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Effect of exogenous iodine on enhancement of oxidative stress in soybean (*Glycine max*) plant and partial expression of 1-Cys peroxiredoxin gene under heat-stress conditions

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ABSTRACT

The present study analyzes the biochemical effects of exogenous iodine on the antioxidant defense system of soybean (*Glycine max* L.) plant when grown under heat stress (35ºC). The parameters studied under oxidative stress generation were superoxide anion production, hydrogen peroxide formation and malondialdehyde production. The enzymatic defense was investigated by analyzing the levels of superoxide dismutase, ascorbate peroxidase, catalase and glutathione reductase. For the partial expression of 1-cys peroxiredoxin, the genomic DNA was extracted and primers were designed to amplify the 3' end region of the PER-1 gene locus 17780502 to 17781727 of genome size 61Mbp, 304 base pairs: PER1-F 5'-ATGCCAGGGATCACACTAGG-3'and PER1-R 5'-ACAAGACAGACCAAGGAGCT-3' and subjected to PCR. The heat stress generated maximum malondialdehyde without application of iodate and production of superoxide anion was maximum in 80 µM iodate concentration. The H_2O_2 production was reported highest in 40 µM iodate concentration, Enzymatic defense was highest in 20 and 80 µM dosages of iodate. We amplified 1-cys peroxiredoxin (1-Cys Prx) gene, an important antioxidant enzyme, in soybean plant using primer of an Arabidopsis 1-Cys Prx gene to confer tolerance against heat stress.

Key words: 1-Cys peroxiredoxin, Heat stress, Iodine, PCR, Soybean

Soybean is one of the most important crops for oil and protein resource. Improvement of stress tolerance will be beneficial for soybean seed production (Wei *et al.* 2009). Brazil is the second soybean producer and exporter in the world (Borrmann *et al.* 2009). In plants, the 1-Cys Prxs are highly expressed during late seed development, and the expression pattern is dormancy related in mature seeds (Brehelin *et al*. 2003). Peroxiredoxins (Prxs), a group of prominent antioxidant enzymes in plants, were first identified in 1996 (Stacy *et al*. 1996) later, many Prxs were cloned and studied in other plants, such as Arabidopsis (Pulido *et al.* 2010), rice (*Oryza sativa*) (Umate 2010), spinach (*Spinacia oleracea*) (Baier and Dietz 1997).

Potassium iodate (KI) has been shown to act as a chemical dessicant (Kumar *et al*. 2012), which simulates the effect of terminal drought by chemical dessicants during grain filling (Kordenaeej *et al.* 2013). A number of plants like lettuce and tomato show an enhanced antioxidant capacity after application of iodine in the form of iodate (Blasco *et al.* 2011, Kiferle *et al*. 2013). In previous studies the effect of application of iodine in the form of potassium iodate on the antioxidant system of soybean seeds were reported (Gupta *et al.* 2015). Prx plays an important role in growth, development and desiccation tolerance in dormant seeds.

This paper describes beneficial effect of exogenous iodine in soybean and partial expression of 1-cys peroxiredoxins under heat stress condition.

MATERIALS AND METHODS

The experiment was conducted in the rainy season of 2013 at the field of Sharda University, Department of Biotechnology, Greater Noida, India. The seeds of soybean were procured from Indian Agriculture Research Institute, Pusa, New Delhi. Seeds were sown in pots carrying soil and cowdung manure in the ratio 3:1. Ten days old seedlings having four-five true leaves were displaced and subjected to heat stress in a growth chamber with conditions of 35°C (day)/33°C (night), 12/12 h (light/dark) photoperiod and light intensity of 72 µmol/m^2/s. The different treatments were irrigated with the Hoagland nutrient solution. Out of the five experimental pots, three were administered doses of

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IO_3^- in the increasing order (20, 40 and 80 µM as KIO_3^-) (Pickering *et al*. 2000). Two pots were kept as control, one was supplied with only heat stress and the other was administered only Hoagland Nutrient solution. The treatment was given regularly for one month (alternate days). At the end of treatment, the seeds were harvested and stored at -20 ºC for further analysis.

