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DIFFERENTIAL RESPONSE OF WHEAT GENOTYPES TO LOW NITROGEN STRESS: VARIATION IN ANTIOXIDANT CAPACITY

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SUMMARY

Two wheat (*Triticum aestivum* L.) genotypes *viz.* Uniculm 'Gigas' Line 492 and Kalyansona were used to study the development of oxidative stress and antioxidant capacity in fully expanded flag leaves in response to low nitrogen (N). The genotypes differed in terms of metabolic constituents, green leaf area, membrane integrity and activities of antioxidant enzymes. Uniculm maintained photosynthetic rate and metabolic constituents in spite of large reduction in total flag leaf area. Low N resulted in increased formation of hydrogen peroxide (H_2O_2) and accumulation of thiobarbituric acid reactive substances (TBARS) in flag leaves of both the genotypes. However, the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) were higher in N stressed plants of Uniculm till maturity. In N stressed Kalyansona, activities of SOD and APX declined early as compared to control N plants. The low N induced generation of reactive oxygen species (ROS) was higher in Kalyansona as compared to Uniculm due to lower APX activity and/or the higher SOD/APX or SOD/CAT ratio. Kalyansona was found to be highly sensitive to low N in comparison to Uniculm. The catalase activity did not increase in low N grown plants of Kalyansona leading to increased hydrogen peroxide in this genotype. The study provides evidence that wheat genotypes respond differentially to N supply.

Key words **: Antioxidant enzymes, nitrogen, oxidative stress, wheat**

INTRODUCTION

An unavoidable consequence of aerobic metabolism is exposure of cells to reactive oxygen species (ROS), *viz.* H_2O_2 (hydrogen peroxide), $O_2^{\cdot-}$(superoxide) and $OH^{\cdot}$(hydroxyl radical). In plants, low concentration of ROS functions in signal transduction leading to activation of defense responses, while higher level causes serious oxidative damage to membrane lipids, DNA and proteins (Ghezzi and Bonetto 2003). Abiotic stress increases the generation of ROS in the cells. In plants, chloroplasts (Davletova *et al*. 2005), peroxisomes (Foyer and Noctor 2000) and mitochondria (Moller and Kristensen 2004) are recognized to be the major points of ROS metabolism.

Oxidative stress in crop plants has been reported in response to several abiotic stresses such as drought (Shahbazi *et al*. 2009), high temperature (Keles and Öncel 2002), salinity (Sairam *et al*. 2005), water logging (Tan *et al*. 2008) and nutrients (Kumagai *et al.* 2009). Nitrogen availability is a determinant factor for the growth and yield of plants. Deficiency or change in form of this essential element results in nutrient imbalance affecting several metabolic pathways (Abrol *et al*. 1999) including increased production of ROS (Dominguez-Valdivia *et al.* 2008). The increased excitation energy under N-deficiency is the result of saturation of the electron transport chain due to limitation of the use of reductants by the Calvin cycle (Bungard *et al.* 2000). Effect of N

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deficiency and antioxidant system has been studied in rice plants in presence of high irradiance (Huang *et al*. 2004), resulting in decreased light-harvesting capacity and increased thermal dissipation of absorbed energy. Cultivar differences in terms of tolerance to photo-inhibition and activities of antioxidant enzymes in flag leaves of rice under low N have been reported (Kumagai *et al.* 2009). Although much evidence has confirmed that N-deficiency induces early leaf ageing in plants (Crafts-Brandner *et al*. 1998), there is little evidence about the initiation and progression of senescence in flag leaves once it is fully expanded.

The rise of ROS imposes oxidative stress on the plants and to prevent their excessive accumulation plants contain protective enzymes such as superoxide dismutase (SOD), peroxidases (POX) and catalase (CAT) (Agarwal *et al*. 2005). In our earlier studies, it was observed that wheat genotypes responded differentially to the N supply in relation to leaf growth and photosynthesis as well as maintenance of metabolic constituents (Sivasankar *et al.* 1998 a, b). The decline in leaf area was due to the decline in mesophyll cell size and surface area as a result of low N availability (Sivasankar *et al.* 1998 b). The objective of present study was to understand the effect of N supply on the physiological and biochemical activities in the flag leaves of contrasting wheat genotypes.

