CORRIGENDUM

➢ List of abbreviations

Reactive oxygen species (ROS)
Deoxyribonucleic acid (DNA)
Superoxide dismutase (SOD)
Ascorbate peroxidase (APX/ APOX)
Catalase (CAT)
Salicylic acid (SA)
Abscisic acid (ABA)
Calcium chloride ($CaCl_2$)
Cell membrane stability (CMS)
Malondialdehyde (MDA)
Trichloroacetic acid (TCA)
Polyvinylpyrrolidone (PVP)
Glutathione oxidized (GSSG)
Nicotinamide adenine di nucleotide phosphate (NADPH)
Ethylene diamine tetra hydrochloric acid (EDTA)
Nitroblue tetrazolium (NBT)
Ultra violet - visible (UV- VIS)
Hydrogen peroxide (H_2O_2)
Polyacrylamide gel electrophoresis (PAGE)
NNN'N Tetramethyl ethylenediamine (TEMED)
Hydrochloric acid (HCl)
Ammonium persulphate (APS)
Sulphuric acid (H_2SO_4)
3,3'- diaminobenzidine (DAB)
2,2- Diphenyl- 1- picrylhydrazyl (DPPH)
Cupper Sulphate ($CuSO_4$)
Sodium Carbonate (Na_2CO_3)
Sodium Hydroxide (NaOH)
Bovine serum albumin (BSA)
Sodium Dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE)
Sodium Dodecyl Sulphate (SDS)
Sodium nitrite ($NaNO_2$)
Sodium Molybdate (Na_2MoO_4)
High performance liquid chromatography (HPLC)
Glutathione reductase (GR)
Enzyme unit (EU)
Peroxidase (POX)
Carotenoid (Car)
Ascorbate(Asc)
Chlorophyll (Chl)

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- Objectives of work has already been given
- Details of HPLC protocol has been provided in Materials and methods
- Fig.6A- Y axis should be **μMH_2O_2 oxidised mg protein^{-1} min^{-1}**
- Fig. 6B – **Y axis** should be **mM O- dianisidine oxidized mg protein^{-1} min^{-1}**
  Fig. 7A- **Y axis** should be **mM Ascorbate mg protein^{-1} min^{-1}**
- Fig.21 – Y axis should be mM MDA accumulated g fresh wt. tissue $^{-1}$
- Page 154 – **Here *et al.* 1997** should be **Hare *et al* 1997**
- Page 172 – **Health and Packer 1968** should be **Heath and Packer 1968**
- Page 4 - In **Babu and Devraj , 2008 - Devraj** should be **Devaraj**
- Page 169- **Boyer JS 1982.** Plant productivity and environment science. **218:** 443-498 should be **Boyer JS 1982.** Plant productivity and environment. *Science*. **218:** 443-448
- Page 175- **Larcher W. 1995.** Ecophysiology and stress physiology of functional groups. In Physiological Plant Ecology Ed. *Springer- Verlag. Berlin.* 340-353 should be **Larcher W. 1995.** Ecophysiology and stress physiology of functional groups. In Physiological Plant Ecology Ed.W. Larcher, *Springer- Verlag. Berlin.* 340-353
- Page 180- **Sambrook J, Friteh EF, Maniatis T. 1989**. Molecular cloning a laboratory manual *2nd* Edition Book 3: 18.60-18.74 should be **Sambrook J, Friteh EF, Maniatis T. 1989**. Molecular cloning a laboratory manual *2nd* N. Y. Cold Spring Harbor Laboratory Press. 1659 p.
- Page 168 – **Bhullar SS, Jenner CF 1985,** last part i.e. " biomembranes. *Methods Enzymol.* **148:** 350-382" should be shifted to Page 176 **Lichtenthaler II K 1987** i.e. it should be **Lichtenthaler II K 1987.** Chlorophylls and Carotenoids pigments of photosynthetic biomembranes. *Methods Enzymol.* **148:** 350-382
- Page 168 – **Rosmarinus officinalis L.** should be ***Rosmarinus officinalis* L.**
- Page 169- **Cicer arietinum L.** should be ***Cicer arietinum* L.**
- Page 170- **In**volvement **of** soluble sugars **in** reactive oxygen species balance and responses to **oxidative stress in plants.** *J Exp Bot.* **57:** 449-459 should be Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *J Exp Bot.* **57:** 449-459
- Page 173- **Zea mays** L. should be ***Zea mays*** L.
- Page 175 177 and 179 - **Arabidopsis** should be ***Arabidopsis***
- Page 177- **Pisum sativum** should be ***Pisum sativum***
- Page 178- **Glycine max** (L.) Merr. should be ***Glycine max*** (L.) Merr.
- Page 182 – **Vitis vinifera** L. should be ***Vitis vinifera*** L.
- Reference of **Arnow 1933** should be **Arnows E. L. 1933.** Colorimetric determination of the component of 2,4- dihydroxyphenyl- alanine- tyrosine mixture. *J Biol Chem.* **118:** 513-537

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List of publications

1. Antioxidant Responses of Four Glycine *max* (L.) Merr. Cultivars to Low Temperature Induced Stress. Journal of Plant Biology.

   Usha Chakraborty and Deepti Pradhan. *Journal of Plant Biology.* Vol. 36 (1&2). 2009, pp: 23-32.

2. High temperature induced oxidative stress in *Lens culinaris*, role of antioxidants and amelioration of stress by chemical pre-treatments.

   U Chakraborty and D Pradhan. *Journal of Plant Interactions.* Volume 6, Issue 1, 2010, pp: 43-53.

3. Biochemical responses of Lentil (*Lens culinaris* Medik.) to elevated temperature stress.

   U Chakraborty and D Pradhan. *Research journal of Pharmaceutical, Biological and Chemical Sciences.* Volume 1 Issue 3, 2010, pp: 575-585.

4. Time-course dependant accumulation of metabolites and expression of anti-oxidative enzymes in different varieties of *Glycine max* under cold temperature stress.

   D Pradhan and U Chakraborty. *North Bengal University Journal of Plant Sciences* Vol. 4, 2010, pp: 25-30.

J. Plant Biol., Vol. **36** (1&2), April–August, 2009, pp. 23–32.

ARTICLE

Antioxidant Responses of Four *Glycine max* (L.) Merr. Cultivars to Low Temperature Induced Stress

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MS received 14 March 2010; accepted 20 March 2010

The present study was undertaken to determine low temperature induced oxidative stress responses in four cultivars of *Glycine max* (L.) Merr. Seedlings were exposed to low temperatures in the range of 5–25°C for 4 h or up to 24 h at 5°C with sampling at 2 h intervals. Among the enzymes, catalase showed a decline in activity from 25°C to 5°C in all cultivars, whereas peroxidase, ascorbate peroxidase, glutathione reductase and superoxide dismutase showed an initial increase before declining. Cold stress enhanced lipid peroxidation at all temperatures. Accumulation of ascorbate, carotenoid and H_2O_2 was enhanced following cold stress but declined after 15–10°C. Total antioxidant activity also showed an increase between 15 and 10°C after which it declined. The activity of the antioxidative enzymes and accumulation of H_2O_2 was also monitored every 2 h up to 24 h at 5°C. The time of responses differed with the different cultivars, but most of the cultivars responded within 4–6 h. Results revealed that cold stress induces antioxidant activity in all the cultivars but the nature of response depended on the cultivars, temperature and period of stress. GR and SOD were significantly over-expressed during stress in all cultivars but activities of POX and APOX were significantly higher in tolerant cultivar.

Keywords: Antioxidative enzymes, antioxidants. cold stress, soybean.

Introduction

Legumes play an important role in human diet as they are the essential sources of protein. Besides being rich source of protein, they are also important for sustainable agriculture as they improve physical and chemical properties of soil and function as mini nitrogen factory. Among the leguminous plants, soybean (*Glycine max* (L.) Merr. is commonly cultivated and used for different purposes. It is an important global crop and native to South East Asia. Cultivation of this plant is greatly hampered due to biotic and abiotic stresses. Soybeans are hot season annuals and the plants are more sensitive to cold season. Optimal temperature requirements for soybean cultivation ranges between 25 and 30°C.

Abiotic stresses such as drought, high and low temperatures, salinity, etc., occur locally and exhibit variation in occurrence, intensity and duration. They generally cause reduced crop productivity. Among these abiotic stresses, temperature is one of the major factors limiting the growth of plants. Temperature stress, either as heat, cold or freezing, is a principal cause for yield reduction in crops (Boyer, 1982) and reactive oxygen species (ROS) generated by these stresses have been shown to injure cell membranes and proteins (Queiroz *et al.*, 1998; Keshavkant and Naithani, 2001; Larkindale and Knight, 2002). Temperature stress can have a devastating effect on plant metabolism, disrupting cellular homeostasis and uncoupling major physiological processes (Suzuki and Mittler, 2006).

Plant species growing in tropical and sub-tropical regions show characteristic damage symptoms of both roots and shoots exposed to chilling temperatures (Raison and Lyons, 1986; Zheng *et al.*, 1995; Queiros *et al.*, 1998). Chilling injury is associated with changes in membrane properties, such as solute leakage, reduced transport across the plasma membrane, malfunction of the mitochondrial respiration and inhibition of photosynthetic activity (Lyons, 1973; Lyons *et al.*, 1979), and induction of active oxygen species (Omran, 1980; Prasad *et al.*, 1994; Radyuk *et al.*, 2009). In genetically engineered tobacco plants, chilling sensitivity has been shown to be correlated with the extent of fatty acid unsaturation of the glycerol lipids of plastid membranes (Murata *et al.*, 1992; Kodarna *et al.*, 1994). During the time of temperature

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stress ROS level can increase dramatically which can result in significant damage to cell structure. Prolonged accumulation of ROS is detrimental and can cause inactivation of enzymes, lipid peroxidation, protein degradation and damage to DNA (Asada, 1999). Controlling ROS production might therefore be a promising avenue of genetic engineering to enhance the tolerance of plants to temperature stress and a combination of temperature stress and high light (Allen, 1995). The extent of oxidative stress in a cell is determined by the amount of superoxide, hydrogen peroxide and hydroxyl radicals. Hydrogen peroxide, though toxic at higher concentrations, also plays significant role as signalling molecules in various functions like guard cell opening, photoprotection, pathogenesis and development (Desikan *et al.*, 2004, 2005; Miller *et al.*, 2007). Therefore the balance of superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities will be crucial for suppressing toxic ROS level in a cell. Changing the balance of scavenging enzymes will induce compensatory mechanisms (Chakraborty *et al.*, 2005).