Seeds after harvesting were measured for their size and mass. Standard blotter technique was followed for seed germination percentage of soybean. For germination percentage, pure soaked seeds of each sample were spread over a wet filter paper in a Petri plate. The entire setup was placed in dark for 72 h. Seeds were observed for germination at an interval of 4 h till 3 days.

For superoxide anion production (O_2^-), 0.1gm seeds were placed in a test tube and poured over with a solution containing 0.05 M PBS (pH 7.8), 0.05% nitroblue tetrazolium (NBT) and 10 mM NaN_3. After 5 min incubation in the dark, 2.0 ml of the solution was taken up from the tubes and heated at 85ºC for 15 min. Then the samples were cooled in room temperature and optical density was measured at a wavelength of 580 nm and the O_2^- content was expressed as A_{580}/gFW. For H_2O_2 estimation, the seeds were ground in 6 ml of ice-cold acetone and homogenized by using centrifuging at 8 000g at 4°C for 30 min 0.5 ml of supernatant was mixed with 1.5 ml mixture of $CHCl_3$ and CCl_4 (1:3, v/v). The mixture was centrifuged at 1 000rpm for 1 min, and the water phase was collected for H_2O_2 determination. 1ml of phosphate buffer and 1ml of 4-2-pyridylazo was added to the supernatant and incubated at 45°C for 20 min. Optical density was measured at 508 nm and concentration was expressed as µmol/g FW. For estimation of MDA production, 100 mg seeds were homogenized with 5 ml of 80% chilled ethanol. Homogenates were centrifuged at 8,000g at 4°C for pellet debris and different aliquots of the supernatant were mixed either with 20% trichloracetic acid or 0.5% thiobarbituric acid. Both mixtures were allowed to react in a water bath at 90°C for 1 h. The samples were prepared as described by (Hodges *et al*. 1999).The optical density was measured at 532 nm using spectrophotometer.

In antioxidant enzymatic assays, four enzymes, viz SOD, catalase, ascorbate peroxidase and glutathione reductase estimated for spectrophotometric superoxide dismutase (SOD, EC 1.15.1.1) enzyme extract was prepared by homogenizing 0.1 gm of sample with 100 mM of phosphate buffer.The reaction mixture was developed by adding the following reagents:0.1 ml of supernatant, 0.2 ml of 200 mM methionine, 0.1 ml of NBT, 0.1 ml of 3 mM EDTA, 1.5 ml of PBS, 0.1 ml of 1.5 M Na_2CO_3 and 0.1 ml of 60µM riboflavin. Reaction mixtures were illuminated for 15 min at light intensity of 5 000 lux. The absorbance of solution was measured at 560 nm by using spectrophotometer (SHIMADZU-UV). For catalase enzyme (CAT, EC 1.11.1.6) was estimated by following the consumption of H_2O_2 at 240 nm for 3 min. The reaction mixture contained 0.1ml of 50 mM potassium phosphate buffer (pH 7.0), 0.1 ml of 0.1 mM EDTA, 0.1 ml of 100 mM H_2O_2 and 0.7 ml of enzyme aliquot (Cakmak and Marschner 1992). The degradation of H_2O_2 was measured at 240 nm by using spectrophotometer. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured by homogenizing 100 mg of seed in 50 mM sodium phosphate buffer (pH 7.0) and centrifuged at 1 000 rpm for 15 min. 0.5 mM ascorbic acid, 0.1 mM H_2O_2 and 200 µl of enzyme extracts. The decrease in absorbance at 290 nm was measured. Activity was calculated using the extinction coefficient (2.8 mM^{-1} cm^{-1}) as described by Nakano and Asada (1987). For Glutathione reductase enzyme, extract was prepared by homogenizing100 mg of seed with 0.067 M of phosphate buffer (pH 7.6). The reaction mixture consisted of 0.2 ml GSSG and 0.1 mM EDTA. The supernatant was pre incubated at 25°C for 5 min. The reaction was initiated by an addition of 1 mM NADPH, and the rate of oxidation of NADPH at 340 nm was measured.

All the experiments were performed in triplicate.Values in the tables indicate mean values ± SD. Differences among treatments were analyzed by Two Way ANOVA, taking $p < 0.05$ as significant according to Fisher's multiple range test.

