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ANTIOXIDANT DEFENCE SYSTEM IN WHEAT SEEDLINGS UNDER SODIUM CHLORIDE STRESS: AN INDUCTIVE ROLE OF HYDROGEN PEROXIDE

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SUMMARY

The effect of exogenously supplied hydrogen peroxide on the enzymes involved in antioxidant defense and other related processes in wheat seedlings under NaCl stress was examined. Treatment with hydrogen peroxide activates the antioxidants especially catalase, ascorbate peroxidase and glutathione reductase, however no such activation was observed in case of superoxide dismutase. Treatment with hydrogen peroxide lowers the endogenous level of hydrogen peroxide which is indicative of the activation of reactive oxygen species scavenging system in concomitant with improved growth of wheat seedlings leading to prevention of damage to membranes. However, hydrogen peroxide does not affect the osmotic potential and contents of mineral ions like K , Na and Cl. This clearly indicates that improved growth of wheat seedlings by hydrogen peroxide treatment does not involve osmotic adjustment. These results substantiate the contention that under salt stress conditions an " inductive pulse" of hydrogen peroxide treatment could partially alleviate the salt stress effects on plants.

Key words: **Antioxidant defence system, enzymes, hydrogen peroxide, ionic composition, salt, wheat**

INTRODUCTION

Abiotic stresses affect plant metabolism, disrupt cellular homeostasis, and uncouple major physiological processes (Arora *et al*. 2002, Srivalli *et al*. 2003). A direct result of stress-induced cellular changes is the enhanced accumulation of toxic compounds in cells that include reactive oxygen species (Suzuki and Mittler 2006). Exposure of plants to stress activates plasma membrane bound NADPH-dependent superoxide synthase (Sagi and Fluhr 2006) producing superoxide ($O_2^{\bullet -}$) which in turn is converted to hydrogen peroxide (H_2O_2) by SOD. H_2O_2 can be converted further by a reaction mediated by metals such as Fe^{3+}, or Cu^{2+} into one of the most damaging and reactive hydroxyl radical ($OH^{\bullet}$) that damage the plant metabolic systems leading to plant senescence and programmed cell death (Halliwell and Gutteridge 1989). Under normal conditions, reactive oxygen species (ROS) are cleared from the cell by the action of superoxide dismutase (SOD), catalase, or ascorbate peroxidase (APX). There are increasing evidences that production of ROS such as $O_2^{\bullet -}$ is a common phenomenon in the aerobic organisms under stress conditions. Plants have evolved a complex antioxidant system to prevent the harmful effects of ROS, which play a major role in stress tolerance. The damage by ROS in any cellular component depends on the antioxidant system, comprising the ascorbate-glutathione cycle, superoxide dismutase and a number of other antioxidant enzymes (Noctor and Foyer 1998).

Morita *et al*. (1999) suggested that H_2O_2 is a cardinal molecule involved in oxidative stress signaling, leading to the induction of cytosolic ascorbate peroxidase. Recently, Foyer and Noctor (2005) suggested that ROS are involved in oxidative signal transduction which in turn trigger the

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antioxidant defence system associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology. Hung *et al*. (2005) advocated that H_2O_2 seems to serve as a common stress signal in plants. Stress signal is transduced via ABA, Ca^{2+} and H_2O_2, which activates a common transcription factor associated with SOD, APX and Catalase (CAT) (Agarwal *et al*. 2005). Such findings definitely provide clues regarding H_2O_2 mediated signal transduction pathway for antioxidants protection against oxidative stress. Keeping this in view, it was considered worthwhile to study the role of H_2O_2 on the enzymes involved in antioxidant defense process and associated tissue ionic composition in wheat under NaCl stress.

MATERIALS AND METHODS

Six uniform and healthy seeds of two wheat cultivars (salt tolerant , KRL 1-4 and salt sensitive, HD 2329) were sown in Petri plates (9 cm diameter) lined with filter paper containing 10 ml of distilled water in a BOD incubator at 25 ± 1°C. After 48 h, seedlings were transferred in fresh set of Petri plates containing four treatment solutions - Control (distilled water), H_2O_2 (100 µM), NaCl (120 mmol/l), H_2O_2 (100 µM) + NaCl (120 mmol/l). All the observations were recorded after 48 h of treatment. Length of seedlings was recorded in cm and dry mass expressed as mg seedlings^{-10} was determined after drying the tissues in an oven at 80 ^oC until constant weight. The membrane injury was assessed by calculating relative stress injury (RSI) according to the method described by Dionisio Sese and Tobita (1998) using the formula RSI = $(EC_a/EC_b) \times 100$. EC_a denotes electrical conductivity taken at room temperature after 5 h incubation in distilled water whereas EC_b denotes electrical conductivity after autoclaving the tissue. Lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) according to the method of Heath and Packer (1968) and expressed in mg MDA /g dry weight. H_2O_2 content of the seedlings was determined according to Evans *et al*. (1999). Osmotic potential was determined by using vapour pressure osmometer (5100-B, Wescor Inc. Logan, Utah, USA) and expressed in mega Pascals (MPa).

