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The developing reproductive 'sink' induces oxidative stress to mediate nitrogen mobilization during monocarpic senescence in wheat

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Abstract

Removal of reproductive 'sink,' i.e., spikelets from wheat, after anthesis delays the rate of flag leaf senescence. Oxidative stress and the oxidative damage to proteins were studied in relation to nitrogen mobilization in wheat plants showing normal and delayed senescence. Wheat plants lacking a reproductive sink showed decreased oxidative stress, lower lipid peroxidation and maintained higher protein, oxidatively damaged proteins, and nitrogen levels as compared to plants with reproductive sink during monocarpic senescence. Oxidative damage to the proteins when not followed by high proteolytic activities led to a slower nitrogen mobilization in wheat plants lacking a reproductive sink. Thus, the influence of the reproductive sink was due to its ability to drive forward the nitrogen mobilization process through high ROS levels which mediated both damage to the proteins and influenced proteolytic activities.

Keywords: Nitrogen; Oxidatively damaged proteins; Reproductive sink; Senescence; Wheat

Senescence is a developmental stage of the leaf or the plant ultimately leading to its death. It is highly coordinated at the molecular, cellular, biochemical, and physiological levels, and involves degradation of chlorophyll, proteins, lipids, and nucleic acids, and a decline in photosynthesis rate [1]. Leaf senescence has been regarded as an important determinant of crop productivity. Senescence is regulated by both endogenous factors such as the reproductive sink, hormones, etc., and environmental factors such as abiotic stresses [2]. In monocarpic plants, the developing reproductive sink (grain/pod) often governs the senescence of the whole plant, especially in the leaves and removal of reproductive sink usually delays senescence [3,4]. In wheat, ear removal regulated the senescence process through delayed onset of senescence [5] or decreased rate of senescence [6,7]. The manner in which the reproductive sink acts as a signal in the regulation and execution process of senescence still remains to be answered [8,9].

Senescence is also associated with an increased production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, and its more toxic derivative hydroxyl radical. These toxic ROS oxidize proteins, lipids, and DNA resulting in lipid peroxidation, cellular damage, and cell death as the antioxidant status of the leaf is also reduced [10,11]. It has been shown earlier by several groups of workers that degradation of stromal proteins especially ribulose-1,5-bisphosphate carboxylase/oxygenase may be initiated by ROS [12–14]. Whether these proteins are modified prior to their degradation is still open to debate [15]. The fact that these proteins contribute to the post-anthesis nitrogen, which is mobilized to the reproductive sink in monocarpic plants, is well known.

Characterization of the processes that regulate monocarpic senescence is important in understanding the senescence phenomenon and mobilization of nitrogen.

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In this study, an attempt was made to study the oxidative stress induced damage caused in flag leaf senescence of wheat plants wherein the senescence process was regulated through removal of reproductive sink.

Materials and methods

Triticum aestivum cv. Kalyansona was sown in the fields of Water Technology Centre, Indian Agricultural Research Institute, New Delhi, India, during the month of November. Recommended dose of fertilizers was applied for wheat at the rate of 60N:40P:40K kg/ha as urea:super phosphate:potash, respectively, at the time of sowing and anthesis. At anthesis, the reproductive sink was reduced by complete removal of the spikelets of both the main shoot and the tillers (about three per plant).

Sampling of the flag leaf was done for the various biochemical analyses from anthesis up to full grain maturity at seven day interval. Plants were well watered throughout the experiment. For the biochemical assays, the leaves were cut into small pieces after measuring their fresh weight, frozen in liquid nitrogen, and stored at −80 °C. Three replicates were maintained for all measurements. The various plant parts were dried in oven at 80 °C for dry matter analyses.

In situ $O_2^{\bullet-}$ was estimated using the nitroblue tetrazolium staining method as described [16]. Flag leaf sections were cut with a blade and vacuum-infiltrated (three cycles of 5 min) in 0.5 mg/mL NBT prepared in 10 mM sodium phosphate buffer, pH 7.8. As a control, SOD (10 U/mL) and 10 mM $MnCl_2$ were added to the staining medium before infiltration. Samples were incubated for 1 h in the dark at room temperature and then cleared in 90% ethanol at 70 °C until complete removal of chlorophyll. $O_2^{\bullet-}$ was visualized as blue colour at the site of NBT precipitation. Samples were stored and examined in 70% glycerol. Since, the colour development was more or less uniform throughout the leaf, discs punched out from the middle portion of the leaves have been taken as representatives.