One gram of control and stressed seedlings of soybean were frozen in liquid nitrogen and total DNA was isolated by CTAB method (Sambrook *et al*. 1989). Primers were designed to amplify the 3' end region of the PER-1 gene locus 17780502 to 17781727 of genome size 61Mbp, 304 base pairs: PER1-F 5'-ATGCCAGGGATCACACTAGG-3' and PER1-R 5'-ACAAGACAGACCAAGGAGCT-3'. The primers were designed using Primer 3 (www.frodo.wi.nit.edu) and primer blasting using www.ncbi.nlm.nih.gov. 25.0 µl of PCR reaction mixture containing 5× PCR buffer (5.0 µl), 10 mM dNTPs (1 µl), 25 mm $MgCl_2$ (1.5 µl), 10 µM forward and reverse primers (0.5 µl each), Taq polymerase (0.35 µl), molecular grade water and 1.0 µl of DNA (50 ng) was used for PCR amplification. PCR was carried out under the following conditions using a Gradient thermocycler (C-1000TM, BIORAD): 94°C for 1 min followed by 35 cycles of 94°C for 30 sec, 48.9°C for 1 min and 68°C for 4 minutes, and terminated by a final elongation at 72°C for 5 min. The PCR product was mixed with 1.0 µl of loading dye. Electrophoresis was carried out using 1.2% agarose gel, prepared in 1× Tris-acetate-EDTA (TAE) buffer and added ethidium bromide (0.5 µg/ml). Electrophoresis was carried out at 60V for 1.5 hours, visualised on Gel Doc TMXR+ gel documentation system (BIORAD) under UV light (300 nm) and photographed using Image Lab version 2.0.1 software (BIORAD) for gel analysis.

RESULTS AND DISCUSSION

Seed yield, size, growth and germination

The number of seed yield per pod was found to be 3 in all the controls and treated plants and size of the seeds increased from 0.5 cm in both the controls to 0.7 cm in treated plants.There was a variation in the mass of the seeds; in control the mass was found to be 0.15g. Whereas,

in plants treated with heat and increasing concentration of iodate, the mass was found to be 0.22g. However, the mass of the seed in control only treated with heat was same as the control supplied only with Hoagland nutrient solution. Colour of all the seeds was found to be greenish yellow. All seeds showed hundred per cent germination (Table 1).

Oxidative stress

Under oxidative stress, parameters investigated were superoxide ion generation, H_2O_2 production and malondialdehyde production. Superoxide anion generation in control plants was more than in the treatment with heat. However, on treatment with IO_3^- along with heat (35^0C), the superoxide anion generation increase considerably, 80 µM showing the highest levels of generation (Fig 1). In the case of H_2O_2 production, the effect of exogenous iodine was not prominent. The production of H_2O_2 was increased under the heat stress, however under IO_3^- application no remarkable change was observed (Fig 1). As per our data, production of H_2O_2 was increased by 33% in heat treatment against control. The application of IO_3^- reduced the production of H_2O_2 in all the three treatments. 40 µM of IO_3^- along with heat showed highest results as compared to control. In the past work, lipid peroxidation was measured by the MDA content. Our findings show that there was an increase of 95% in the MDA content of seeds of soybean in heat treatment. However, the combination dosages of heat and IO_3^- reduced the MDA content against heat treatment (Fig 1).

Table 1 The effect of iodate supplementation and heat stress on seed yield, size, growth and germination in soybean seeds

Treatment	Germination	Size	Mass	Colour
Control	100%	0.5cm	0.15g	Greenish yellow
Heat	100%	0.5cm	0.14g	Greenish yellow
20 µm IO_3^- + Heat	100%	0.7cm	0.20g	Greenish yellow
40 µm IO_3^- + Heat	100%	0.6cm	0.19g	Greenish yellow
80 µm IO_3^- + Heat	100%	0.7cm	0.22g	Greenish yellow

