Indian Journal of Biochemistry & Biophysics
Vol. 55, April 2018, pp. 114-124

Modulation of proline metabolism under drought and salt stress conditions in wheat seedlings

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Received 07 April 2016; *revised 09 January 2018*

Drought and salinity are the major environmental constraints that limit plant growth and productivity. In the present investigation, shoots of seven day old plantlets of nineteen wheat genotypes (PBW621, PBW660, PBW175, HD3086, WH1105, HD2967, C306, C273, C518, C591, Type 11, Excalibar, Gladius, Drysdale, Babax, Krichauff, Kharchia, Krl 1-4 and Krl 19) were evaluated for proline metabolism and its cross-talk with various biochemical parameters under water deficit, water withholding and salinity stress conditions. Principle component analysis categorized the genotypes into four groups: *i.e.* drought tolerant (Excalibar, Krichauff, Babax, Drysdale, Gladius and C306), salt tolerant (Kharchia, Type11, Krl1-4 and Krl19), low stress tolerant (C273, C518 and C591) and susceptible (HD2967, PBW621, WH1105, HD3086, PBW660 and PBW175). Tolerant genotypes possessed increased proline content and 1,1 diphenyl-picryl hydrazyl (DPPH) radical scavenging activity along with the reduced magnitude of thiobarbituric acid reactive species in parallel with decreased H_2O_2 content. Proline accumulation in shoots of tolerant genotypes under stress conditions may be an adaptative strategy, as it supplies energy for growth and lowers the generation of free radicals and reduces the lipid peroxidation linked membrane damage resulting in their stabilization. Glutamate dehydrogenase might have played a dominant role in ammonium assimilation and glutamate biosynthesis, leading to an increased glutamate pool, which *via* pyrroline-5-carboxylate synthetase activity led to enhanced proline accumulation in tolerant genotypes under stress conditions. Water withholding condition induced the stimulation of proline synthesis *via* increased glutamate dehydrogenase (GDH), pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) activities with inhibition of oxidation *via* reduced proline dehydrogenase activity to a large extent as compared to water deficit and salt stress conditions. Our results highlight that in certain genotypes, GDH under water deficit, P5CS and PDH under salt stress and P5CR under water withholding stress condition were responsible for stress tolerance and could be used as a selectable marker.

Keywords: DPPH, Proline, Radical scavenging activity, Salinity, TBARs, *Triticum aestivum*, Water deficit

Water deficit induced by drought and salinity is the most harmful environmental factor to which plants are frequently exposed during their life cycle[1]. The limited availability of water caused by drought or salinity induces osmotic stress, therefore both stresses adversely affects the physiology of plants[2]. Reactive oxygen species (ROS) including superoxide radicals (O_2^-), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2) are through natural products of cell metabolism, but abiotic stresses enhance their rapid production and accumulation[3,4]. Among all ROS, H_2O_2 may act as structural defense signal molecule but a high level of H_2O_2 is cytotoxically leading to oxidative stress. Overproduction of ROS above constitutive level is potentially harmful to all cellular compounds and negatively influence cell metabolism[5].

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Membrane damage is also taken as a stress parameter to determine the level of lipid destruction. It has been recognized that products of lipid peroxidation are formed from polyunsaturated precursors that include small hydrocarbon fragments such as ketones, malondialdehyde (MDA) and compounds related to them[6]. Some of these compounds react with thiobarbituric acid to form coloured products called thiobarbituric acid reactive substances (TBARs)[7]. Acclimation to stress conditions is achieved by maintaining the lower level of H_2O_2 content and reduced lipid peroxidation. The stress tolerant plants show reduced H_2O_2 content and TBARs in contrast to sensitive ones[5].

The content of free proline has also been reported to increase in plants growing under abiotic stress conditions, and it has been proposed that proline accumulation can serve as an adaptive mechanism to abiotic stresses in higher plants[8]. The physiological

effect of proline accumulation may be expressed in sustained photosynthesis and osmoregulation and prevention of proteins, including enzymes, from degradation. Proline can also serve as a rapidly available source of nitrogen, carbon, and reduction equivalents during the recovery from stress[9]. The predominant mechanism of proline accumulation is its *de novo* synthesis from glutamate, although the decrease in catabolism and enhanced proteolysis may also be implicated[10]. In plants, proline is synthesized mainly from glutamate, which is reduced to glutamate-semialdehyde (GSA) by pyrroline-5-carboxylate synthetase (P5CS) enzyme which spontaneously converts it to pyrroline-5-carboxylate (P5C). Pyrroline-5-carboxylate reductase (P5CR) further reduces the P5C intermediate to proline[11,12]. Proline catabolism occurs in mitochondria *via* the sequential action of proline dehydrogenase (PDH) producing P5C from proline which converts P5C to glutamate[13].

In spite of several studies, the influence of proline metabolism on abiotic stresses is not clear. So biochemical consequences of drought and salt stress-mediated changes in defense mechanism especially in conjunction with proline metabolism are worthy of investigation. In the present study, we aimed at investigating the activities of the enzymes as well as the content of proline and its cross-talk with other biochemical parameters in the shoots of nineteen diversified wheat genotypes. Through this investigation, we expected to gain insight into the responses of proline metabolism and its potential roles under water withholding, water deficit, and salt stress conditions.

Materials and Methods

Plant material and water stress treatments

Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana, Punjab, India supplied the seeds of nineteen wheat genotypes *viz.* C306, C273, C591, C518, Type11, Excalibar, Krichauff, Babax, Gladius, Drysdale, Kharchia, Krl1-4, Krl19, PBW175, HD2967, PBW621, WH1105, HD3086 and PBW660. Seeds were surface sterilized with 0.1% mercuric chloride for 1 min and rinsed thoroughly with distilled water. These genotypes were grown in petri plates on germination paper moistened with 5 mL of distilled water at 25 ± 1°C in continuous dark conditions under control, water deficit, water withholding, and salt stress conditions. Water deficit conditions were generated by using 8% polyethylene glycol (PEG-6000) solution and to generate water withholding conditions, water was withheld for two days. Salinity stress conditions were maintained by using 300 mM NaCl solution. The seedlings were watered with distilled water regularly for three days and at the fourth day with PEG solution in water deficit and NaCl solution in salt stress whereas water was withheld for two days in water withholding condition. In the control as well stressed seedlings, growth parameters, biochemical parameters and enzymatic analysis were performed on shoots in triplicate.

Growth parameters

The shoot lengths were measured at seventh day of post germination (DPG) under control, water deficit, water withholding and salt stress conditions with meter scale. Fresh biomass of shoots was measured on the same day using weighing balance. For the dry weight analysis, roots were dried at 60°C in the oven till constant weight.

Extraction and estimation of H_2O_2

For extraction, 500 mg of fresh shoot tissue was homogenized in 1.5 mL of ice cold 0.1% TCA. Homogenate was passed through layers of cheesecloth and then centrifuged at 10000 g at 4°C for 15 min. The supernatant after centrifugation was used for estimation[14].