The characteristic features of these wheat genotypes are that, the Uniculm, as the name implies, produces single culm with occasional one or two tillers, which were removed as and when they emerged, broad-sized leaves, and is responsive to N in terms of growth. Kalyansona is a widely cultivated, tiller producing and high yielding variety. However, to compare these two genotypes, tillers were removed in Kalyansona also, as and when they emerged so as to maintain only main shoot in both the genotypes.

MATERIALS AND METHODS

Plant material and growth conditions: Wheat genotype *viz*. Uniculm ('U') 'Gigas' (Atsmon and Jacobs 1977) wheat line 492 and Kalyansona ('K') were grown in pots (30 x 30 cm) containing 8 kg sandy loam soil at two N levels *viz*. 30 Kg N ha^{-1} (low N; LN) and 120 Kg N ha^{-1}(control; CN) applied as urea. Half of the N was applied as basal dose and rest in two equal splits at 30 and 45 days after sowing (DAS). Entire phosphorous and potassium were applied as basal doses of 60 Kg ha^{-1} and 40 Kg ha^{-1} in the form of single super phosphate and muriate of potash, respectively. Plants were raised in net house during winter season under natural condition of light, temperature and humidity. The mean maximum and minimum temperatures during the growing season were 25.6°C and 9.8°C, respectively. Three replicates were sampled for flag leaf from full expansion onwards till senescence for various physiological and biochemical parameters. The data presented is mean of two years experiment with same set up. The experiment was laid out in completely randomized design. All the experiments were repeated twice with three replicates (n = 6) and data presented are mean ± standard errors (SE).

Photosynthesis, chlorophyll and nitrogen estimation: The green area of individual flag leaves was measured using green leaf area meter (Delta T Devices, Burwell, UK). Net photosynthetic (P_N) rate was measured using portable infrared gas analyzer (IRGA, Model LI-6200; LICOR, USA) in flag leaves from full expansion till maturity at an interval of ten days. Chlorophyll (CHL) and carotenoid contents were estimated by non-maceration method (Hiscox and Israelstom 1978). Absorbance was recorded at 645 and 663 nm for chlorophylls and 470 nm for total carotenoid contents. Total soluble proteins were estimated by Bradford (1976). Total N in the dried leaf samples and grains was estimated using N- auto analyzer (Gerhardt, Germany).

Estimation of hydrogen peroxide: Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex (Rao *et al*. 1997). Leaf material (1g) was ground with liquid nitrogen and 10 ml chilled acetone was added. To this, 4 ml titanium reagent (1g titanium dioxide and 10 g potassium sulphate were mixed and digested with 150 ml of concentrated sulphuric acid, the digested mixture was diluted to 1.5 L with distilled water) and 5 ml of concentrated ammonium solution was added to precipitate the titanium hydrogen peroxide complex. Reaction mixture was centrifuged at 10,000 g for 10 min (model J2-21 Beckman). Precipitate was dissolved in 10

ml H_2SO_4 (2M). Absorbance was measured at 415 nm, with H_2O_2 as a standard.

TBARS estimation: The level of lipid peroxidation was measured in terms of thiobabituric acid reactive substances (TBARS) content (Heath and Packer 1968). Leaf samples (0.5 g) were homogenized in 10 ml of 0.1% trichloroacetic acid (TCA) and centrifuged (15,000 g). To 2 ml of aliquot, 4 ml of 0.5% thiobarbituric acid (TBA) and 20% TCA was added. The mixture was heated at 95ºC for 30 min and cooled immediately. The absorbance was recorded at 532 nm. The value for the nonspecific absorption at 600 nm was subtracted. The TBARS content was calculated using its absorption coefficient of 155 $mM^{-1}cm^{-1}$.

