Research Article

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Changes in catalase, peroxidase activities and soluble proteins in wheat leaves on thiourea and H_2O_2 treatments.

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Different thiourea (10 & 20 mM) and H_2O_2 (10 & 20 mM) treatment effects on antioxidant enzymes (catalase and peroxidase) and soluble protein was investigated in wheat leaves. A gradual decrease in leaf protein content in control leaves was observed. Under exposure of 10mM H_2O_2 stress, a gradual decrease in leaf protein content was observed with a peak at 7^{th} day of stress. This gradual decrease in leaf protein content was completely prevented by 20 mM thiourea treatment. Catalase activity was increased under oxidative stress by 10 mM H_2O_2 and after both thiourea treatments (10 & 20 mM). However, collective application of thiourea and H_2O_2 lowered the leaf catalase activity. Leaf peroxidase activity was significantly increased under oxidative stress induced by H_2O_2 and after thiourea treatment as compared to control. After 20mM H_2O_2 and 10 mM thiourea treatment more pronounced increase in peroxidase activity was observed. Briefly, 10 mM H_2O_2 treatment increased the antioxidants and protein level while 20 mM H_2O_2 adversely affect the catalase activity in leaves. Moreover, thiourea treatments enhanced the leaf catalase and peroxidase activities as protective effect.
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Key words: Thiourea, peroxidase, catalase, wheat, antioxidants.

Wide range of environmental factors including drought, UV stress, herbicide action and oxygen shortage can induce oxidative stress (Blokhina *et al.,* 2003). During oxidative stress produced reactive oxygen species (ROS) i.e. superoxide anion and hydrogen peroxide (H_2O_2) are thought to be involved in several areas of plant physiology and development. Under many metabolic processes such as chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems, reactive oxygen species (ROS) i.e. superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), and the hydroxyl free radical (OH^-) are produced even under optimal conditions (Asada, 1997). Furthermore, during abiotic stress conditions like drought and salt stress (Lin and Wang, 2002) excessive ROS are produced which give rise to oxidative damage at cellular level (Zhang *et al.,* 2003).

Due to any imbalance between free radical generation and antioxidant defense system efficiency, free radicals leads to oxidation of various macromolecules like membrane lipids, proteins, and DNA. Normal physiology of cell disrupted by this macromolecular oxidation and ultimately leads to cellular pathogenesis (Blokhina *et al.,* 2003).

Plant cells posses a highly efficient antioxidative defence system, including both nonenzymic and enzymic constituents to counteract the reactive oxygen species. This antioxidant defence system contains low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols), enzymes regenerating the reduced forms of antioxidants and ROS-interacting enzymes SOD, peroxidase and catalase (Blokhina *et al.,* 2003). Within the enzymatic defense system Superoxide dismutase, ascorbate peroxidase and catalases are the key players. During stresses like UV radiations, pathogens, low temperature, drought and heavy metals enhanced peroxidase activity protect the cells from H_2O_2 stress (Scalet *et al.,* 1995). H_2O_2

generated through superoxide dismutation also induce cytosolic ascorbate peroxidase production under oxidative stress (Morita *et al.,* 1999).

Catalase a front line antioxidant enzyme which by dismutation, degrade H_2O_2 to water and oxygen occurs in all aerobic organism. Plant catalyses are generally localized in micro bodies. Usually plant contains multiple isozymes and small families of catalase genes, which exhibit differential pattern of organ specific and development expression (Willekens *et al.,* 1997).

Peroxidases are of importance in a variety of cellular functions. The existence of an inverse correlation between cell wall peroxidase and growth is well documented (Chanda and Singh, 1997; Zheng and Vanhuystee, 1992). The high peroxidase activity has been observed in hypocotyls of seedlings treated with heavy metals (Hg or Cr), which might indicate the initiation of a disruption in the biochemical processes that precede the appearance of visible symptoms of toxicity (Parnar and Sumitra, 2005).

Thiourea has been proved to be the best scavenger of hydroxyl radicals which can be produced due to superoxide and H_2O_2 reaction. Superoxide dismutase and ascorbate peroxidase activities are decreased in light and darkness due to H_2O_2-induced stress (Lin and Kao, 1998). Thiourea (0.1-10 mM) can provide dose-dependent protection against protein oxidation. Thiourea also significantly inhibits copper-catalyzed oxidation of ascorbate. Reduction of cytochrome-C due to xanthine/xanthine oxidase superoxide-generating system is completely inhibited by thiourea. Thus, hydrogen peroxide, hydroxyl radicals and superoxide radicals are directly scavenged by thiourea (Kelner *et al.*, 1990).