H_2O_2 was estimated by adding 0.5-1.0 mL of supernatant to 2 mL of a reaction mixture containing 4 mM of potassium iodide and 0.1 mM of potassium phosphate buffer (pH 7.0). Test tubes were incubated at room temperature in dark for 1 h. Absorbance was read at 390 nm against reagent blank. The amount of H_2O_2 was calculated by preparing standard curve of 50-200 nM of H_2O_2. H_2O_2 was expressed as μM of H_2O_2 g^{-1} dry weight.

Extraction and estimation of thiobarbituric acid reactive substances

The concentration of lipid peroxide products was determined in the shoot tissues in terms of thiobarbituric acid reactive substances (TBARs) content[15]. 0.5 g fresh tissues were homogenized in 0.1% TCA and mixed with 5 mL of TBA solution containing 0.5% (w/v) TBA in 20% TCA. The mixture was heated at 90°C for 30 min, cooled on ice and centrifuged at 10000 g for 15 min. The color was measured at A_{532} nm and A_{600} nm. An extinction coefficient 155 mM^{-1} cm^{-1} was used to quantify lipid peroxide content and expressed as μM TBARs g^{-1}dry weight

Extraction and estimation of proline

Proline was estimated according to the method of Bates *et al.*[16]. Above 500 mg shoot tissue was homogenized with 3% sulfosalicylic acid and the contents were centrifuged at 10000 g. A volume of 2 mL of glacial acetic acid and 2 mL of acid ninhydrin was added to 2 mL of tissue homogenate and incubated for 1 h in boiling water bath followed by cooling in an ice bath. About 4 mL of toluene was then added and mixed vigorously. The chromophore containing toluene was aspirated from the aqueous phase and the absorbance was measured at 575 nm.

Extraction and estimation of DPPH radical scavenging activity

Tissue (100 mg) was homogenized in 2 mL methanol and centrifuged at 10000 g for 20 min. 1 mL supernatant was added to 3 mL ethanol solution of DPPH radical[17]. The mixture was shaken vigorously for 1 min by vortexing and left to stand at room temperature in the dark for 30 min. Thereafter, the absorbance of the sample (sample A) was measured using the UV spectrophotometer at 517 nm against ethanol blank. A negative control (control A) was taken after adding DPPH solution to 0.2 mL of the respective extraction solvent. The percent of DPPH discoloration of the sample was calculated according to the equation:

$$\% \text{ discolouration} = [1 - (\text{sample A} / \text{control A})] \times 100$$

Determination of stress tolerance index (STI) of wheat genotypes

Drought tolerance index was measured by a modification of formula as described by Fischer & Maurer[18] as follows:

$$STI = \frac{(Ys)(yn)}{(Yn\tilde{})2}$$

Where Ys, DPPH radical scavenging activity/content of H_2O_2, TBARS or proline/shoot length of given genotype in stress condition; Yn, DPPH radical scavenging activity/content of H_2O_2, TBARS or proline/shoot length of given genotype in non-stress condition; Yn˜, mean DPPH radical scavenging activity/content of H_2O_2, TBARs or proline/shoot length of given genotype in non-stress condition.

Level of stress resistance was determined by using the median value of STI for each parameter studied under respective stress. The genotypes having STI ≥ median value for proline content, DPPH scavenging activity, length and biomass of shoot and ≤ median value for the content of H_2O_2 and TBARs were marked positive for stress resistance level.

Enzymatic assay for proline metabolism

Shoot tissue (100 mg) was homogenized in pre-chilled pestle and mortar in the extraction medium containing 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 10 mM 2-mercaptoethanol, 1% (w/v) polyvinylpolypyrrolidone, 5 mM $MgCl_2$ and 0.6 M KCl. The homogenate was centrifuged at 12000 g for 20 min at 4°C, the resulting supernatant was kept at 20°C and used for enzymatic assays[19].

GDH activity was assayed according to the method of Akihiro *et al.*[20]. The assay mixture comprised of 50 mm $(NH_4)_2SO_4$, 13 mM α-ketoglutarate, 0.25 mM NADPH and 1 mM $CaCl_2$ in 100 mM Tris-HCl buffer (pH 8). Absorbance was read at 340 nm and GDH activity was expressed as μmol $min^{-1}mg^{-1}$ protein. The P5CS activity was estimated as described by Silva-Ortega *et al.*[21]. The reaction mixture (3 mL) contained 100 mM Tris-HCl buffer (pH 7.2), 25 mM $MgCl_2$, 75 mM sodium glutamate, 5 mM ATP, and 0.2 mL of enzyme extract. The reaction was initiated by the addition of 0.4 mM NADPH. The activity was measured as the rate of consumption of NADPH monitored by the decrease in absorbance at 340 nm. The activity of P5CR was assayed following the method described by Lutts *et al.*[22]. The assay mixture contained 50 mM Tris-HCl buffer (pH 7.0), 1 mM dithiothreitol, 0.25 mM NADH and 1 mM D-pyrrolline-5-carboxylic acid. The reaction was started with the addition of 0.1 mL enzyme extract and the decrease in the absorbance of NADH was monitored at 340 nm for 3 min using a spectrophotometer. P5CR activity was determined by using an extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADH. PDH activity was examined by monitoring the $NADP^+$ reduction at 340 nm in 0.15 M Na_2CO_3 buffer (pH 10.3) containing 15 mM proline and 1.5 mM $NADP^+$[23].

Statistical analysis

Data were statistically analysed by multifactor ANOVA (CPCS1). Values are presented as a means ± SD (n = 3) and are represented as bars in the graph. Multivariate Principle Component Analysis was applied to categorize the genotypes[24].

Results

Growth and biomass

Water deficit, water withholding, and salt stress conditions caused a significant variation in the shoot length and biomass of all wheat genotypes (Fig.1 A-C). For instance, C 306, C591, C518, Type

