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Time-course accumulation of metabolites and expression of anti-oxidative enzymes in *Glycine max* under temperature stress

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Abstract
One month old seedlings of four cultivars of *Glycine max* (L.) Merrill were subjected to a cold stress of 5°C for a period of 0-24 h and analyses were done every 2 h. Results revealed that among the antioxidative enzymes, catalase and ascorbate peroxidase showed an initial decline upto 4 h after which there was an increase. The other 3 enzymes- superoxide dismutase, glutathione reductase and peroxidase exhibited increased activities during the early periods of stress followed by a decline. In all cases, 2 peaks were obtained- one during the early periods of stress, and a second during the late period. Decrease in catalase and ascorbate peroxidase activities were concomitant with increase in accumulation of H_2O_2 during initial stress. Accumulation of small antioxidants – ascorbate and carotenoids showed differences among the 4 cultivars. In JS 71-05 and JS 335 both these antioxidants decreased initially, whereas in the other 2 cultivars- Rossio and NRC 37, an initial increase was evident. In these 2 cultivars, besides the 3 antioxidative enzymes, ascorbate and carotenoids also may play a role in conferring tolerance. Total phenols increased initially in all cultivars. Protein accumulation during the different periods of stress also varied with the cultivars. It is quite clear from the results that the time of over expression of enzymes or accumulation of antioxidants varies with different cultivars and this differential time related response may be involved in tolerance.

Keywords: cold stress, soybean, antioxidants, antioxidative enzymes

Among the abiotic stresses temperature is one of the most important environmental stress that a plant encounters and it is also a major factor limiting the growth of plants. Temperature stress as heat, cold or freezing is a principal cause for yield reduction in crops (Boyer,1982) and ROS (reactive oxygen species) generated by these stresses have been shown to injure cell membranes and proteins which lead to oxidative stress (Larkindale and Knight, 2002). The effects of chilling on cell membranes have been looked at as an oxidative stress that results in the production of highly reactive substances, such as hydrogen peroxide and oxygen free radicals (Elstner and Oswald 1994, Prasad *et al.* 1994 a, b). Not only temperature but also the rate of temperature changes (Steffen *et al.* 1989) and duration of exposure determine the degree of injury (Rajashekar *et al.* 1983). Tropical and subtropical plant species are prone to injury at chilling temperatures of 0-15°C (Zhang *et al.* 1995)

Leguminous oil seed crop like *Glycine max* (L.) Merr. are hot season annuals and the plants are more sensitive to cold season. It is assumed that at low temperatures, the antioxidant systems of heat-loving plants fail to overcome the increasing level of ROS and peroxides arising there from; such failure is an initial stage of injury (Hariadi and Parkin, 1993).

The present study was undertaken to investigate how the responses of 4 cultivars of soybean to cold temperature, with special emphasis on antioxidative responses, proceed with increasing duration of stress at a specific temperature.

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Materials and Methods

Plant material and induction of cold stress

The seeds of 4 different cultivars of soybean (Rossio, NRC 37, JS 335, JS 71-05) were obtained from the National Centre for Soybean Research, Indore, M.P. and one (Rossio) from ICAR Gangtok. Viability was checked in laboratory and seedlings of the different cultivars were then raised from this stock of seeds. Seeds were soaked overnight in distilled water after surface sterilization with 0.1% $HgCl_2$ and grown in petriplates. For experimental purposes small seedlings were transferred to pots containing sandy loam soil mixed with farmyard manure. Plants were watered regularly and maintained properly. One month old seedlings were exposed to low temperature of 5°C for 0-24 hours, during cold temperature treatment, plants were kept in plant growth chamber, with controlled humidity and light.

Antioxidant enzyme extraction and assays

For extraction of enzymes, leaf samples were initially ground to powder in liquid nitrogen and then extracted with 0.05 M sodium phosphate buffer, pH 6.8-7.2 (peroxidase, ascorbate peroxidase and catalase) and 0.1 M potassium phosphate buffer, pH 7.6 (glutathione reductase and superoxide dismutase) using polyvinylpyrrolidone under ice cold conditions. The homogenates were then centrifuged at 10,000 rpm for 15 min. Supernatants were used as crude enzyme extracts.