In this view, the present study was planed to reveal the changes in antioxidant enzymes i.e. catalase, peroxidase activities and soluble protein contents in wheat leaves that occur on thiourea and H_2O_2 treatments.

MATERIALS AND METHODS

Seeds of wheat (*Triticum aestivum* L) variety MH-97 with uniform size were used in the experiment. Seeds in three replicates (35 seedlings per replicate) were germinated in darkness for 24 hours at 25±1°C on wet (with H_2O) filter paper in petridishes. Germinated seeds were then covered with a lid to minimize the evaporation, and growth was continued in an incubator for 24 h at 25±1°C. Hydrogen peroxide (10 mM and 20 mM H_2O_2) and thiourea (10 mM and 20 mM) were applied on 4th day of germination. Treatment details are presented in the table 1. Except for control, water as the medium was replaced with treatment solutions and the growth of the seedlings was continued at 25±1°C for 8 days. Initial leaf samples were collected after every 24 hours (5th, 6th, 7th and 8th day of treatment) and used for different biochemical analyses.

Extraction of antioxidant enzymes: For extraction of enzymes, fresh leaves (0.5 g) were ground in extraction buffer (specific for different enzymes) and centrifuged at 15,000×g for 20 min at 4 oC. The supernatant was separated and used for the determination of different enzyme activities. Details are given below.

Estimation of antioxidant enzymes: For the estimation of catalase and peroxidase leaves (0.5 g) were homogenized in medium composed of 50 mM potassium phosphate buffer, pH 7.0 and 1 mM dithiothreitol (DTT). Activities of peroxidase (POD) and catalase (CAT) were measured using the method of Chance & Maehly (1955) with some modification.

Catalase (CAT) activity: For measurement of CAT activity assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H2O2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Decrease in absorbance of the reaction solution at 240 nm was recorded after every 20 sec. An absorbance change of 0.01 units min-1 was defined as one unit CAT activity. Enzyme activities were expressed on protein basis.

Peroxidase (POD) activity: For measurement of POD activity assay solution (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding the enzyme extract. Increase in absorbance of the reaction solution at 470 nm was recorded after every 20 sec. One unit POD activity was defined as an absorbance change of 0.01 units min-1.

Table1: Different hydrogen peroxide and thiourea treatments used in the study.

Sr. No.	Symbol	Treatment
1	Cont.	H_2O
2	10 mM HP	10 mM H_2O_2
3	20 mM HP	20 mM H_2O_2
4	10 mM TU	10 mM Thiourea
5	10 mM HP + 10 mM TU	10 mM H_2O_2 and 10 mM thiourea
6	20 mM HP + 10 mM TU	20mM H_2O_2 and 10 mM thiourea
7	20 mM TU	20 mM Thiourea
8	10mM HP + 20 mM TU	10mM H_2O_2 and 20 mM thiourea
9	20 mM HP + 20 mM TU	20 mM H_2O_2 and 20 mM thiourea

TU: thiourea, HP: Hydrogen Peroxide

Soluble protein contents: Total soluble protein content of leaf was measured by dye binding assay as described by Bradford (1976).

Statistical analysis: Experiments were conducted in triplicates. First of all descriptive statistics were applied to analyze and organize the resulting data. F-test was applied to find differences in variance among samples. The significance of differences between means for different parameters was measured using Student's t-Test (two tailed) at 0.01 and where applicable at 0.05 significance level. All the statistical calculations were performed using computer software Microsoft Excel 2002.

RESULTS

Influence of different thiourea treatments on antioxidant enzymes and soluble protein contents under hydrogen peroxide induced oxidative stress and non stressed condition was investigated. In control, catalase activity was extremely lower and almost same after 5^{th} to 6^{th} day however after that a gradual increase in catalase activity was observed from 7^{th} to 8^{th} day. Similarly, under both levels of oxidative stress (10 mM and 20 mM H_2O_2), catalase activity was extremely lower and almost same after 5^{th} to 7^{th} day. However, after 8^{th} day, a sharp and many fold increase in catalase activity was observed (figure 1).