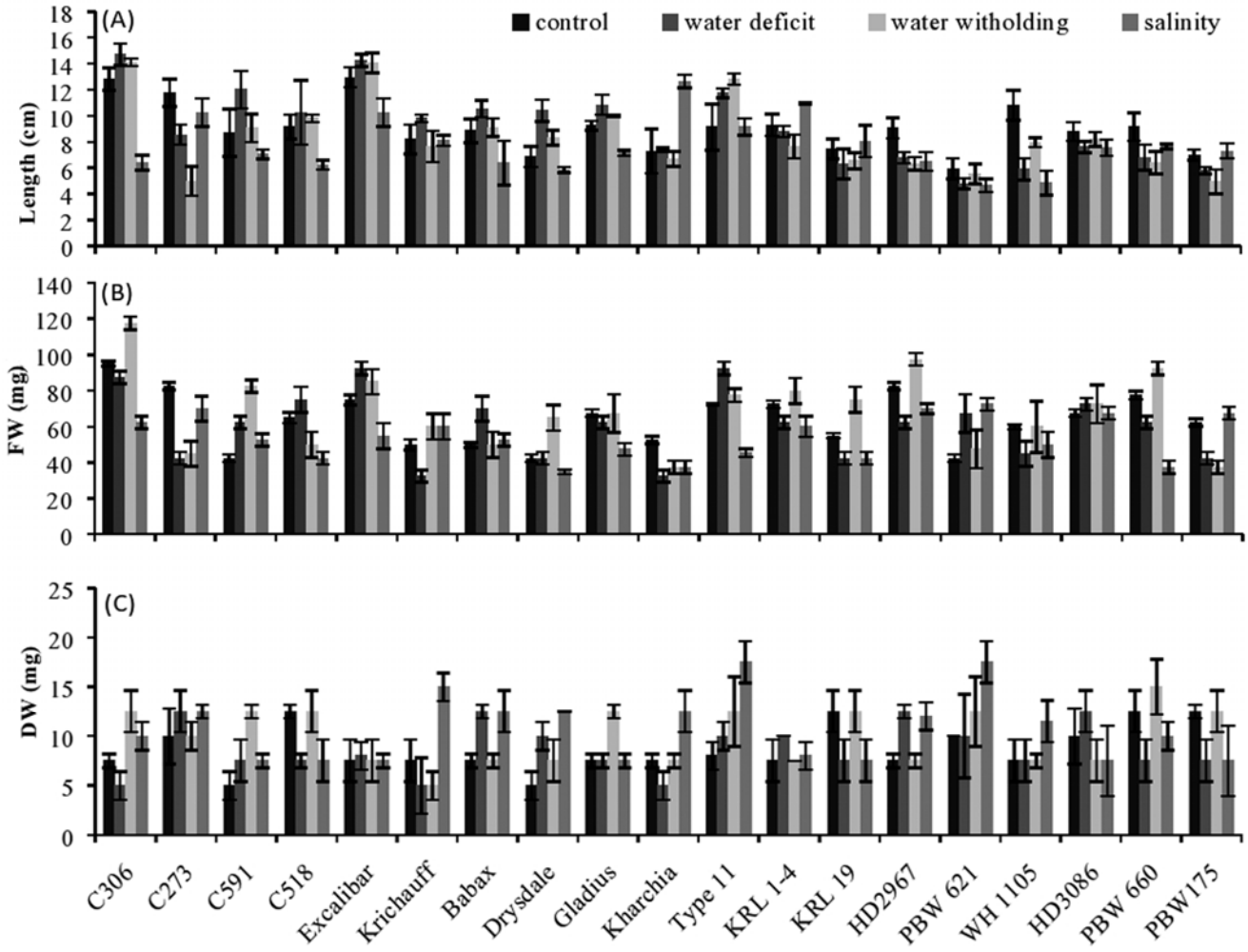

Fig. 1 — Effect of water deficit, water withholding and salinity stress on physiological parameters in the shoots of wheat genotypes . Shoot length (G = 0.863289, T = 0.396104, G X T = 1.72658) (A), Fresh weight (G = 0.00503471, T = 0.00231008E, G X T = 0.0100694) (B), and Dry weight (G = 0.0019694, T = 0.000903622, G X T = 0.00393880) (C) where G is genotypes, T is treatment and G X T is genotype and treatment interaction. [The values of G, T and G X T are the critical differences at 5% level of difference while vertical bars on graph represent standard errors.]

11, Excalibar, Krichauff, Babax, Drysdale and Gladius genotypes showed a significant increase in shoot length and fresh biomass under water deficit and water withholding conditions. However, under salt stress condition, genotypes Kharachia, Krl1-4 and Krl19 showed a marked increase in shoot lengths and C591, Kricahuff, Babax, PBW621 and PBW175 exhibited a dramatic increase in the fresh biomass. On the other hand, underwater withholding condition, a significant increase in the dry biomass was observed in C591, C306, C518, Drysdale, Gladius, Krl19, Type11, PBW660, PBW621 and PBW175 genotypes. Whereas, a dramatic increase in dry biomass was observed in C306, C591, C273, Kricahuff, Babax, Drysdale, Gladius, Kharchia, Type11, Krl1-4, HD 2967, PBW621 and WH1105 among all studied genotypes under salinity stress condition. Among all stress conditions, salinity stress posed deleterious effects on length and biomass of shoots.

Biochemical parameters

An increase in the accumulation of proline induced by osmotic stress was markedly under water withholding than water deficit and salt stress condition (Fig. 2A). Changes in proline concentration were found 2-3.4 fold higher in the shoots of genotypes Gladius, Babax, Excalibar, Type11, C306, C518, C591, C273, Drysdale, PBW660 and Krichauff under water deficit and water withholding conditions. However, under salinity stress Kharchia, Krl 1-4 and Krl 19 showed 2-2.6 fold increase in the proline content where maximum fold increase was found in

