Effect of seed soaking with thiols on the antioxidant enzymes and photosystem activities in wheat subjected to water stress

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Abstract

Photosystem 1 and 2 and antioxidant enzyme activities were determined in wheat (*Triticum aestivum* L. cv. Sonalika) leaves. Seedlings from both control seeds and seeds soaked in solutions like dithiothreitol, thioglycollic acid and thiourea were subjected to water stress induced by polyethylene glycol. Photosystem 1 and 2 activities were less inhibited by water stress due to seed soaking with sulphydryl compounds. The changes in activities of antioxidant enzymes induced by water stress were higher in seedlings from thiol-pretreated seeds than from water-soaked seeds.

Additional key words: oxygen evolution, peroxidase, photosynthesis, polyethylene glycol, seed pretreatment, sulphydryl compounds.

Introduction

The coordinated action of several antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APOX), guaiacol peroxidase (GPOX), glutathione reductase (GR) and glutathione-S-transferase (GST) is activated when plants are exposed to different kinds of stress such as salinity, drought and high irradiance in order to scavenge reactive oxygen species (ROS). Superoxide is converted to H_2O_2 by the action of SOD, whereas CAT converts H_2O_2 to H_2O and molecular O_2. H_2O_2 is also detoxified by peroxidases with the help of glutathione as reductant (Noctor and Foyer 1998), which plays an important role as antioxidant in cells. The lipid peroxidation by-products such as organic peroxides are eliminated from the cells by glutathione peroxidase and GR. Besides antioxidant protection, intracellular and extracellular redox states of thiols play a critical role in stabilizing the protein structure, function, regulation of enzyme activity and control of the activity of transcription factors (Deneke 2000, Sen 2000).

Sulphydryl group is a dominant chemical group influencing metabolic reactions in the plant both under normal as well as stress conditions. Cellular protein denaturation and loss of membrane integrity are the major factors responsible for dehydration injury to plants under water deficit conditions. Loss of reactive sulphydryl groups of membrane proteins is one of major factors playing an important role in water stress injury. As the -SH group has diverse bio-regulatory roles in crop plants (Sahu and Singh 1995), it is quite likely that thiols apart from playing a role in productivity can also be involved in the antioxidant defense response of the plant. Seed soaking in sulphydryl compound solutions should therefore improve stress tolerance of plants. Thiourea improves the productivity of crops such as maize (Sahu and Solanki 1991, Sahu *et al.* 1993) and wheat (Sahu and Singh 1995).

The aim of the present study was to understand the protective effect of thiols such as dithiothreitol (DTT), thioglycollic acid (TGA) and thiourea (TU) by seed soaking on the primary photochemical reactions of both photosystem (PS); PS 1 and PS 2. Additionally, emphasis was given to study the enhancement in activities of antioxidant enzymes such as CAT, SOD, APOX, GPOX, GR and GST in developing wheat seedlings against oxidative water stress.

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Abbreviations: ε - coefficient of absorbance; APOX - ascorbate peroxidase; CAT - catalase; Chl - chlorophyll; DCQ - 2,6-dichloro-*p*-benzoquinone; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; GPOX - guaiacol peroxidase; GR - glutathione reductase; GSH - glutathione reduced form; GSSG - glutathione oxidised form; GST - glutathione-S-transferase; MeV - methyl viologen; $NADP^+$ - nicotinamide adenine dinucleotide oxidised form; NADPH - nicotinamide adenine dinucleotide phosphate reduced form; NBT - nitroblue tetrazolium; PEG - polyethylene glycol; PS 1, PS 2 - photosystems 1 and 2; ROS - reactive oxygen species; PVP - polyvinylpolypyrrolidone; SOD - superoxide dismutase; TGA - thioglycolic acid; TU - thiourea.
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Materials and methods

Plants, growth conditions and experimental treatments: Wheat (*Triticum aestivum* L. cv. Sonalika) seedlings were grown in cotton soaked with half-strength Hoagland solution (Hoagland and Arnon 1938) under continuous irradiance of 500 μmol m^{-2} s^{-1} at 23 ± 1 °C. Water stress was applied to 5-d-old seedlings by adding 10 % polyethylene glycol-6000 (PEG-6000) into Hoagland solution. Seedling age was determined from the day of radicle emergence (Misra and Biswal 1980). The experiment consisted of four sets of samples: *1*) control seedlings; *2*) seedlings subjected to 10 % PEG stress; *3*) seedlings whose seeds were previously soaked for 6 h in 0.07 mM DTT, 1.4 mM TGA and 6.6 mM TU instead of distilled water and air dried, and *4*) a set similar to thiol pretreated samples but subjected to 10 % PEG. Leaves were sampled on the 14 and 18 d after the plant has undergone stress.