Extraction of protein for enzyme assays: Protein extract for the superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) was prepared by homogenizing 0.5 g leaf material in phosphate buffer (0.1 M, pH 7.5, 10 mM EDTA, protease inhibitor mix, 1x). The extraction buffer for APX activity was supplemented with 1 mM ascorbic acid. The extracts were centrifuged for 15 min at 20,000 g. The supernatant was used for enzyme assays.

SOD assay: It was based on the measurement of inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm (Dhindsa *et al.* 1980). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 µM EDTA, 4 µM riboflavin and required amount of enzyme extract. The reaction was started by adding riboflavin and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal color, served as control. A non-irradiated complete reaction mixture served as blank. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm (Giannopolitis and Ries 1977).

APX and CAT assay: APX activity was assayed by recording the decrease in ascorbate content at 290 nm (Nakano and Asada 1981). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM EDTA, 0.1 mM H_2O_2 and 0.1 ml of protein extract in total volume of 3 ml. The reaction was started with the addition of H_2O_2 and absorbance was recorded at 290 nm for 30s. Catalase (CAT) activity was determined by monitoring the disappearance of H_2O_2 at 240 nm (å = 40 mM^{-1} cm^{-1}) according to the method of Aebi (1984). The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 33 mM H_2O_2 and enzyme extract.

RESULTS AND DISCUSSION

Leaf area and net photosynthetic rate in relation to N supply: The flag leaf area and net photosynthetic rate (P_N) were significantly higher at full expansion (taken as day zero) in both the genotypes (Fig. 1A & 1B). The green leaf area of flag leaves started to decline in both Uniculm and Kalyansona, however, in Uniculm, the overall flag leaf area at full expansion reduced drastically under LN as compared to flag leaves of control N plants.

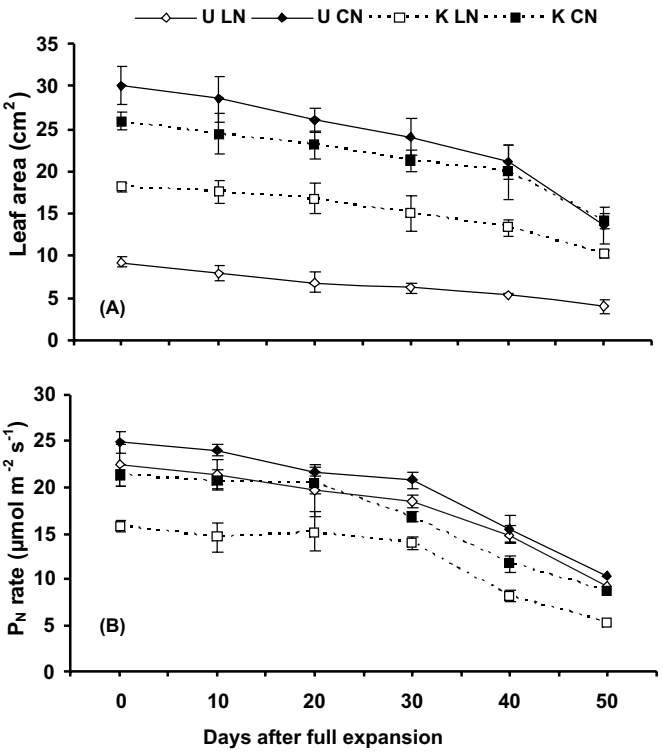

Fig. 1. Effect of nitrogen supply on (A) changes in leaf area (B) net photosynthetic rate in flag leaves of wheat genotypes. Uniculm low N, ULN; Uniculm control N, UCN; Kalyansona low N, KLN; Kalyansona control N, KCN. The values are mean of ± SE.