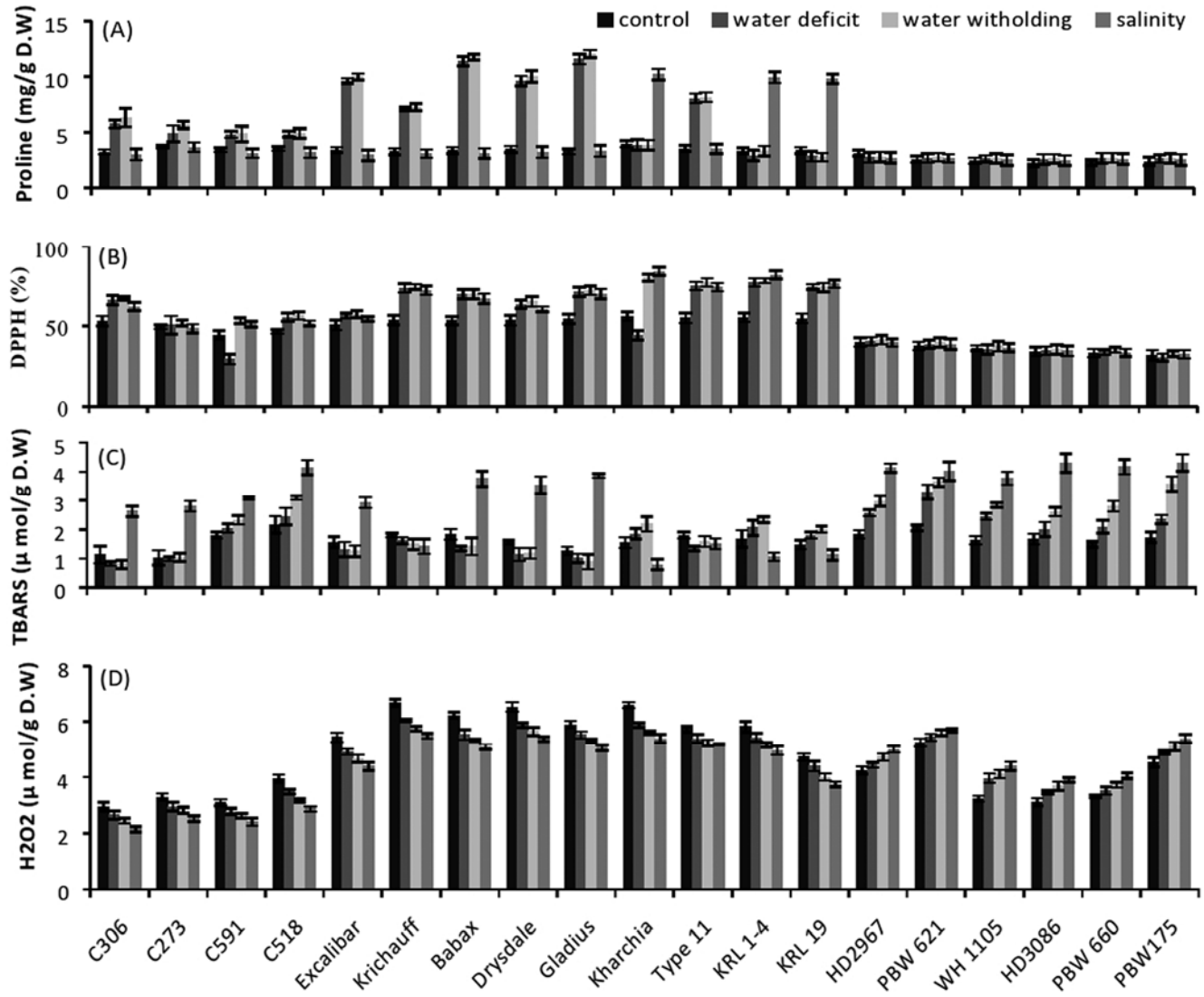

Fig. 2 — Effect of water deficit, water withholding and salinity stress on biochemical parameters of wheat genotypes. Proline (G = 0.405731, T = 0.186162, G X T = 0.811462) (A), DPPH (G = 6.91965, T = NS, G X T = 13.8393) (B), TBARS (G = 0.191432, T = 0.0878349, G X T = 0.382864) (C), and H_2O_2 (G = 0.0119863, T = 0.00549970, G X T = 0.0239727) (D) where G is genotypes, T is treatment and G X T is genotype and treatment interaction. [The values of G, T and G X T are the critical differences at 5% level of difference while vertical bars on graph represent standard errors.]

Kharchia (Fig. 2A). Our results are in agreement with the study of Song *et al.*[25] where similar proline accumulation pattern was observed under different stress conditions in wheat seedlings. As a higher level of proline was also found in the roots of these genotypes (Excalibar, C306, Drysdale, Babax, Gladius, Krichauff and Kharchia, Krl1-4 and Krl 9) as compared to those which could not tolerate the harsh conditions (HD2967, PBW621, WH1105, HD3086, PBW660 and PBW175). Increased proline content in the stressed plants may be an adaptative strategy to overcome the stress conditions as it supplies energy for growth and survival and thereby helps the plant to tolerate stress[26,27].

The antioxidant potential of wheat genotypes is measured in terms of percentage scavenging of DPPH[28]. DPPH radical scavenging activity was found in the range of 31.7-85%. The maximum increase in DPPH radical scavenging activity was observed in the Kharchia under salt stress (85%) and water withholding stress (80%) conditions (Fig. 2B). A significant increase in the activity was observed in C306, C518, Babax, Drysdale and Type11 genotypes under water deficit and water withholding stress conditions whereas Krl 1-4 and Krl 19 exhibited higher activity under salt conditions. The increased DPPH radical scavenging activity might have

contributed towards the stress tolerance mechanism in quenching free radicals for the better performance in Excalibar, C306, Drysdale, Babax, Gladius, Krichauff and Kharchia, Krl1-4 and Krl19 genotypes. A similar increase in the level of DPPH activity has been correlated with tolerance to different stress conditions in wheat genotypes[5].

TBARs is widely used as a marker for evaluating oxidative lipid injury and its concentration varies in response to abiotic stresses[29]. An increase in the TBARs content of wheat seedlings induced by osmotic stress was markedly higher under salt stress condition as compared to water deficit and water withholding condition (Fig. 2C). Among nineteen genotypes, TBARs content was found to be lowest in the shoots of Kharchia under salinity stress condition. However, the content of TBARs was observed to be lower in Krl1-4 and Krl19 genotypes under salinity stress and in C306, C273, Gladius, Drysdale and Excalibar under water deficit and water withholding conditions. The similar reduction in TBARs content under stress conditions was also reported in wheat seedlings[30]. The lowered TBARs content in genotypes *viz.* C306, C273, Gladius, Drysdale and Excalibar (under water deficit and water withholding condition), Kharchia, Krl1-4 and Krl19 (under salt stress condition) indicates reduced oxidative damage to their membrane, which may be responsible for their better performance under respective stress conditions. It is reported that rate of lipid peroxidation (in terms of TBARs content) indicates the sensitivity of plant to stress condition[31]. On the other hand, genotypes HD2967, PBW621, WH1105, HD3086, PBW660 and PBW175 among nineteen genotypes exhibited an increase in TBARs content under all studied stress conditions, depicting extensive lipid peroxidation which revealed their salt and drought susceptible nature.

A significant decrease in the contents of H_2O_2 was observed in genotypes *viz.* C306, C273, C591, C518, Exaclibar, Krichauff, Babax, Drysdale, Gladius, Kharchia, Type11, Krl1-4, and Krl19. While, HD 2967, PBW621, WH1105, HD3086, PBW660 and PBW175 genotypes showed a dramatic increase in the contents of root H_2O_2 under all studied stress conditions (Fig. 2D). Our results are in agreement with the studies of Kumar *et al.*[32] where a similar increase in the contents of H_2O_2 was observed under stress conditions.

Stress tolerance index and stress resistance level

Based on STI for the various parameters of shoots (Table 1), the level of stress resistance was

Table 1 — Stress tolerance index in wheat seedlings. WD, water deficit; WW, water withholding; and S, salt stress condition