Chloroplast isolation, photosystem (PS) 1 and 2 activities: Chloroplasts were isolated from wheat leaves following the method of Izawa and Good (1968). PS 1 activity was measured polarographically in a Clark-type O_2 electrode (*Gilson Scientific Instruments*, St. Louis, USA) at 21 °C in rate-saturating red light as methyl viologen (MeV) mediated O_2 uptake by chloroplast suspension equivalent to 20 μg chlorophyll (Chl) cm^{-3} as described by Izawa (1980). PS 2 activity was measured as oxygen evolution by chloroplasts [20 μg(Chl) cm^{-3}] using either 0.3 mM 2,6-dichloro-*p*-benzoquinone (DCQ) or 0.4 mM potassium ferricyanide [$K_3Fe(CN)_6$] as electron acceptors as described by Nayak *et al.* (2003).

Enzyme extractionand assays: Leaf samples (0.5 g fresh mass) were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 % polyvinylpolypyrrolidone (PVP). The homogenate was filtered through four layers of cheesecloth and then centrifuged at 4 °C for 20 min at 15 000 *g*. The supernatant was collected and an appropriate aliquot dilution of the crude extract was used for enzyme assays. All operations for enzyme extraction were performed at 0 - 4 °C and the enzyme assays were carried out at room temperature (23 ± 1 °C) unless otherwise stated.

CAT activity was measured by following the decomposition of H_2O_2 at 240 nm (coefficient of absorbance, ε = 39.4 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 as described by Chance and Maehly (1955) for 2 min. Enzyme activity was expressed as μmol of H_2O_2 decomposed mg^{-1} (protein) min^{-1}.

Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) as detailed by Becana *et al.* (1986). The reaction mixture (3.0 cm^3) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 14.3 mM methionine, 82.5 μM NBT and 2.2 μM riboflavin. The system was placed 30 cm from six 15 W fluorescent tubes. The reaction was run for 30 min and stopped by switching off the lights. The reduction in NBT was followed by reading absorbance at 560 nm. One unit of SOD (U) was defined as the amount of enzyme that produced a 50 % inhibition of NBT reduction under the assay conditions as Giannopolitis and Ries (1977).

APOX activity was assayed by following the decrease in absorbance at 290 nm due to ascorbate oxidation (ε = 2.8 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 0.1 mM EDTA for 1 min according to the method of Nakano and Asada (1981). Enzyme activity was expressed as mmol(ascorbate oxidised) mg^{-1}(protein) min^{-1}.

For GPOX, the oxidation of guaiacol was measured by following the increase in absorbance at 470 nm (ε = 26.6 mM^{-1} cm^{-1}) for 1 min. The assay mixture contained 50 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 10 mM guaiacol and 10 mM H_2O_2 as described by Chance and Maehly (1955). GPOX activity was expressed as mmol(tetraguaiacol formed) mg^{-1}(protein) min^{-1}.

GR activity was measured by monitoring the decrease in absorbance at 340 nm (ε = 6.2 mM^{-1} cm^{-1}) for 1 min. The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM GSSG, 0.1 mM EDTA, 3 mM $MgCl_2$ and 0.15 mM NADPH as described by Shaedle and Bassham (1977). Activity was expressed as μmol (NADPH oxidised) mg^{-1}(protein) min^{-1}.

GST activity was measured as per Mannervik and Guthenberg (1981) by following the changes in the absorbance at 340 nm for 1 min in a mixture containing 100 mM sodium phosphate buffer (pH 6.5), 1 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene. The activity of GST was expressed as μmol(2,4-dinitrophenyl glutathione formed) mg^{-1}(protein) min^{-1} (ε = 9.8 mM^{-1} cm^{-1}).