The P_N was significantly reduced in Kalyansona compared to Uniculm when grown at LN. The P_N in Uniculm grown at low N was comparable to Kalyansona under CN. The flag leaves in Uniculm grown at LN senesced at slower rate as compared to Kalyansona. This helped Uniculm to maintain its photosynthetic rate similar to that of plants grown with sufficient N. The reduction in total flag leaf area at full expansion, dry weight and thickness were reported to be influenced by N nutrition in both Kalyansona and Uniculm (Sivasankar *et al.* 1998a, b, Guru *et al*. 1999). Reduction in area of flag leaves was due to production of less number of mesophyll cells under low N (Sivasankar *et al*. 1998b). Reduction in P_N, and stomatal conductance was also reported in flag leaves of two rice cultivars grown under N stress (Kumagai *et al*. 2009). Reduction in carboxylation efficiency could be explained on the basis of Rubisco content and activity in these genotypes. It has been shown in our earlier studies that the Rubisco concentration at full lamina expansion declined to 8 and 36% in flag leaves of wheat (Sivasankar *et al.* 1998a) and rice (Huang *et al.* 2004), respectively grown at low nitrogen.

Chlorophyll and carotenoid content: Total chlorophyll (CHL) content was significantly higher at full flag leaf expansion and lowest at 30 days after full expansion (Fig. 2A). In LN grown Kalyansona, a 33% decline in total 'CHL' was observed in flag leaves as compared to CN plants. However, N stress did not affect total 'CHL' content in flag leaves of Uniculm. Similar trend was noticed for total carotenoid concentration which declined with the age of flag leaves. The carotenoid concentration in flag leaves of Kalyansona grown at LN decreased by 15-28% compared to CN at various stages of observation while in Uniculm there was no significant effect of applied N on carotenoid content (Fig. 2B). Degradation of photosynthetic pigments and proteins consisting of slow and rapid stages is the typical characteristic of leaf senescence (Deng *et al.* 2001). Higher 'CHL' in Uniculm under N stress signifies lower pigment bleaching in this genotype, probably due to the presence of more carotenoids. It is known that pigments bound to thylakoids are stable, while free pigments are labile and sensitive to oxidative degradation (Dalal and Chopra 1999).

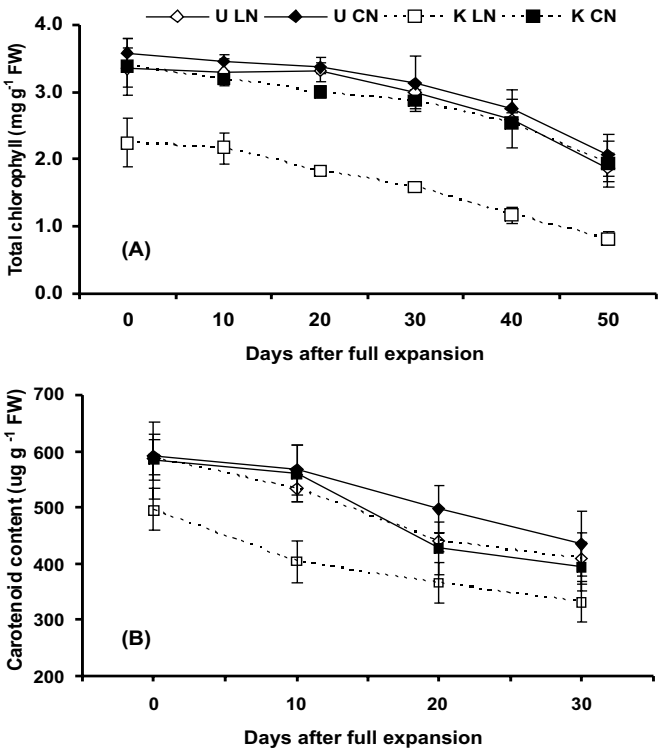

Fig. 2. Effect of nitrogen supply on changes in (A) total chlorophyll (B) total carotenoid content from full expansion till senescence in flag leaves of wheat genotypes. Uniculm low N, ULN; Uniculm control N, UCN; Kalyansona low N, KLN; Kalyansona control N, KCN. The values are mean of ± SE.