Genotypes	Length			Fresh weight			Dry weight			Proline			MDA			H_20_2			DPPH		
	WD	WW	S	WD	WW	S	WD	WW	S	WD	WW	S	WD	WW	S	WD	WW	S	WD	WW	S
C306	2.28	2.19	0.99	2.03	2.73	1.45	1.23	1.54	0.61	1.86	2.07	0.97	0.35	0.34	1.12	0.34	0.31	0.27	1.59	1.62	1.49
C273	1.21	0.71	1.46	1.54	1.51	1.28	2.05	2.05	1.62	1.77	2.11	1.37	0.37	0.39	1.08	0.42	0.40	0.36	1.13	1.16	1.08
C591	1.26	0.95	0.74	0.65	0.85	0.54	0.83	1.02	0.61	1.65	1.67	1.06	1.38	1.57	2.09	0.37	0.35	0.32	0.58	1.07	1.02
C518	1.13	1.08	0.69	0.88	0.67	0.61	1.54	1.23	1.43	1.71	1.73	1.12	2.01	2.54	3.39	0.60	0.54	0.49	1.16	1.18	1.08
Excalibar	2.24	2.20	1.61	1.70	1.56	1.01	1.23	1.66	0.67	3.31	3.44	1.00	0.77	0.73	1.73	1.17	1.11	1.05	1.29	1.31	1.24
Krichauff	0.97	0.76	0.80	1.04	1.36	0.52	1.12	1.22	0.67	2.33	2.40	1.01	1.10	1.01	0.96	1.77	1.69	1.61	1.80	1.81	1.76
Babax	1.13	0.97	0.68	0.85	0.84	0.52	1.54	1.72	0.55	3.84	3.98	1.05	0.92	0.97	2.58	1.52	1.46	1.39	1.69	1.70	1.62
Drysdale	0.86	0.69	0.48	0.78	0.67	0.36	0.82	1.10	0.32	3.35	3.48	1.12	0.66	0.68	2.07	1.69	1.62	1.54	1.54	1.58	1.46
Gladius	1.21	1.11	0.80	1.60	1.46	0.78	1.29	1.54	0.92	3.81	3.96	1.08	0.48	0.42	1.85	1.43	1.38	1.31	1.75	1.78	1.72
Kharchia	0.65	0.59	1.12	0.41	0.48	1.28	0.61	0.73	1.66	1.54	1.53	4.07	1.07	1.26	0.45	1.71	1.62	1.56	1.12	2.04	2.14
Type11	1.30	1.42	1.01	1.64	1.37	0.80	1.31	1.64	0.92	2.87	2.90	1.25	0.91	1.07	1.02	1.36	1.32	1.31	1.87	1.92	1.85
Krl1-4	0.99	0.86	1.23	1.11	1.06	1.76	0.67	0.55	1.17	0.97	1.11	3.33	1.30	1.48	0.67	1.39	1.32	1.27	1.93	1.96	2.06
Krl 19	0.56	0.59	0.72	0.57	0.57	1.01	1.54	1.13	2.97	0.96	0.93	3.33	0.99	1.11	0.62	0.91	0.83	0.77	1.85	1.84	1.89
HD2967	0.74	0.69	0.71	1.26	1.12	0.69	0.73	0.67	0.43	0.85	0.87	0.83	1.78	2.06	2.86	0.82	0.88	0.93	0.73	0.74	0.71
PBW 621	0.34	0.40	0.33	0.41	0.38	0.31	0.54	0.49	0.39	0.69	0.71	0.68	2.55	2.82	3.11	1.25	1.29	1.31	0.65	0.67	0.65
WH1105	0.77	1.04	0.63	0.66	0.57	0.45	0.73	0.67	0.30	0.63	0.65	0.61	1.50	1.74	2.31	0.56	0.58	0.62	0.57	0.60	0.59
HD3086	0.81	0.87	0.80	0.71	0.67	0.62	0.80	0.61	0.24	0.57	0.59	0.55	1.26	1.64	2.70	0.47	0.50	0.53	0.52	0.54	0.52
PBW 660	0.75	0.71	0.84	1.18	1.16	0.82	0.53	0.42	0.21	0.61	0.62	0.59	1.17	1.57	2.34	0.51	0.54	0.58	0.50	0.52	0.49
PBW175	0.49	0.42	0.62	0.65	0.57	0.48	0.31	0.27	0.09	0.62	0.63	0.59	1.52	2.32	2.80	0.97	1.02	1.07	0.43	0.47	0.46

calculated for all the nineteen genotypes. The genotypes *viz.* Excalibar, Krichauff, Babax, Gladius, Krl 1-4 and Krl19 possessed much higher STI ≥ median value for most of the parameters studied *i.e.* proline content, DPPH scavenging activity, length and biomass of root or ≤ median value for content of H_2O_2 and MDA were proposed to have higher stress tolerance toward water deficit, water witholding and salinity conditions. C306 and Kharchia exhibited higher stress tolerance exclusively under water stress and salinity stress respectively. The genotypes *viz.* HD2967, PBW621, WH1105, HD3086, PBW660 and PBW175 were proposed to be highly susceptible on the basis of lower resistance level.

Activities of proline metabolizing enzymes

Osmotic stress caused a significant increase in GDH, P5CS and P5CR activity in shoots of all genotypes in comparison to the control (Fig. 3A-C). The maximum increase in the activity was observed under water deficit conditions among all stress

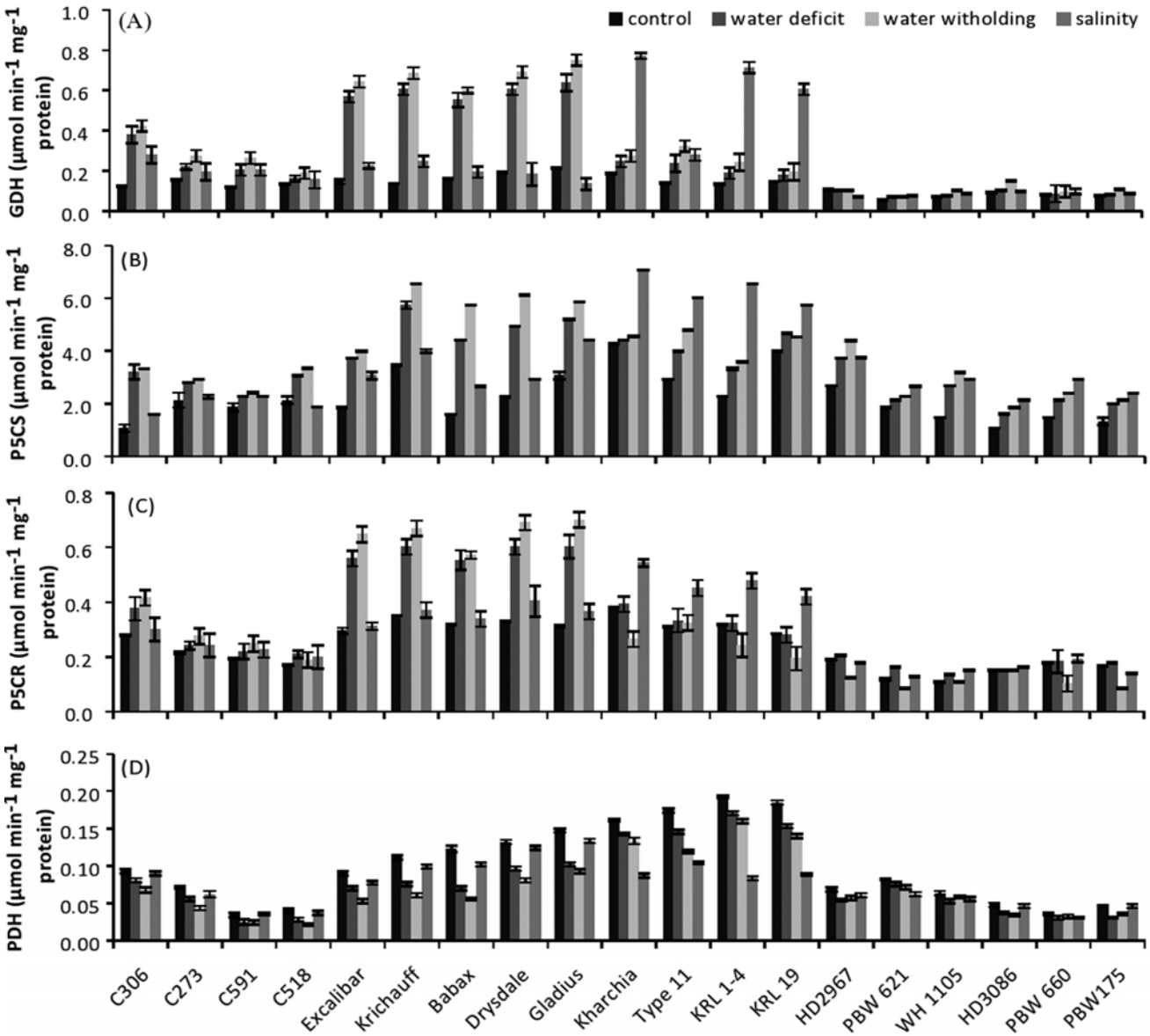

Fig. 3 — Effect of water deficit, water withholding and salinity stress on the activities of proline metabolizing enzymes of wheat genotypes. (A) GDH (G = 0.0713407, T = 0.0327334, G X T = 0.142681) (B) P5CS (G = 1.32324, T = NS, G X T = 2.64647) (C) P5CR (G = 0.0624154, T = 0.0286382, G X T = 0.124831) (D) PDH (G = 0.0176580, T = NS, G X T = 0.0353160) where G is genotypes, T is treatment and G X T is genotype and treatment interaction. [The values of G, T and G X T are the critical differences at 5% level of difference while vertical bars on graph represent standard errors.]

