Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.8017

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (4):** 327-335 (2016)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Evaluation of Proline Metabolizing Enzymes in Pigeonpea under PEG Induced Drought Stress

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Received: 10.06.2016 | Revised: 18.07.2016 | Accepted: 20.07.2016

ABSTRACT

Drought stress triggered by changes in the external water potential is an environmental commonplace in the life of a higher plant. The compatible osmolytes, such as proline plays an important role and act as osmoprotectants during stress. Though several attempts have been made to establish a relationship between proline accumulation and drought tolerance, not much is known about the regulation of proline metabolism in pigeonpea grown under drought conditions. It is, therefore, of equal importance to study the regulation of this biochemical phenomenon in plants during drought stress. In the present research, we anaylzed the activities of proline and proline metabolizing enzymes in two previously screened tolerant and susceptible pigeonpea cultivar under Polyentylene glycol induced drought stress. The proline accumulation data revealed that the tolerant cultivar GRG-295 accumulated higher proline content at different time intervals of drought stress. The comparative analysis of proline metabolizing enzymes like ornithine aminotranferase, P-5-C reductase, P-5-C dehydrogenase and Proline dehyrogenase were also carried out and it was revleaed that the accumulation of these enzymes were relatively higher in GRG-295 as compare to TAT-9903during different time interval of drought stress. The free proline content was gradually increased upon increase in stress duration in both the cultivars and significant higher activity was observed in GRG-295 as compare to TAT-9903. The activity of Ornithine aminotransferase and P-5-C reductase was increased upon increasing the stress period (upto 6 hour) and thereafter declined in bothe the cultivars while decrease in activity of proline oxidase and P-5-C dehydrogeanse was observed in both the cultivars upon increase in stress duration. The higher activities of these enzymes suggest their adaptive role in drought tolerant in pigeonpea. The present study indicated that accumulation of proline may provide a biochemical adaptation for plants during drought stress.

Key words: *Pigeonpea, Proline, Proline oxidase, Ornitihine aminotransferase, P5C reductase*

INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is an important grain legume crop of rain fed agriculture in the semi-arid tropics. The Indian sub-continent, eastern Africa, and Central America, in that order, are the world's three main pigeonpea-producing regions.

Cite this article: Ranjan, T., Rajani, K., Kumar, A. and Kumar, R.R. Evaluation of Proline Metabolizing Enzymes in Pigeonpea under PEG Induced Drought Stress, *Int. J. Pure App. Biosci.* **4(4):** 327-335 (2016). doi: http://dx.doi.org/10.18782/2320-7051.8017

Pigeonpea is cultivated in more than 25 tropical and sub-tropical countries, either as a sole crop or intermixed with such cereals as sorghum, pearl millet, maize, or with other legumes, e.g., groundnut. In India, it is grown in an area of 3.63 mha^{-1} with a production of 2.12 mt and productivity of 584 kg ha^{-1} (Sameer Kumar et al., 2014).