The present study was undertaken to investigate how four cultivars of soybean respond to cold temperatures with special reference to their antioxidative mechanisms, to compare these cutivars for tolerance and to determine specific biochemical parameters, if any, which could be linked to tolerance.

Materials and methods

Plant material and induction of cold stress

The seeds of three different cultivars of soybean (JS 335, JS 71-05 and NRC 37) were obtained from the National Centre for Soybean Research, Indore, India and one (Rossio) from ICAR Gangtok, India. 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 petri plates. For experimental purposes, one-week-old seedlings were transferred to pots containing sandy loam soil mixed with farmyard manure. Plants were watered regularly and maintained properly. One-month-old seedlings of the different cultivars were exposed to low temperature of 25°C–5°C for 4 h, and for different hours at 5°C. During cold temperature treatment, plants were kept in plant growth chamber, at 70–75% humidity and 12 h photoperiod.

Antioxidant enzyme extraction and assays

For extraction of enzymes, mature leaf samples were initially ground to powder in liquid nitrogen and then extracted with 50 mM sodium phosphate buffer [peroxidase, catalase (pH 6.8) and ascorbate peroxidase (pH 7.2) and 100 mM potassium phosphate buffer, pH 7.6 (glutathione reductase and total 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.

Assay

Peroxidase (POX: EC. 1.11.17). For determination of peroxidise activity, 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 200 mM sodium phosphate buffer (pH 5.4), 45 µl of 30% H_2O_2, 100 µl of O-dianisidine (5 mg/ml methanol) and 1.7 ml of distilled water. 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 (Chakraborty *et al.*, 1993). Specific activity was expressed as ΔA_{460} mg 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 (1984) with some modification. The reaction mixture consisted of 0.01 ml of enzyme extract, 0.01 ml of 0.5 mM ascorbic acid, 0.01 ml of 30% H_2O_2 and 2.97 ml of sodium phosphate buffer (pH 7.2). Enzyme activity was finally 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). Enzyme extract (40 µl) was added to 3 ml of 30% H_2O_2 in phosphate buffer (0.4 ml of 30% H_2O_2 to 100 ml of phosphate buffer, pH 7.0) and the breakdown of H_2O_2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H_2O_2 was used as reference. The enzyme activity was expressed as ΔA_{245} mg protein^{-1} min^{-1}.

Superoxide dismutase (SOD: EC 1.15.1.1). SOD 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. Each 3 ml of the assay mixture constituted of 0.1 ml enzyme extract, 1.5 ml phosphate buffer (100 mM, pH 7.6), 0.1 ml Na_2CO_3 (1.5 mM), 0.1 ml NBT (2.25 mM), 0.2 ml methionine (200 mM), 0.1 ml EDTA (3 mM), 0.1 ml riboflavin (0.06 mM) and 0.8 ml of distilled water. The reaction tubes containing enzyme samples were illuminated with 15 W fluorescent lamp for 10 min. The other set of tubes lacking enzymes were also illuminated and served as control. A non-irradiated complete reaction mixture served as blank. The absorbance of samples was measured at 560 nm and 1 unit of 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). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.6), 100 mM EDTA, 0.1 mM NADPH, 0.6 mM glutathione (oxidised form, GSSG) with 0.1 ml of enzyme extract. The reaction was initiated by the addition of NADPH at 25°C. Enzyme activity was finally expressed as µmols NADPH oxidized mg protein^{-1} min^{-1}.

Estimation of protein content

Protein content in each of the extract was estimated following the method of Lowry *et el.* (1951) using BSA as standard.

Determination of hydrogen peroxide

The hydrogen peroxide was extracted by following the method of Jena and Choudhuri (1981) by homogenizing 50 mg mature intermediate leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The homogenate was then centrifuged at 6000 *g* for 25 min. To determine H_2O_2 level, 3 ml of extracted solution was mixed with 1 ml of 0.1% Titanium sulphate in 20% H_2SO_4 (w/v), and the mixture was then centrifuged at 6000 *g* for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. Concentration of H_2O_2 was calculated using the extinction coefficient (0.28 µmol^{-1} cm^{-1}).

Determination of peroxidation of membrane lipids

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa *et al.* (1981). Mature intermediate leaf tissue was homogenized in 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged. 0.5 ml of the supernatant was mixed with 2 ml of 20% TCA containing 0.5% of (v/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was read at 532 nm and 600 nm. The concentration of MDA was calculated by means of an extinction coefficient of 155 mM^{-1} cm^{-1}.

DPPH (1,1-diphenyl-2-picryl hydrazyl) analysis for antioxidant

Mature intermediate leaves were powdered and 1 g of the powder was combined with 4 ml of methanol, centrifuged at 4000 rpm for 10 min, supernatant was collected and placed in a 60°C water bath for 25 min (Blois, 1958). The semi-dried extract was gathered and for estimation, 0.2 g of the extract was dissolved in 10 ml of methanol. Two ml of DPPH solution with a concentration of 0.025 g of DPPH in 1000 ml of methanol was mixed with 40 µl of extract solution and the initial reading was taken. After a 30 min incubation at room temperature, the reaction solution was examined at 515 nm by using a spectrophotometer (UV-VIS Spectrophotometer, Model 118 Systronics). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation

$$\text{Inhibition } \% = \frac{AT_0 - AT_{30}}{AT_0} \times 100.$$

(AT_0 is the absorbance of DPPH at time zero. AT_{30} is the absorbance of DPPH after 30 min of incubation.)

Results

Effect of low temperatures on antioxidative enzymes

Enzymes were extracted from the leaves of seedlings of the cultivars – Rossio, NRC 37, JS-335 and JS 71-

Table 1. Effect of low temperature stress on catalase activities of soybean cultivars.

Temperature (°C)	CAT activity (ΔA_{245} mg protein^{-1} min^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.368 ± 0.013	0.075 ± 0.003	0.086 ± 0.007	0.320 ± 0.010
20	0.176 ± 0.009	0.058 ± 0.004	0.063 ± 0.005	0.265 ± 0.021
15	0.090 ± 0.008	0.052 ± 0.009	0.043 ± 0.004	0.113 ± 0.009
10	0.064 ± 0.006	0.037 ± 0.002	0.030 ± 0.003	0.083 ± 0.007
5	0.038 ± 0.002	0.028 ± 0.004	0.022 ± 0.002	0.040 ± 0.008

CD ($P = 0.05$) treatments – 0.126; cultivars – 0.113

Average of three separate experiments; ± SE.

Table 2. Changes in peroxidase activities of soybean cultivars exposed to low temperatures.

Temperature (°C)	POX activity (ΔA_{460} mg protein^{-1} min^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.180 ± 0.011	0.159 ± 0.009	0.176 ± 0.040	0.417 ± 0.036
20	0.200 ± 0.008	0.209 ± 0.010	0.198 ± 0.009	0.442 ± 0.020
15	0.243 ± 0.040	0.352 ± 0.032	0.330 ± 0.031	0.686 ± 0.070
10	0.692 ± 0.022	0.319 ± 0.015	0.227 ± 0.018	0.518 ± 0.050
5	0.339 ± 0.012	0.290 ± 0.043	0.222 ± 0.015	0.300 ± 0.040

CD ($P = 0.05$) treatments – 0.182; cultivars – 0.163

Average of three separate experiments; ± SE.

Table 3. Effect of low temperature stress on ascorbate peroxidase activities of soybean cultivars.

Temperature (°C)	APOX activity (ΔA_{290} mg protein^{-1} min^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.232 ± 0.019	0.162 ± 0.053	0.270 ± 0.027	0.567 ± 0.030
20	0.546 ± 0.064	0.284 ± 0.022	0.420 ± 0.034	0.680 ± 0.029
15	0.786 ± 0.046	0.433 ± 0.039	0.739 ± 0.045	0.717 ± 0.077
10	0.896 ± 0.052	0.360 ± 0.017	0.530 ± 0.048	0.225 ± 0.020
5	0.429 ± 0.022	0.150 ± 0.020	0.096 ± 0.055	0.240 ± 0.018

CD ($P = 0.05$) treatments – 0.257; cultivars – 0.230

Average of three separate experiments; ± SE.

05 subjected to temperatures of 25, 20, 25, 10 and 5°C for a period of 4 h and assayed. Sampling was done soon after imposition of stress. Assay of activities revealed that in all cultivars, there was a decline in activity of catalase with decrease in temperature. Among the four cultivars, Rossio and NRC-37 had higher constitutive catalase activities, but at 5°C, all had similar low activities (Table 1). The decrease in the activity of catalase was around 9-fold in the above two cultivars, but around 3-fold in the other two. Activities of all the other enzymes, i.e. peroxidase, ascorbate peroxidase, glutathione reductase and superoxide dismutase showed an initial increase followed by a decline. In JS 335, JS 7105 and NRC 37, the activities increased till 15°C, but with further decrease in temperature, the enzyme activities also declined. However, in Rossio the activities increased till 10°C and there was a decline at 5° (Tables 2–5). Statistical analysis (ANOVA) revealed that among the five enzymes, SOD and GR increased significantly at 20, 15 and 10°, whereas CAT activity decreased significantly from 15° onwards.