conditions. GDH activity was reported in the range of (0.1 -0.6 µmol NADH $min^{-1}mg^{-1}$protein) and (0.1-0.8 µmol NADH min^{-1} mg^{-1} protein) under water deficit and water withholding conditions respectively (Fig. 3A). Gladius showed maximum fold (3 and 4) increase in the activity of GDH under water deficit and water withholding condition respectively. On the contrary, under salinity stress condition, the GDH activity was reported in the range of (0.1-0.8 µmol NADH min^{-1} mg^{-1} protein) where Kharchia exhibited maximum fold (4) increase in the activity. A similar increase in the GDH activity has been reported under conditions of ample ammonia supply or the adverse environment as reported by Lu *et al.*[33].

The activity of P5CS was also reported to rise under all studied stress conditions. P5CS activity was reported in the range of (1.6-5.7 µmol NADH min^{-1} mg^{-1} protein) and (1.9-6.5 µmol NADH min^{-1} mg^{-1} protein) under water deficit and water withholding conditions respectively (Fig. 3B). Krichauff showed maximum fold (1.6 and 1.8) increase in the activity under water deficit and water withholding condition, respectively. On the other hand, under salt stress condition, the activity of P5CS was found in the range of 1.6-6.5 µmol NADH $min^{-1}mg^{-1}$ protein, where Kharchia showed a maximum of 1.65 fold increase in the activity. These results are found in parallel with the investigation carried out by Filippou *et al.*[34] in *Ailanthus altissima*. Free proline accumulation might be a consequence of the higher activity of P5CS in genotypes *i.e.* Excalibar, C306, Drysdale, Babax, Gladius, Krichauff and Kharchia, Krl1-4 and Krl19.

The activity of P5CR increased 2 fold under water deficit, water withholding and salt stress conditions (Fig. 3C). The maximum increase in the activity was observed underwater withholding condition among all stress conditions. Water deficit condition led to an increase in the activity of P5CR in the range (0.3-0.6 µmol NADPH min^{-1} mg^{-1} protein) with the minimum increase being reported in WH 1105 and maximum in Gladius, Drysdale , and Krichauff (Fig. 3C). However, under water withholding conditions, a significant increase in the P5CR activity ranging from 0.3 to 0.7 µmol NADPH min^{-1} mg^{-1} protein was observed among studied genotypes except for Kharchia, HD2967, PBW621, Krl1-4, Krl19 and PBW660. The maximum activity was reported in Gladius and minimum being reported in C518. On the contrary, under salinity stress condition, the P5CR activity was reported in the range of 0.1-0.5 µmol NADPH min^{-1} mg^{-1} protein with the maximum being reported in Kharchia and minimum in PBW 621. A similar increase in the activity of P5CR was observed in leaves of *Cicer arietinum* L. under salt stress[35]. However, the genotypes PBW175 and HD2967 showed a marked decline in the activity of P5CR under salinity stress.

Contrary to proline synthesizing enzyme discussed above, PDH activity showed a significant decline in the shoots of nearly all studied genotypes under water deficit, water withholding and salt stress conditions (Fig. 3D). Under waterdeficit condition, 8-43% decline in the activity of PDH was observed being maximum reduction reported in Babax. On the other hand under water withholding condition, 7-54% decrease in the activity was observed where PBW660 and Babax genotypes showed lowest and highest reduction in the activity of PDH, respectively. However, under salinity stress conditions, a significant decline in PDH activity was found to be in the range of 12-56% with maximum reduction observed in Krl1-4 and minimum in PBW660 (Fig. 3D). A similar decline in the activity of PDH was reported in *Oryza sativa* L. under salt stress[36].

Agglomerative hierarchical clustering and principle component analysis

Agglomerative hierarchical clustering by principal component analysis categorized the nineteen genotypes on similarity basis into two major clusters: MC-I and MC-II. MC-I was subdivided into two clusters A and B while MC-II was divided into C and D subclusters (Fig. 4). It was observed that MC-I constituted most of the genotypes having high-stress tolerance index and MC-II with genotypes exhibiting low-stress tolerance index. The A cluster of MC-I mainly comprised of genotypes *i.e.* C306, Excalibar, Krichauff, Babax, Drysdale and Gladius that are tolerant towards water scarcity conditions while B cluster chiefly constituted genotypes *i.e.* Kharchia, Type11, Krl1-4 and Krl19 that more tolerant towards salinity stress. The small subclusters C of MC-II basically contained the genotypes *i.e.* C273, C518 and C591 that possesed low-stress tolerance index. The genotypes within this small cluster were low tolerant towards water and salinity stress. Furthermore, it was the D cluster of MC-II that chiefly constituted the genotypes that exhibit much lower stress tolerance index and were susceptible towards the stress conditions.

