Effect of thiourea on proteins, catalase, guaiacol-peroxidase and protease activities in wheat leaves under H_2O_2 induced oxidative stress

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Abstract

The effect of different thiourea (TU) concentrations (10 and 20 mM) on antioxidants, protease activity and protein content with and without H_2O_2 (50 mM) induced stress was investigated in wheat leaves. A gradual decrease in protein content with a peak at 72 hours was observed in stressed as well as control leaves. This gradual decrease in leaf protein content at different time intervals was completely prevented by 20 mM TU treatment. Leaf protease activity was increased due to oxidative stress by H_2O_2 while it decreased after 20 mM TU treatment under stressed and non-stressed condition. In general, catalase (CAT) activity increased under oxidative stress and after both thiourea treatments. However, at 72 hours, CAT activity reduced along with simultaneous increase in peroxidase activity under H_2O_2 induced stress. Actually, stress induced reduction in CAT activity at 72 hours was compensated with peroxidase. Oxidative stress and TU treatments generally raised the peroxidase activity. TU treatment followed by oxidative stress condition also increased the leaf peroxidase activity irrespective of applied concentration. Again, H_2O_2 and TU treatment induced reduction in CAT activity at 96 hours was compensated with a prompt increase in peroxidase activity. Collectively, H_2O_2 treatment increased the antioxidant and protease activities. Moreover, thiourea treatments prevented the protein loss (20mM TU), decreased the protease activity and enhanced the CAT and peroxidase activities which resulted in protective effects. Dose dependent effects of TU treatments were observed mostly.

Keywords: thiourea; peroxidase; catalase; senescence; wheat; antioxidants

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Introduction

Oxidative stress is induced by a wide range of environmental factors including drought, UV stress, pathogen invasion (hypersensitive reaction), herbicide action oxygen shortage (Blokhina et al., 2003) and salinity (Raza et al., 2007; Hameed et al., 2008; 2010). A central role for Reactive Oxygen Species (ROS) themselves with H_2O_2 playing an important role is being recognized in the signal transduction cascade of defense responses against pathogen attack and abiotic stress (Vancamp *et al.* 1998; Morita *et al.* 1999).

ROS, such as the superoxide anions and hydrogen peroxide (H_2O_2) produced during

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oxidative stress are thought to be involved in several areas of plant physiology and development. Even under optimal conditions, many metabolic processes, including chloroplastic, mitochondrial, and plasma membrane-linked electron transport systems, produce reactive oxygen species (ROS) such as the superoxide radical, hydrogen peroxide (H_2O_2), and the hydroxyl free radical (Asada, 1997). Furthermore, the imposition of abiotic stress conditions, i.e., drought and salt stress can give rise to excessive concentrations of ROS, resulting in oxidative damage at the cellular level (Lin and Wang, 2002; Hameed et al., 2011).

To counteract the toxicity of reactive oxygen species, a highly efficient antioxidative defense system, including both nonenzymic and enzymic constituents, is present in plant cells (Hameed et al., 2008; 2009; 2010). The formation of ROS is prevented by an antioxidant system: low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols), enzymes regenerating the reduced forms of antioxidants, and ROS-interacting enzymes such as SOD, peroxidase and catalase (Blokhina et al., 2003; Hameed et al., 2009). Superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase are the main players within the enzymatic defense system. Induction of cytosolic APX under oxidative stress is caused by H_2O_2 generated through superoxide dismutase (Morita et al., 1999). Similarly, proteases are responsible for selective breakdown of regulatory proteins, which controls key aspects of plant growth, development, and defense (Schaller, 2004). The rapidly growing amount of information indicates that proteases participate in turnover of proteins during response to abiotic stresses (Grudkowska and Zagdanska, 2004).