Catalase (CAT) activity was estimated by the method of Aebi (1983)and expressed as units mg^{-1} protein min^{-1}. Ascorbate peroxidase (APX) activity was assayed by the method of Nakano and Asada (1981) and expressed as nmoles g^{-1}fw. Peroxidase (POX) activity was measured by measuring the oxidation of guaiacol at 470 nm (Rao *et al*. 1996) and was expressed as units mg^{-1} protein min^{-1}. Glutathione reductase (GR) activity was estimated by the method of Goldberg and Spooner (1983) and was expressed as units mg^{-1} protein min^{-1}. Superoxide dismutase activity was estimated according to the method of Jiang and Huang (2001) and expressed as units mg^{-1} protein min^{-1}. Ascorbic acid content was measured by the method of Schopfer (1966) and was expressed in nmoles g^{-1}fw. Sodium, potassium and chloride contents were determined on microprocessor based Ion Analyzer (Elico, India) using ion specific electrode (Na, K and Cl).

RESULTS AND DISCUSSION

Seedling length and dry mass: The length and dry mass of NaCl treated seedling decreased considerably as compared to untreated seedlings and the genotype KRL 1-4 was more resistant to salt as compared to HD 2329 (Fig. 1 A & B). It is interesting to note that addition of H_2O_2 improved length as well as dry mass of seedlings under salt stress and unstressed conditions, however, magnitude of effect was more under saline conditions. The improvement in length and dry biomass of seedlings was marginal in KRL 1-4 than HD 2329. The decline in length and dry weight of seedlings under salt stress could be attributed to the reduced mobilization of the reserve food materials from endosperm to growing axis (Sharma *et al*. 1994). However, the pivotal point broughtforth by the present investigation is that the presence of H_2O_2 in the medium caused a stimulation of growth in all the cases.

Relative stress injury and lipid peroxidation: The relative stress injury (RSI) increased upon imposition of NaCl stress (Fig. 1C). More RSI was noted in sensitive HD 2329 than the salt resistant KRL 1-4. Presence of H_2O_2 in the medium lowered the level of RSI in case of all the treatments. The efficacy of H_2O_2 was more pronounced in cultivar HD 2329. Lipid peroxidation in

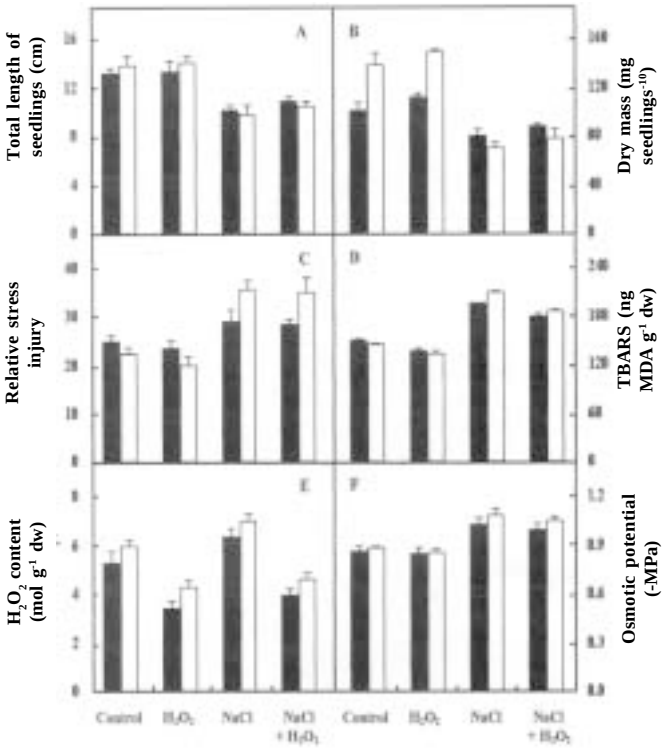

Fig. 1. Effect of NaCl and H_2O_2 on (A) seedling length, (B) dry mass, (C) relative stress injury, (D) lipid peroxidation (TBARS), (E) H_2O_2 content and (F) osmotic potential in two cultivars of wheat. ■ Denotes cultivar KRL 1-4, □ denotes cultivar HD 2329. Vertical bars on top represent standard deviation (SD).