Being a legume, pigeonpea enriches the soil through symbiotic nitrogen fixation. A short-day plant with a deep root system, pigeonpea tolerates drought, but is highly sensitive to water logging. The crop is cultivated on marginal land by resource- poor farmers, who commonly grow traditional medium- and long-duration (5-11 months) landraces. Pigeonpea experiences severe drought condition during its developmental phases. The response of the plant during drought stress is much complex. One of the most common induced responses in all organisms undergoing water deficit is the production and/or accumulation of so called compatible osmolytes. Some osmotically active, neutral organic compounds such as sugars (polyols), certain amino acids, and quaternary ammonium compounds being generated during stress (Ahanger et al., 2014). By lowering water potentials, the accumulation of compatible osmolytes allows additional water to be taken up from the environment, thus buffering the immediate effect of water shortages within the organism. Compatible osmolytes continue to accumulate during prolonged water deficit, and it has been proposed that they may help to stabilize protein tertiary structure as cells dehydrate (Low, 1985). The amino acid proline is perhaps the most widely distributed compatible osmolyte. In organisms from bacteria to maize, there is a strong correlation between increased cellular proline levels and the capacity to survive both water deficit and the effects of high environmental salinity. In plants, the role of proline may not be restricted to that of a compatible osmolyte. For example, proline synthesized during water deficit may serve as an organic nitrogen reserve that can be utilized during recovery. There is also evidence that the degradation of proline in mitochondria (which takes place all the time) is directly coupled to the respiratory electron transport system and ATP production (Elthon and Stewart, 1981). Proline degradation could therefore improve the energy status of cells recovering from water deficit (Lawlor, 1995). Accumulation of proline could be due to de novo synthesis or decreased degradation or both. Proline is synthesized not only from glutamate, but also from arginine/ornithine (Kavi Kishor et al., 2005). The pathway from glutamate is the primary route for the synthesis of proline under conditions of osmotic stress and nitrogen limitation, while the pathway from ornithine predominates at high levels of available nitrogen (Yoshiba et al., 1997). The first two steps of proline biosynthesis are catalyzed by a bifunctional enzyme pyrroline-5-carboxylate synthetase (P5CS) which also behaves as a limiting step in proline biosynthesis. Proline is then produced from the action of Pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2) following nonenzymatic ring closure of L-glutamic semialdehyde to P5C. Ornithine, the alternate precursor can be transaminated to P5C by ornithine aminotransferase (OAT), a mitochondrial enzyme. Catabolism is the oxidation of organic nutrients into simple end molecules to extract energy. For proline, the catabolic pathway involves two enzymes, L-proline dehydrogenase (PRODH, EC 1.5.99.8) and L-pyrroline-5-carboxylate dehydrogenase (P5CDH, EC 1.5.1.12). PRODH is an FAD-dependent enzyme that catalyzes the oxidation of proline to P5C. As in proline biosynthesis, equilibrium is established between P5C and its hydrolysis product L-glutamic semialdehyde. The latter molecule is the substrate for the second enzyme of proline catabolism, P5CDH, which catalyzes the oxidation of the semialdehyde to glutamate. P5CDH is an NAD -dependent enzyme having a catalytic Cys. (Verbruggen N and Hermans, 2008; Lehmann et al., 2010)

The present research was proposed to investigate the role of proline metabolizing

enzymes like; proline oxidase, ornithine transferease, P5CR and P5CS under drought stress condition.

MATERIALS AND METHODS

Plant Growth and Stress induction

The seeds of GRG 295 (drought tolerant) and TAT 9903 (drought sensitive) pigeonpea cultivars reported by Kumar et al. (2011) were used for the present study. The seeds of two different varieties were treated with 0.1% $HgCl_2$ for 1 min and washed with distilled water for three to four times. The pots of portable size were obtained and checked for proper aeration. Then 1:1 ratio of sand and soil was mixed properly and added in each pots. About 20 seeds were sowed in each pot of both varieties. On the first day just moistened and later were watered everyday according to plants necessity. The seeds were allowed to grow for a month and after that the direct stress induction was given as according to Kandpal et al (1981). The stress was induced with the help of PEG-6000. 40% PEG solution was prepared and then plants were dipped into the solution along the roots and stress was induced for 0, 6, 12, 18 and 24 hrs under 100W bulb. After the given stress period, the activities of various proline metabolizing enzymes, free proline content and relative water content were carried out.

Relative Water Content

Relative water content of leaves after each stress treatment was measured and it was compared with control. The leaves were dipped in 50 ml of distilled water for 6 hours and allowed to gain turgidity. The turgid weight were recorded and dried in oven at 80^0C for 24 hrs and dry weight of sample was recovered. RWC was quantified and expressed in percentage using the following formula:

$$\text{RWC} = \frac{Fresh\ wt. - Dry\ Wt.}{Turgid\ Wt. - Dry\ Wt.} \text{ x } 100$$

Free Proline Content

The extraction and estimation of free proline of the leaves was done according to Bates et al., (1973). 500mg of plant material was homogenized in the mortar with pestle using 10ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered with four layered muslin cloth and the filtrate was collected. The extraction was repeated twice and all the filtrate were pooled and made up to known volume. 2ml of filtrate was taken into a test tube and 2ml of acid ninhydrin and 2ml of glacial acetic acid were added. The tubes were incubated at 100^0C for 1hr in boiling water bath. The tubes after incubation were transferred to an ice bath to terminate the reaction. 4ml toluene was added to the contents of the tubes and mixed thoroughly using a test tube stirrer for 15sec. Chromophore containing toluene was aspirated from aqueous phase. Then the absorbance of the solution was measured at 520nm UV-Visible spectrophotometer against toluene as blank. Proline was measured from the standard curve prepared with authentic proline and its amount was calculated on their dry weight basis.