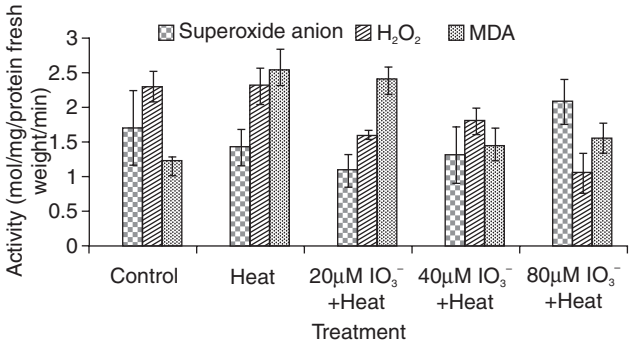

Fig 1 Effect of IO_3^- on superoxide anion, hydrogen peroxide (H_2O_2 and malondialdehyde (MDA) concentration in seeds of soybean plants under heat stress. Data represent (± SE) of value for one control and three treatments with three replications

Under stress conditions, transfer of electrons leads to the free radical generation. These electrons damage DNA, lipids and proteins. Chloroplast is the site of maximum production of superoxide anion through the Mehler reaction and H_2O_2. In our experiment, superoxide anion generation was decreased by 31% in heat stress against control. However, with the application of IO_3^-, its levels were reduced at 20 µM and 40 µM concentrations. 80 µM highest concentration of IO_3^- showed an increase in the superoxide anion generation, similar to control treatment.

H_2O_2 can serve as substrate for numerous enzymes such as catalase, which, though located in the peroxisomes where the H_2O_2 concentration is very high, is absent in the cytosol and chloroplasts, and thus the H_2O_2 is eliminated by peroxidases. These include APX, which is considered one of the most important enzymes in the reduction of this reactive molecule (Foyer and Noctor 2009).

In the case of H_2O_2 production, the effect of exogenous iodine was not prominent. The production of H_2O_2 increased under the heat stress, however under IO_3^- application no remarkable change was observed. The data shows an increment of 33% in production of H_2O_2 for heat treatment against control. The application of IO_3^- reduced the production of H_2O_2 in all the three dosages.

MDA is a biochemical indicator of stress, since it inhibits biomass production and reduces the possibilities of adaptation of the plant to salt stress (Hernandez and Almansa 2002). In our experiment, lipid peroxidation was determined by measuring the concentration of MDA in cells. MDA is formed through auto oxidation and enzyme degradation of polyunsaturated fatty acids in cells. Our findings show that there was an increase of 95% in the MDA content of seeds of soybean in heat treatment. However, the combination dosages of heat and IO_3^- reduced the MDA content against heat treatment.

Antioxidant enzymatic assay

Plants have developed an extensive network of antioxidant enzymes to combat the effect of heat stress. Our experiments indicate that there was a considerable increase in the concentration of SOD in heat treatment. However, application of exogenous iodine triggered the activity of SOD. The SOD activity was found maximum in the dosage of 20 µM IO_3^- (Fig 2).

APX activity showed very little fluctuation showing a decrease of only 11% in heat against control. CAT showed an increase in its activity in all the dosages, reaching its maximum value in heat treatment against control. It showed an increase of 78% in heat treatment against control.The activity of GR decreased in the Heat treatment (Fig 3).

Metalloenzyme SOD is the most effective intracellular enzymatic antioxidant against the toxic effects of elevated levels of ROS. SOD reduces O_2 to H_2O_2 and hence decrease the chances of production of OH via Haber-Weiss reaction

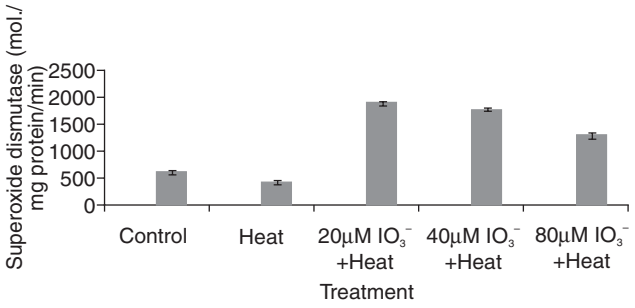

Fig 2 Effect of IO_3^- application on superoxide dismutase actvities in seeds of soybean plants under heat stress. Data represent (± SE) of value for one control and three treatment each of three replicates