terms of TBARS content increased in NaCl-stressed seedlings as compared to unstressed seedlings (Fig. 1D). Magnitude of lipid peroxidation was more in cultivar HD 2329 than cultivar KRL 1-4. Addition of H_2O_2 in the medium brought down the level of lipid peroxidation considerably in both the cultivars.These results suggest that NaCl stress had an increasing effect on relative stress injury. The enhancement of TBARS in terms of MDA content under NaCl stress corroborated with the findings of Bliss *et al.* (1984) who have reported that cell membranes are the site for primary or secondary salt effects. Changes in permeability due to lesion formation, transport of both organic and inorganic solutes cause structural alteration and ion leakage. The interesting point that emanates from the present study is that H_2O_2 caused a partial reversal of NaCl induced membrane leakiness as was apparent from both relative stress injury and activity of TBARS in both the cultivars. Although no direct evidence of beneficial effects of H_2O_2 on the membrane leakage are available in the literature, yet such an effect could be attributed to the H_2O_2 signal-mediated defence triggering system (Hung *et al.* 2005) that reduced membrane damage.

Hydrogen peroxide content: Endogenous hydrogen peroxide is an indicator of ROS status of plant tissue which is explicitly increased in the NaCl stressed seedlings in both the cultivars. It was worthwhile to note that levels of H_2O_2 were more in sensitive cultivar HD 2329 under stress as well as non-stress conditions. The most important observation was that exogenous H_2O_2 treatment resulted in lowering of the level of endogenous H_2O_2 in both cultivars (Fig. 1E). Levine *et al.* (1994) also putforth that H_2O_2 is one of the crucial reactive oxygen species produced in response to different environmental stresses including salt and ionic stress. However, the interesting observation is the lowering of endogenous levels of H_2O_2 in all the treatments on exogenous application. H_2O_2 might act as an inducer of its scavenging enzymes like CAT and other enzymes which in turn resulted in lowering the endogenous level of H_2O_2 (Sairam and Srivastava 2000).

Osmotic potential: Osmotic potential of seedlings became more negative in NaCl stressed seedlings as compared to control (Fig. 1F). Both the cultivars behaved almost in similar fashion, but a slightly more negative osmotic potential was observed in cultivar HD 2329.These results are in accord with Nandwal *et al.* (2000) who also reported more negative osmotic potential in various plant parts under salinity in *Vigna radiata.* There is no direct evidence suggesting involvement of H_2O_2 in osmotic adjustment and resultant improvement in growth under salinity.

Antioxidative defence system: Imposition of NaCl stress caused reduction in the CAT activity (Fig. 2A). Addition of the H_2O_2 in the medium caused a well marked rise in the CAT activity as compared to control in both the cultivars. It was seen that CAT activity was higher in KRL 1-4 than HD 2329 under stress as well as non-stress conditions. The decrease in catalase (CAT) activity under NaCl stress (Fig. 2A) could be attributed to CAT being a H_2O_2 detoxifying enzyme and mostly associated with peroxisomes where it removes H_2O_2 formed during photorespiration (Apel and Hirt 2004).