H_2O_2 can react with superoxide to form more reactive hydroxyl radical. Thiourea has been proved to be the best scavenger of hydroxyl radicals (Lin and Kao, 1998). Thiourea can completely prevent H_2O_2-promoted senescence and the H_2O_2-induced decrease in SOD and APX activities in light and darkness (Lin and Kao, 1998). Thiourea can also exert antioxidant effects unrelated to hydroxyl radical scavenging. Thiourea has been reported to protect against copper-mediated protein oxidation through the chelation of cuprous copper and the formation of a redox-inactive thiourea-copper complex (Zhu et al., 2002). It has been reported that the field application of thiourea along with phosphorus significantly increased the grain yield and net returns in green gram (*Vigna radiata var*. *aureus* (L.) Wilczek) (Singh and Rathore, 2003). Thiourea (0.1-10 mM) can provide dose-dependent protection against protein oxidation. It also significantly inhibits copper-catalyzed oxidation of ascorbate (Zhu, 2001). It also competitively inhibits the reduction of cytochrome-C by the xanthine/xanthine oxidase superoxide-generating system, and the release of iron from ferritin by superoxide radicals. Thus, thiourea is direct scavenger of superoxide radicals as well as hydroxyl radicals and hydrogen peroxide (Kelner et al., 1990).

In this view, the present study was planned to reveal the effect of thiourea on catalase, guaiacol-peroxidase, and protease activities and protein content in wheat leaves under oxidative stress induced by exogenous application of H_2O_2 and non-stressed conditions.

Material and Methods

Seeds of wheat (*Triticum aestivum* L) variety MH-97 with uniform size were selected for experiment. Seeds in three replicates (35 seedlings per replicate) were germinated for 24 hours at 25±1°C on wet (with H_2O) filter paper in Petri dishes. 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 (50 mM) and thiourea (10 mM and 20 mM) treatments were applied on 4th day of germination. 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 96 hours. Thiourea treatments precede hydrogen peroxide treatment by 4 hours. As both thiourea and H_2O_2 were added to the growth medium, its actual concentrations in the leaves were unknown. Leaf samples were collected after every 24 hours (24, 48, 72 and 96 hours) and used for different biochemical analyses.

Catalase (CAT) and guaiacol peroxidase (POD)

For the estimation of catalase (CAT) and peroxidase (POD) leaves (0.5 g) were homogenized in medium composed of 50 mM potassium phosphate buffer, pH 7.0 and 1 mM dithiothreitol (DTT) and centrifuged at 15,000×*g* for 20 min at 4 ^{o}C. The supernatant was separated and used for enzyme estimations. Activities of POD and CAT were measured using the method of Chance and Maehly (1955) with some modification. 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}. For measurement of CAT activity assay solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM H_2O_2 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. Protein concentration of the enzyme extract was measured by dye binding assay as described by Bradford (1976).

Protease activity

For estimation of protease, samples (0.5 g) were extracted in 50 mM potassium phosphate buffer of pH 7.8. Protease activity was determined by the casein digestion assay described by Drapeau, (1974). By this method one unit is the amount of enzyme which releases acid soluble fragments equivalent to 0.001 A280 per minute at 37 °C and pH 7.8. Enzyme activities were expressed on protein basis.

Statistical analysis

Experiments were conducted in triplicates. The 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 thiourea treatments on antioxidant enzymes, protease and soluble protein contents under oxidative stress (H_2O_2) and non-stressed conditions was investigated. In control, CAT activity was extremely low and remained almost the same at 24 to 48 hours (Fig. I a). However, a gradual increase in CAT activity was observed from 72 to 96 hours. Similarly, under oxidative stress, CAT activity was extremely low and almost the same at 24 to 72 hours. Though, at 96 hours a sharp and manifold increase in CAT activity was observed. When compared to control, CAT activity was not affected by H_2O_2 stress during initial 48 hours. Whereas, CAT activity was lowered down under stress at 72 hours. In contrast, CAT activity significantly ($p<0.01$) rose at 96 hours under H_2O_2 stress. Thiourea treatments significantly influenced the CAT activity in dose specific manner. CAT activity increased gradually in 20 mM thiourea treated and non-treated control leaves with a very sharp increase at 96 hours. Treatment with 20 mM thiourea did not modulate the CAT activity during initial 72 hours, while activity was high in treated leaves at 96 hours as compared to non-treated control. Leaf CAT activity was significantly ($p<0.01$) increased at 24 and 48 hours by 10mM thiourea treatment compared to non-treated control. CAT activity was lower ($p<0.01$) in thiourea treated leaves at 96 hours under stress, compared to non-treated stressed leaves.