Total soluble protein and nitrogen content: Concentration of total soluble proteins was higher in control plants of both Uniculm and Kalyansona during the initial period of flag leaf expansion. Total soluble protein in flag leaves of LN grown Uniculm plants declined by 7 to 15% whereas, in Kalyansona, the decrease ranged between 21 to 36% compared to their respective controls (Fig. 3A). However, the N concentration declined in flag leaves from full expansion to maturity and minimum values were recorded at 50 days after full expansion (Fig. 3B). Uniculm recorded higher N concentration in flag leaves at all the stages of sampling as compared to Kalyansona. In Kalyansona at LN, the total reduced N in flag leaf tissues was 40% less as compared to CN plants at 10 days after full expansion.

Reductions in P_N in Kalyansona along with reduced N content imply that photosynthetic rate is positively

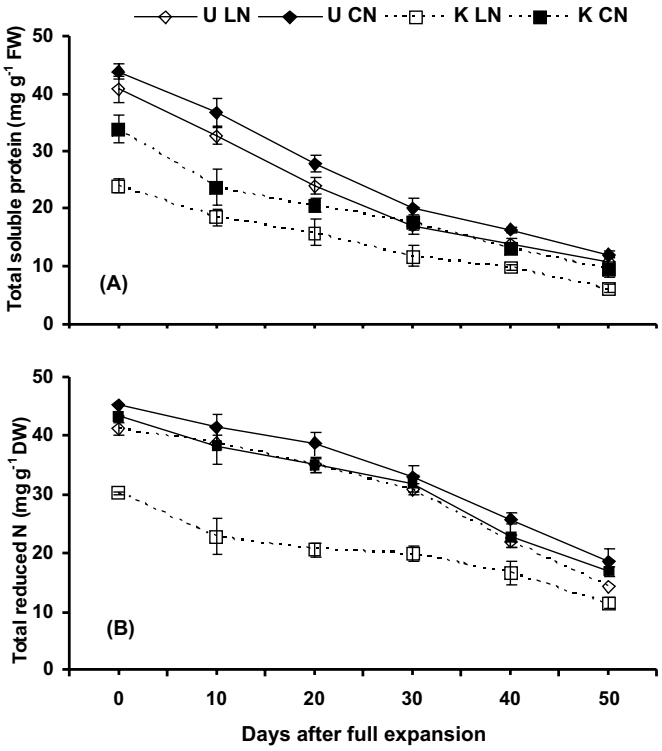

Fig. 3. Effect of nitrogen supply on changes in (A) reduced nitrogen concentration (B) total soluble proteins from full expansion till senescence in the flag leaves of wheat genotypes. Uniculm low N, ULN; Uniculm control N, UCN; Kalyansona low N, KLN; Kalyansona control N, KCN. The values are mean of ± SE.

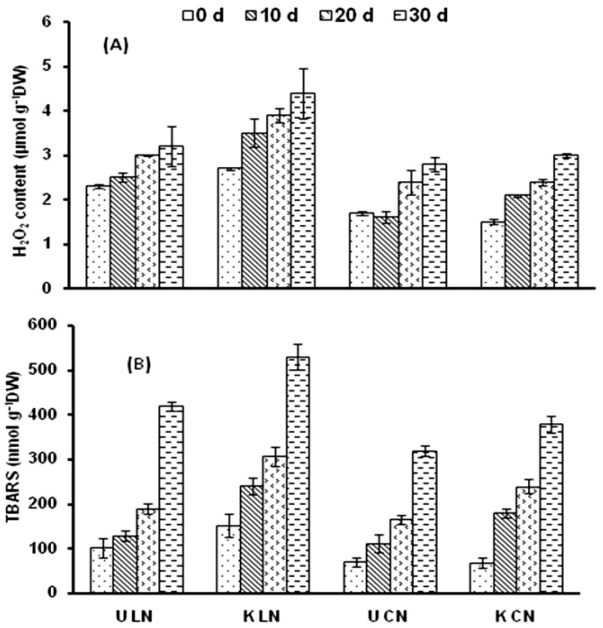

Fig. 4. Effect of nitrogen supply on (A) accumulation of hydrogen peroxide (B) lipid peroxidation from full expansion (taken as day zero) in the flag leaves (at ten days interval after full expansion) of wheat genotypes. Uniculm low N, ULN; Kalyansona low N, KLN; Uniculm control N, UCN; Kalyansona control N, KCN. The values are mean of ± SE.