Table 1: Accumulation of H_2O_2 in different cultivars of soybean at different time periods of cold treatment

Time*	H_2O_2 content (mM/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
0	1.384	1.049	1.222	1.084
2	2.108	1.161	1.411	1.268
4	2.125	1.678	1.661	1.691
6	3.108	2.488	1.947	1.887
8	2.106	2.381	3.214	1.643
10	2.089	2.328	2.25	1.286
12	2.053	2.238	1.822	1.268
14	1.628	2.22	1.643	1.233
16	1.138	1.5	1.553	1.22
18	2.048	1.358	1.483	2.125
20	2.822	1.358	2.286	2.214
22	1.411	2.75	2.322	3.105
24	1.286	3.697	2.911	3.161

*Treatment time in hours; treatment at 5°C; average of 3 replicates

Peroxidase (POX: EC. 1.11.17)

Peroxidise activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118 SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakrabotry *et al.*, 1993). Specific activity was expressed as ΔA_{460nm} protein $^{-1}$ min $^{-1}$

Ascorbate peroxidase (APOX : EC.1.11.1.11)

Activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada *et al* (1987) with some modification. Enzyme activity was expressed as ΔA_{290}mg protein $^{-1}$ min $^{-1}$

Catalase (CAT: EC.1.11.1.6)

Catalase activity was assayed as described by Chance and Machly (1955) by estimating the breakdown of H_2O_2 which was measured at 240 nm in a spectrophotometer. The enzyme activity was expressed as ΔA_{245}mg protein $^{-1}$ min $^{-1}$.

Superoxide dismutase (SOD: EC 1.15.1.1)

Activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981) with some modification. The absorbance of samples were measured at 560nm and 1 unit and activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

Glutathione reductase (GR: EC 1.6.4.2)

Glutathione reductase activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was finally expressed as μM NADPH oxidized mg protein^{-1} min^{-1}.

Isozyme analysis

Isozyme analysis was carried out by PAGE following standard techniques. Following electrophoresis, gels were treated

Quantification of hydrogen peroxide

The hydrogen peroxide was extracted and estimated following the method of Jena and Choudhuri (1981) using titanium sulphate. Concentration of H_2O_2 was calculated using the extinction coefficient (0.28μmol^{-1}cm^{-1})

Non- enzymatic antioxidants

Ascorbate

Ascorbate was extracted and estimated by following the method of Mukherjee and Choudhuri (1983). The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler (1987). Extraction was done in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480nm, 663nm and 645nm in a VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

Phenols

Phenol was extracted by following the method of Mahadeven and Sridhar (1982). Total phenol was estimated by following the method of Bray and Thorpe (1954).

Protein quantification

Total soluble protein extracted in 0.05M sodium phosphate buffer were used as crude protein extract for quantification and analysis analysis of protein pattern. Proteins were estimated following the method of Lowry (1951).

Results

Effect of low temperature treatments on antioxidative enzymes

The seedlings were exposed to 5°C for a period of 24 h and sampling was done every 2 h as described in Materials and Methods. Catalase activities showed an

Table 2: Accumulation of carotenoids in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Carotenoid content (mg/g fresh wt.)			
	Rossio	JS-335	JS-71-05	NRC37
2	0.02	0.044	0.012	0.017
4	0.02	0.019	0.003	0.018
6	0.02	0.019	0.004	0.019
8	0.04	0.021	0.026	0.02
10	0.05	0.031	0.033	0.02
12	0.07	0.042	0.045	0.021
14	0.08	0.061	0.049	0.022
16	0.09	0.029	0.057	0.023
18	0.04	0.027	0.084	0.024
20	0.01	0.027	0.029	0.028
22	0.01	0.025	0.027	0.019
24	0.01	0.025	0.027	0.019

*Treatment time in hours; treatment at 5°C; average of 3 replicates

interesting trend. Initially, activity declined in all cultivars upto 4 h, with the decline being very steep in Rossio and NRC 37. After 4 h, there was an increase in activity till 22 h in Rossio, and 16 h in NRC 37. In JS 335 and JS 71-05, a sort of plateau was observed between 10-14 h and then there was a further decline (Fig.1).