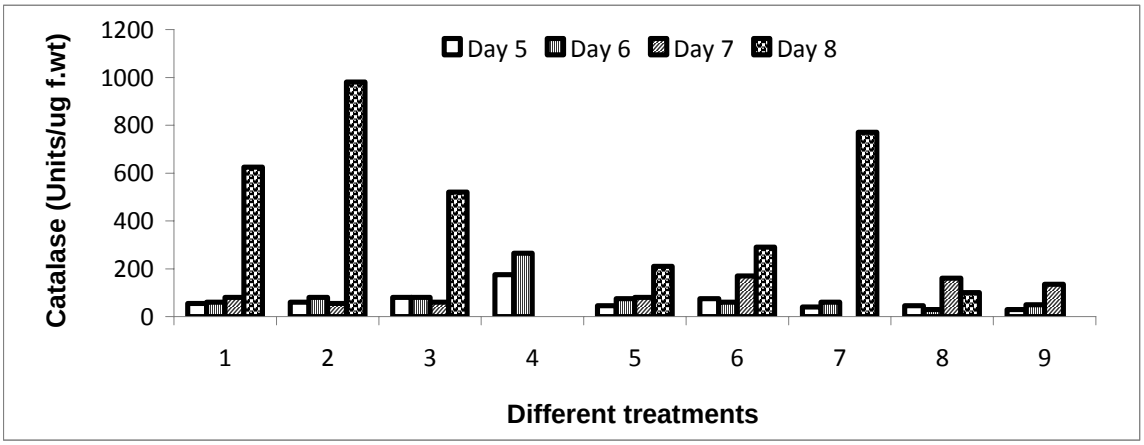
Under non stressed condition, the leaf catalase activity was increased from 5^{th} to 6^{th} day on 10 mM thiourea treatment followed by non- detectably low catalase activity from 7^{th} to 8^{th} day of treatment. In case of 20 mM thiourea treatment under non- stressed condition, catalase activity was extremely lower and almost same after 5^{th} to 6^{th} day followed by non- detectably low catalase activity after 7^{th} day of treatment. Here after a significant and many fold increase in activity was seen after 8^{th} day of 20 mM thiourea treatment (figure 1).

In absence of thiourea treatment very small increase in catalase activity was observed from 5^{th} to 7^{th} day of oxidative stress by 10 mM H_2O_2. However, after 8^{th} day many fold increase in leaf catalase activity was seen. Under oxidative stress by 10 mM H_2O_2 and 10 mM thiourea treatment, a slight increase in leaf catalase activity was observed with the passage of time. Moreover, catalase activity was non- detectable on 6^{th} day after 20 mM thiourea treatment. After 7^{th} day of 20 mM thiourea treatment there was significant increase in catalase activity followed by decrease in activity during next 24 hours (8^{th} day).

In absence of thiourea treatment, no change in catalase activity was observed from 5^{th} to 7^{th} day of oxidative stress by 20 mM H_2O_2. However, after 8^{th} day many fold increase in leaf catalase activity was seen. In case of thiourea treatment (10 mM), after 5^{th} to 6^{th} day of oxidative stress (20 mM H_2O_2), there was no change in catalase activity. However, from 6^{th} to 8^{th} day there was a gradual increase in catalase activity. A gradual increase in catalase activity was observed after 5^{th} to 7^{th} day of 20 mM thiourea treatment. However 8^{th} day after 20 mM thiourea treatment catalase activity was non-detectably lower (figure 1).

In control after 6^{th} day first slight increase in peroxidase activity was observed then after 7^{th} day a very slight decrease in activity was found. Again very sharp increase in leaf peroxidase activity was seen after 8^{th} day. Under oxidative stress by 10 mM H_2O_2 a sharp increase in leaf peroxidase activity was observed during the whole stress duration.

Figure: 1. Effect of stress and Thiourea treatments on catalase level in wheat leaves.