correlated with laminae N concentration (R^2=0.93). The reduction in N content of flag leaves in Kalyansona at low N was associated with decline in concentration of soluble proteins, photosynthetic pigments and N content, thereby leading to decrease in photosynthesis.

Concentration of hydrogen peroxide and thiobarbituric acid reactive substances (TBARS): Hydrogen peroxide (H_2O_2) accumulation and lipid peroxidation measured as its degradation product TBARS in flag leaves increased with age as well as under low N in both the genotypes (Fig. 4A & 4B). Increase in H_2O_2 in flag leaves of Uniculm was lower as compared to H_2O_2 levels in flag leaves of Kalyansona grown at LN. In Uniculm, the changes in lipid peroxidation were similar at both the levels of N supply. Increased lipid peroxidation is caused by increased ROS (Prochazkova *et al.* 2001). Higher production of H_2O_2 in leaves has been reported in N, phosphorus and sulphur deficient maize plants leading to enhanced lipid peroxidation (Tewari *et al.* 2007).

Activities of the antioxidant enzymes: Uniculm flag leaves had higher CAT and APX activities at low N level at all stages of observation in comparison to CN grown plants (Fig. 5A & 5B). There was no significant increase in CAT activity in flag leaves of Kalyansona grown at LN. However, the APX activity was maximum at 10 days after full expansion of flag leaves in both the genotypes irrespective of N supply. In Kalyansona, at later stages i.e., 20 and 30 days after full expansion of flag leaves, a sharp decline in APX activity was recorded in LN grown plants as compared to CN plants. The mean total SOD activity averaged over N levels and stages of observation was higher in Uniculm compared to Kalyansona (Fig. 5C). Initially at zero days, the SOD activity in LN plants were non-significant in both the genotypes, however, it decreased in Kalyansona by 1.4- and 4.0-fold at 20 and 30 days after full expansion of flag leaves, respectively, compared to Uniculm LN

