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Differential response of proline metabolizing enzymes in drought tolerant and drought sensitive varieties of wheat (*Triticum aestivum* L.) during grain development

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Abstract
Wheat (*Triticum aestivum* L.) plants exposed to drought stress show unfavorable changes at the cellular and molecular level. Under draught stress plants adjust osmotic mechanism by production of proline in the cell. Proline metabolizing enzymes are crucial for selection of draught stress tolerance by accumulation of compatible osmolytes. The present study depicts the activities of two major proline metabolizing enzymes *viz.* Δ^1 pyrroline-5-carboxylate synthetase and Δ^1pyrroline -5-carboxylate reductase are increased under drought stress compared to irrigated condition in both leaves and developing grains of wheat varieties. Plants accumulate proline under drought stress condition which is normally catabolized to pyrroline-5-carboxylate by proline oxidase. The activity of proline oxidase is decreased under drought stress both in leaves and developing grains there by provides osmoprotection and made plants tolerant to drought.

Keywords: Differential, proline, metabolizing, tolerant and drought, *Triticum aestivum* L.

Introduction
Wheat is the most widely cultivated food crop and is eaten in various forms by more than one thousand million human beings in the world. In India, it is second important staple food crop after rice in respect of area and production and it occupies about 12 per cent of total wheat production of the world. Wheat compares well with other important cereals in its nutritive value. It contains more protein than other cereals and is of special significance. Wheat crop contributes substantially to the national food security by providing more than 50 per cent of the calories to the people who mainly depend on it. Due to its wider adaptability it can be grown under various agro-climatic conditions.

Plants are exposed to many biotic and abiotic stresses that affect their growth and productivity. These stresses may cause unfavorable changes at the cellular and molecular levels in plants. Among various stresses, drought stress is one of the major limitations to crop productivity. Improving drought tolerance and productivity is one of the most difficult tasks for cereal breeders. The difficulty arises from the diverse strategies adopted by the plants themselves to combat drought stress depending on the timing, severity and stage of crop growth. One of the consequences of drought stress is the excessive generation of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen. Although ROS can function as signaling molecules for plant growth, development and defence, the uncontrolled production of ROS can be detrimental. ROS can inactivate biomolecules and initiate auto catalytic peroxidation of the membrane and other macro molecules such as photosynthetic pigments, protein, lipids and nucleic acids, resulting in the loss of membrane integrity and some functional modifications. Thus, their levels must be closely and carefully monitored inside the cell.

Osmotic adjustment is a mechanism to maintain water relations under osmotic stress. Synthesis and accumulation of proline has been advocated for use as a parameter of selection of drought stress tolerance (Beemarao *et al.* 2007) [2]. High levels of proline enables a plant to maintain low water potentials and accumulation of compatible osmolytes involved in osmoregulation. Proline accumulation in stressed plant is a well documented phenomenon, but little is understood in plant stress biology, further a cause and effect relationship between proline and drought has not been fully established (Mimoun *et al.* 2006; Gobinathan *et al.* 2009) [15, 5]. Proline has been demonstrated to ameliorate dehydration induced perturbations in proteins (Kavi *et al.* 1995) [10] and a protective agent for cytoplasmic enzymes as well a reservoir

of nitrogen and carbon source for post stress growth. Accumulation of proline can be correlated with cell damage, therefore it is probably one of the consequences of stress as a result of reciprocal regulation of two pathways where, upregulation of proline synthesizing enzymes and down regulation of proline degrading enzymes (Rayapati *et al.* 1989; Kavi *et al.* 1995; Ashish *et al.* 2008) [20, 10, 1]. The relationship between antioxidants level and stress tolerance has been demonstrated in transgenic plants. Studies on proline biosynthetic enzymes indicated that the gene *P5CS* which encodes Δ^1-pyrroline-5-carboxylase synthetase is induced by dehydration in *Arabidopsis* (Yoshu *et al.* 1995) [30]. Δ^1-pyrroline-5-carboxylate reductase activity increased under salt stress in foxtail millet (Veeranagamallaiah *et al.* 2007) [26]. The level of proline degrading enzyme proline oxidase activity was inhibited in the roots by drought stress when compared to control in all okra varieties (Bheemarao *et al.* 2007) [2]. The activities of proline biosynthetic enzymes (Δ^1-pyrroline-5-carboxylate reductase and ornithine amino transferase) increased considerably in *Brassica juncea* in tolerant lines under salt stress while the activity of proline degrading enzyme (proline oxidase) decreased (Madan *et al.* 1995) [13].