Ascorbate peroxidase activities also showed an initial decline till 4-6 h in all cutivars. In all cultivars activities further increased till 14- 18 h of treatment and declined again after that (Fig.2). In case of peroxidase activity, Rossio showed enhanced activity till 12 h, a decline thereafter and a further increase after 20 h. In all the other 3 cultivars, activities initially increased till 6 h and then declined (Fig.3). Glutathione reductase and superoxide dismutase activities increased significantly in Rossio till 8-10 h and then declined. In the other 3 cultivars, there was an initial increase in activities till 4-6 h following which there was a decline (Figs. 4 and 5).

Accumulation of H_2O_2

This was also monitored for 24 h and results taken every 2 h. It was observed that in all cultivars, two peaks were evident in the accumulation pattern- one during the early hours (6-8 h) and the other towards the end , ie., 20 h in Rossio and 24 h in all other 3 cultivars (Table 1).

Effect of low temperature on small antioxidants

Total phenols increased initially in all 4 tested cultivars, followed by a decline and another increase following longer duration of stress. However, while in JS 335, JS 71-05 and in NRC 37, decline started after 4-6 h, in Rossio it was maintained till 10 h (Table 2). In case of ascorbate, in 2 of the cultivars, JS 335 and JS 71-05, a decline in accumulation was obtained even during the early periods, and only after 20 h of stress an increase was obtained. However, in Rossio and NRC 37, there was an initial increase till 4-6 h, followed by a decline and another increase later on (Table 3). Similar trend was also obtained in case of accumulation of carotenoids- i.e., in JS 335 and JS 71-05, a decline in accumulation was obtained till 8 h after which there was an increase.

Table 3: Accumulation of ascorbate in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Ascorbate content (mM/g fresh wt.)			
	Rossio	JS-335	JS-71-05	NRC37
2	6.13	1.60	1.48	1.70
4	8.58	1.25	1.48	2.26
6	5.83	1.60	1.20	2.80
8	4.17	1.00	1.20	2.30
10	2.77	0.73	1.03	1.33
12	1.35	0.4	0.60	5.02
14	0.85	0.33	0.90	5.14
16	5.14	0.14	0.90	5.70
18	8.16	0.63	1.50	4.70
20	9.48	0.30	0.73	4.40
22	8.95	0.23	0.65	3.50
24	8.13	0.23	0.59	3.30

*Treatment time in hours, treatment at 5°C. Average of 3 replicates

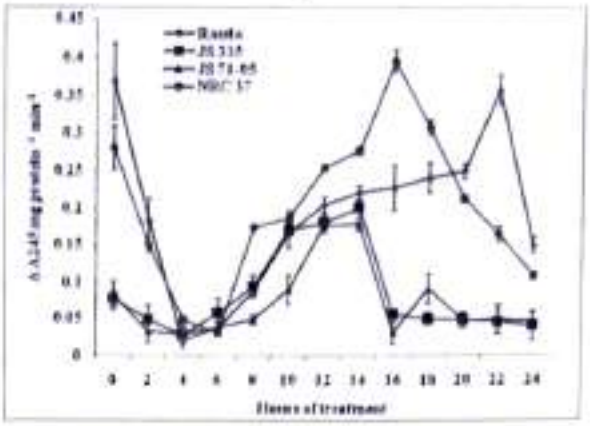

Fig. 1: Catalase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

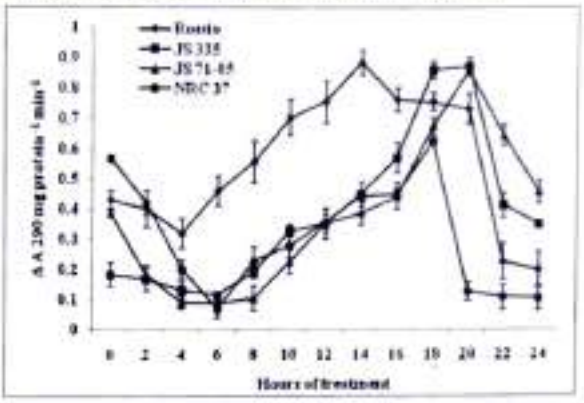

Fig. 2: Ascorbate peroxidase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