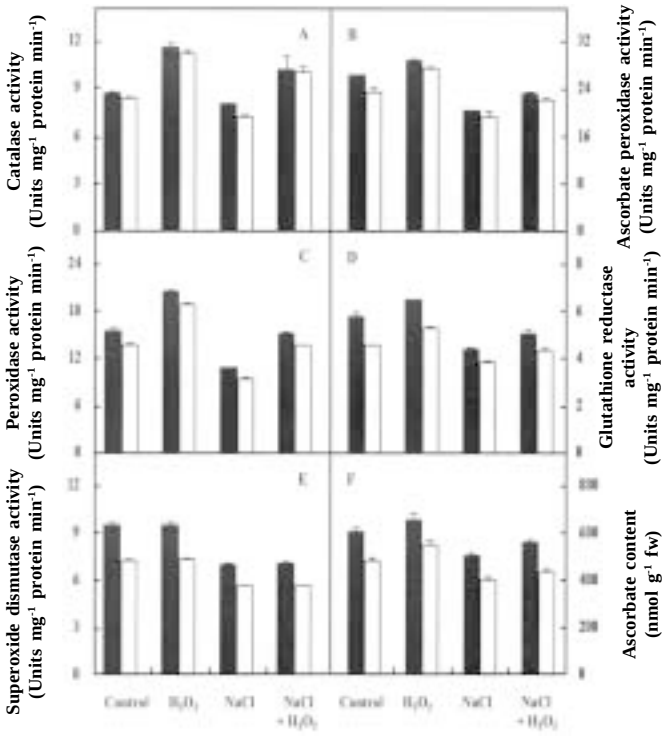

Fig. 2. Effect of NaCl and hydrogen peroxide on activity of (A) catalase, (B) ascorbate peroxidase, (C) peroxidase, (D) glutathione reductase, (E) superoxide dismutase and (F) content of ascorbic acid in two cultivars of wheat. ■ Denotes cultivar KRL 1-4, □ denotes cultivar HD 2329. Vertical bars on top represent standard deviation (SD).

Even, Corpas *et al.* (1993) reported a significant decrease in the enzyme activity during salt stress in both salt tolerant and salt sensitive cultivars of pea leaves. Swaraj *et al.* (1995) showed a decrease in the activity of CAT in *Cajanus cajan* and similarly, Dhindsa *et al.* (1981) also observed a decrease in CAT activity and suggested that it was a consequence of cumulative membrane deterioration due to the acceleration of lipid peroxidation. Likewise, the APX activity was drastically decreased in response to NaCl stress over unstressed control in both the cultivars (Fig. 2B). H_2O_2 treatment caused an activation of APX in control as well as NaCl treated seedlings. Invariably, again the enzyme activity was higher in KRL 1-4 than HD 2329. These results are in consonance with the results of Comba *et al*. (1998) who observed more APX activity at 50 than 200 mmol/l NaCl in soybean root nodules. It was suggested that decreased enzyme activity might be due to imbalance between the production of active oxygen species and the quenching ability of antioxidants that got upset and resulted in oxidative damage. Exactly similar trends were observed with respect to POX activity in KRL 1-4 and HD 2329 seedling as that of CAT and APX. NaCl treatment caused a reduction in enzyme activity and H_2O_2 treatment partially alleviated the effect of NaCl. KRL 1-4 depicted a higher activity of peroxidase than HD 2329 (Fig. 2C).These results are in agreement with Bhattacharjee and Mukherjee (1997) and Santos *et al*.(2001) who also observed a decrease in POX activity under salt stress in rice and sunflower, respectively. Wang *et al.* (1999) also noticed a decrease in the activities of POX, CAT and APX enzymes in fig cell lines under salt treatment and it was suggested that these enzymes might be imparting salt tolerance to fig cells. GR activity decreased considerably in both the cultivars when NaCl was present in the medium (Fig. 2D). Differences within the cultivars in KRL 1-4 and HD 2329 were clearly significant as KRL 1-4 maintained higher activity. Exogenously applied H_2O_2 caused a significant stimulation in the activity of GR under NaCl stress condition. Comba *et al.* 1998 also observed that soybean root nodules when exposed to 200 mmol/l NaCl concentration, the GR activity decreased. Likewise Hernandez *et al.* (2000) found that in sensitive cultivar of pea showed diminished activity of the GR, however, reverse was evident in case of tolerant pea cultivar. Even high salinity (150 mmol/l NaCl) decreased all the antioxidative parameters (except CAT and DHAR) in sensitive clone of potato (Benavides *et al.* 2000). Activity of SOD was also found to be decreased in NaCl treated seedling as compared to untreated seedlings (Fig. 2E). Unlike all other enzymes, presence of H_2O_2 in the medium could not bring about any noticeable stimulation in the activity of SOD rather it caused a slight decline. KRL 1-4 sustained a higher activity of SOD and showed a lesser decline under NaCl treatment. These results are in consistent with Dionisio–sese and Tobita (1998) who exihibited an elevated level of SOD activity in salt tolerant rice and lower activity was observed in sensitive plants. Similar decrease in SOD activity with salinity stress was also reported in *Cajanus cajan* (Swaraj *et al.* 1995) and wheat (Liu *et al.* 1995). Kayupova and Klyshev (1984) have ascribed such a decrease in SOD activity to destruction/inactivation of SOD by NaCl by