Other determinations: Chlorophyll content was determined using 80 % acetone filtrates of chloroplast suspension as described by Arnon (1949) and protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Results

PS 1 activity was not affected much by water stress. However, PS 1 activity was higher in 14-d-old seedlings from seeds pretreated with DTT, TGA and TU by 34.87, 40.49, and 31.78 %, respectively. This increase was

Table 1. Effect of seed soaking with 0.07 mM DTT, 1.4 mM TGA and 6.6 mM TU on the PS 1 (MV → H_2O) and PS 2 (H_2O → $K_3Fe(CN)_6$ or H_2O → DCQ) activities [nmol(O_2) mg^{-1}(Chl) s^{-1}] in chloroplasts isolated from wheat seedlings subjected to 10 % PEG-induced water stress for 14 or 18 d. Means ± SE of three independent sets of experiments.

PS		Time [d]	Control	PEG	DTT	TGA	TU	DTT + PEG	TGA + PEG	TU + PEG
PS 1		14	45 ± 3.6	43 ± 3.3	61 ± 3.0	64 ± 2.7	59 ± 3.9	58 ± 3.3	63 ± 4.2	62 ± 4.7
		18	47 ± 2.7	44 ± 3.8	59 ± 3.6	63 ± 4.4	64 ± 4.4	61 ± 3.3	60 ± 3.3	61 ± 2.7
PS 2	$K_3Fe(CN)_6$	14	69 ± 5.3	28 ± 2.7	111 ± 5.5	115 ± 4.4	83 ± 3.6	86 ± 3.1	83 ± 3.6	83 ± 3.3
		18	30 ± 4.2	14 ± 1.7	49 ± 4.2	44 ± 3.9	47 ± 4.7	29 ± 1.1	29 ± 1.1	28 ± 1.4
	DCQ	14	38 ± 2.2	11 ± 0.8	50 ± 3.6	53 ± 3.1	47 ± 5.0	25 ± 1.1	26 ± 1.1	24 ± 0.6
		18	17 ± 0.8	22 ± 0.1	19 ± 1.7	21 ± 0.8	21 ± 1.1	8 ± 0.3	7 ± 0.2	7 ± 0.2