Table 4. Superoxide dismutase activities of soybean cultivars exposed to low temperatures.

Temperature (°C)	SOD activity (EU mg protein^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.132 ± 0.014	0.080 ± 0.009	0.072 ± 0.002	0.232 ± 0.009
20	1.460 ± 0.023	1.400 ± 0.013	0.880 ± 0.024	1.960 ± 0.012
15	1.800 ± 0.012	1.560 ± 0.010	1.640 ± 0.035	2.120 ± 0.043
10	2.080 ± 0.046	0.840 ± 0.012	0.480 ± 0.014	1.720 ± 0.025
5	0.680 ± 0.012	0.240 ± 0.009	0.080 ± 0.004	0.456 ± 0.010

CD (P = 0.05) treatments – 0.469; cultivars – 0.419

Average of three separate experiments; ± SE.

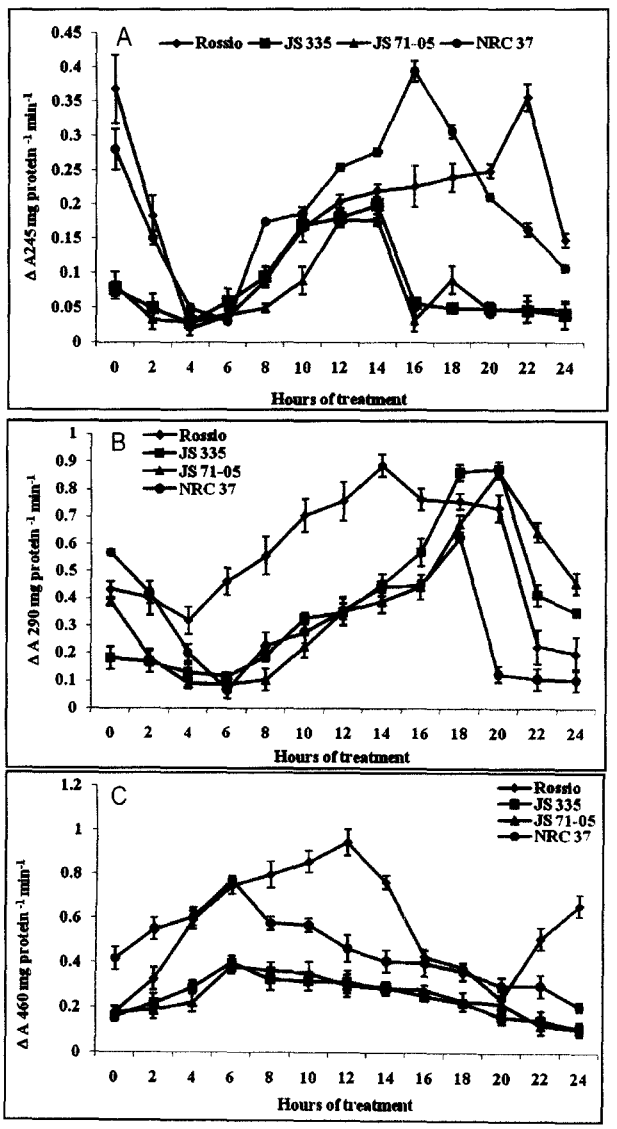

Figure 1. Catalase (A), ascorbate peroxidase (B) and peroxidase (C) activities in soybean cultivars subjected to low temperature of 5°C for a period of 24 h.

For further analysis, the seedlings were exposed to 5°C for 24 h and sampling was done every 2 h. Catalase activities showed an interesting trend. Initially, the activity declined in all cultivars up to 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 and 14 h and then a further decline was noticed (Figure 1).

Ascorbate peroxidase activities also showed an initial decline till 6 h in three cultivars and till 12 h in Rossio. In all cultivars, activities further increased till 18 h of treatment and declined again after that (Figure 1B). 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 three cultivars, activities initially increased till 6 h and then declined (Figure 1C). Glutathione reductase and superoxide dismutase activities increased significantly in Rossio till 8–10 h and then declined. In the other three cultivars, an initial increase in activities was noticed till 4–6 h following which a decline was observed (Figure 2A and B).

Levels of H_2O_2

Determination of levels of H_2O_2 at different temperatures revealed that with decrease in temperature, accumulation was enhanced, till 15° in cultivars NRC 37, JS 335 and JS 71-05, and till 10° in Rossio (Table 6).

Accumulation of H_2O_2 was also monitored for 24 h and results taken every 2 h. It was observed that in Rossio, two peaks were evident in the accumulation pattern – one at 6 h and the other at 20 h. In the other three cultivars, after an increase till 6–8 h there was

Table 5. Effect of low temperature stress on glutathione reductase activities of soybean cultivars.

Temperature (°C)	GR activity (μmols NADH oxidized mg protein^{-1} min^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.862 ± 0.048	0.380 ± 0.064	0.880 ± 0.074	0.788 ± 0.072
20	2.280 ± 0.170	2.460 ± 0.178	1.840 ± 0.098	2.840 ± 0.245
15	3.120 ± 0.135	2.840 ± 0.188	2.480 ± 0.175	3.620 ± 0.227
10	3.740 ± 0.314	1.500 ± 0.116	1.340 ± 0.158	1.830 ± 0.180
5	1.030 ± 0.166	0.592 ± 0.056	0.412 ± 0.032	1.630 ± 0.169

CD ($P = 0.05$) treatments – 0.822; cultivars – 0.736

Average of three separate experiments; ± SE.

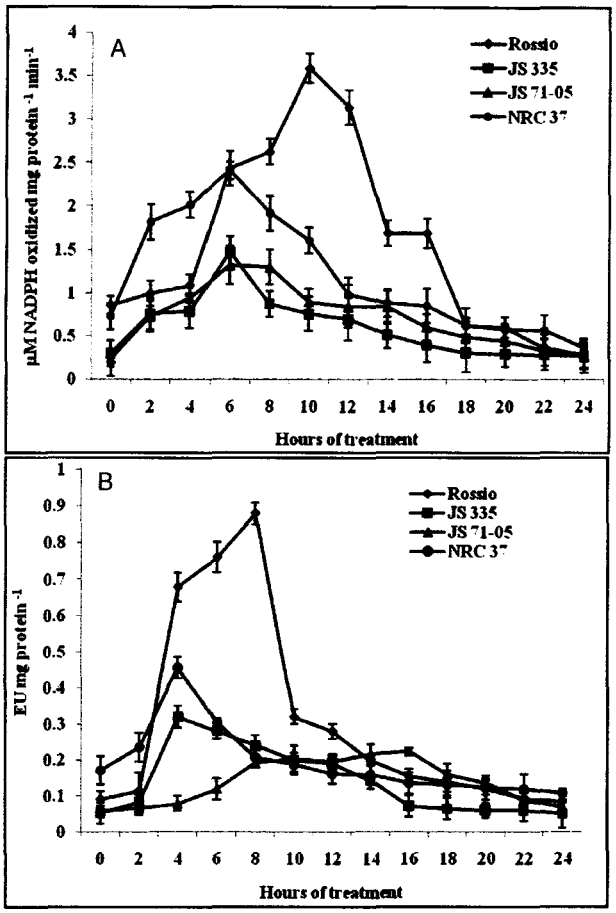

Figure 2. Effect of exposure to low temperature of 5°C for a period of 24 h on glutathione reductase (A) and superoxide dismutase (B) activities in soybean cultivars.

decline, and a further increase after 20 h which was maintained till 24 h (Figure 3).

Lipid peroxidation

Lipid peroxidation was measured in terms of MDA during the different low temperatures. Cold stress increased MDA accumulation at all low temperatures in all cultivars (Table 7). Initially, MDA content was not significantly different from control in any of the cultivars, but at 10° and 5°C, significant differences from control were observed.

Total antioxidant activity

Total antioxidant activity was measured in terms of percentage of inhibition of DPPH. Antioxidant activity increased under temperature stress till 15–10°C (Table 8). Overall antioxidant activity during cold stress was significantly enhanced in comparison to control. Among the cultivars, Rossio showed significantly higher activities than the other cultivars.

Discussion

The antioxidant responses of one-month-old soybean seedlings were determined in four cultivars exposed to varying cold temperatures. Analysis of five antioxidant enzymes revealed that activities of four enzymes, i.e. superoxide dismutase, peroxidase, ascorbate peroxidase and glutathione reductase were initially enhanced with decrease in temperature. But after a threshold temperature, activities declined. Of the four cultivars, three showed similar trend, where the activities started declining after 15°C. However, in Rossio, increase in activity continued till 10°C. It is clear that in all cultivars, increase in the cold-induced stress led to an initial response where the antioxidant activity was enhanced to withstand the stress. Catalase activity in all cultivars decreased with a decrease in temperature. Decrease in activity of catalase was correlated with an increased build up of H_2O_2 in the tissues. However, H_2O_2 accumulation was highest at 10°C in Rossio and at 15°C in other cultivars. It is quite probable that H_2O_2, being involved in signalling, would be initially built up, until it reaches a toxic

Table 6. Accumulation of H_2O_2 in soybean cultivars under low temperature stress.

Temperature (°C)	H_2O_2 content (μ mols g fresh wt tissue^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	16.71 ± 1.024	12.86 ± 0.932	14.36 ± 0.837	13.91 ± 0.916
20	19.71 ± 0.890	14.36 ± 0.897	18.86 ± 0.649	13.93 ± 0.829
15	25.50 ± 2.368	21.64 ± 0.954	24.90 ± 1.175	29.00 ± 1.127
10	30.20 ± 1.159	20.36 ± 1.058	20.36 ± 1.050	22.10 ± 1.084
5	25.50 ± 2.130	16.40 ± 0.984	19.93 ± 0.816	10.29 ± 0.933

Average of three separate experiments; ± SE.