Leaf POD activity increased gradually with time under stress and non-stressed conditions (Fig. I b). Comparing leaf POD activity under non-stressed and stressed condition, it remained equal up to 48 hours while significantly ($p<0.01$) increased under stress at 72 and 96 hours. Both thiourea treatments raised the POD activity as compared to control at almost all time intervals. At 96 hours, difference was highly

significant ($p<0.01$) after 20 mM thiourea treatment as compared to non-treated control. After both thiourea treatments, under H_2O_2 induced stress, the POD activity increased from 24 to 48 hours followed by a decrease in activity at 72 hours. However at 96 hours a significant increase in POD activity was observed under treated condition. Leaf POD activity was significantly ($p<0.05$) higher after both thiourea treatments as compared to non-treated control during initial 48 hours. However at 96 hours, leaf POD activity increased only after 20mM thiourea treatment as compared with non-treated stressed leaves. POD activity increased initially up to 48 hours after 20 mM thiourea treatment, followed by a decrease at 72 hours and once again a significant increase at 96 hours.

Protease activity level decreased

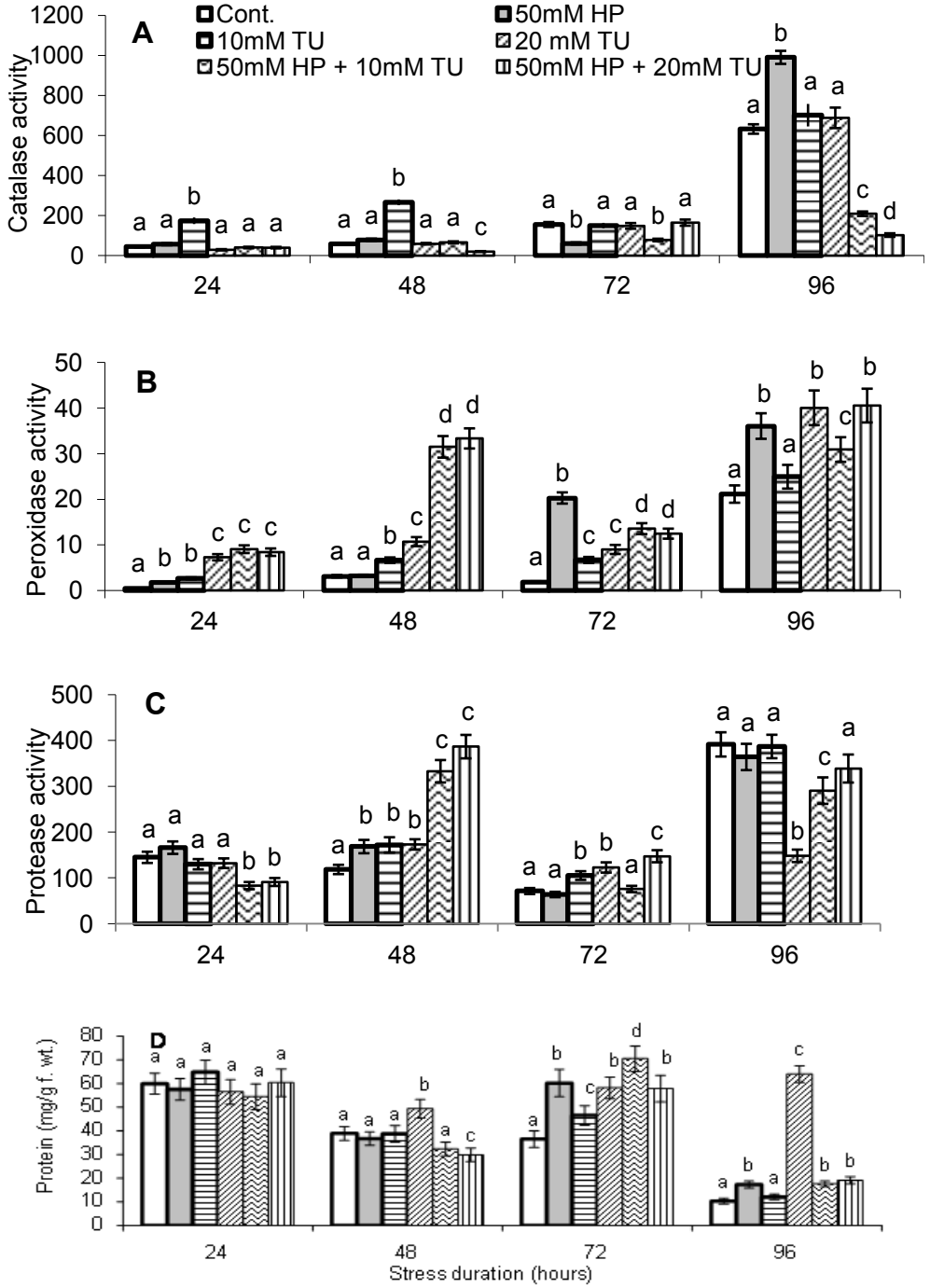

Fig. I. Effects of thiourea on leaf catalase (a), guaiacol-peroxidase (b), protease (c) activities, and protein contents (d) under H_2O_2 induced oxidative stress and non-stress conditions. HP: H_2O_2, TU: thiourea