`1= T_0C: Control (H_2O), **2= T_0S_1:** Stressed (10 mM Hydrogen peroxide), **3= T_0S_2:** Stressed (20 mM Hydrogen peroxide), **4= T_1C:** 10 mM Thiourea, **5= T_1S_1:** 10 mM Hydrogen peroxide and 10 mM Thiourea, **6= T_1S_2:** 20 mM Hydrogen peroxide and 10 mM Thiourea, **7= T_2C:** 20 mM Thiourea, **8= T_2S_1:** 10 mM Hydrogen peroxide and 20 mM Thiourea, **9= T_2S_2:** 20 mM Hydrogen peroxide and 20 mM Thiourea.

Similarly under second level of oxidative stress (20 mM H_2O_2), the gradual increase in peroxidase activity was seen up to 7th day, after that decrease in activity was observed at 8th day (figure 2).

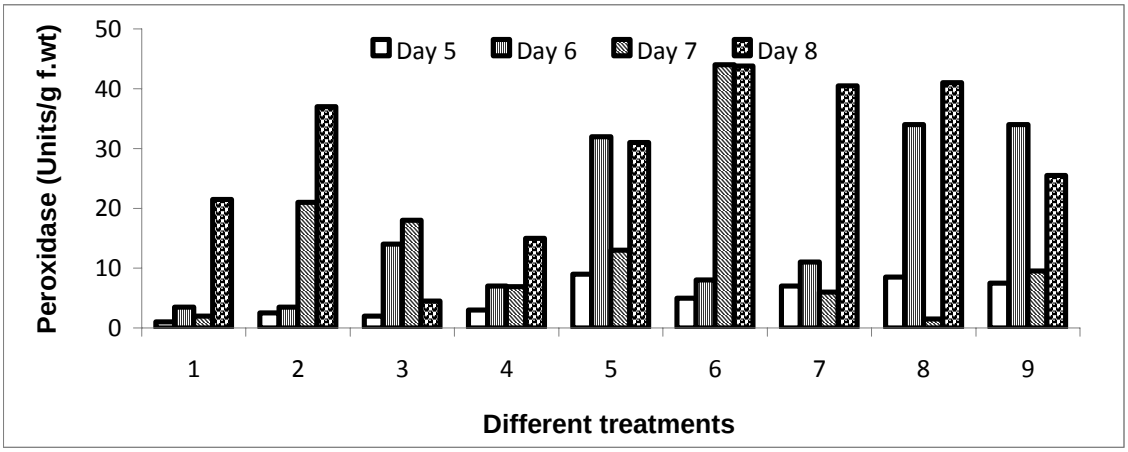
A steady increase in leaf peroxidase activity was observed during the whole period of 10 mM thiourea treatment. However in case of 20 mM thiourea treatment initially an increase in peroxidase activity after 6th day followed by a decrease in activity after 7th day and once again a many folds increase after 8th day was observed. In control a prompt increase in peroxidase activity was observed with passage of time under oxidative stress by 10 mM H_2O_2 (figure 2).

After 10 mM thiourea treatment under oxidative stress by 10 mM H_2O_2, the peroxidase activity was increased from 5th to 6th day followed by a decrease in activity after 7th day. Again after 8th day a significant increase in peroxidase activity was observed. Similarly after 20 mM thiourea treatment under oxidative stress by 10 mM H_2O_2, the peroxidase activity was increased from 5th to 6th day followed by decrease resulting into non- detectably low activity after 7th day. Again after 8th day significant increase in peroxidase activity was observed. Again after 8th day a significant increase in peroxidase activity was observed. In non- treated control a gradual increase in peroxidase activity was observed up to 7th day. After 8th day there was a significant decrease in peroxidase activity (figure 2). Under oxidative stress by 20 mM H_2O_2 there was a sharp and significant increase in leaf peroxidase activity in 10 mM thiourea treated leaf up to 7th day, followed by no change in peroxidase activity at 8th day. Initially an increase in peroxidase activity was observed 6th day after 20 mM thiourea treatment and then a decrease in activity was observed after 7th day. Once again a significant increase in leaf peroxidase activity was observed after 8th day (figure 2).

Effect of different levels of oxidative stress induced by exogenous application of hydrogen peroxide on leaf protein contents in wheat was studied. A gradual decrease in protein contents was observed in control with passage of time. Under first level of oxidative stress (10 mM H_2O_2), there was a significant decrease in protein concentration from 5th to 6th day followed by a significant increase after 7th day of stress. Again during next 24 hours of stress (8th day) there was many fold decrease in protein contents (figure 3).