Materials and Methods

Seeds of two wheat varieties WH 1105 (Drought sensitive) and WH 1025 (Drought tolerant) were sown in micro plots of Chaudhary Charan Singh Haryana Agricultural University farm, Hisar for drought and irrigated condition. Normal irrigation schedules were given for irrigated condition where as drought was created by giving pre sown irrigation only to the fields. Leaf and grain samples were collected after anthesis starting from 7, 14, 21 and 28 days by keeping an interval of seven days. Leaf and grain extracts were prepared and were used for assaying three proline metabolizing enzymes.

Extraction

For the extraction of Δ^1 – Pyrroline-5-carboxylate synthetase (P5CS) and Δ^1 – Pyrroline-5-carboxylate reductase (P5CR) the extraction medium used contained 0.1 M potassium phosphate buffer (pH 7.4) having 1 mM pyridoxal-5-phosphate, 1 mM EDTA, 10 mM β-mercapto ethanol and 1% polyvinyl pyrrolidine (PVP) while for proline oxidase 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5% triton X-100 was used. The tissue was thoroughly washed and extracted by grinding in a chilled mortar-pestle with the respective extraction medium. The resulting slurry was filtered through four layer of cheese cloth and the filtrate was centrifuged for 10 min. at 10,000 × g at 4 ºC. This supernatant was used for the enzyme assays.

Assay of enzymes

Pyrroline-5-carboxylate: Pyrroline-5-carboxylate (P5C) was prepared in simple aqueous solution from its 2, 4-dinitrophenylhydrazine derivative (Mezl & Knox, 1976). 30 mg of 2, 4-dinitrophenylhydrazone and 0.2 ml of acetophenone for each milligram of hydrazone were added to 5.0 ml of 0.25 N HCl. The suspension was shaken for 30 min. on a Burrel shaker until the entire colour dissolved in acetophenone layer. This layer was removed and the aqueous layer extracted again with an equal volume of toluene. The amount of pyrroline-5-carboxylate was estimated by the method decribed by Wiilams & Frank (1975) [27], where pyrroline-5-carboxylate reacts with 20 mM O-aminobenzaldehyde in 5% TCA (w/v). One ml of O-aminobenzaldehyde solution was added to 2 ml of sample, followed by centrifugation at 30,000 ×g for 10 min. Absorbance at 443 nm of the clear supernatant was determined after 40 minutes of total incubation. The O-aminobenzaldehyde reagent was prepared immediately before use.

Δ^1 – Pyrroline-5-carboxylate synthetase (P5CS) (EC 2.7.2.11)

The enzyme was assayed by the method of Hu *et al.* (1992) [8]. The reaction mixture (1 ml) consisted of 75 mM glutamate, 100 mM Tris-HCl buffer (pH 7.2), 20 mM $MgCl_2$, 5 mM ATP and 0.4 mM NADPH. The reaction was initiated by adding NADPH and the decrease in absorbance was measured at 340 nm in spectrophotometer.