H_2O_2 was extracted according to the procedure of Veljovic-Jovanovic et al. [17]. 0.2 g fresh leaf material was ground in liquid nitrogen and extracted with 2 ml of 5% trichloroacetic acid. Four percentage of insoluble PVPP was added at the time of grinding. The homogenate was centrifuged at 18,000*g* for 5 min, and the supernatant was passed through a Dowex anion exchange resin (1 × 8–400, Sigma-Aldrich Co., St. Louis, MO, USA) equilibrated with 2 ml of 5% TCA to remove the coloured compounds. After centrifugation for 5 min at 1000*g*, the extracts were neutralized to pH 5.6 with K_2CO_3. One unit of ascorbate oxidase was added to the samples, which were then incubated for 10 min at room temperature. The reaction mixture contained 50 μL test solution, 50 μL of 0.5 mM luminol in 0.2 M NH_4OH (pH 9.5), and 800 μL of 0.2 M NH_4OH in 1.0 mL test tubes which were placed in Luminoskan TL Plus luminometer (Labsystems). Chemiluminescence was initiated by the addition of 100 μL of 0.5 mM $K_3Fe(CN)_6$ in 0.2 M NH_4OH and the photons emitted were counted over 5 s. H_2O_2 content was determined using a calibration curve.

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances in leaf homogenates, prepared in 20% TCA containing 0.5% of 2-thiobarbituric acid, and heated at 95 °C for 25 min [18]. Malondialdehyde (MDA) ($\varepsilon = 155\ mM^{-1}\ cm^{-1}$) content was determined spectrophotometrically at A_{532} and corrected for non-specific turbidity at A_{600}.

Total nitrogen in flag leaf, grains, and shoots was quantified in dried samples by Kel Plus, Distyl-EM using the Kjehldahl digestion procedure. Total protein was calculated as total N (mg/g FW) × 6.25 [19].

Frozen leaf samples were ground in a mortar with liquid nitrogen and extracted (3 ml/0.25 g fresh weight) in 30 mM Tris buffer, pH 7.8, containing 1 mM ascorbic acid, 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT and 0.5 mM PMSF [20]. PVPP was added at the time of grinding (0.05 g/gFW). The homogenate was centrifuged at 10,000*g* for 20 min. Total soluble proteins in the supernatant were measured [21].

Carbonyl groups in the proteins were derivatized as described [22]. Samples with a concentration of 10 μg of total soluble proteins were mixed with an equal volume of 12% SDS and then with two volumes of 20 mM dinitrophenylhydrazine dissolved in 10% trifluoroacetic acid (TFA). As controls, samples were treated only with 10% TFA. The mixture was incubated for 25 min at room temperature and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris/30% (v/v) glycerol. The volumes mentioned above always refer to the volume of the sample, before the addition of the derivatizing agent.

Derivatized samples and controls were loaded onto a 10% SDS–PAGE [23]. For Western blot analysis, proteins were electrotransferred at 4 °C onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) at 50 V for 2 h in a Transblot unit (Bio-Rad, Richmond, CA, USA). Immunodetection was carried out with rabbit anti-DNP (Sigma-Aldrich Co.). The titre obtained was 1:2000. The blot was incubated with the primary antibody and immunoreactive protein bands were visualized using anti-rabbit IgG (whole molecule) alkaline phosphatase conjugated antiserum with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate. The molecular weight marker was a pre-stained marker ranging from 20 to 118 kDa and gave blue coloured bands on the blot (MBI Fermentas). The molecular weights of the oxidatively damaged proteins were calculated using the TotalLab software (Biosystematica, UK).

Results and discussion

Manipulation of the reproductive 'sink' in monocarpic plants can accelerate or delay leaf senescence and is a useful tool in studying the regulation of senescence [24]. Removal of all spikes from multiculm wheat plants delays the rate of senescence [6]. The plants with spikelets removed (S−) showed delayed senescence and sampling of the flag leaf could be done up to 32 days after anthesis (DAA) whereas for control plants, i.e., plants with intact spike (S+), sampling was terminated at 28 DAA. At harvest, S− and S+ plants had a shoot dry weight of 7.68 ± 1.20 and 3.20 ± 0.49, respectively. Thus, there was a 58.3% increase in the dry weights of the shoots of the S− plants, which could act as alternate sinks, as compared to the S+ plants.

There was a decline in flag leaf N in S+ plants with a concomitant increase of 46% in grain N (Fig. 1A). There was hardly any mobilization of flag leaf N in S− plants with the levels being more or less the same from anthesis up to 32 DAA. Total proteins increased at 7 DAA in S+ plants after which there was a decline up to grain maturity (Fig. 1B). There was a slow and gradual increase in total proteins in S− plants with the levels being maximum at 32 DAA. There was a decline in total soluble proteins in both sets of plants from anthesis onwards (Fig. 1C). However, S− plants maintained higher levels of proteins as compared to S+ plants, with the difference being maximum at 28 DAA. The decline in soluble protein levels coincided with the increase in the insoluble protein levels, both of which thus contributed to the