gradually in non-treated, non-stressed control leaves during 24 to 72 hours followed by an elevated level at 96 hours (Fig. I c). Almost the same was true for stressed seedlings as similar trend (decrease at 72 hours and then increase at 96 hours) was observed. Comparing leaf protease activity under control and stressed conditions, overlapping trend was observed. However at 48 hours under stressed condition, protease activity was slightly higher as compared with that of control followed by overlapping trend at 72 and 96 hours of stress. Leaf protease activity increased at 48 hours after 10 mM and 20 mM thiourea treatments followed by a decrease at 72 hours. After 20 mM thiourea treatment, protease activity was significantly lower at 96 hours as compared with non-treated samples. Comparison between non-treated and treated samples revealed that there was no difference in protease activity in all samples at 24 hours. However during 48 to 72 hours, protease activity was higher ($p<0.05$) after both thiourea treatments as compared with non-treated samples. A cyclic increase (48 hours), decrease (72 hours), and increase (96 hours), in leaf protease activity was observed after both thiourea treatments under oxidative stress by 50 mM hydrogen peroxide. However, the magnitude of protease activity was higher in samples treated with 20 mM thiourea. Comparing leaf protease activity after thiourea treatment with non-treated stressed control, the activity was clearly lower in treated samples after 24 hours while significantly higher after 48 hours for both treatments. However, activity was higher ($p<0.01$) only in 20 mM thiourea treated samples at 72 hours as compared to non-treated stressed control. Moreover, protease activity was lower ($p<0.01$) in 10 mM thiourea treated samples at 96 hours as compared with non-treated control.

A gradual decrease in protein contents was observed in control with time (Fig. I d). Oxidative stress by 50 mM H_2O_2, caused a significant increase in soluble protein at 72 and 96 hours. Increase in leaf protein content at 72 hours by stress was very prominent as compared with that of control. Collectively, there was a gradual decrease in protein content with increasing time; however, a peak was observed at 72 hours under stressed condition. As mentioned above, a gradual decrease in protein content was observed in control at different time intervals (Fig. IV). Treatment with 20 mM thiourea prevented this gradual decrease in protein content observed in control. As a result, soluble protein content was significantly ($p<0.05$) higher at 48, 72 and 96 hours after thiourea treatment (20 mM) as compared to control. Maintained protein content indicates that 20 mM thiourea treatment prevents the leaf senescence. However such protective effect was not observed after 10 mM thiourea treatment as in this case, trend was almost overlapping with that of control except an increase at 72 hours. Under oxidative stress by 50 mM H_2O_2, thiourea treatments did not modulate the soluble protein content; level was increased only at 72 hours after 10 mM thiourea treatment. A peak in protein content at 72 hours was common in all type of samples. Leaf protein content was significantly higher ($p<0.05$) after 20 mM thiourea treatment at 24 and 96 hours as compared to non-treated control.

Discussion

Activation of the plant antioxidant system by H_2O_2 plays an important role in the induced tolerance against oxidative stress (Gechev et al., 2002). The H_2O_2 can induce stress tolerance but it depends on the applied dose. 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). Similarly, it was also evident from the present study that prolonged oxidative stress induced by H_2O_2 ultimately raised the CAT activity at 96 hours while POD activity was raised at 72 and 96 hours. Two points can be inferred from these findings. Firstly, oxidative stress induced by hydrogen peroxide in growth medium can modulate the antioxidant defense system in wheat leaves. In this connect, H_2O_2 has been regarded as a signaling molecule and a regulator of the expression of some genes in cells, i.e., genes encoding antioxidants, cell rescue/defense proteins, and signaling proteins such as kinase, phosphatase, and transcription factors (Hung et al., 2005). Moreover, an increase in POD activity under oxidative stress induced by exogenous hydrogen peroxide has also been