Δ^1 – Pyrroline-5-carboxylate reductase (P5CR) (EC1.5.1.2)

The enzyme activity was assayed by the method of Lutts *et al.* (1999). The reaction mixture (1 ml) consisted of 50 mM Tris-HCl buffer (pH 7.0), 1 mM dithiothreitol, 0.25 mM NADH and 1 mM pyyroline-5-carboxylate. The reaction was initiated by adding 0.1 ml of enzyme extract and the decrease in absorbance of NADH was monitored at 340 nm. P5CR activity was determined by using an extinction coefficient of 6.2 mM cm^{-1} for NADH. One unit of P5CR is defined as the amount of enzyme required to oxidize one nanomole of NADH per min.

Proline Oxidase (PO) (EC 1.5.99.8)

Proline oxidase was assayed by the following method of Strecker (1971) [22]. The reaction mixture contained 15 mM L-proline, 0.01 mM cytochrome C, 0.1 mM potassium phosphate buffer (pH 8.0) 0.5% (v/v) triton X-100 and an enzyme extract in a total volume of 1 ml. After incubation at 37 ºC for 30 min. the reaction was terminated by adding 1.0 ml of 10% trichloroacetic acid and colour 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 at 440 nm. Enzyme activity was calculated using a molar extinction coefficient of 2.71 x 10^3 M^{-1} cm^{-1} for pyrroline-5-carboxylic acid.

Results

Effect of drought stress proline metabolizing enzymes

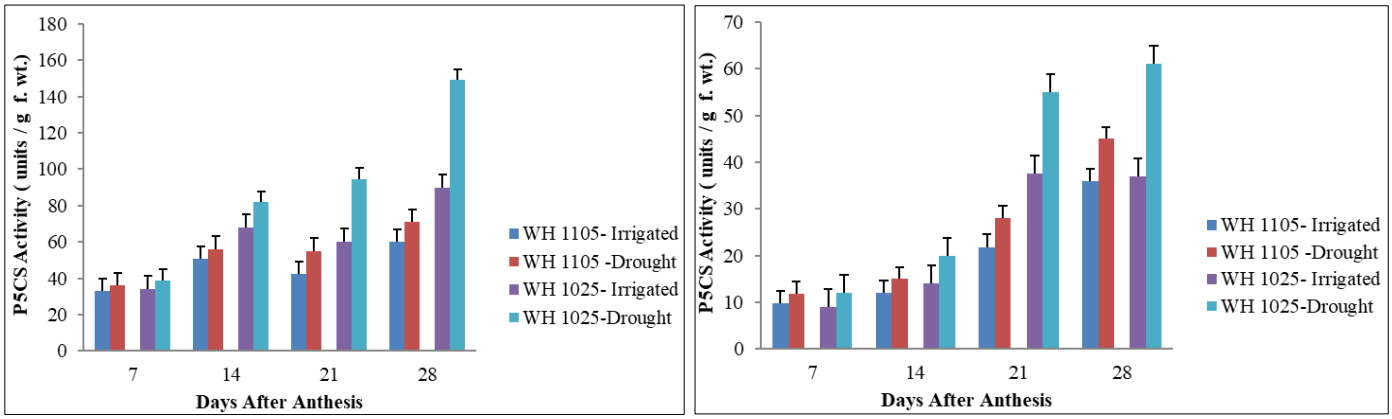
Δ^1-Pyrroline-5-carboxylate synthetase (P5CS)

The activity of P5CS increased in leaves and developing grains of the both wheat varieties grown under drought stress condition compared to irrigated condition during different stages of grain development (Fig. 1A).

The per cent increase of P5CS activity exhibited was 9.09, 9.80, 29.89 and 18.33 at 7^{th}, 14^{th}, 21^{st} and 28^{th} days after anthesis respectively in the leaves of WH 1105 under drought stress compared to irrigated condition. While in leaves of WH 1025 the per cent increase exhibited was 14.71, 20.59, 57.90 and 65.56 at 7^{th}, 14^{th}, 21^{st} and 28^{th} days after anthesis respectively. However the magnitude of per cent increase under drought stress was more in leaves of WH 1025 than WH 1105 during various stages of grain development.