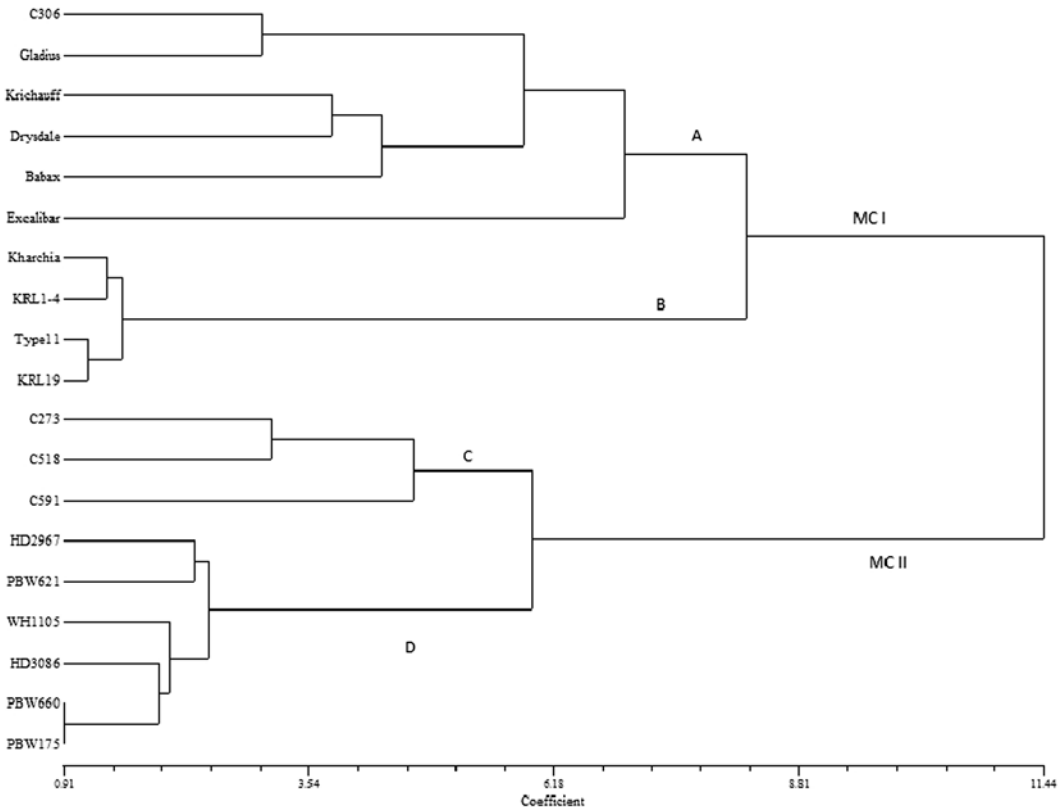

Fig. 4 — Hierarchical agglomerative clusturing

Discussion

Inhibition of shoot growth is a common response to water scarcity and salinity but the extent to which seedling can counteract the stress depends upon the inbuilt genetic characters of the cultivars. From growth parameter studies and from earlier studies[37] we conclude that salt stress stimulated the osmotic stress to a higher degree than water deficit and water withholding stress conditions. The reduction in shoot growth under salt stress in comparison with water stress conditions was probably due to toxic effect of high NaCl (300 mM) concentration as used in this study and earlier reports[38]. It may be inferred that the genotypes exhibiting longer shoots with higher biomass as observed in Excalibar, C306, Drysdale, Babax, Gladius, Krichauff under water deficit and water withholding condition and Kharchia, Krl 1-4 and Krl 19 under salt stress condition might be tolerant towards respective stress conditions. Similar observations on biomass and stress tolerance in wheat genotypes were also reported by Marcin´ska *et al.*[39].

Stress resistance levels and principal component analysis categorized the genotypes into four groups *i.e.* drought tolerant (C306, Excalibar, Krichauff, Babax, Drysdale, and Gladius), salt tolerant (Kharchia, Type11, Krl1-4 and Krl19), mid tolerant (C273, C518 and C591) and susceptible (HD2967, PBW621, WH1105, HD3086, PBW660 and PBW175). This categorization was based on the effective relation of proline metabolism and DPPH radical scavenging activity with contents of TBARs and H_2O_2 of genotypes under stress conditions. The genotypes exhibiting high tolerance had reduced magnitude of TBARs content in parallel with decreased H_2O_2 content and increased proline content and DPPH radical scavenging activity. An higher level of proline and DPPH radical scavenging activity in radicles have been correlated with enhanced tolerance[27]. It is reported that rate of lipid peroxidation (in terms of TBARs content) indicates the sensitivity of plant to stress condition[30]. The decreased TBARs content indicate reduced oxidative damage to the membrane, which thereby attributes towards stress tolerance. The genotypes exhibiting intolerance had a high level of TBARs content in their shoots which might result from lack of stress-dependent upregulation of antioxidant system as described by De Azevado *et al.*[40]. Thus, increased proline content in shoots along with DPPH radical scavenging activity in the tolerant genotypes may be

an adaptative strategy to overcome the stress conditions as it supplies energy for growth and lowers the generation of free radicals and reduces the lipid peroxidation linked membrane damage resulting in their stabilization[26, 27].

Enhancement of proline content in tolerant genotypes may be due to the variety of causes. Increased activities of GDH might have played a predominant role in ammonium assimilation and glutamate biosynthesis, leading to an increased glutamate pool for proline synthesis in tolerant genotypes as compared to susceptible genotypes. Increased GDH activity and its significant role in the synthesis of glutamate have been reported in many plant species[41,42]. As P5CS activity was reported higher than P5CR, so it seems obvious that P5CS activity might be in tight relation to free proline content indicating that glutamate is further converted to proline under stress conditions, and P5CS might be the rate-limiting factor in this pathway. A similar increase in the activity of GDH, P5CS and P5CR was observed in leaves of *Cicer arietinum* under salt stress[34]. Proline dehydrogenase converts proline to glutamate. A declining PDH activity was though reported in all studied genotypes under all stress conditions, the reduction was comparatively higher in tolerant genotypes which might have also influenced the proline accumulation. Among all stress conditions studied, water withholding stress induced the stimulation of proline synthesis (*via* increased GDH, P5CS and P5CR activity) with an enhanced inhibition of oxidation (reduced PDH activity) to a large extent than water deficit and salt stress condition. Therefore a close correlation between these enzymes and proline content under water withholding stress condition as compared to water deficit and salt stress condition was further confirmed.

Conclusion

Salt stress condition affected the physiology of seedlings of nineteen studied genotypes to a higher degree as compared to water deficit and water withholding condition due to higher contents of TBARs. Based on the principal component analysis and stress tolerance index the nineteen genotypes were categorized into four groups *i.e.* drought tolerant (Excalibar, Krichauff, Babax, Drysdale, Gladius and C306), salt tolerant (Kharchia, Type11, Krl1-4 and Krl19), low stress tolerant (C273, C518 and C591) and susceptible (HD 2967, PBW 621, WH 1105, HD 3086, PBW 660 and PBW175). Accumulation of proline in tolerant genotypes under stress was found as a result of the reciprocal regulation of two pathways: increased expression of proline synthetic enzymes (GDH, P5CS and P5CR) and repressed activity of proline degrading enzyme *i.e.* proline dehydrogenase. Apparently, increased proline content might have contributed towards enhanced membrane stability by overcoming oxidative stress, especially in tolerant genotypes. Future studies on these genotypes can be explored by breeders or plant biotechnologists in building superior lines by crossing or introgression of these genes for metabolites or enzyme.

References

1 Hussain M & Mumtaz S, Climate change and managing water crisis: Pakistan's perspective. *Rev Environ Health*, 29 (2014) 71.

2 Golldack D, Li C, Mohan H & Probst N, Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Front Plant Sci*, 5 (2014) 151.

3 Ahmad P, Hakeem KR, Kumar A, Ashraf M & Akram NA, Salt-induced changes in photosynthetic activity and oxidative defense system of three cultivars of mustard (*Brassica juncea L.*). *Afr J Biotechnol*, 11 **(**2012) 2694.

4 Ahmad P & Umar S, *Oxidative stress: role of antioxidants in plants.* (New Delhi: Studium Press Pvt. Ltd.) 2011.

5 Rao A, Ahmad SD, Sabir SM, Awan SI, Hameed A, Abbas SR, Shehzad M, Khan MF, Shafique S & Ahmad Z, Detection of saline tolerant wheat cultivars (*Triticum aestivum* L.) using lipid peroxidation, antioxidant defense system, glycinebetaine and proline contents. *J Anim Plant Sci*, 23 (2013) 1742.

6 Garg N & Manchanda G, ROS generation in plants: Boon or bane? *Plant Biosyst*, 143 (2009) 81.

7 Hameed A, Hussain T, Gulzar S, Aziz I, Gul B & Khan MA, Salt tolerance of a cash crop halophyte *Suaeda fruticosa*: biochemical responses to salt and exogenous chemical treatments. *Acta Physiol Plant*, 34 (2012) 2331.

