 6, which was 2.49, 2.82, 2.13 and 2.64 fold more compared to 10 days water stress. The increases in proline level suggest greater osmoprotection under drought stress.




C.D. at 5%, I= 1.98, II= 1.73, III= 1.91; **I-** First stage, **II-** Second stage and **III-** Third stage

**1.** ICMA 94555 x J 2454, **2.** ICMA 94555 x J 2340, **3.** ICMA 94555 x J 998, **4.** ICMA 94555 x J 2405, **5.** ICMA 94555 x J 108, **6.** ICMA 94555 x J 2290, **7.** ICMA 94555 x J 2440, **8.** ICMA 94555 x J 104

**Fig. 1. Proline content (µg $g^{-1}$ fw) in eight hybrids of pearl millet at three different stages of terminal drought stress**

Second stage of stress, (20 days after boot leaf stage), exhibited increase in all the enzymes and proline content in hybrid nos. 2, 4, 5 and 6. APOX and CAT showed maximum increase at second stage of stress, and hence better management of $H_2O_2$ levels. With further increase in water stress intensity (30 days after boot leaf stage) there was a shift, suggesting a greater role of SOD and POX in the management of oxidative stress. These results suggest that coordinated defense mechanism is required to protect plants from oxidative damage. Among the eight hybrids tested, four hybrids, *viz.* ICMA 94555 x J 2340 (2), ICMA 94555 x J 2405 (4), ICMA 94555 x J 108 (5), ICMA 94555 x J 2290 (6) showed acclimation to terminal water stress and showed enhanced antioxidant enzyme activity required to protect plants from stress induced oxidative damage.

## ACKNOWLEDGMENTS

The authors are thankful to Millet scientist, Millet Research Station, Jamnagar for providing seed material for this study.

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