Table 7. Effect of low temperatures on lipid peroxidation of soybean cultivars.

Temperature (°C)	MDA content (μ mols g fresh wt^{-1})			
	Rossio	JS 335	JS 71-05	NRC 37
25	0.032 ± 0.005	0.029 ± 0.002	0.036 ± 0.002	0.029 ± 0.001
20	0.038 ± 0.009	0.029 ± 0.003	0.037 ± 0.005	0.045 ± 0.002
15	0.045 ± 0.007	0.042 ± 0.003	0.067 ± 0.005	0.061 ± 0.007
10	0.050 ± 0.006	0.069 ± 0.005	0.088 ± 0.006	0.073 ± 0.007
5	0.058 ± 0.002	0.083 ± 0.008	0.094 ± 0.006	0.096 ± 0.006

Average of three separate experiments; ± SE.

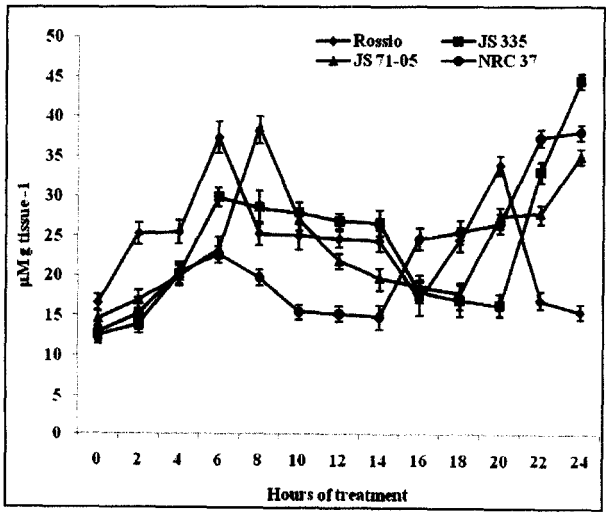

Figure 3. Accumulation of H_2O_2 in soybean cultivars subjected to low temperature of 5°C for a period of 24 h.

level, at which point it would be broken down. Previous reports have also confirmed that stresses lead to a decrease in catalase activities (Fadzillah *et al.*, 1996; Tongden and Chakraborty, 2005), which could be correlated to accumulation of H_2O_2. Queiroz *et al.* (1998) reported 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). Lukatin (2001) 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 suggesting that different mechanisms may be involved 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, whereas that of susceptible cultivar decreased after chilling and remained low throughout the chilling period. However, Radyuk *et al.* (2009) reported that under low temperature stress, total SOD activity exceeded the initial value by

Table 8. Antioxidant activities in soybean cultivars exposed to low temperatures.

Temperature (°C)	% inhibition of DPPH			
	Rossio	JS 335	JS 71-05	NRC 37
25	18.34 ± 0.924	17.67 ± 0.862	11.27 ± 0.937	16.50 ± 0.960
20	26.30 ± 1.695	22.50 ± 1.897	25.63 ± 0.859	23.80 ± 1.290
15	31.51 ± 1.668	37.99 ± 1.956	34.60 ± 1.125	36.90 ± 1.272
10	40.35 ± 1.069	28.53 ± 1.582	22.20 ± 1.156	29.60 ± 1.035
5	31.99 ± 1.630	21.97 ± 0.949	19.16 ± 0.918	20.40 ± 1.093

Average of three separate experiments; ± SE.

15%. In the present study also, SOD activities increased most significantly during low temperature. However, this was more or less similar in the different cultivars. Payton *et al.* (2001) also reported that elevating levels of APOX or GR improved recovery of cotton from chilling in transgenic plants. Among the enzymes, peroxidase and ascorbate peroxidase increased by about 4-fold in one of the cultivars, Rossio, whereas in other three cultivars the increase was about 2-fold. This cultivar could also maintain higher levels of antioxidative enzymes till 10°C, and the decline was evident only after this temperature. This cultivar, more commonly cultivated in higher altitudes, was more tolerant to lower temperatures than the other cultivars, which are normally grown in the plains.

In the present study, besides analysis of enzyme activities of plants subjected to varying temperatures at one time period, analysis of enzyme activities for 24 h at 5°C was also monitored every two hours. Results revealed that both catalase and ascorbate peroxidase showed an initial decline in activity for 4–6 h before being enhanced. Thus, during the early period of 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. The decline in activity of CAT and APOX 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 signalling 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. 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.

Membrane damage is one of the most important consequences of cellular injury by temperature stress. Membrane injury was detected in terms of lipid peroxidation of membranes indicated by accumulation of malonaldehyde. In the present study, increased accumulation of MDA was observed in all cultivars subjected to the cold stress, but least was observed in cv. Rossio. Several authors have confirmed that peroxidation of membranes contributes to oxidative stress induced by chilling or high temperatures (Queiroz *et al.*, 1998; Jiang and Huang, 2000; Larkindale and Huang, 2004; Huang and Guo, 2005; Kumar and Yadav, 2009). Finally, the overall antioxidant activity of the cell was determined by DPPH reduction assay. It was observed that the total antioxidant activity also increased initially and then declined. The total antioxidant activity of one of the cultivars, Rossio, was significantly higher than the others.

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. Activities of POX and APOX were higher in the tolerant cultivar, indicating a major role of peroxide detoxification in tolerance to cold stress. However, SOD and GR were involved in the overall detoxification mechanisms as their levels increased significantly. 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 other cultivars. Higher activities of antioxidative

enzymes during chilling, along with accumulation of other antioxidants, and lower lipid peroxidation could be associated with tolerance in soybean.

Acknowledgement

Financial assistance received from the University Grants Commission, New Delhi, is gratefully acknowledged.

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Journal of Plant Interactions
Vol. 6, No. 1, March 2011, 43–52

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ORIGINAL ARTICLE

High temperature-induced oxidative stress in *Lens culinaris*, role of antioxidants and amelioration of stress by chemical pre-treatments

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(Received 7 June 2010; final version received 2 August 2010)

Six varieties of lentil (*Lens culinaris* Medik.) – Asha, Subrata, IPL 406, IPL 81, Lv and Sehore – were exposed to temperatures ranging from 30–50°C which resulted in retarded germination and seedling growth at higher temperatures. Tolerance index and membrane stability tests revealed Sehore and Lv to be susceptible to elevated temperatures while IPL 406, IPL 81, Asha and Subrata were tolerant. Catalase, ascorbate peroxidase and superoxide dismutase showed an initial increase before declining at 50°C, while peroxidase and glutathione reductase activities declined at all temperatures. Lipid peroxidation significantly increased in all varieties. In the tolerant varieties, there was an initial decrease in accumulation of H_2O_2 followed by an increase from 40°C onwards; however, in the susceptible varieties, accumulation was enhanced at all high temperatures. Ascorbate and glutathione also showed initial increase followed by a decline. Total antioxidant activity was at a maximum at 35–40°C in the tolerant varieties and at 30°C in the susceptible ones. Oxidative stress induced by high temperature was ameliorated by treatment with salicylic acid, abscisic acid or $CaCl_2$, of which salicylic acid was the most effective.

Keywords: *Lens culinaris*; high temperature; oxidative stress; antioxidants; salicylic acid

Introduction

Elevated temperature stress is one of the major factors limiting the growth of plants as it adversely affects normal physiological processes such as photosynthesis, respiration, membrane stability and protein metabolism (Georgieva 1999). A major mechanism of injury is by the generation of reactive oxygen species such as superoxides, hydrogen peroxide and hydroxyl radicals which damage cellular components (Noctor and Foyer 1998; Liu and Huang 2000; Breusegem *et al.* 2001). During the time of temperature stress, reactive oxygen species level can increase dramatically which can result in significant damage to cell structure. Prolonged accumulation of reactive oxygen species (ROS) is very harmful and can cause inactivation of enzymes, lipid peroxidation, protein degradation and damage to DNA. In order to limit oxidative damage under stress condition, plants have developed a series of enzymatic and non-enzymatic detoxification systems that break down the highly toxic reactive oxygen species to less reactive molecules (Sairam and Tyagi 2004). Antioxidant enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase function in detoxification of superoxide and H_2O_2 (Mittler 2002). Protective roles of the antioxidant enzymes in temperature stress have been previously reported for a number of plants (Almeselmani *et al.* 2006; Babu and Devraj 2008). Antioxidant metabolites like glutathione, ascorbic acid, tocopherol and carotenoids also protect plants against oxidative stress (Sairam *et al.* 2000). It has also been reported that increase in temperature leads to ion leakage and this could be used as an index for screening genotypes against heat stress (Deshmukh *et al.* 1991).

A promising area for increasing resistance of crops to thermal stress is by the use of chemical treatments like salicylic acid, abscisic acid and calcium chloride (Larkindale and Knight 2002; Chakraborty and Tongden 2005; He *et al.* 2005). Acquisition of thermotolerance is likely to be of particular importance to plants that experience daily temperature fluctuations and are unable to escape to more favorable environments. Lentil (*Lens culinaris*), a legume, is a bushy annual plant grown for its lens-shaped seeds. Lentils contain high levels of proteins, including the essential amino acids isoleucine and lysine, and are an essential source of inexpensive protein in many parts of the world for those who adhere to a vegetarian diet and are widely used in India. Apart from a high level of proteins, lentils also contain dietary fiber, folate, vitamin B_1, and minerals. Lentils are relatively tolerant to drought and are grown throughout the world. They are cool season annuals and are sensitive to high temperatures. About a third of the worldwide production of lentils is from India, most of which is consumed in the domestic market. However,

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ISSN 1742-9145 print/ISSN 1742-9153 online

DOI: 10.1080/17429145.2010.513484
http://www.informaworld.com

productivity is seriously hampered by narrow genetic base of the presently cultivated varieties and losses due to the biotic and abiotic factors (Tickoo *et al.* 2005).