8 Kaur G & Asthir B, Proline: a key player in plant abiotic stress tolerance. *Biol Plant*, 59 (2015) 609.

9 Hellmann H, Funck D, Rentsch D & Frommer WB, Hypersensitivity of an *Arabidopsis* sugar signaling mutant toward exogenous proline application. *Plant Physiol*, 122 (2000) 357.

10 Kavi-Kishor PB, Sangam S, Amrutha RN, Sri-Laxmi P, Naidu KR, Rao KRSS, Rao S, Reddy KJ, Theriappan P & Sreenivasulu N, Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Curr Sci*, 88 (2005) 424.

11 Miller G, Honig A, Stein H, Suzuki N, Mittler R & Zilberstein A, Unraveling delta1-pyrroline-5-carboxylate proline cycle in plants by uncoupled expression of proline oxidation enzymes. *J Biol Chem*, 284 **(**2009) 26482.

12 Verbruggen N & Hermans C, Proline accumulation in plants: a review. *Amino Acids*, 35 (2008) 753.

13 Szabados L & Savoure A, Proline: a multifunctional amino acid. *Trends Plant Sci*, 15 **(**2010) 89.

14 Ashraf M, Biotechnological approach of improving plant salt tolerance using antioxidants as markers. *Biotech Adv*, 27 (2009) 84.

15 Heath RL & Packer L, Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys*, 125 (1968) 189.

16 Bates L, Waldren RP & Teare ID, Rapid determination of free proline for water-stress studies. *Plant Soil*, 39 (1973) 205.

17 Blois MS, Antioxidant determinations by the use of a stable free radical. *Nature*, 181 (1958) 1199.

18 Fisher RA & Maurer R, Drought resistance in spring wheat cultivars. I. Grain yield responses in spring wheat. *Aust J Agric Sci*, 29 (1978) 897.

19 Chen CT, Chen L, Lin CC & Kao CH, Regulation of proline accumulation in detached rice leaves exposed to excess copper. *Plant Sci*, 160 (2001) 283.

20 Akihiro T, Koike S, Tani R, Tominaga T, Watanabe S, Iijima Y, Aoki K, Shibata D, Ashihara H, Matsukura C, Akama K, Fujimura T & Ezura H, Biochemical mechanism on GABA accumulation during fruit development in tomato. *Plant Cell Physiol*, 49 (2008) 1378.

21 Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Aguero JA, Aguado-Santacruz GA & Jiménez-Bremont JF, Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. *Plant Phys Biochem*, 46 (2008) 82.

22 Lutts S, Majerus V & Kinet JM, NaCl effects on proline metabolism in rice (*Oryza sativa* L.) seedlings. *Physiol Plant*, 105 (1999) 450.

23 Ruiz JM, Sanchez E, Garcia PC, Lopez-Lefebre LR, Rivero RM & Romero L, Proline metabolism and NAD kinase activity in greenbean plants subjected to coldshock. *Phytochem*, 59 **(**2002) 473.

24 Wold S, Esbensen E & Geladi P, Principle component analysis. *Chemom Intell Lab Syst*, 3 (1987) 37.

25 Song SQ, Lei YB & Tian XR, Proline metabolism and cross-tolerance to salinity and heat stress in germinating wheat seeds. *Russ J Plant Physiol*, 52 (2005) 793.

26 Abdul-Jaleel C, Sankar B, Sridharan R & Rajaram P, Soil salinity alters growth, chlorophyll content and secondary metabolites accumulation in *Catharanthus roseus*. *Turk J Biol*, 32 (2008) 79.

27 Gill SS & Tuteja N, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem*, 48 (2010) 909.

28 Kang HM & Saltveit ME, Antioxidant enzymes and DPPH radical scavenging activity in chilled and heat shocked rice (*Oryza sativa* L.) seedling radicles. *J Agric Food Chem*, 50 (2002) 513.

29 Moller IM, Jensen PE & Hansson A, Oxidative modifications to cellular components in plants. *Annu Rev Plant Biol*, 58 (2007) 459.

30 Mandhania S, Madan S & Sawhney V, Antioxidant defense mechanism under salt stress in wheat seedlings. *Biol Plant*, 50 (2006) 227.

31 Jain MG, Mathur SK & Sarin NB, Ameliorative effects of proline on salt stress induced lipid peroxidation in cell lines of groundnut (*Arachis hypogea* L.). *Plant Cell Rep*, 20 (2001) 463.

32 Kumar RR, Sharma SK, Gadpayle KA, Singh K, Sivaranjani R, Goswami S & Rai RD, Mechanism of action of hydrogen peroxide in wheat thermotolerance - interaction between antioxidant isoenzymes, proline and cell membrane. *Afr J Biotechnol*, 11 (2012) 14368.

33 Lu B, Yuan Y, Zhang C, Ou J, Zhou W & Lin Q, Modulation of key enzymes involved in ammonium assimilation and carbon metabolism by low temperature in rice (*Oryza sativa* L.) roots. *Plant Sci*, 169 (2005) 295.

34 Filippou P, Bouchagier P, Skotti E & Fotopoulos V, Proline and reactive oxygen/nitrogen species metabolism is involved in the tolerant response of the invasive plant species *Ailanthus altissima* to drought and salinity. *Environ Exp Bot*, 97 (2014) 1.

35 Baher N, Agronomic effectiveness of mycorrhizal *Cicer arietinum* L. plants on mechanism of proline in imparting protection, against NaCl Stress. *Adv Environ Biol*, 9 (2015) 286.

36 Bagdi DL & Shaw BP, Analysis of proline metabolic enzymes in *Oryza Sativa* under NaCl stress. *J Environ Biol*, 34 (2013) 667.

37 Ashraf M & Ali Q, Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus* L.). *Environ Exp Bot*, 63 (2008) 266.

38 Glyn BA, Mc Kenzie BM, Hallett PD & Valentine TA, Root elongation, water stress, and mechanical impedance: a review of limiting stresses and beneficial root tip traits. *J Exp Bot*, 62 **(**2011) 59.

39 Marcin´ska I, Czyczyło-Mysza I, Skrzypek E, Filek M, Grzesiak S, Grzesiak MT, Janowiak F, Hura T, Dziurka M, Dziurka K, Nowakowska A & Steve AQ, Impact of osmotic stress on physiological and biochemical characteristics in drought-susceptible and drought-resistant wheat genotypes. *Acta Physiol Plant*, 35 (2013) 451.

40 De Azevedo NADD, Prisco JT, Eneas J, De Abreu CEB & Gomes EF, Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environ Exp Bot*, 56 (2006) 87.

41 Mattioni C, Gabbrielli R, Vangronsveld J & Clijsters H, Nickel and cadmium toxicity and enzymatic activity in Ni-tolerant and non-tolerant populations of *Silene italica* Pers. *J Plant Physiol*, 150 (1997) 173.

42 Gill SS, Khan NA, Anjum NA & Tuteja N, Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. In: *Plant Nutrition and Abiotic Stress Tolerance III, Plant Stress 5 (Special Issue 1)* (Ed Anjum NA & Lopez-Lauri F, Ikenobe: Global Science Books Ltd., UK) 2011, 1.