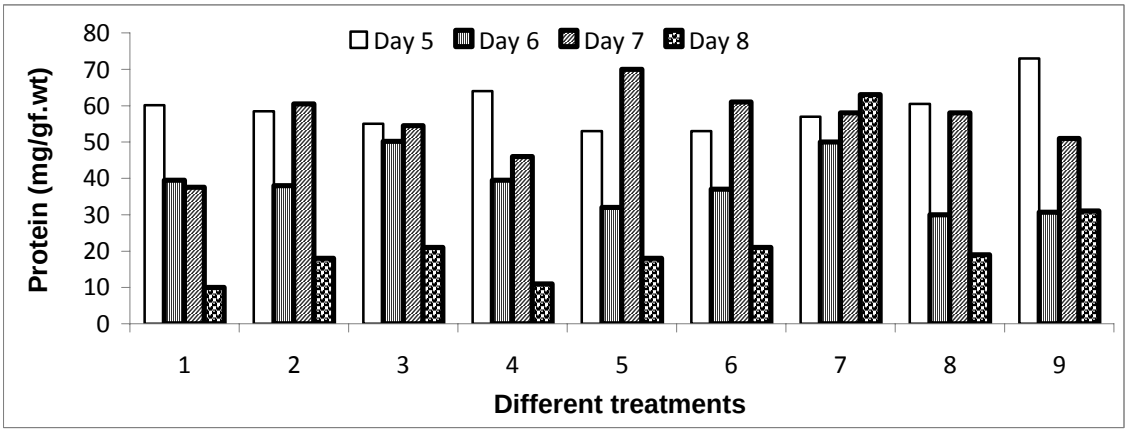
Under second level of oxidative stress (20 mM H_2O_2), there was a non- significant difference in protein concentration after 5th , 6th and 7th day followed by a significant decrease during next 24 hours of stress (8th day). Effect of different thiourea treatments on leaf protein contents under normal condition in wheat was studied. A gradual decrease in protein contents was observed in control with passage of time. At (10 mM) thiourea treatment under normal condition there was a

Figure: 2. Effect of stress and Thiourea treatments on peroxidase level in wheat leaves.



1= T_0C: Control (H_2O), **2= T_0S_1:** Stressed (10 mM Hydrogen peroxide), **3= T_0S_2:** Stressed (20 mM Hydrogen peroxide), **4= T_1C:** 10 mM Thiourea, **5= T_1S_1:** 10 mM Hydrogen peroxide and 10 mM Thiourea, **6= T_1S_2:** 20 mM Hydrogen peroxide and 10 mM Thiourea, **7= T_2C:** 20 mM Thiourea, **8= T_2S_1:** 10 mM Hydrogen peroxide and 20 mM Thiourea, **9= T_2S_2:** 20 mM Hydrogen peroxide and 20 mM Thiourea.

Figure: 3. Effect of stress and thiourea treatments on protein level in wheat leaves.



1= T_0C: Control (H_2O), **2= T_0S_1:** Stressed (10 mM Hydrogen peroxide), **3= T_0S_2:** Stressed (20 mM Hydrogen peroxide), **4= T_1C:** 10 mM Thiourea, **5= T_1S_1:** 10 mM Hydrogen peroxide and 10 mM Thiourea, **6= T_1S_2:** 20 mM Hydrogen peroxide and 10 mM Thiourea, **7= T_2C:** 20 mM Thiourea, **8= T_2S_1:** 10 mM Hydrogen peroxide and 20 mM Thiourea, **9= T_2S_2:** 20 mM Hydrogen peroxide and 20 mM Thiourea.

significant decrease in protein concentration from 5^{th} to 6^{th} day followed by a slight increase after 7^{th} day of treatment under normal condition. Again during next day of treatment (8^{th} day) there was sharp decrease in protein contents (figure 3).