The present investigation was undertaken to determine the effect of high temperatures on anti-oxidative enzymes, antioxidants, lipid peroxidation and membrane stability on different varieties of *Lens culinaris* and to identify the most tolerant varieties. Besides, amelioration of high temperature stress by pre-treatment with some chemicals has also been attempted.

Materials and methods

Plant material and induction of high temperature stress

The seeds of six different varieties of *Lens culinaris* Medik. – Asha, Subrata, IPL 81, IPL 406, Lv and Sehore – were obtained from the Indian Institute of Pulses Research, Kanpur, Uttar Pradesh, and Oil and Pulses Research Centre, Behrampur, West Bengal. The above varieties have been released and grown in different geographical regions of the country: Asha, Subrata in the Eastern region, Lv in North Eastern regions and IPL 81, 406 and Sehore from the North West. Hence, these were selected to study varieties obtained from different locations. Viability was checked in the laboratory and seedlings of the different varieties were then raised from this stock of seeds. Seeds were surface sterilized with 0.1% $HgCl_2$ (w/v) for 3 min and washed 3–4 times thoroughly with sterile distilled water; after which surface sterilized seeds were soaked overnight in sterile distilled water and grown in Petri dishes at 20°C. For experimental purposes, small seedlings were transferred to pots containing sandy loam soil mixed with farmyard manure. Plants were watered regularly twice a day – early in the morning and evening – and maintained properly by weeding once a week. One month-old seedlings were exposed to the different elevated temperatures of 30, 35, 40, 45 and 50°C for 4 h in plant growth chamber at 65–70% RH, 16 h photoperiod and irradiance of 400 µmol $m^{-2}s^{-1}$. Immediately after temperature treatment, sampling was carried out for various analyses.

Chemical pre-treatments

For chemical pre-treatments, one month-old seedlings of the different varieties were sprayed with solutions of salicylic acid (100 µM), ABA (50 µM) or $CaCl_2$ (10 mM) separately twice a day (early in the morning and in the evening) for a week after which seedlings were exposed to 50°C as described.

Determination of tolerance index (TI) of seedlings

Variation in heat tolerance of the seedlings was calculated as the tolerance index (TI) which gives the percentage of shoot and/or root fresh biomass (g/plant) of treated (FW_t) over untreated control (FW_c) plants according to the following equation as suggested by Metwally *et al.* (2005):

$$TI\ (\%) = (FW_t/FW_c \times 100) - 100.$$

Determination of cell membrane thermostability

Membrane thermostability was tested by cell membrane stability (CMS) test with the pinnules obtained from seedlings following the method of Martineau *et al.* (1979). The injury was determined by following the equation:

$$\text{Relative injury (RI) (\%)} = \{1 - [1 - (T_1/T_2)]/[1 - (C_1/C_2)]\} \times 100,$$

where T and C refer to the conductance in treatment and control tubes and subscripts 1 and 2 refer to reading before and after autoclaving, respectively.

Determination of peroxidation of membrane lipids

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa *et al.* (1981). Mature intermediate leaf tissue was homogenized in 2 ml of 0.1% (w/v) tricholoroacetic acid (TCA) and centrifuged for 10 min at 10,000 rpm at 4°C. Supernatant (0.5 ml) was mixed with 2 ml of 20% trichloroacetic acid containing 0.5% of (v/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min, quickly cooled and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant was read at 532 and 600 nm. The concentration of MDA was calculated by means of an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Antioxidant enzyme extraction and assays

For extraction of enzymes, mature leaf samples were initially ground to powder in liquid nitrogen and then extracted with 50 mM sodium phosphate buffer (peroxidase, catalase (pH 6.8) and ascorbate peroxidase (pH 7.2)) and 100 mM potassium phosphate buffer, pH 7.6 (glutathione reductase and total superoxide dismutase) using polyvinylpyrrolidone under ice cold conditions. The homogenates were then centrifuged at 10,000 rpm for 10 min at −4°C. Supernatants were used as crude enzyme extracts.

Assay

Peroxidase (POX: EC. 1.11.17)

For determination of peroxidase activity, 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 200 mM sodium phosphate buffer (pH 5.4), 45 µl of 30% H_2O_2, 100 µl of O-dianisidine (5 mg/ml methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (Model 118 SYSTRONICS) at 460 nm by monitoring the oxidation of O-dianisidine

in the presence of H_2O_2 (Chakraborty *et al.* 1993). Specific activity was expressed as mmol O-dianisidine mg 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 (1984) with some modification. The reaction mixture consisted of 0.01 ml of enzyme extract, 0.5 mM ascorbic acid, 30% H_2O_2 (v/v) and 0.05 M sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as mmol ascorbate mg protein^{-1}min^{-1}.

Catalase (CAT: EC.1.11.1.6)
Catalase activity was assayed as described by Chance and Machly (1955). Enzyme extract (40 µl) was added to 30% H_2O_2 (v/v) in phosphate buffer and the breakdown of H_2O_2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H_2O_2 was used as reference. The enzyme activity was expressed as µmol H_2O_2 mg protein^{-1}min^{-1}.

Superoxide dismutase (SOD: EC 1.15.1.1)
SOD activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.* (1981). Each 3 ml of the assay mixture constituted of 0.1 ml enzyme extract, 100 mM, pH 7.6 phosphate buffer, 1.5 mM Na_2CO_3, 2.25 mM NBT, 200 mM methionine, 3 mM EDTA, 0.06 mM riboflavin and distilled water. The reaction tubes containing enzyme samples were illuminated with a 15 W fluorescent lamp for 10 min. The other set of tubes lacking enzymes were also illuminated and served as control. A non-irradiated complete reaction mixture served as blank. The absorbance of samples was measured at 560 nm and 1 unit of 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). The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.6), 2 mM EDTA, 0.1 mM NADPH, 0.6 mM glutathione (oxidized form, GSSG) with 0.1 ml of enzyme extract. The reaction was initiated by addition of NADPH at 25°C. Enzyme activity was finally expressed as µmols NADPH oxidized mg protein^{-1}min^{-1}.

Estimation of protein content
Protein content in each of the extract was estimated following the method of Lowry *et al.* (1951) using BSA as standard curve.

Extraction and estimation of non-enzymatic antioxidants

Ascorbate
Ascorbate was extracted and estimated by following the method of Mukherjee and Choudhuri (1983). Leaves were homogenized in a cold mortar and pestle on ice using 10 ml of 6% tricholoacetic acid and filtered. To 4 ml of the extract, 2 ml of 2% dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% thiourea (in 70% ethanol) were added. The mixture was kept in boiling water bath for 15 min and cooled at room temperature; 5 ml of 80% (v/v) sulphuric acid was added to the mixture at 0°C. The absorbance at 530 nm was recorded. 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 performed in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480, 663 and 645 nm in a VIS spectrophotometer and the carotenoid content was calculated using standard formula.

Estimation of total antioxidant activity

Mature intermediate leaves were powdered and 1 g of the powder was combined with 4 ml of methanol, centrifuged at 4,000 rpm for 10 min, supernatant was collected and placed in a 60°C water bath for 25 min (Blois 1958). The semi-dried extract was gathered and for estimation, 0.2 g of the extract was dissolved in 10 ml of methanol. Two ml of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) solution with a concentration of 0.025 g of DPPH in 1000 ml of methanol was mixed with 40 µl of extract solution and the initial reading was taken. After 30-min incubation at room temperature, the reaction solution was examined at 515 nm by using a spectrophotometer (UV-VIS Spectrophotometer, Model 118 Systronics). The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

$$\text{Inhibition } \% = \frac{A\,T_0 - A\,T_{30}}{A\,T_0} \times 100,$$

where A T_0 was the absorbance of DPPH at time zero, and A T_{30} was the absorbance of DPPH after 30 min of incubation. Total antioxidant activity was thus measured as free radical scavenging ability in terms of inhibition of absorbance by DPPH.

Quantification of hydrogen peroxide

Hydrogen peroxide was extracted following the method of Jena and Choudhuri (1981) by homogenizing 50 mg mature intermediate leaf tissue with 3 ml of phosphate buffer (50 mM, pH 6.5). The

homogenate was then centrifuged at 6,000 *g* for 25 min. To determine H_2O_2 level, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium sulphate in 20% H_2SO_4 (w/v), and the mixture was then centrifuged at 6,000 *g* for 15 min. The intensity of the yellow color of the supernatant was measured at 410 nm. Concentration of H_2O_2 was calculated using the extinction coefficient (0.28 $\mu mol^{-1}cm^{-1}$)

Statistics

Standard error of means, ANOVA and *t*-test of significance were determined wherever appropriate.

Results

Tolerance and membrane stability

The application of the elevated temperature stress resulted in near wilting of the seedlings. Tolerance index was determined for the six varieties at 50°C and on the basis of lowest values (−21.48 and 25.38, respectively) Sehore and Lv were found to be least tolerant. All the other four varieties (IPL 406, IPL 81, Asha and Subrata) exhibited values ranging from −10 to −15. Cell membrane stability of all the varieties was determined at the temperature range of 20°C (control) −50°C. Membrane stability was expressed as% relative injury and results revealed that% RI increased with increasing temperatures in all the varieties, with maximum being at 50°C. Both Sehore and Lv had% RI in the range 85–86%, while the others it ranged from 58–68% (Figure 1).