In developing grains of WH 1105 the P5CS activity was increased under drought stress (Fig. 1B). The per cent increase exhibited was 20.83, 25.08, 28.09 and 25.20 at 7^{th}, 14^{th} 21^{st} and 28^{th} days after anthesis respectively; similarly in

WH 1025 the per cent increase exhibited was 33.33, 42.86, 46.36 and 64.86 at 7th, 14th, 21st and 28th days after anthesis respectively under drought stress. However the P5CS activity was found to be higher in developing grains of WH 1025 than WH 1105 during various stages of grain development.



(A) (B)

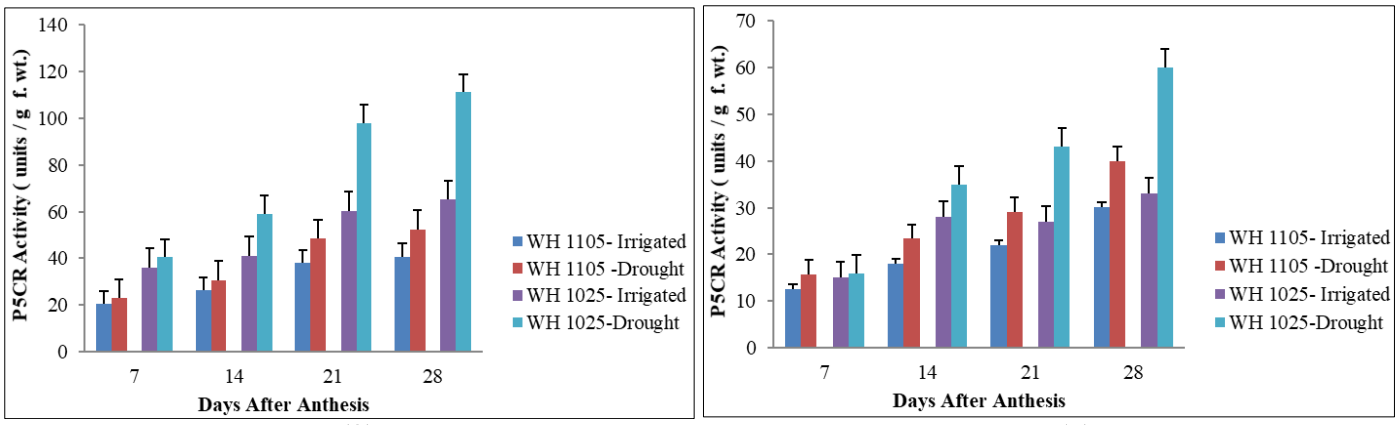
Fig 1: Effect of drought stress on Δ1-Pyrroline-5-carboxylate synthetase activity in leaves (A) and developing grains (B) of wheat at different days after anthesis

Δ1-Pyrroline-5-carboxylate reductase (P5CR)

The activity of P5CR increased in leaves of the both wheat varieties grown under drought stress condition as compared to irrigated condition during different stages of grain development (Fig. 2A). The per cent enhancement in activity of P5CR ranged from 12.28 to 28.69 with respect to different grain developmental stages in leaves of WH 1105. While in WH 1025 the per cent increase in P5CR activity was more than WH 1105 with the increased activity ranged between 12.80 to 70.45 per cent from 7th to 28th days after anthesis.

In developing grains of WH 1105, the P5CR activity was increased under drought stress condition (Fig. 2B). The per cent increase under drought stress showed an initial decrease (25.39 to 24.72) from 7th to 14th days after anthesis and later increase (32.63 to 33.04) from 21st to 28th day after anthesis. While in WH 1025, the P5CR activity was increased under drought stress condition (6.67 to 81.99 per cent) from 7th to 28th days after anthesis with an increasing trend.