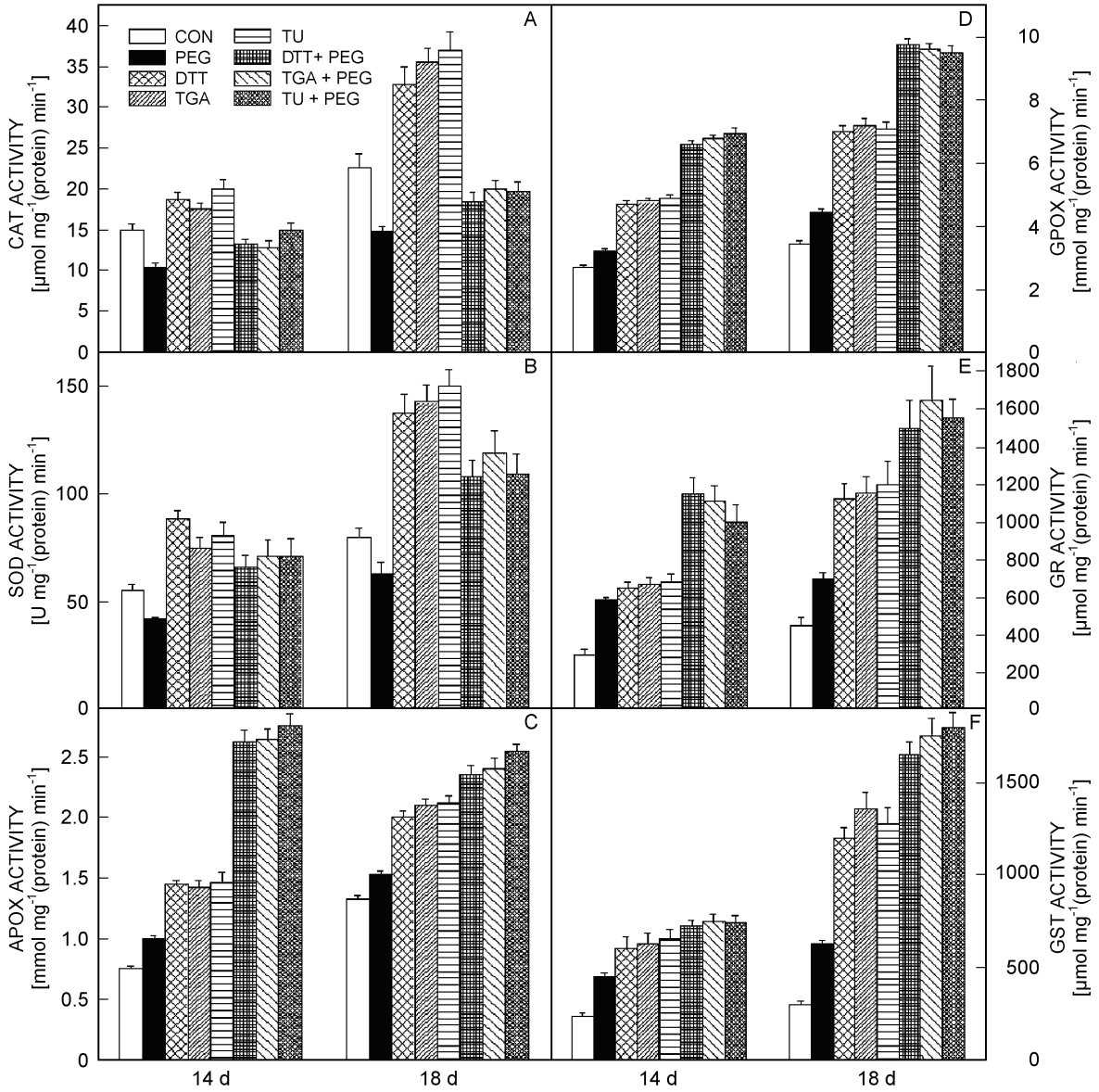

Fig. 1. Effect of seed soaking with different thiols on CAT (*A*), SOD (*B*), APOX (*C*), GPOX (*D*), GR (*E*) and GST (*F*) activities in leaves from wheat seedlings subjected to water stress. Experimental treatment notations include: CON, control and PEG, water stress; DTT, TGA and TU, seeds soaked in 0.07 mM DTT, 1.4 mM TGA and 6.6 mM TU respectively. Means ± SE of three independent set of experiments.

27.21, 33.13, and 36.09 % over that of the control in DTT, TGA and TU pretreated set on the day 18. Even under PEG-induced water stress, PS 1 activities were higher in the thiol pretreated set (34.89, 46.51, and 44.19 %, respectively on the day 14 and 38.64, 36.36, and 38.64 % on the day 18; Table 1).

PS 2 activities were affected by water stress (Table 1). With $K_3Fe(CN)_6$ as the electron acceptor, drop in PS 2 activity by PEG stress was 60 and 54.55 % on the day 14 and 18, respectively. However, in case of thiol pretreatments, there was no drop in PS 2 activity in case of TU pretreated set under water stress whereas it was only 22.75 and 27.71 %, respectively, in case of DTT and TGA pretreatments on the day 14. Drop in PS 2 activity of the 18-d-old seedlings was 38.86, 35.00, and 41.18 %, respectively, in case of DTT, TGA and TU pretreated set. When DCQ was used as the electron acceptor, decrease in PS 2 activity was 70.37 and 86.89 % on the days 14 and 18, respectively. However, after DTT, TGA and TU pretreatments decrease in the PS 2 activity by 14-d water stress was 50.00, 52.63, and 48.24 % and by 18-d water stress it was 60.00, 65.33, and 67.53 %, respectively.

CAT activity was found to decrease due to water stress by 30.67 and 34.59 % after 14 and 18 d, respectively. Due to DTT, TGA and TU pretreatments this decrease in CAT activity was 29.49, 27.73, and 25 %, respectively, on the day 14. However, this drop was higher than that observed on the day 18 (Fig. 1*A*)

SOD activity was also found to decrease under PEG-induced water stress by 23.64 and 21.25 % on the day 14 and 18, respectively. But in case of TGA and TU pretreatments this decrease was lower by 5.31 and 11.58 %, respectively on the day 14. However, it was significant only in case of TGA pretreatment (16.58 %) on the day 18 (Fig. 1*B*).

APOX activity was increased in PEG stressed seedlings by 33.33 and 15.91 % on the day 14 and 18, respectively. This activity was higher (by 80.69, 85.61, and 88.90 %) in DTT, TGA and TU pretreated set on the day 14 whereas it was only 17.5 and 20.85 % higher in case of DTT and TU pretreated set on the day 18 (Fig. 1*C*).

GPOX activity increased similarly as APOX activity, 18.52 and 28.99 % on the day 14 and 18, respectively. In case of DTT, TGA and TU pretreatments it was 40.43, 41.67, and 41.84 %, respectively, on the day 14, whereas on the day 18 the increase in activity was 39.29, 33.33 and 33.80 %, respectively (Fig. 1*D*).