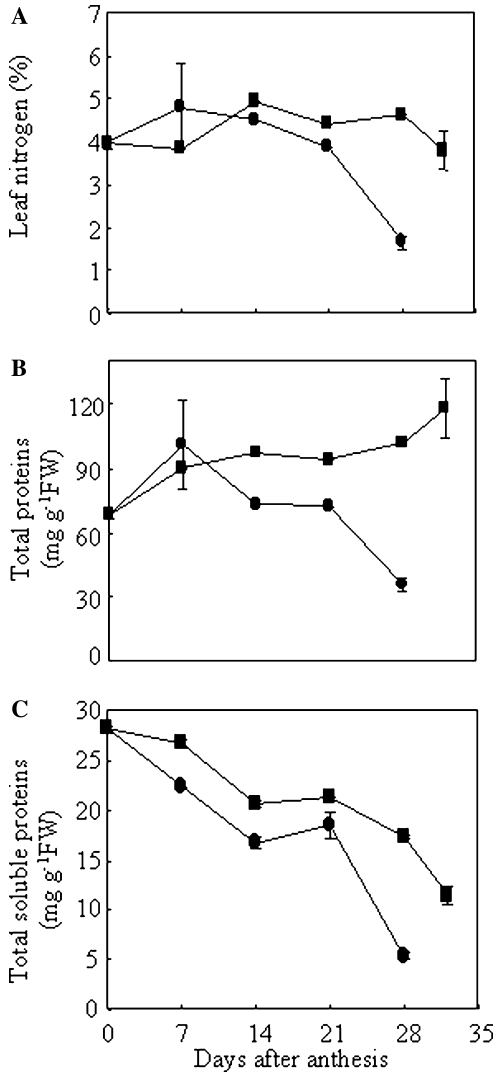

Fig. 1. Effect of reducing sink intensity on nitrogen (A), total protein (B), and total soluble proteins (C) in the wheat cv Kalyansona. Control plants with intact spikes (●); plants with spikelets removed (■). Vertical bars indicate SE ($n = 3$). In some cases, error bars are smaller than the symbols.

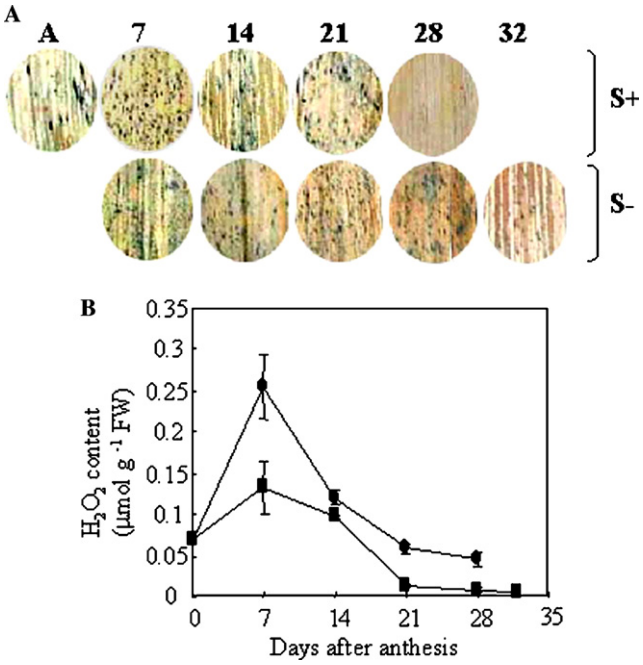

Fig. 2. Effect of reducing sink intensity on superoxide levels (A) and H_2O_2 content (B) in the wheat cv Kalyansona. Symbols as in Fig. 1. Vertical bars indicate SE ($n = 3$). In some cases, error bars are smaller than the symbols.

accumulated N in the flag leaves of S− plants (Fig. 1). In wheat, it has been shown earlier that nitrogen accumulation in plants with no reproductive sink is due to the increased levels of soluble proteins and, at the later stages of senescence due to increased levels of insoluble proteins [19].

Increased oxidative stress in the flag leaf was observed due to increased levels of superoxide and H_2O_2 which was higher in S+ plants as compared to S− plants (Fig. 2). An increase in ROS has been found to increase the expression of senescence-enhanced genes [25]. There was an initial increase in the in situ superoxide levels at 7 DAA in the controls after which the levels showed a decline (Fig. 2A). In S− plants, the increase in the superoxide levels was observed at the later stages of senescence with the maximum being at 28 DAA. S+ plants showed higher H_2O_2 levels as compared to S− plants throughout grain development (Fig. 2B). There was an initial increase in H_2O_2 levels at 7 DAA in both sets of plants after which the levels showed a decline.