Lipid peroxidation

Lipid peroxidation, irrespective of varieties, increased significantly following exposure to high temperatures (Figure 2). However, at higher temperatures, MDA accumulation was about 50% greater in Sehore and Lv in comparison to the other four varieties (IPL 406, IPL 81, Asha and Subrata).

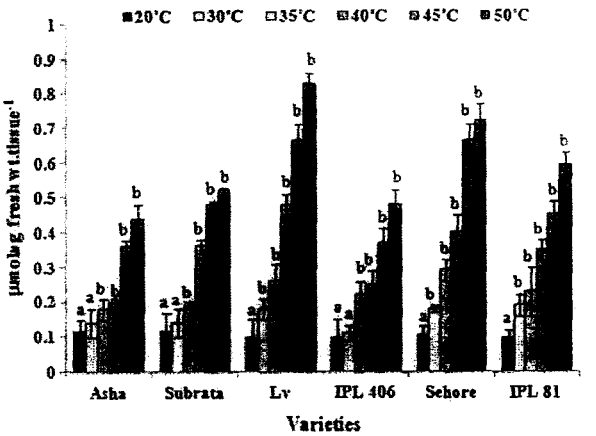

Figure 2. Effect of elevated temperatures on lipid peroxidation (expressed as MDA content) in six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

Antioxidative enzymes

Activities of all five tested antioxidative enzymes – POX, APOX, CAT, SOD and GR –registered varying responses. CAT, SOD and APOX showed an initial increase until 35, 40 and 45°C, respectively, but declined at 50°C in four varieties IPL 406, IPL 81, Asha and Subrata, while in Sehore and Lv it declined after 30°C or 35°C. Maximum activity of CAT was observed at 35°C in all four varieties IPL 406, IPL 81, Asha and Subrata, followed by a significant decline at 45°C and 50°C (Figure 3). In the case of SOD, maximum activity was observed at 40°C in the four varieties IPL 406, IPL 81, Asha and Subrata, which were significantly higher than that of control.

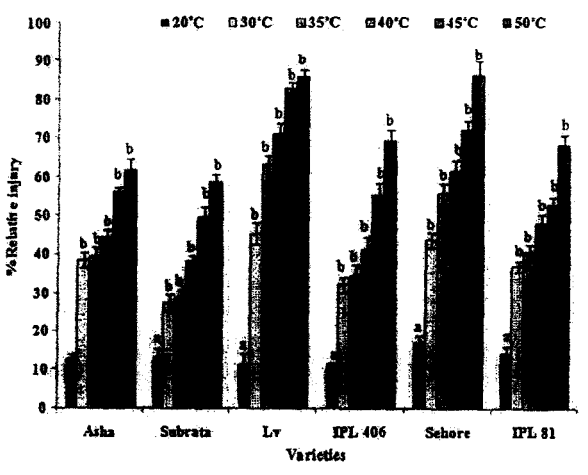

Figure 1. Effect of elevated temperatures on cell membrane stability of six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

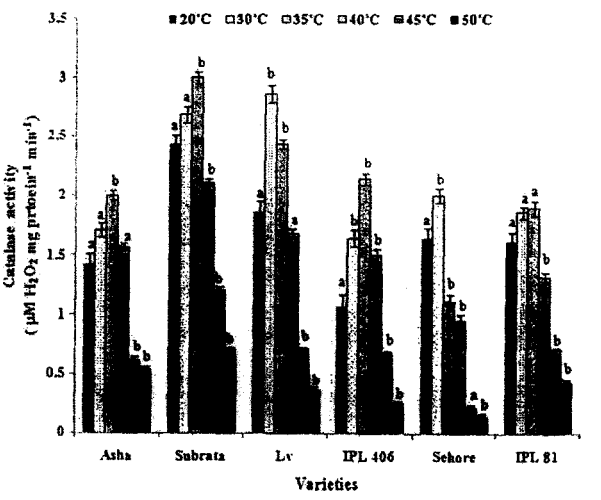

Figure 3. Catalase activities in six varieties of lentil subjected to high temperature treatments. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control (20°C) ($p < 0.01$).

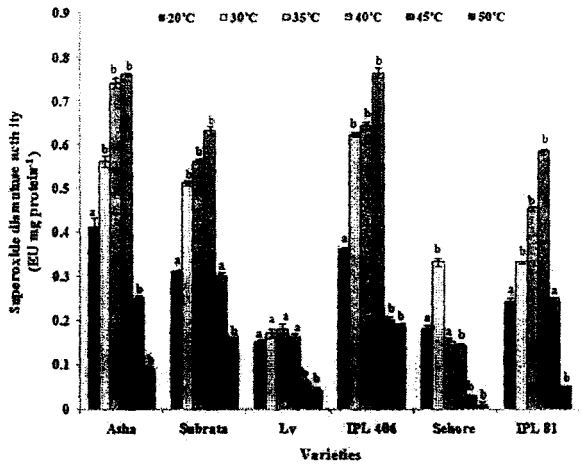

Figure 4. Superoxide dismutase activities in six varieties of *Lens culinaris* subjected to high temperature treatments. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

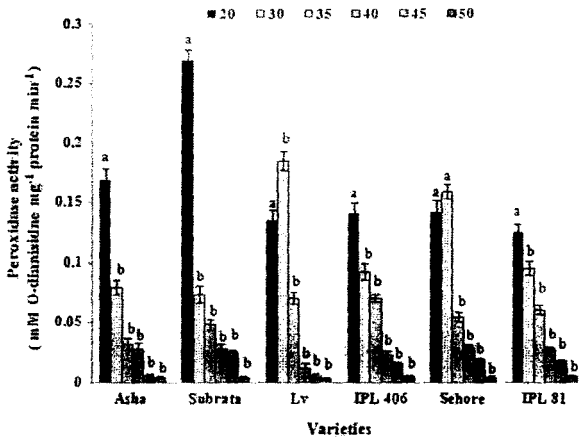

Figure 6. Peroxidase activities in different varieties of *Lens culinaris* subjected to high temperature treatments. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

However, in Sehore and Lv an initial increase at 30°C was followed by a continuous decline until 50°C (Figure 4). APOX activities increased significantly in all six varieties initially. In Sehore, it declined after 30°C, while in Lv it declined after 35°C. In the other four varieties – IPL 406, IPL 81, Asha and Subrata – APOX activity continued to increase until 45°C after which a steep decline occurred at 50°C. At 50°C again, activities in all six varieties activities had fallen to a very low level (Figure 5). Activities of POX showed a significant decline at all elevated temperatures in IPL 406, IPL 81, Asha and Subrata, while in the other two varieties (Sehore and Lv) there was an initial increase at 30°C before declining (Figure 6). GR, on the other hand, declined in all six varieties at all elevated temperatures (Figure 7). ANOVA also revealed that treatment effects on different enzymes varied. In the case of CAT, activities from 35°C onwards were significantly different from those at 20°C, 30°C and 35°C, while activities at 45°C and 50°C were not significantly different. POX activities at 20°C and 30°C were significantly different from those at higher temperatures while from 35°C onwards they were not significantly different among themselves. Activities of SOD and GR differed significantly between higher temperatures from 40°C onwards and those at temperatures up to 35°C.

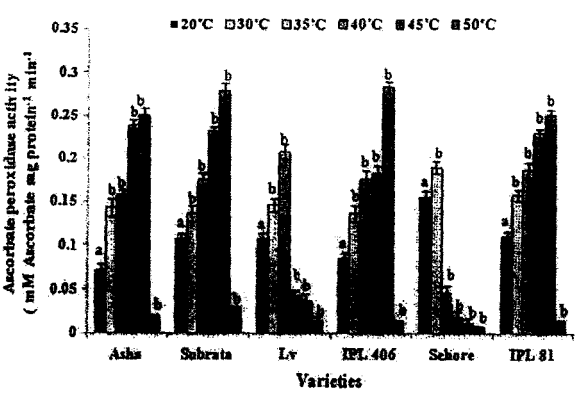

Figure 5. Ascorbate peroxidase activities in six varieties of *Lens culinaris* subjected to high temperature treatments. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

Antioxidants

Accumulation of ascorbate and carotenoids also showed similar trend to that of the enzymes. An initial increase in the four varieties IPL 406, IPL 81, Asha and Subrata, at 35–40°C was followed by a decline, while in the two varieties Lv and Sehore, there was a decline at all elevated temperatures (Figures 8 and 9).

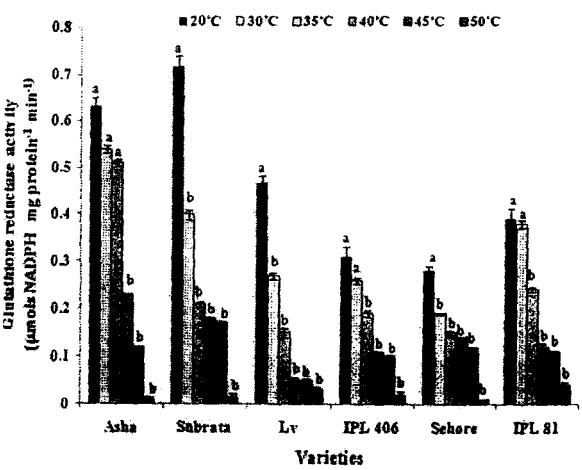

Figure 7. Glutathione reductase activities in different varieties of *Lens culinaris* subjected to high temperature treatments. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

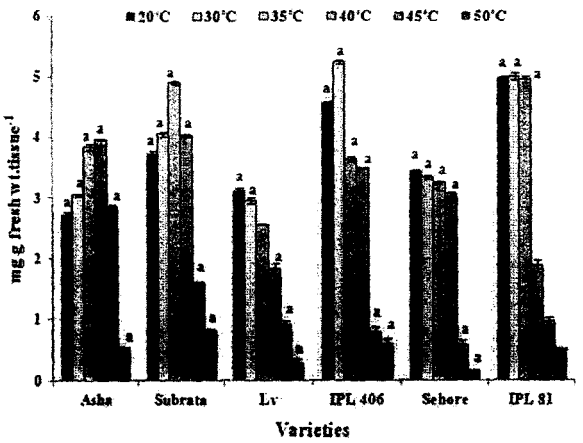

Figure 8. Effect of elevated temperatures on ascorbate contents in six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control (20°C) ($p < 0.01$).