Proline oxidase (PO): Results in Fig.3 (A and B) shows the activity of proline oxidase in leaves of two wheat varieties under irrigated and drought condition during grain developmental stages (Fig. 3A). The proline oxidase activity was decreased under drought stress condition as compared to irrigated condition in leaves of WH 1105. The per cent reduction ranged from 3.65 to 33.33 from 7th to 28th days after anthesis. The leaves of WH 1025 also showed a similar pattern where the activity of proline oxidase decreased under drought stress as compared to irrigated condition during grain development. The per cent reduction varied from 3.19 to 39.76 from 7th to 28th days after anthesis. The proline oxidase activity decreased more in leaves of WH 1025 than WH 1105 under drought stress.



(A) (B)

Fig 2: Effect of drought stress on Δ1-Pyrroline-5-carboxylate reductase activity in leaves (A) and developing grains (B) of wheat at different days after anthesis

The activity of proline oxidase decreased in developing grains of both wheat varieties under drought stress condition as compared to irrigated condition (Fig. 3B). The per cent reduction varied from 8.36 to 41.02 from 7th to 28th days after anthesis in WH 1105 while in WH 1025 the per cent reduction varied from 34.46 to 23.77 from 7th to 14th days after anthesis and later varied from 38.89 to 40.30 from 21st to 28th days after anthesis. The proline oxidase activity in developing grains decreased more in WH 1025 than WH 1105 under drought stress.

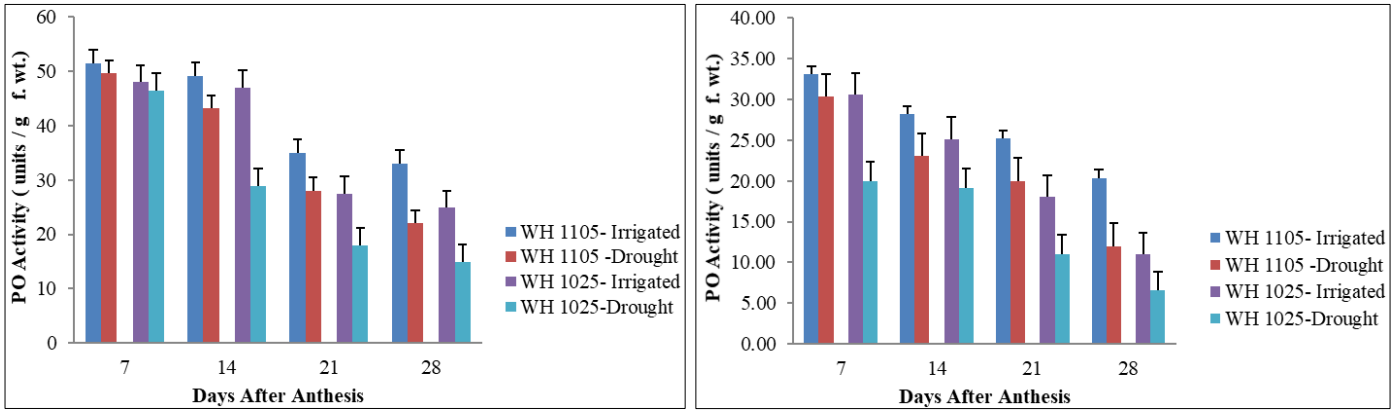


Fig 3: Effect of drought stress on proline oxidase activity in leaves (A) and developing grains (B) of wheat at different days after anthesis.