Lipid peroxidation increased during senescence of both controls and treated plants and was slightly lower in S− plants as compared to S+ plants (Fig. 3A). Carbonyl groups are taken as presumptive evidence of oxidative modification [22]. Oxygen free radicals catalyze the oxidative modification of animal proteins and mark them for degradation by proteases [26]. The derivatized carbonyl groups as detected by the immunoblot showed a large number of oxidatively damaged proteins ranging from 154.4 to 14.4 kDa (Fig. 3B). The damage, on the whole, was observed to be greater in S− plants as compared to S+ plants. The maximum damage was observed in a 47 kDa protein. Although, these proteins have to be further identified using specific antibodies, it is tempting to speculate that the 47 kDa protein could be a putative PS II 680 chlorophyll apoprotein which is conserved amongst several plant species [27,28]. In both sets of plants, the maximum damage was observed at 7 DAA, after which there was a decrease in the damaged proteins. The control blot, where samples were incubated with only 10% TFA, did not show any band except for the marker lane (data not shown).

At first instance, decreased oxidative stress, lower lipid peroxidation, higher soluble protein levels seem to contradict the higher oxidative damage observed to the proteins in the S− plants (Figs. 1C, 2 and 3). However,

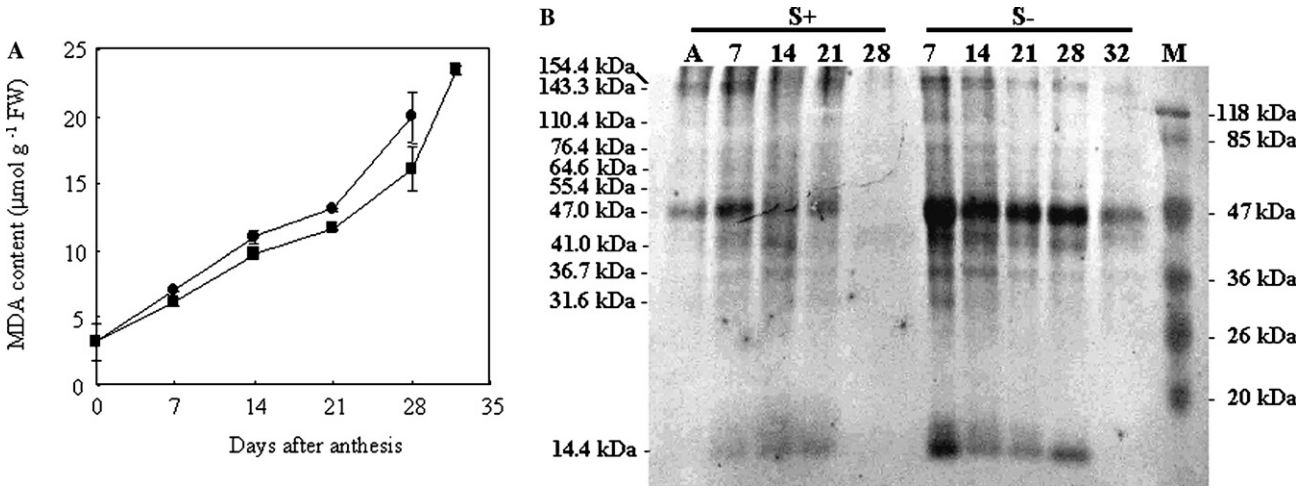

Fig. 3. Effect of reducing sink intensity on lipid peroxidation (A) and oxidative damage to the proteins (B) in the wheat cv Kalyansona. Symbols as in Fig. 1. Vertical bars indicate SE ($n = 3$). In some cases error bars are smaller than the symbols.

the presence of damaged proteins at any given time in the cell is also a function of the proteolytic activities at that point of time. Oxygen free radicals are also known to mediate proteolysis by increasing the activity of endopeptidases [29]. It has been shown earlier that removal of reproductive sink led to lower proteolytic activities, which was observed in this wheat variety as well [7,30]. Proteolytic activities are responsible for nitrogen mobilization during monocarpic senescence in wheat plants [31]. Thus, oxidative damage when not followed by sufficient proteolytic activities leads to a slower nitrogen mobilization leading further to its accumulation in the flag leaf. When more amount of the protein of S+ plants was loaded on the gel and immunoblotted with anti-DNP, the number of proteins showing oxidative damage was the same as those of S− plants (data not shown). This showed that the S+ and S− plants differed only in the extent of the proteins damaged during monocarpic senescence. Thus, in S− plants, ROS levels were sufficient to cause damage to the proteins but not enough to induce high proteolytic activities.

In conclusion the present study showed that desinked wheat plants showed decreased oxidative stress, lower lipid peroxidation, and maintained higher protein, oxidatively damaged proteins and nitrogen levels as compared to plants with reproductive sink during monocarpic senescence. This is the first report which shows the influence of the reproductive sink which is due to its ability to drive forward the mobilization process through high ROS levels which mediates both damage to the proteins and influences proteolytic activities.

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