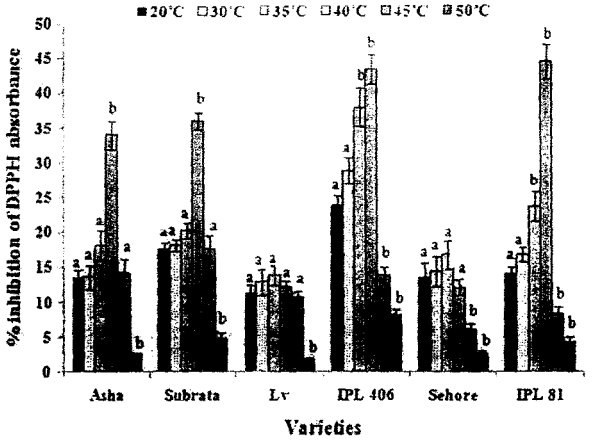

Figure 10. Effect of elevated temperatures on free radical scavenging activity in six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

Total antioxidant activity

Measurement of total antioxidant activity at elevated temperatures in all six varieties revealed that in the four varieties, IPL 406, IPL 81, Asha and Subrata, the highest antioxidant activity was obtained at a temperature of 40°C, which was significantly higher than that of control, while in Sehore and Lv, a non-significant increase in antioxidant activity was obtained at 30°C and 35°C (Figure 10). In the four tolerant varieties IPL 406, IPL 81, Asha and Subrata, exposure to 40°C resulted in an almost doubling of free radical scavenging activity.

Effect of elevated temperatures on H_2O_2 accumulation

Accumulation of H_2O_2 showed an interesting trend. In the four varieties IPL 406, IPL 81, Asha and Subrata, there was an initial decrease in accumulation until 40°C, after which accumulation started increasing. In the two varieties of Sehore and Lv however, an increased accumulation was obtained at all high temperatures (Figure 11).

Effect of pre-treatment with chemicals

Pre-treatment of seedlings with solutions of SA, ABA and $CaCl_2$ followed by exposure to 50°C revealed that all three chemicals could provide protection against oxidative stress. Activities of CAT, POX, APOX, SOD and GR, which had decreased to very low levels at 50°C, were enhanced after pre-treatments to levels near normal. Among the three chemicals, SA was most effective in the case of CAT, SOD and APOX, while $CaCl_2$ was most effective in the enhancement of POX and GR (Table 1). Lipid peroxidation was decreased by the pre-treatments. Accumulation of carotenoids and ascorbate showed significant enhancement in the four tolerant varieties when exposed to 50°C after the chemical pre-treatments (Table 2).

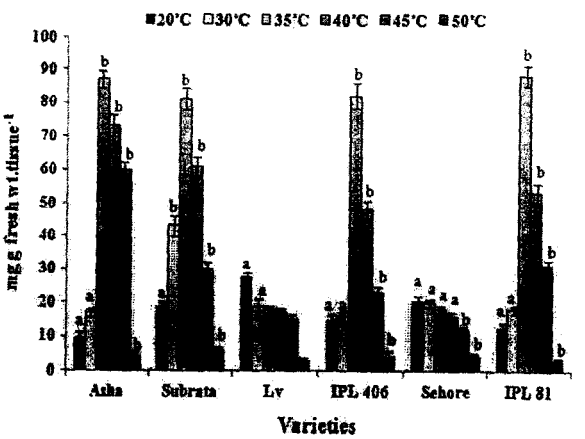

Figure 9. Effect of elevated temperatures on carotenoid contents in six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences in respect to control (20°C) ($p < 0.01$).

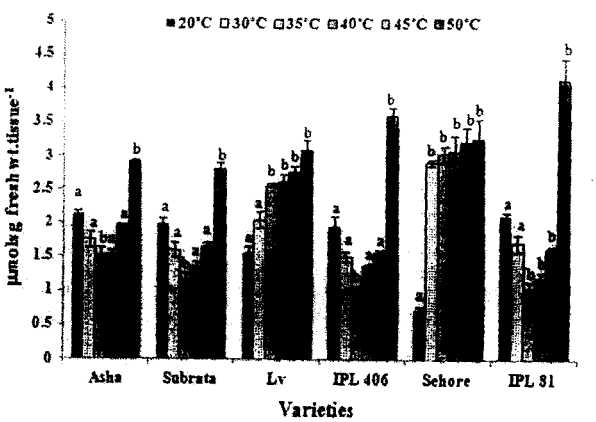

Figure 11. Effect of elevated temperatures on accumulation of H_2O_2 in six varieties of *Lens culinaris*. Results are expressed as mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control (20°C) ($p < 0.01$).

Table 1. Effect of pre-treatments with chemicals on activities of antioxidative enzymes in six varieties of *Lens culinaris*.

Varieties	Treatments*	CAT	SOD	APOX	POX	GR
Asha	Control	0.143±0.031	0.41±0.021	0.071±0.008	0.168±0.067	0.63±0.021
	50	0.054±0.011	0.09±0.005	0.018±0.007	0.001±0.003	0.01±0.011
	SA	0.157±0.032	0.41±0.008	0.107±0.002	0.134±0.006	0.54±0.022
	ABA	0.057±0.010	0.23±0.006	0.057±0.001	0.122±0.007	0.72±0.032
	$CaCl_2$	0.071±0.009	0.31±0.003	0.036±0.001	0.154±0.005	0.73±0.014
Subrata	Control	0.243±0.011	0.31±0.001	0.107±0.002	0.92±0.050	0.72±0.022
	50	0.071±0.009	0.16±0.001	0.029±0.001	0.001±0.008	0.02±0.009
	SA	0.225±0.006	0.47±0.004	0.179±0.010	0.096±0.113	0.47±0.008
	ABA	0.071±0.010	0.20±0.003	0.05±0.009	0.147±0.032	0.31±0.007
	$CaCl_2$	0.107±0.021	0.14±0.002	0.125±0.008	0.128±0.168	0.42±0.017
IPL 406	Control	0.107±0.019	0.36±0.003	0.086±0.005	0.141±0.095	0.31±0.021
	50	0.025±0.007	0.06±0.001	0.011±0.006	0.002±0.003	0.02±0.008
	SA	0.1±0.006	0.33±0.008	0.15±0.010	0.131±0.015	0.30±0.011
	ABA	0.046±0.005	0.15±0.004	0.079±0.003	0.138±0.016	0.36±0.013
	$CaCl_2$	0.075±0.004	0.19±0.001	0.118±0.006	0.140±0.018	0.31±0.022
IPL 81	Control	0.161±0.005	0.24±0.007	0.107±0.002	0.125±0.021	0.39±0.022
	50	0.043±0.003	0.05±0.001	0.014±0.001	0.002±0.009	0.04±0.008
	SA	0.154±0.003	0.25±0.020	0.164±0.012	0.137±0.008	0.31±0.005
	ABA	0.079±0.006	0.11±0.009	0.1±0.013	0.106±0.003	0.22±0.011
	$CaCl_2$	0.068±0.004	0.11±0.010	0.089±0.009	0.144±0.006	0.45±0.014
Sehore	Control	0.164±0.013	0.18±0.007	0.154±0.011	0.142±0.016	0.28±0.013
	50	0.014±0.004	0.01±0.006	0.007±0.001	0.011±0.008	0.01±0.003
	SA	0.157±0.009	0.22±0.008	0.182±0.009	0.140±0.011	0.39±0.009
	ABA	0.118±0.002	0.15±0.003	0.093±0.005	0.142±0.021	0.15±0.012
	$CaCl_2$	0.086±0.004	0.16±0.002	0.107±0.004	0.149±0.015	0.39±0.015
Lv	Control	0.186±0.020	0.15±0.014	0.039±0.014	0.153±0.011	0.47±0.015
	50	0.036±0.007	0.04±0.008	0.018±0.003	0.004±0.009	0.03±0.009
	SA	0.171±0.003	0.34±0.006	0.135±0.005	0.127±0.214	0.42±0.016
	ABA	0.1±0.001	0.13±0.009	0.05±0.001	0.125±0.312	0.47±0.018
	$CaCl_2$	0.082±0.002	0.16±0.007	0.061±0.009	0.142±0.218	0.46±0.019

Notes: Results are expressed as mean of three replicates (10 plants each). ± =SE. Different letters indicate significant differences with respect to control (20°C) ($p<0.01$). *Treatments were followed by exposure at 50°C. Enzyme activities expressed as: CAT =μmol H_2O_2 mg protein^{-1}min^{-1}; APOX =mmol ascorbate mg protein^{-1}min^{-1}; POX =mmol O-dianisidine mg protein^{-1}min^{-1}; SOD =EU mg protein^{-1}; GR =μmols NADPH oxidized mg protein^{-1}min^{-1}.