In non treated leaf there was no significant change in leaf protein contents after 5^{th}, 6^{th} and 7^{th} day of oxidative stress by 20 mM H_2O_2. But from 7^{th} day to 8^{th} day of oxidative (20 mM H_2O_2) there was a sharp decrease in protein concentration in non treated leaf. Moreover after 10 mM thiourea treatment leaf protein concentration first decreased after 48 hours, than increase after 7^{th} day and finally again decreased after 8^{th} day of oxidative stress by 20 mM H_2O_2. After 20 mM thiourea treatment a significant decrease in protein concentration was observed from 5^{th} to 6^{th} days of oxidative stress (20 mM H_2O_2). However increasing trend in protein contents was observed after 7^{th} day of oxidative stress, followed by significant decrease in protein contents after 8^{th} day, in thiourea treated (20 mM) leaf (figure 3).

DISCUSSION

H_2O_2 can play an important role in the induced tolerance against oxidative stress by

activation of the plant antioxidant system in a dose dependent manner i.e. moderate doses of H_2O_2 enhanced the antioxidant status and induced stress tolerance, while higher concentrations caused oxidative stress and symptoms resembling a hypersensitive response (Gechev *et al*., 2002). Similar was also evident from the present study as catalase activity increased under oxidative stress by H_2O_2 while severe oxidative stress by 20 mM H_2O_2 adversely affected (lowered) the leaf catalase activity in aged leaves. These results are further justified by the description of Baek and Skinner, (2003) that the freezing injury has been shown to involve the attack of ROS and cold acclimation may involve a decrease in the expression level of catalase enzyme. Repression of catalase transcription might be caused by the accumulation of H_2O_2 (Yi *et al*., 2003). Similarly suppressed catalase translates (activity) in present study under oxidative stress caused by exogenous hydrogen peroxide might be due to endogenous increase or accumulation of H_2O_2. There is a report that catalase activity decreased progressively with increasing salt concentration (Garratt *et al*., 2002) that also supports our present observations.

Reduced catalase activity can be compensated by alternative H_2O_2-scavenging mechanisms such as increased ascorbate peroxidase and glutathione peroxidase levels (Willekens *et al*., 1997). This observation provides explanation for reduced catalase activity and simultaneous increase in peroxidase activity under oxidative stress in present study. Further it has been reported that double antisense plants lacking the two major hydrogen peroxide-detoxifying enzymes, ascorbate peroxidase (APX) and catalase (CAT), activate an alternative/redundant defense mechanism that compensates for the lack of APX and CAT (Rizhsky *et al.,* 2002). This also point towards activation of alternative defence mechanisms under stress.

In higher plants, peroxidase plays an important role in the metabolism of H_2O_2. Because in present experiment, leaf peroxidase level increased under H_2O_2 induced oxidative stress, one can assume that H_2O_2 acting as second messenger enhanced the level of this antioxidant enzyme. It is also important to mention here that combined thiourea treatment and oxidative stress further increased the leaf peroxidase activity showing that treatment with ROS scavenger thiourea counteracts the adverse effects of oxidative stress induced by hydrogen peroxide.

Increase in peroxidase activity under oxidative stress induced by exogenous hydrogen peroxide has been reported in rice leaves (Lin and Kao, 1998). Similarly, the level of peroxidase activity was significantly higher as compared to the control up to 7^{th} day of stress. However at 8^{th} day the peroxidase activity was suppresses under oxidative stress induced by 20 mM hydrogen peroxide. This may be explained as H_2O_2, during the initial stress period acts as a signaling molecule and enhance the activity of peroxidase but as stress period prolongs it causes sever oxidative stress and suppressed the activity of peroxidase enzyme. Previous reports also highlighted that moderate doses of H_2O_2 enhanced the antioxidant status, while higher concentrations caused oxidative stress and symptoms resembling a hypersensitive response (Gechev *et al*., 2002).

Proteins are main cellular components susceptible to damage by free radicals (Blokhina *et al*., 2003). Furthermore, it has been reported that sea water treatments increased the protein content of the developing grains. Similarly, in present study, leaf protein content was increased under oxidative stress induced by hydrogen peroxide. This increase in soluble protein contents might has resulted due to memebrane reconfugration and resysnthesis of membrane bound enzymes as proviuosly reported in peanut (Jeng and Sung, 1994).

We concluded that oxidative stress induced by 10 mM H_2O_2 raised the antioxidant activities while 20 mM H_2O_2 treatment suppresses the defence molecules. Treatments with thiourea increased the leaf peroxidase and catalase activities with a preventive effect on protein down regulation (senescence). In general, thiourea treatment can enhance the antioxidant defense under oxidative stress by hydrogen peroxide.

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