Enzyme assays

Preparation of homogenate for activities of Ornithine aminotransferase and pyrroline-5-carboxylate dehydrogenase

The leaves were ground in a cold pestle and mortar with glass powder (1:1 wt/wt) and 0.1M potassium phosphate buffer (pH 7.4) containing 1 mM pyridoxal-5-phosphate; 1 mM EDTA; and 10 mM ß-mercaptoethanol. The resulting slurry was filtered through cheese cloth yielding about 20 ml of liquid. The filtrate was then centrifuged for 10 min at 12,000g. The supernatant was collected and used for enzyme assays.

Preparation of the homogenate for measuring the activities of proline oxidase and pyrroline-5-carboxylate dehydrogenase

The leaf tissue was ground in a cold pestle and mortar with glass powder (1:1 wt/wt) and 0.1? potassium phosphate buffer (pH 7.4) containing 0.5% (v/v) triton X-100. The resulting slurry was filtered through cheese cloth and then centrifuged at 1000 g for 10 min. The supernatant was used for enzyme assays.

Ornithine aminotransferase assay

The assay mixture consisted of 0.1M potassium phosphate buffer (pH 8.0); 50 mM ornithine; 20 mM α-ketoglutarate; 1 mM pyridoxal-5'-phosphate and enzyme in a total volume of 1.0 ml. After incubation at 37°C, the reaction was terminated by adding 1.0 ml of 10% trichlorocacetic acid. The colour was developed by adding 2 ml of 0.5% o-aminobenzaldehyde in 95% ethanol. The mixture was further incubated for 10 min and then centrifuged to remove the denatured proteins. The control tube was devoid of the enzyme extract. The product formed was determined by measuring the absorbance at 440nm in a Pye-Unicam spectrophotometer. Enzyme activity units were calculated by using a molar extinction coefficient of 2.71×10 M cm for pyrroline-5-carboxylic acid.

Proline oxidase assay

The assay mixture consisted of 15 mM L-proline; 0.01 mM cytochrome c. 0.1M potassium phosphate buffer (pH 8.0); 0.5% (v/v) triton X-100; and enzyme extract in a total volume of 1.0 ml. The incubation was carried out at 37°C for 30 min and the reaction terminated by adding 1.0 ml of 10% trichloroacetic acid. The color was developed by incubating the reaction mixture with 2.0 ml of 0.5% o-aminobenzaldehyde in 95% ethanol for 10 min. The denatured proteins were removed by centrifugation and absorbance measured against a control at 440 nm. The enzyme extract was not added in the control. Enzyme activity units were calculated as described for ornithine aminotransferase.

Pyrroline-5-carboxylate dehydrogenase assay

The enzyme activity was monitored by following the increase in absorbance of NADH formed at 340 nm. The reaction mixture consisted of 1 mM pyrroline-5-carboxylate; 0.6 mM NAD ; 0.1 M potassium phosphate buffer (pH 8.0); 0.5% (v/v) triton X-100 and enzyme extract in a total volume of 3.0 ml. The incubation was carried out at room temperature (20-25°C). The enzyme activity was expressed as nmol of NADH formed/min/g fresh weight.

Pyrroline-5-carboxylate reductase assay

The enzyme activity was followed by measuring the decrease in absorbance at 340nm due to oxidation of NADH. The reaction mixture consisted of 1 mM pyrroline-5-carboxylate; 0.12 mM NADH; 0.1 M potassium phosphate buffer (pH 7.4) and enzyme extract in a total volume of 3 ml. The reaction mixture was incubated at room temperature and the decrease in absorbance at 340 nm was followed. The enzyme activity was expressed as nmol of NADH oxidized/min/g fresh weight.

RESULTS AND DISCUSSION

GRG 295 and TAT 9903 cultivar seeds previously screened for their tolerance to PEG induced osmotic stress by Kumar et al (2011a).Their studies suggested that GRG 295 had a significant germination percentage under PEG stress condition but TAT9903 showed good germination response under control conditions but germinated poorly on decreasing osmotic potential of PEG solutions.