Discussion

The responses of the six varieties of lentil, normally a cool season crop, to high temperatures were determined in the study under report. It was observed that exposure to a temperature of 50°C for 4 h led to significant changes in physiological and biochemical processes. The six tested varieties showed varying degrees of tolerance to high temperatures and could be categorized into tolerant and susceptible varieties. Two of the varieties, namely Sehore and Lv, had low tolerance index values at 50°C, while the other four – Asha, Subrata, IPL 406 and IPL 81 – had higher tolerance indices. In a study by Porch (2006) a stress tolerance index and stress susceptibility index were used to evaluate the genotypic performance of 14 genotypes of common bean under variable temperature conditions. In the present study, the results of tolerance index were also confirmed by a membrane stability test. It was observed that relative injury to membranes increased with increasing temperatures in all the varieties; nevertheless, it was significantly higher in two of the varieties which had low tolerance indices. Several previous authors have also confirmed the importance of using thermostability of cell membranes for screening heat-tolerant genotypes (Agarie *et al.* 1995; Talwar *et al.* 2002). Almeselmani *et al.* (2006) also reported that there was a significant increase in the membrane injury index in all genotypes of wheat under high temperatures and late plantings. Besides ion leakage, peroxidation of membrane lipids also significantly contributed to membrane damage. Lipid peroxidation was evident in all varieties at higher temperatures, with maximum at 50°C. It was also observed that two varieties, namely Sehore and Lv, had significantly higher MDA accumulation than the other four. Free radical-induced peroxidation of lipid membranes is a reflection of stress-induced damage at the cellular level and increase in the level of MDA, produced during peroxidation of membrane lipids, is often used as an

Table 2. Effect of pre-treatments with chemicals on accumulation of malondialdehyde, hydrogen peroxide and antioxidants in six varieties of *Lens culinaris*.

Varieties	Treatments*	MDA	H_2O_2	Car	Asc
Asha	Control	0.12±0.08	2.13±0.06	010±1.21	2.73±0.03
	50	0.44±0.06	2.90±0.03	004±1.15	0.53±0.01
	SA	0.10±0.03	1.64±0.04	035±2.13	4.47±0.03
	ABA	0.14±0.02	1.71±0.08	012±1.09	1.28±0.01
	$CaCl_2$	0.15±0.01	1.80±0.07	029±2.08	1.25±0.03
Subrata	Control	0.12±0.05	1.98±0.10	019±1.32	3.74±0.02
	50	0.52±0.01	3.10±0.11	006±1.14	0.82±0.03
	SA	0.11±0.01	1.01±0.09	042±2.18	3.52±0.02
	ABA	0.13±0.01	0.83±0.05	039±2.97	2.35±0.03
	$CaCl_2$	0.15±0.03	0.93±0.04	026±1.95	2.42±0.05
IPL 406	Control	0.10±0.05	1.94±0.15	015±1.44	4.55±0.02
	50	0.48±0.04	3.58±0.12	002±1.77	0.60±0.09
	SA	0.10±0.02	1.03±0.09	020±1.18	3.08±0.06
	ABA	0.11±0.02	1.37±0.09	019±1.21	1.23±0.01
	$CaCl_2$	0.13±0.01	1.16±0.06	019±1.06	0.96±0.05
IPL 81	Control	0.10±0.02	2.08±0.05	013±1.22	4.95±0.02
	50	0.59±0.04	4.10±0.32	003±1.16	0.51±0.02
	SA	0.09±0.03	1.95±0.12	019±1.78	3.07±0.05
	ABA	0.08±0.02	1.58±0.07	017±1.08	0.92±0.08
	$CaCl_2$	0.14±0.01	1.83±0.04	021±1.56	1.04±0.06
Sehore	Control	0.11±0.02	1.73±0.06	021±1.10	3.43±0.04
	50	0.72±0.07	3.25±0.30	009±1.16	0.16±0.01
	SA	0.11±0.01	1.08±0.05	019±1.09	1.15±0.08
	ABA	0.08±0.01	1.16±0.04	018±1.23	1.35±0.04
	$CaCl_2$	0.09±0.01	1.21±0.09	019±1.98	1.80±0.01
Lv	Control	0.11±0.05	1.55±0.09	013±1.01	3.12±0.03
	50	0.83±0.05	3.08±0.08	003±1.08	0.28±0.07
	SA	0.11±0.03	1.82±0.06	023±2.09	1.97±0.06
	ABA	0.10±0.01	2.10±0.21	022±2.18	1.75±0.04
	$CaCl_2$	0.10±0.01	2.00±0.14	024±2.11	1.62±0.03

Notes: Results are expressed as mean of three replicates (10 plants each). ± = SE. Different letters indicate significant differences with respect to control (20°C) ($p<0.01$).*Treatments were followed by exposure at 50°C. MDA = μ mols g fresh wt.tissue^{-1}; H_2O_2 = μ mols g fresh wt.tissue^{-1}; Car = μg g fresh wt.tissue^{-1}; Asc = mg g fresh wt.tissue^{-1}.

indicator of oxidative damage (Jain *et al.* 2001). In a previous study, Babu and Devraj (2008) reported an increase in accumulation of MDA in French bean during temperature stress.

It is well known that diverse environmental stresses differentially affect plant processes that lead to loss of cellular homeostasis accompanied by the formation of reactive oxygen species (ROS) which cause damage to membranes, lipids, proteins and nucleic acids (Srivalli *et al.* 2003). Under normal growing conditions, the oxidative damage to cellular components is balanced by the efficient processing of ROS through a well coordinated and rapidly responsive antioxidant system. Under stress conditions, this balance tilts in favor of production of more ROS leading to damage. A plant's ability to withstand such stresses would depend on its ability to detoxify the ROS by enhanced activities of antioxidative enzymes. The results of the present study revealed that two of the enzymes – APOX and SOD – showed an increase in activities until 40–45°C in four varieties and at 30°C in the other two. The most significant increase was obtained in the case of SOD and APOX. It is clear that an increase in temperature leads to an increased expression of these antioxidative enzymes until a particular temperature after which they decline. The temperature until which increased activities are maintained varies in the tolerant and susceptible varieties. In the tolerant varieties, they could maintain increased activities at higher temperatures in comparison to the susceptible ones. The results of the present study confirm a previous report by Almeselmani *et al.* (2006) who reported a significant increase in the activity of SOD in all tested genotypes of wheat, although it was greater in tolerant genoytpes.

Several previous authors have also reported involvement of SOD in temperature stress tolerance (Upadhyaya *et al.* 1990; Jagtap and Bhargava 1995; Davidson *et al.* 1996). Gupta and Gupta (2005) also reported that SOD activity in two wheat genotypes increased with increase in temperature although the magnitude was comparatively lower in the susceptible

genotype. In the present investigation it was also observed that POX and GR declined at high temperatures in all varieties although in Sehore and Lv there was an initial increase in POX activity before declining. Similar results have also been reported by Jiang and Huang (2001). CAT activities increased to some extent but declined either after 35°C or 30°C. Activities of CAT were correlated to accumulation of H_2O_2. In the case of H_2O_2 accumulation, it was found that in four of the varieties, namely IPL 406, IPL 81, Asha and Subrata, there was an initial decrease in accumulation, which was followed by an increase from 40°C onwards. In the other two varieties – Sehore and Lv – it increased at all higher temperatures. Thus, it is quite clear that in tolerant varieties, initially CAT activities increase along with a concomitant decrease in accumulation of H_2O_2, indicating the initial ability to scavenge H_2O_2. Previous studies have also reported a decrease in CAT activities along with an increase in H_2O_2 (Blokhina *et al.* 2003; Babu and Devraj 2008). Though accumulation of H_2O_2 could be correlated to decreased CAT activity, in the present study increased accumulation of ascorbate was also observed initially, along with an increase in APOX activity. Besides ascorbate, carotenoids also showed increased accumulation in temperature stressed plants until 40–45°C followed by a decline. Total antioxidant activity, measured by free radical scavenging, was increased in temperature-stressed plants, with the maximum being at 40°C in four varieties and at 30–35°C in the other two. In all varieties, the total antioxidant activities declined significantly at 50°C. Kang and Saltveit (2002) reported that heat-shocked rice seedlings had greater DPPH scavenging activity than control. In the present study, it was noted that the DPPH scavenging activities at 40°C were almost double in comparison to control in the four varieties Asha, Subrata, IPL 406 and IPL 81.

Since all tested metabolic activities were significantly lowered in the six varieties following an exposure to 50°C for 4 h, it was decided to determine whether pre-treatments by chemicals could afford protection. While it was observed that the inherent anti-oxidant activities of the cell which were enhanced following temperature stress up to 40–45°C could provide protection to a certain degree, no protection could be accorded at temperatures beyond that even in tolerant varieties. However, treatments with SA, ABA or $CaCl_2$ protected plants against damage at 50°C. This was observed in the case of antioxidative enzymes, accumulation of antioxidants as well as lesser membrane damage. The ability of such chemicals to afford transient protection against temperature stress has been reported previously (Jiang and Huang 2004; Larkindale and Huang 2004; Chakraborty and Tongden 2005).

The results of the present study clearly reveal that of the six tested varieties, four (IPL 406, IPL 81, Asha and Subrata) were fairly tolerant to high temperature stress, while two (Lv and Sehore) were susceptible. This was evident in their tolerance indices, membrane stability tests, membrane damage and accumulation of lesser antioxidants. Among the antioxidative enzymes, SOD and APOX play important roles in tolerance while GR and POX were not involved. Catalase was involved in detoxifying H_2O_2 in the tolerant varieties. Besides antioxidative enzymes, other antioxidants like ascorbate and carotenoids were also involved in tolerance. Protection against high temperature-induced damage was provided by pre-treatment with chemicals.

The results of the present study clearly indicate significant differences in how the varieties respond to oxidative stress and the tolerance mechanisms. The most significant activities related to tolerance could be utilized as biochemical markers. Furthermore, amelioration of high temperature-induced oxidative stress by pre-treatment with chemicals opens up the possibility of improving the ability of plants to grow at higher temperatures.

Acknowledgements

Financial assistance received from University Grants Commission, New Delhi, grant no. 32–364/2006 (SR) for carrying out this work is gratefully acknowledged.

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