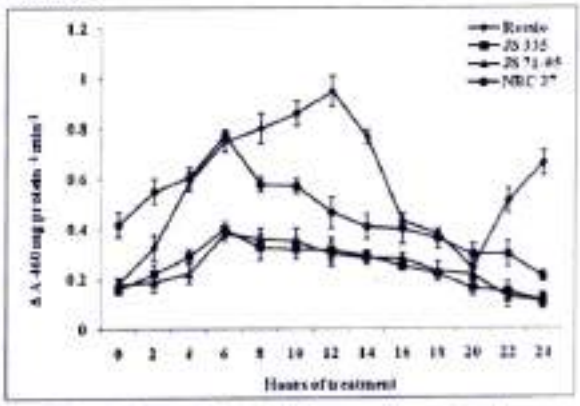

Fig. 3: Peroxidase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

However, in Rossio and NRC 37, there was an initial increase till 4-6 h, followed by a decline and another increase later on (Table 4).

Effect of cold stress on protein accumulation

Pattern of protein accumulation in seedlings of soybean over the period of stress varied among the four cultivars. In 3 of the cultivars, a decrease in protein accumulation was evident till 14-16 h after which there a slight increase followed by further decline. However, in Rossio, an increase in accumulation was evident initially till 6 h followed by a decline and another peak was obtained at 20 h of cold treatment (Table 5). SDS-PAGE

Table 4: Accumulation of phenols in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Phenol content (mg/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
2	2.4	2.3	3.8	2.8
4	2.5	2.9	3.8	2.9
6	2.6	2.9	2.85	2.2
8	3.6	2.7	2	1.6
10	4.4	1.7	1.85	0.8
12	3.0	1.6	1.95	1.0
14	2.4	1.4	3.9	1.4
16	2.0	2.8	4.05	1.8
18	1.9	3.5	5.3	2.8
20	1.8	3.9	5.3	3.4
22	2.7	3.3	4.4	4.7
24	2.6	2.9	3.4	3.6

*Treatment time in hours; treatment at 5°C. Average of 3 replicates

analysis of proteins revealed the accumulation of few new proteins during cold stress.

Discussion

The time-course dependent biochemical responses of soybean seedlings, specially the antioxidant responses were determined in 4 cultivars exposed to a cold temperature of 5°C for a period of 24 h, with analyses being done every 2 h. Among five antioxidative enzymes tested, results revealed that both catalase and ascorbate peroxidase showed an initial decline in activity for 4-6 h before being enhanced. This decline in activity could be correlated with an increase in accumulation of H_2O_2 detected during the early hours. It is quite clear that in the early period of oxidative stress, there is an accumulation of H_2O_2 which, besides being an ROS, is also involved in signaling (Chakraborty, 2005). It is now clear that ROS, besides being toxic molecules causing damage to proteins and DNA, are also involved in signaling substances for guard cell functioning, photoprotection, pathogenesis and development (Desikan *et al.* 2004; Einset *et al.* 2007).

With increase in the duration of cold stress, catalase and ascorbate peroxidase activities increase resulting in breakdown of H_2O_2. Thus, during the early period of

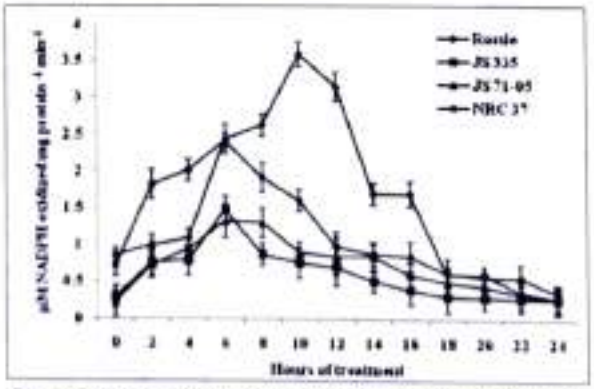

Fig. 4: Glutathione reductase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