PEG is commonly used to stimulate osmotic stress effects in petridishes to control water potential in seed germination studies. The non toxic PEG solution is used because of high molecular weight and cannot pass through plant cell walls. (Emmerich and Hardegree,1990; Kaydan et al, 2007). PEG has also been used to impose water stress by exposing the root system of plants to resolve the problem for understanding plant tolerance and oxidative stress in response to water deficit. Kandpal et al. (1981), Zgallai et al. (2005) and Kumar et al (2011a&b) previously used PEG 6000 to create water stress in plants ,they reported that PEG solution cause water stress by withdrawal of water from plants without any toxicity at plant as well as seedling level.

Relative Water Content

It was observed that the relative water content progressively decreased by increasing duration of the stress in both the cultivars. About 92% relative water content was noted in GRG 295 and TAT 9903 at control plants. After 6 hours stress the RWC content was about 60% in GRG295 while it was 56% in

TAT 9903. After 12 hr of stress only 10% of RWC content was observed in GRG 295 while 7% was observed in TAT 9903 (Fig- 1)

Decrease in RWC both drought tolerant and susceptible cultivars has reported in Rice by Choudhary et.al (2005). Our result is also supported by Kandpal et. al (1981). Kumar et.al (2011) observed decrease in RWC more in susceptible genotype than the tolerate one 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 maintains cell turgor under water stress environment to give relative high yield.

Free Proline Content

The results of the free proline content is depicted in Fig. 2. Increased level in proline contents in both cultivars were observed after 6hrs and 12hrs of water stress as compare to the control plants. A sharp decrease of proline content was observed after 18 and 24hrs of water stress. Our results suggested that, the proline synthesis was higher up to 12hrs of water stressed plants and proline degradation starts after 12hrs of stress plants. The proline content of different time intervals of PEG treatment is shown in the Fig. 2. The increase in proline content was higher in GRG 295 as compared to TAT 9903. Severe progressive stress in pigeon pea leads to about 25 folds more accumulation of proline while 6 folds increase was observed in progressive mild stress (Kumar et.al 2011). Zgallai et.al (2005) observed 10 fold increase in proline accumulation under PEG water stress in tomato plants. Proline content increase significantly in seedling of rice genotype upto 5hrs and increases for higher in tolerant plants as compared to susceptible one (Choudhary et al., 2005).

It is reported that proline accumulation may help in maintaining high WRC for growth and cellular functions. Proline concentration is directly propotional to salinity level or to intensity of water stress condition (Yang et al, 2000)

Proline Metabolizing Enzyme Assay

The specific activity of OAT was increased in GRG 295 cultivar upto 6 hour and after that a significant decrease in activity was observed at 12 and 24 hours of stress condition. A maximum of 1.75 fold increase was observed at 12 hours of stress treatment as compare to TAT 9903. In TAT 9903, a decrease in OAT activity was noted after 6 hours of stress treatment but a sharp increase in OAT activity was observed after 12 hours of stress treatment (Fig. 3). The detection of OAT enzyme suggests that an alternative pathway of proline synthesis in pigeon pea may exist at seedling level. An increase in OAT activity in Ragi leaves were detected after 2 hours of PEG stress treatment by Kandpal et al. (1981). Increase in OAT activity along with an elevation in the level of free proline was also reported in wheat under cold stress (Charest and Phan, 1990) and in *Brassica juncea* under salt stress

In plants proline is not only synthesised from glutamate but from Arginine /Ornithine. In plants, GSA is derived directly from ornithine by the enzyme ornithine amino transferase (Kavi Kishore et.al, 2005). In *V. aconitifolia*; OAT activity was reduced by 60% with removal of pyridoxal phosphate but the level increase in plants supplied excess nitrogen. These results suggest that OAT pathway operates when plants are supplied with high nitrogen (Delauney and Verma, 1993). Pigeon pea is legume plant and so it has capacity to fix atmospheric nitrogen. In Arabidopsis the ornithine pathway appeared to be playing an important role in proline accumulation during osmotic stress.

The enzyme activity of P-5-C reductase was significantly increased under stress condition in both the cultivars. In GRG 295 2.25 fold increase in P-5-C reductase activity are observed after 12 hours of stress condition thereafter decrease in enzyme activity. In TAT 9903 1.39 fold increased in enzyme activity was observed after 12 hours of stress condition as compared to control. The increase in P5CR activity suggest that enhanced synthesis of proline may be occurring due to increased activity of P5CR as

well as OAT during water stress condition (Fig. 4).