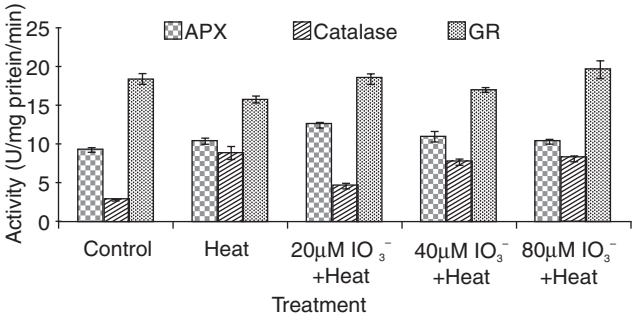

Fig 3 Effect of IO_3^- on ascorbate peroxidase (APX), catalase and glutathione reductase (GR) activities in seeds of soybean under heat stress. Data represent (± SE) of value for one control and three treatments each of three replicates

(Foyer and Noctor 2009).Our experiments show that the SOD activity increased remarkably on the application of IO_3^-. 20 μM IO_3^- Heat treatment showed the maximum activity of SOD (Fig 2).

Ascorbate peroxidase (APX) utilizes ascorbate as a specific electron donor, as ascorbate is a very important reducing substrate for H_2O_2 detoxification in the photosynthetic organism.The APX activity reached the highest values at the dosages of 20 μM and 40 μM IO_3^-. Catalases detoxify hydrogen peroxide by converting it to water and oxygen molecules, and are mostly confined in peroxisomes. In case of catalase activity, our findings are in agreement with the findings of Leyva *et al*. 2011 where they showed the beneficial effects of low concentration of iodine (>40 μM) in lettuce plant under 100 mM NaCl stress. Catalase activity showed an increase in its activity under heat(Fig 3). Catalase showed an increase in its activity in Heat, 40 μM, 80 μM the dosages, reaching its maximum value in Heat treatment against control. Our experiments show that all the combination dosages of IO_3^- and Heat boosted the activity of GR with respect to control treatment, 80μM IO_3^- showing the highest activity. The activity of glutathione reductase (GR) decreased in the Heat treatment (Fig.3). Dai *et al*. 2004 have shown that iodine when supplied in the form of IO_3^- has positive effects on the biomass of plants used for their edible leaves. The data almost in agreement with Dai's findings showing that supply of iodine in the form of IO_3^- increased the antioxidant response of soybean seed proteins.

Partial expression of 1-Cys peroxiredoxin gene

The desiccation-induced antioxidant gene encoding 1-Cys peroxiredoxin (XvPer1) shows 70% sequence identity to *Arabidopsis* seed specific dormancy related 1-Cys peroxiredoxin (Ndima *et al.*2010). The nucleotide sequence of 300bp, representing the full-length DNA, with a predicted molecular mass of 24.4 kDa and pI of 6.44. The Prx gene family is ubiquitously distributed in all organisms from bacteria to higher plants. It is a small gene family with only 10 genes in *Arabidopsis* and 11 genes in rice. Previous studies demonstrated that the transcription of Prx genes are in response to various kinds of stresses, such as low or high light, salinity, heavy metals, nutrient deprivation, temperature extremes and chemical effectors (Dietz 2011).Our experiment indicate that the 1-Cys Prx gene present in soybean are induced by heat stress and application of iodine further acts as a boosting agent in the partial expression of this gene. Along with heat stress, 40 μM concentration of iodine shows the maximum amplification of the gene suggesting that the exogenous application of iodine leads to amplification of 1-Cys Prx gene and hence enhanced resistance to the stress

To the best of our knowledge, it is the first report of amplification of 1-Cys Prx gene present in soybean using *Arabidopsis* 1-Cys Prx primers under heat stress. 1-Cys Prx is an active antioxidant enzyme that plays a central role in the dithiol-disulfide redox regulatory network of the plant. This gene employ a thiol-based catalytic mechanism to reduce H_2O_2, alkyl hydroperoxide, and peroxinitrite. The present study also highlights the activity of other antioxidant enzymes under heat stress. Further research is required for investigating the chaperone activity and functional characterization of 1-Cys Prx gene in soybean under heat stress.

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