Discussion

The accumulation of proline is one of the adaptive mechanisms that plants operate for survival under stress (Hare *et al.* 1998; Kavi *et al.* 2005) [7, 9]. Under environmental stress, proline acts as an antioxidant by utilizing NADPH for the reduction of glutamate to pyrroline-5-carboxylate (P5C) and further from P5C to proline and generates $NADP^+$, which is otherwise prevented due to reduced rate of Calvin cycle resulting in production of singlet oxygen and accumulation of ROS. This generated $NADP^+$ is used as an electron acceptor and for restoration of Calvin cycle under stress conditions (Hare & Cress, 1997) [6]. Several studies have indicated that Δ^1 pyrroline -5 carboxylate synthetase (P5CS) is critical enzyme in proline biosynthesis under salt and water stress (Yamada *et al.* 2005) [28]. The stimulation of proline biosynthesis has been associated with increased P5CS mRNA levels (Savoure *et al.* 1995; Yamchi *et al.* 2007) [21, 29]. The present investigation depicts that P5CS activity increased under drought stress as compared to irrigated condition in leaves and developing grains of both wheat varieties at different stages after anthesis. However, more increase in P5CS was observed in WH 1025 than WH 1105 (Fig. 1). The results are in accordance with previous studies on rice where increase in P5CS activity was observed in both susceptible and tolerant genotypes and the increase was higher in tolerant genotypes (Choudhary *et al.* 2005) [3]. An increased activity of P5CS in transgenic tobacco plants resulted in accumulation of proline up to 10-18 folds over control plants and better growth under dehydration stress (Kavi *et al.* 1995; Sumithra & Reddy, 2004) [10, 24]. Similarly, Asish *et al.* (2008) [1] reported that P5CS activity significantly increased with duration of drought stress and highest activity was observed at 14 days after of stress.

Fig. 2 shows that Δ^1 pyrroline -5-carboxylate reductase (P5CR) activity increased in leaves and developing grains of both the wheat varieties grown under drought and irrigated conditions. However, more increase in P5CR activity was observed in WH 1025 than WH 1105. An increased activity of P5CR under salt stress condition has been reported by several authors (Sudhakar *et al.* 1993; Madan *et al.* 1995; Ramanjulu & Sudhakar, 2001) [23, 13, 19]. In parallel to these studies, the present study also observed an increase in activity of P5CR which was a positive sign to an increased accumulation of proline under drought stress. However, studies with tobacco cells expressing salt tolerance and over expression of the P5CR shows that enzyme activity was not correlated with differential salinity tolerance (La-Rosa *et al.* 1991) [11]. Sumithra & Reddy, (2004) [24] reported an elevation in the activity of P5CR in cowpea seedlings under water deficit.

In catabolic route, proline oxidase catalyzes proline oxidation to P5C which is maintained in a pH dependent non-enzymatic equilibrium with glutamate semialdehyde (GSA) (Tanner, 2008) [25]. In all the plants studied to date, transcription and enzymatic activity of proline oxidase decreases during osmotic stress and rapidly increase during consecutive recovery period. These changes are inversely correlated with cellular levels of free proline (Peng & Verma, 1996; Mattioni *et al.* 1997; Nakashima *et al.* 1998) [18, 14, 17]. In the present study, it was observed that proline oxidase activity decreased in leaves and developing grains of both the wheat varieties under drought stress as compared to irrigated condition. Enhanced activity of proline oxidase was observed in WH 1105 than WH 1025 at different developmental stages of grain under drought stress (Fig. 3). The results presented here coincide with earlier report concerning water stress in tomato (Fujita *et al.* 2003) [4]. Similarly, Beemarao *et al.* (2007) [2] also showed that under water deficit condition, proline metabolizing enzymes like proline oxidase decreased in all *Abelmoschus esculentus* varieties. A decrease in the activity of proline oxidase had also been reported by Madan *et al.* (1995) [13] in *Brassica juncea* under salt stress.

Conclusions

Activities of proline synthesizing enzymes *viz.* Δ^1 pyrroline-5-carboxylate synthetase and Δ^1 pyrroline-5-carboxylate reductase increased under stress in leaves and developing grains with much more increase in WH 1025 than WH 1105 at different grain developmental stages. The per cent increase in WH 1025 was maximum at 28th day after anthesis, while it was minimum at 7th day after anthesis in WH 1105. In the absence of stress, leaves and developing grains of WH 1105 had higher proline oxidase activity but under stress the proline oxidase activity decreased drastically with respect to different grain developmental stages. In general, antioxidative and proline synthesizing enzymes exhibited comparatively higher activity in WH 1025.

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