removing/replacing the metal moiety from the enzyme. Decreased SOD activity resulted in higher accumulation of H_2O_2, thereby, affecting all subsequent steps of ascorbate–glutathione cycle (Polle 2001). Ascorbate content decreased in the NaCl treated seedling of both the cultivars (Fig. 2F). In the presence of H_2O_2, an increase in the ascorbate content was observed. However, KRL 1-4 had higher ascorbate content in comparison to HD 2329.These results could be ascribed to rapid oxidation and slow synthesis rate of ascorbic acid or decreased conversion rates of monodehydroascorbate and dehydroascorbate to ascorbate (Hernandez *et al*. 2000). Furthermore, Hernandez *et al.* (2000) also observed high levels of ascorbate in salt tolerant and lower levels in salt sensitive cultivars of pea and foxtail millet in response to salt stress (Sreenivasulu *et al*. 2000). Ascorbate is known to be oxidized by a direct reaction with superoxide or by serving as a reductant of α-chromoxyl radical of oxidized α-tocopherol, which in turn, disrupts lipid peroxidation reactions by reacting with superoxide and results in scavenging hydroxyl, peroxyl and alkoxyl radicals (Halliwell and Gutteridge 1989).

Na, K and Cl and K/Na: Na content was almost negligible in unstressed seedlings irrespective of the cultivars but NaCl treatment caused a tremendous increase in Na content (Fig. 3A) It is worth noting that Na content was more in HD 2329 than KRL 1-4. On the other hand, potassium (K) content was almost unaffected in KRL 1-4 while in HD 2329, it slightly decreased upon imposition of NaCl stress (Fig. 3B). As obvious, K/Na ratio also declined in NaCl treated seedlings (Fig. 3C). Chloride (Cl) content showed similar trends as that of Na (Fig. 3D). Cultivar KRL 1-4 accumulated lesser Na and Cl in NaCl treated seedlings and maintained better K/Na ratio as compared to cultivar HD 2329. Although reports on H_2O_2– induced mineral imbalances have not been seen in the literature, these could be ascribed to disturbances in ionic homeostasis. The beneficial effects of exogenously applied H_2O_2 could involve the restoration of favorable ionic balance of the cultivars which got disturbed due to NaCl treatment.

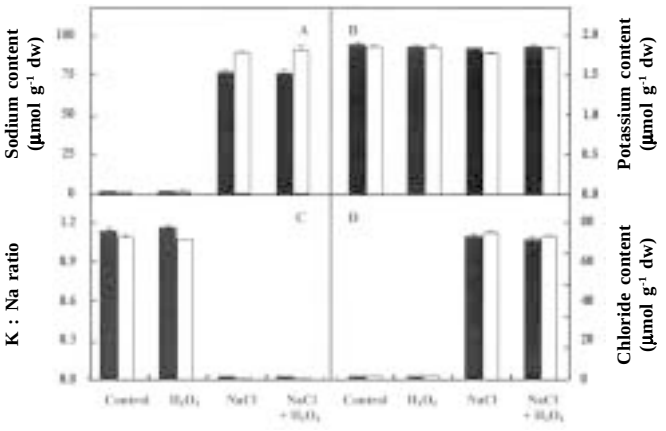

Fig. 3. Effect of salinity and hydrogen peroxide on (A) sodium, (B) potassium, (C) K: Na ratio and (D) chloride content in two cultivars of wheat. ■ Denotes cultivar KRL 1-4, □ denotes cultivar HD 2329. Vertical bars on top represent standard deviation (SD).

It could be inferred that H_2O_2 is a membrane permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Levine *et al.* 1994). It is also known to induce several other genes and proteins involved in stress defenses like CAT (Scandalios *et al.* 1997) and peroxidase (Prasad *et al.* 1994). Agarwal *et al*. (2005) found that H_2O_2 effectively increased the activities of H_2O_2 scavenging enzymes in addition to stimulation of growth as has also been observed in the present study (Fig1A). It is further suggested that stress signal is transduced via ABA, Ca^{2+} and H_2O_2 which might be responsible for the activation of some common transcription factors associated with antioxidant defence mechanism against the generated ROS by NaCl treatment. Recently, Wahid *et al*. (2007) suggested that H_2O_2 signals the activation of antioxidants in seed, which persists in the seedlings to offset the ion-induced oxidative damage. These changes led to the expression of stress proteins and improved physiological attributes, which supported the seedling growth under salinity. Likewise, improved heat stress tolerance in *Agrostis stolonifera* with pre-treatment with H_2O_2 was due to the induction of better antioxidant protection (Larkindale and Huang 2004). H_2O_2 alone or in combination with glutamine may also act as an intercellular and systemic signaling system to achieve tolerance to various environmental stresses (Foyer *et al.* 1997, Morita *et al.* 1999).

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