Accumulation and developmental regulation of the transcript encoding P5CR in Arabidopsis suggests that it plays an important role in proline synthesis in rapidly dividing cells and /or in cells undergoing changes in osmotic potential (Kavi Kishore et al, 2005). The results presented in this study show that the avtivities of OAT and P5CR increased by about 3.5 fold (Kandpal et al., 1981).

Decrease in the activity of proline oxidase as well as P5C dehydrogenase was observed at all stress period in both cultivars. The result represented in the Fig. 5 and 6 exhibit that the activities of proline oxidase and P5CDH was decreased by about 3 fold and 3.24 fold in GRG 295 and TAT 9903 respectively. Level of proline degrading enzyme proline oxidase activity was inhibited in the roots by drought stress when compared to control in all the bhendi varities (Sankar et.al., 2007). Remarkable decrease in PO enzyme activity in stress leaves of *Eleusine Corocana* (Kandpal et.al., 1983)

Proline catabolism is repressed under osmotic stress, once the stress is withdrawn proline is oxidised to P5C by proline oxidase, the first enzyme of proline degradation pathway. P5C is converted to Glutamate by P5C dehydrogenase (Kavi Kishore et al, 2005). The activities of enzyme responsible for the degradation of proline probably result in the increase levels of proline.

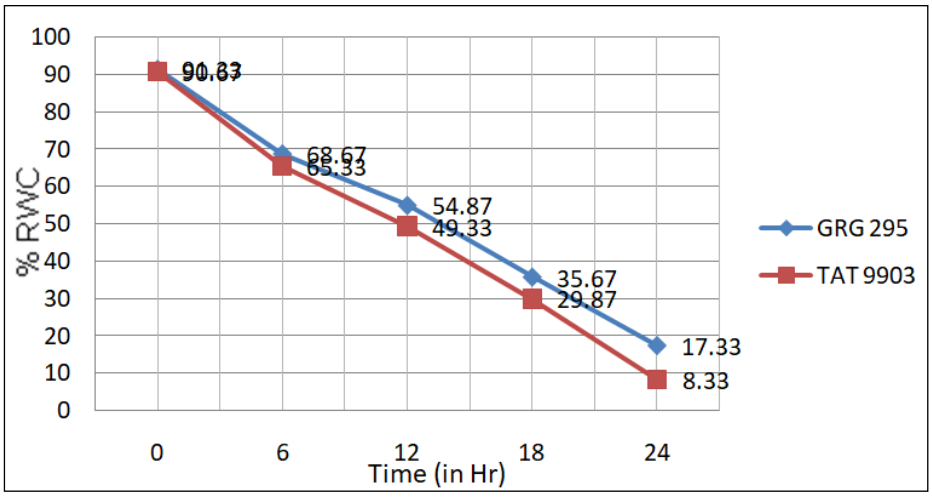

Fig. 1: Graph Showing the percentage of Relative Water Content after different hour of drought stress periods induced by PEG

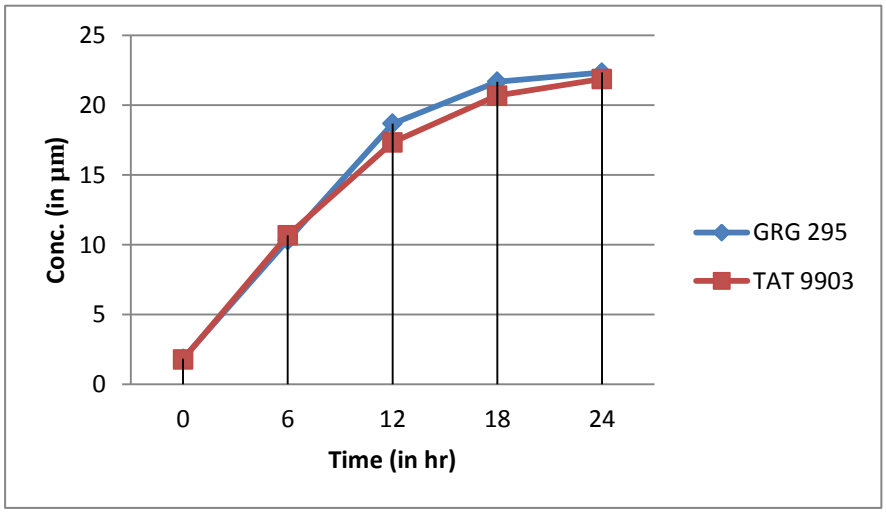

Fig. 2: Graph Showing the activity of proline after different hour of water stress periods induced by PEG