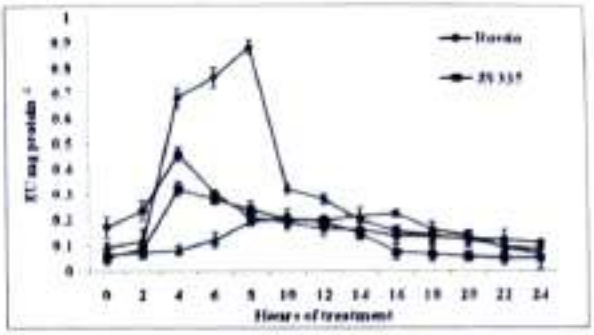

Fig. 5: Superoxide dismutase activities in different cultivars of soybean subjected to a temperature of 5°C for different durations

stress, protection against cold-stress is provided by activities of peroxidase, superoxide dismutase and glutathione reductase which are enhanced initially and lead to a certain degree of protection against oxidative stress. Prolonged period of stress or increasing the stress intensity, however, leads to a decline in activity indicating that the plants succumb to oxidative stress after an initial resistance. It was reported by Queiroz *et al.* (1998) that in coffee seedlings subjected to chilling stress of 15-10°C, ascorbate peroxidase and catalase activities did not change, in contrast with the activities of guaicol peroxidase, which increased. Cellular damage caused by superoxide and lipid peroxidation might be reduced or prevented by protective mechanisms like free radical processing by enzymes such as SOD, CAT, POX and APOX (Asada and Takahashi, 1987). Ascorbate peroxidase gene expression and activity has been reported to be rapidly induced by various stress conditions including chilling (Prasad *et al.* 1994, Keshavant and Naithani, 2001). Lukatkin (2002) compared SOD activity in various plant species differing in their cold-resistance during chilling. According to him, in resistant cultivars, chilling sharply activated SOD production. It has been reported that under stress conditions different plants and tissues respond to SOD induction differently with regard to SOD induction suggesting that different mechanisms may be involved

Table 5: Accumulation of proteins in different cultivars of soybean at different time periods of cold temperature treatment

Time*	Protein content (mg/g tissue)			
	Rossio	JS-335	JS-71-05	NRC37
2	151	144	200	224
4	157	104	192	210
6	290	102	188	204
8	129	84	182	200
10	113	84	176	184
12	103	176	192	136
14	100	261	192	152
16	130	200	224	184
18	130	200	240	160
20	150	168	256	144
22	120	148	250	116
24	110	140	240	108

*Treatment time in hours; treatment at 5°C. Average of 3 replicates

in protection against oxidative stress (Blokhina *et al.* 2003). Huang and Guo (2005) reported that, under chilling conditions, SOD activity of tolerant rice cultivar remained similar to control, while that of susceptible cultivar decreased after chilling and remained low throughout the chilling period. In the present study also, the nature of responses varied among the cultivars. Rossio could maintain antioxidant responses for more prolonged period than the other cultivars. NRC 37 also showed responses more or less similar to Rossio, while the other 2 cultivars varied.

Besides, antioxidative enzymes, small antioxidants such as phenols, ascorbate and carotenoids are also involved in stress tolerance. In the present study, accumulation of these was also determined at specific time intervals. It was observed that while total phenols initially increased in all cultivars, increased accumulation was maintained in Rossio for longer periods than in the other 3. The other 2 antioxidants- ascorbate and carotenoids decreased in JS 335 and JS 71-05 with increase in the duration of cold stress up to 8h after which they increased. In Rossio and NRC 37, the responses were different, with an initial increase, followed by a decline. It seems probable that in Rossio, and NRC 37, to some extent, which are more tolerant than the other two, small antioxidants also play a role initially, whereas, in the more susceptible cultivars, they are not involved in protection.

In conclusion, it may be stated that soybean plants respond to cold-stress by an initial enhancement of antioxidant activity which however, declines with prolonged stress. Among the cultivars, Rossio, which is grown in the hilly regions exhibited more tolerance towards cold-stress as evidenced by maintenance of antioxidant activities for longer periods in comparison to the other cultivars. Higher activities of antioxidative enzymes during chilling, along with accumulation of other antioxidants, could be associated with tolerance.

Acknowledgement

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