reported in rice leaves (Lin and Kao, 1998). Secondly, a comparatively long exposure of hydrogen peroxide was required for up regulation of CAT activity as for POD activity in wheat leaves. It is also important to bring up here that CAT activity reduced at 72 hours under oxidative stress overlap with simultaneous increase in POD activity at this time interval. This point to that oxidative stress induced drop in CAT activity was compensated with parallel increase in the POD that is an alternate hydrogen peroxide detoxifying enzyme. Similar changes in the defense mechanism has been reported previously indicating that reduced CAT activity can be compensated by alternative H_2O_2-scavenging mechanisms such as increased APX and glutathione peroxidase levels (Willekens et al., 1997). Further, it has been reported that in double antisense plants lacking the two major hydrogen peroxide-detoxifying enzymes, APX and CAT activate an alternative/redundant defense mechanism that compensates for the lack of APX and CAT (Rizhsky et al., 2002).

Thiourea treatments raised the POD activity in the wheat leaves and increase was evident up to 72 hours and 96 hours after 10 mM and 20 mM treatments. This indicates that effect of thiourea on POD activity is dose dependent being longer for higher dose and vice versa. Moreover, only lower dose (10 mM) of TU was able to increase the CAT activity limited up to 48 hours then dropped back to control level at 72 hours. This points towards a dose dependant effect of TU treatment on CAT as well. The CAT activity was less responsive to TU treatment compared to POD. These observations point out a protective effect of TU as a general antioxidant instead of a specific hydroxyl radical scavenger. This is in line with a previous report that TU provided protection against copper-mediated oxidation of protein (albumin) by chelation of cuprous copper and the formation of a redox-inactive thiourea-copper complex (Zhu et al., 2002). However, this previous study was limited to protein oxidation protection, while enzyme activities like CAT or POD were not investigated.

Long exposure of oxidative stress by hydrogen peroxide and TU treatments also increased the leaf soluble proteins content. This increase in soluble protein content might have resulted due to the enhanced production of antioxidant enzymes, i.e., POD and CAT. In this connection it has been reported that stress condition like salinity can cause the induction of a new proteins that may be antioxidants and dehydrins (Hameed et al., 2010). Therefore, other possibility is the expression of stress proteins that raised the overall quantitative protein status in the leaves of stressed seedlings.

Proteases are involved in signaling pathways (Coffeen and Wolpert, 2004) and in the response to biotic and abiotic stresses (Grudkowska and Zagdanska, 2004). Proteases are responsible for selective breakdown of regulatory proteins, which controls key aspects of plant growth, development, and defense (Schaller, 2004). The same was true for the present study where protease level increased due to stress at 48 hours. Moreover, TU treatments also elevated the protease level in the leaves at this time interval. Higher protease level seems to be for degradation and recycling of damaged, misfolded and potentially harmful proteins. Proteolytic degradation of these damaged, misfolded and potentially harmful proteins may have provided free amino acids required for the synthesis of new proteins as reported previously (Schaller, 2004; Hieng et al., 2004). It was clearly evident from this study that 20 mM thiourea treatment lowered the protease activity in wheat leaves as compared to control at 96 hours under non-stressed condition. This drop in the protease activity overlaps with significant increase in the POD activity at this interval. There is the possibility that in the presence of better antioxidant defense there was less damage to proteins and requirement of protease also decreased. Further, it has been reported that thiourea prevented the hydroxyl radical and peroxyl radical induced degradation of a stress induced citrus dehydrin, i.e., CuCOR19 (Hara et al., 2004). Actually this citrus dehydrin scavenge the hydroxyl radical generated by the Fe^{2+}/H_2O_2 system and peroxyl radical generated from 2, 2'-azobis (2-amidinopropane) (AAPH) (Hara et al., 2004). Except at 48 hours, under oxidative stress, thiourea application either lowered or non-significantly affected the protease activity. Further, a dose dependent effect of TU was evident as higher thiourea concentration (20

mM) was more effective in lowering the protease activity (at 96 hours).

In conclusion, oxidative stress induced by H_2O_2 amplified the antioxidant enzyme activities. Treatments with thiourea further increased the leaf POD and CAT activities with a preventive effect on proteins and in return a reduction in protease activity. Thiourea treatments were able to enhance the antioxidant defense under oxidative stress by hydrogen peroxide. TU effects were mostly dose dependent.

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