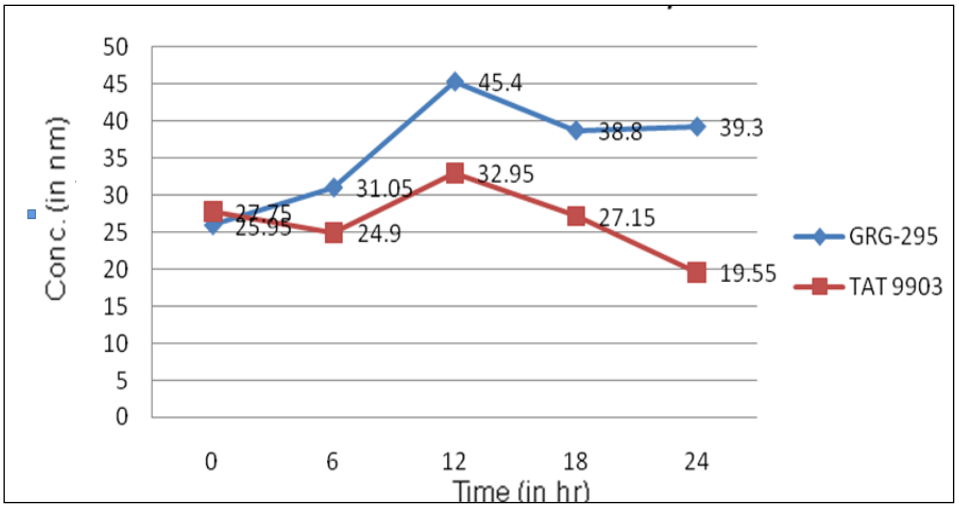

Fig. 3: Graph showing the activity of Ornithine aminotransferase after different hour of water stress periods induced by PEG

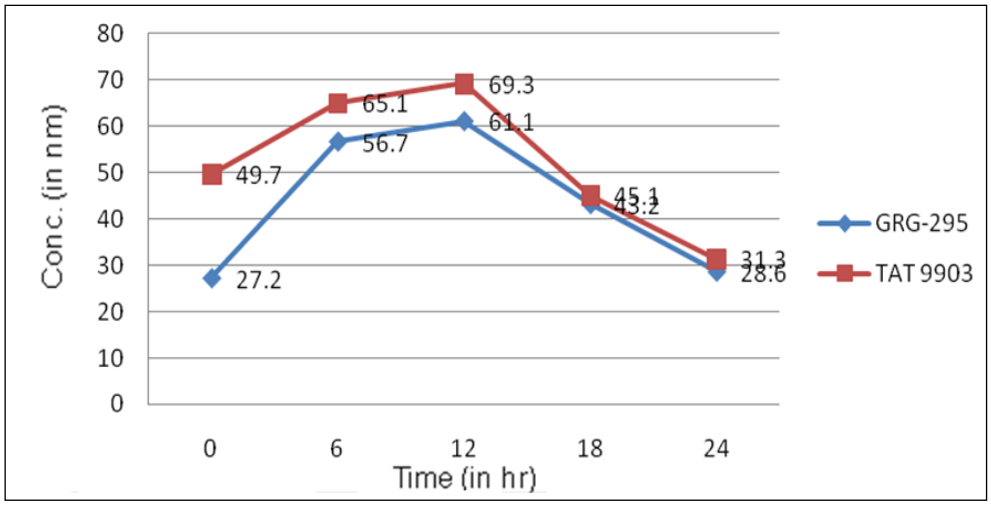

Fig. 4: Graph showing the activity of P5C reductase after different hour of drought stress induced by PEG