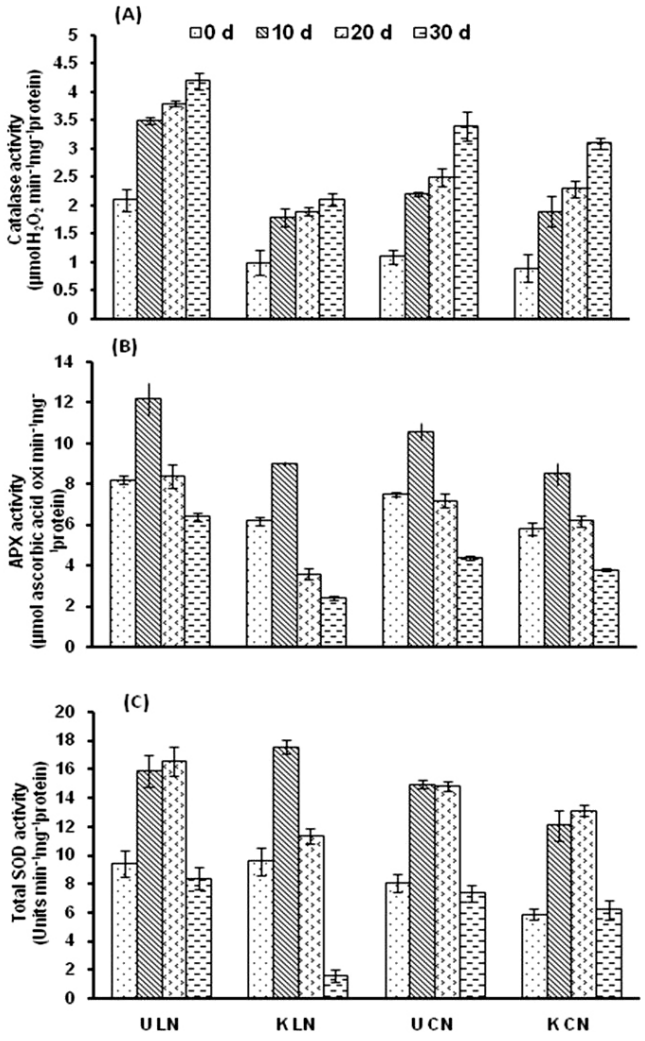

Fig 5. Effect of nitrogen supply on activity of enzymes (A) catalase (B) ascorbate peroxidase (C) total superoxide dismutase in the fully expanded flag leaf (taken as day zero and up to 30 days after full expansion) of wheat genotypes. Uniculm low N, ULN; Kalyansona low N, KLN; Uniculm control N, UCN; Kalyansona control N, KCN. The values are mean of ± SE.

grown plants. Studies on transgenic CAT-1 deficient tobacco plants revealed that CAT was essential for protection of ascorbate/glutathione pools from oxidation in order to maintain redox balance in cells (Takahashi *et al.* 1997). It is possible that as no increase in CAT activity was noticed in Kalyansona at low N, the availability of reduced ascorbate may have become rate limiting step during antioxidant defense (Foyer and Noctor 2000). This could be the reason for rapid decline of APX activity in flag leaf of Kalyansona under N stress. At molecular level, low N causes a 5-fold increase in ascorbic acid and a severe drop in CAT and APX activities which triggers distinct redox changes and induces oxidative stress (Kandlbinder *et al.* 2004). SOD activity is also increased in tissues of tolerant genotypes in response to diverse abiotic stresses (Zhang 2007, Sairam *et al.* 2002).

The ratio between ROS producers and scavengers indicates efficiency of the antioxidant system. Higher SOD/APX and SOD/CAT ratios were obtained in flag leaf of low N grown Kalyansona indicating that there was more production of H_2O_2, which is not completely scavenged probably causing higher membrane damage due to lipid peroxidation. The levels of H_2O_2 in flag leaf of Uniculm at low N were comparable with that of control N plants. The increased activity of APX and CAT in Uniculm might have resulted in membrane stability (Fig. 4A, 4B and 5A, 5B) in comparison to Kalyansona. These results indicate that the N stress induced generation of ROS was higher in Kalyansona as compared to Uniculm, resulting from the lower APX activity and/or the higher SOD/APX (1.83 in LN grown Kalyansona as compared to average of 1.4 in CN grown plants of both the genotypes as well as LN grown Uniculm plants) or SOD/CAT ratio (6.55 in LN grown Kalyansona as compared to the ratio of 3.9 LN grown plants of Uniculm). Previous studies revealed that increased accumulation of H_2O_2 in stress sensitive plants as compared to stress tolerant plants was associated with higher SOD/APX ratios under various stress conditions, such as salt stress (Mittova *et al*. 2003), chilling stress (Zhou *et al*. 2006) and N stress (Kumagai *et al*. 2009).

Uniculm is able to maintain its metabolic components in spite of large reduction in flag leaf area due to increased antioxidant capacity till maturity, which protects the membrane integrity and scavenges the excess ROS produced in the tissues. Increased activity of SOD, APX as well as CAT in LN plants of Uniculm suggested that a major quantity of H_2O_2 was being scavenged by these routes. Kalyansona was found to be highly sensitive to low N stress in comparison to Uniculm, probably due to the inefficient ROS scavenging capacity leading to the oxidative damage and overall metabolic reduction in N stressed plants. This may be

possibly due to lack of increase in CAT activity, though Kalyansona maintained higher green leaf area. Enhanced H_2O_2 scavenging capacity due to increased CAT activity in the genotypes could be one of the strategies to cope with nutritional stress.

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