The rise in GR activity by water stress was two-fold on the day 14, whereas it increased by 55.56 % on the day 18. However, in case of DTT, TGA and TU pretreatments the rise in GR activity was less, *i.e.*, 76.71, 65.74, and 46.92 %. Similarly on the day 18, GR activity was increased by 33.25, 43.37, and 29.58 % in DTT, TGA and TU pretreated set, respectively (Fig. 1*E*).

The increase in GST activity by water stress was 95.57 % on the day 14, whereas it increased two-fold on the day 18. However, in case of DTT, TGA and TU pretreatments the increase in activity was less, *i.e.*, 20.93, 19.13, and 13.74 %, respectively. Similarly on the day 18, GR activity was increased by 37.52, 28.74, and 41.12 % in DTT, TGA and TU pretreated set (Fig. 1*F*).

Discussion

The PS 2 activity drops under PEG-induced water stress though the PS1 activity is relatively stable (Table 1). PS 1 activity was increased by thiol pretreatments even under water stress conditions. Due to water stress, when 60 % loss in PS 2 activity was observed in untreated samples; the thiol-pretreated samples had only lost 27 % of the activity (Table 1*B*). Thus a partial recovery of around 30 and 20 % in the PS 2 activity (ferricyanide or DCQ as acceptors) could be possible due to thiol pretreatment. So far, there are no reports on the effect of seed soaking with thiol compounds on the primary photochemistry of plants, but it has been reported that foliar spray of TU increased both canopy photosynthesis and photosynthetically active leaf area in maize (Sahu *et al.* 1993). Recently, it has been shown that seed soaking with TU enhances net photosynthesis and leaf area in clusterbean under water stress conditions (Burman *et al.* 2004). DTT has also been reported to stimulate CO_2 assimilation in the dark (Werdan *et al.* 1975).

Photosynthesis decreases under water deficit conditions resulting in inhibition of electron transfer, which in turn leads to the formation of ROS that can lead to photooxidative damage (Asada 1999). As a consequence, plants have evolved cellular adaptive responses like up-regulation of antioxidant defense system (Horling *et al.* 2003). PEG stress was found to activate the antioxidant enzymes except CAT and SOD. Due to thiol pretreatments an increase in activities of antioxidant enzymes was seen. The activities of antioxidant enzymes were higher in case of thiol pretreated sets even under water stress. However, in some cases the increase in activity is lower than that induced by PEG stress alone. This clearly shows that the thiols are able to enhance the antioxidant system, which increases the plant capability to counteract oxidative stress. Thiols can act as a thiol/disulphide component of redox buffer, free radicals scavengers and chelators of metal ions and their biological activity is attributed to the presence of the sulphydryl group (Meister and Anderson 1983). Since, the redox states of thiols are also involved in regulation of enzyme activity and transcription factors, it is quite possible that seed pretreatment with external thiols might result in an up-regulation of the antioxidant defense system of the plant. Significantly higher activities of antioxidant enzymes in the thiol pretreatments clearly demonstrate more efficient antioxidant system functioning. There are only very few reports on the effect of thiols on the antioxidant system . It has been reported that treatment with 1 mM N-acetyl

cysteine did not alter the total SOD activity or catalase activity under UV-B stress in *Chlorella vulgaris* or soybean leaves. However, supplementation with 1 mM N-acetyl cysteine kept the ascorbyl and lipid radical content to a basal level following UV-B treatment (Malanga *et al.* 1999). In a similar kind of a study, it was shown that treatment with dimethyl thiourea was able to trap H_2O_2 and also decrease the expression of APOX in maize roots (De Agazio and Zacchini 2001). Dimethyl thiourea has been known to specifically scavenge hydroxyl radicals, whereas thiourea has been widely used to study the role of hydroxyl radicals in metal-mediated biological damage both *in vitro* and *in vivo*, though most of the data available are from animal studies (Zhu *et al.* 2002). To our knowledge, the results presented here are the first reports on the activation of the antioxidant defense system and also recovery of the primary photochemical reactions under drought stress in wheat following seed treatments with bioactive thiols. Better understanding of the biochemical aspects of these tolerance mechanisms imparted due to seed pre-treatments with thiols will help increasing the yield of wheat under water - limited environment.

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