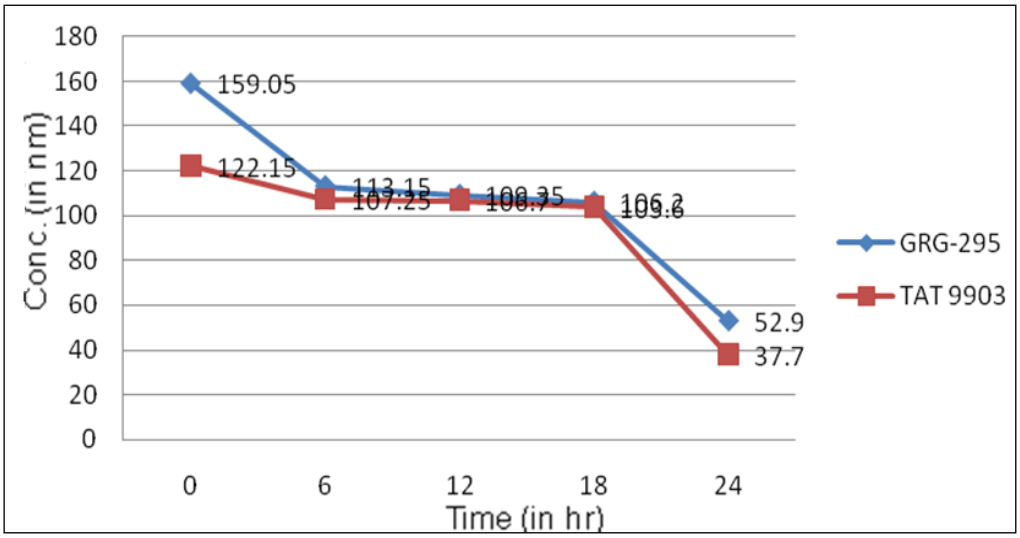

Fig. 5: Graph showing the activity of Proline Oxidase after different hour of drought stress induced by PEG

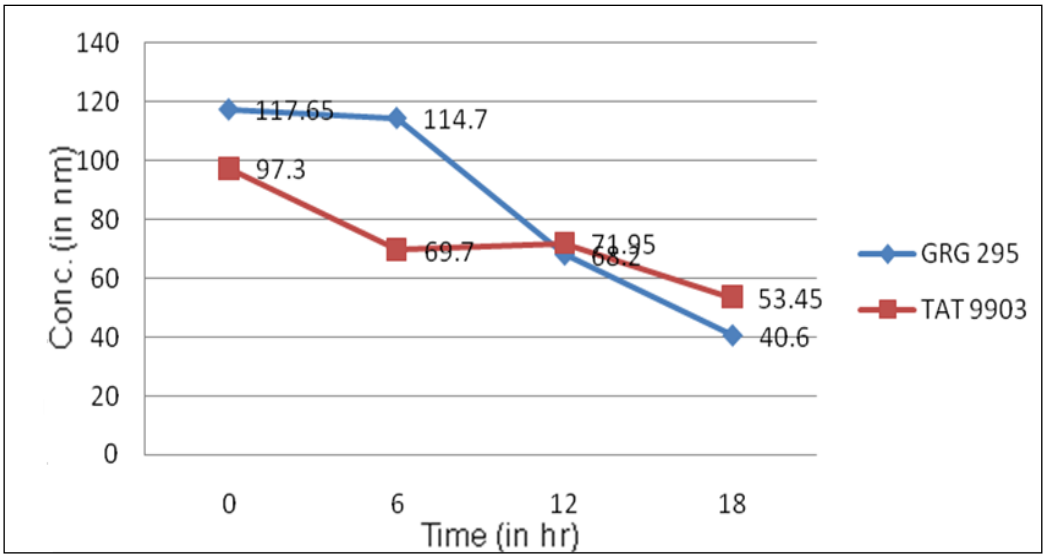

Fig. 6: Graph showing the activity of P5C dehydrogenase after different hour of drought stress periods induced by PEG

CONCLUSION

In our present investigation, proline and proline metabolizing enzymes were studied in normal and PEG induced water stress conditions of both GRG-295 and TAT-9903 cultivars. The results clearly indicated that proline expression level was increased under stress conditions in comparably resistant pigeonpea cultivar than the susceptible one, which may play a protective role in combating higher stress. The study of biochemical analysis of proline metabolizing enzymes suggests that the Ornithine pathway was also active in pigeonpea at seedling level along with the glutamate pathway. The results delineate the pathway for the biosynthesis and degradation of proline, emphasize the role of proline in the metabolism of plants subjected to water stress and suggest possible reasons for the increased levels of proline observed during water deprivation. The osmoprotectant Proline plays a cardinal role under drought stress condition. It can be indicated that accumulation of proline may provide a biochemical adaptation for plants during water stress. Although, our study is in preliminary stage, a detail studies on proline and proline metabolizing enzymes at field level under drought stress condition will provide concrete information on protective role of proline under stress conditions.

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