Discussion

Plants which are exposed to various environmental stresses throughout the course of their life span have an inbuilt ability to adjust to seasonal and other environmental variables. Temperature stress is one of the most common environmental stress to which plants are exposed and this includes both elevated temperature and chilling stress. Although most of the biochemical factors for stress tolerance acquisition are present in all species, the difference is how fast and how persistent this machinery activated, and how the stress is perceived and how the signals are further transduced into a series of responses(Here *et al.* 1997; Kaur and Gupta 2005).

In nature plants are generally exposed to the combination of stresses and in many cases their metabolic responses are similar under varying stresses. The ability to withstand and to acclimate to super optimal and sub optimal temperatures results from both prevention of heat damage and repair of heat sensitive components (Larkindale *et al.* 2005). The degree of susceptibility of different plant species and of different genotype within the species is often varied. In the present study, the responses of six varieties of lentil crop to high temperatures were determined. Similarly the responses of four varieties of soybean, generally a summer crop, to low temperature stress were also determined. In lentil the six tested varieties showed varied degree of tolerance to high temperatures and could be categorised into tolerant and susceptible varieties. Two of the varieties viz. Sehore and Lv had low tolerance index value at 50°C while the other four varieties – Asha , Subrata, IPL 406 and IPL 81 had higher tolerance indexes. In the study by Porch (2006), a stress tolerance index and stress susceptible index were used to evaluate the genotypic performance of 14 genotypes of common bean under variable temperature conditions. In another study by Terzioglu *et al.* (2006), the thermal tolerance of *Aegilops biuncialis* and *Triticum durum* cultivars were determined by growth experiment and cell viability test. No genotypic difference could be determined in this experiment, though significant genotypic differences were obtained in acclimated seedlings. In the present study, the tolerance was also confirmed by cell membrane thermostability test and it was observed that, though the relative injury increased with increase in temperature in all the varieties, Sehore and Lv had much higher relative injury. Several previous authors have also confirmed the importance of using thermostability test of cell membrane for screening heat tolerant genotypes (Agarie *et al.* 1995; Talwar *et al.* 2002; Wahid and Shabbir 2005) 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 planting. It is quite clear that integrity and function of biological membranes are sensitive to high temperature, as heat stress alters the tertiary and quaternary structure of membrane proteins (Wahid *et al.* 2007). Such alteration enhances the permeability of membrane as evident from increased loss of electrolytes. Electrolyte leakage is influenced by factors such as genotypic difference, plant age etc.

In case of soybean subjected to low temperature stress, results of the present study showed that the tested varieties did not show much differences in terms of morphological symptoms during cold stress. Besides, even though short duration of cold stress did not induce much change, leaf hardening and other morphological symptoms were evident from 8h onwards. There are reports that tropical and sub tropical plants show characteristics of damage symptoms when exposed to chilling temperatures (Raison and Lyons, 1986; Queiroz *et al.* 1998). Various phenotypic symptoms in response to cold stress include poor germination, stunted seedlings, reduced leaves, leaf expansion and wilting. The major negative effect of cold stress is that it induces severe membrane damage (Yadav 2010).

Lipid peroxidation of membranes is another factor which adversely affects membrane properties during temperature stress. Lipid peroxidation is generally determined as accumulation of malondialdehyde (MDA) at the membrane and in the present study it was observed that both high and low temperature stress induced lipid peroxidation in all varieties. The degree of lipid peroxidation was dependent on the temperature as well as the variety. Present results are in conformity with previous results where lipid peroxidation has been reported to be related to temperature stress. Free radical induced peroxidation of lipid in membranes is a reflection of stress induced damage at the cellular level and increase in the level of MDA during peroxidation of membrane lipids is often used as an indication of oxidative damage (Elstner and Oswald 1994; Zhang *et al.* 2010). Babu and Devaraj (2008) reported an increase in accumulation of MDA in fresh bean during temperature stress. Queiroz *et al* (1998) suggested that the decrease in membrane fluidity in coffee seedling detected at 10 °C is probably due to increased levels of lipid peroxidation.

It is well known that diverse environmental stresses differentially affect plant processes that lead to loss of cellular homeostasis accompanied by the formation reactive oxygen species (ROS) which cause damage to membrane lipids, proteins and

nucleic acids (Srivalli *et al* 2003). Under normal growing conditions the oxidative damage to the cellular component is balanced by the efficient processing of ROS through a well co-ordinated and rapidly responsive antioxidant system. Under stress condition this balance tilts in favour of production of more ROS leading to damage. The plant's ability to withstand such stress depends on detoxification of the ROS by enhanced activity of antioxidative enzymes. Thus the cellular damage caused by super oxide and lipid peroxidation might be reduced or prevented by protective mechanisms including free radical processing by enzymes such as superoxide dismutase, catalase, peroxidase and ascorbate peroxidase. Analysis of five antioxidant enzymes in the four varieties of *Glycine max* exposed to varying cold temperatures revealed that activities of four enzymes superoxide dismutase, glutathione reductase, peroxidase and ascorbate peroxidase were initially enhanced with decrease in temperature. But after a threshold temperature activities decline. Of the four varieties, three showed similar trend where activity showed decline after 15°C whereas in Rossio increase in activity continued till 10 °C. It is quite clear that in all the varieties increase in cold induced stress led to an initial response where the antioxidative activity was enhanced to withstand the stress. Catalase activities in all varieties decreased with the decrease in temperature. Previous reports have also confirmed that certain stress lead to a decrease in catalase activities (Fadzillah *et al* 1996 ; Chakraborty and Tongden 2005).

There are several earlier reports on the role of antioxidative enzymes during chilling stress. Ascorbate peroxidase gene expression and activity has been reported to be rapidly induced by various stress conditions including chilling (Prasad, *et al.*, 1994; Keshavkant and Naithani, 2001). Lukatkin (2002) compared SOD activity in various plant species differing in their cold – resistance during chilling. According to him, in resistant varieties, 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 15%. In the present study also, SOD activities increased most significantly during low

temperature. However, this was more or less similar in the different varieties. 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 varieties, Rossio, whereas in other three varieties the increase was about 2-fold. These varieties could also maintain higher levels of antioxidative enzymes till 10°C, and the decline was evident only after this temperature. This variety, more commonly cultivated in higher altitudes, was more tolerant to lower temperatures than the other varieties, 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 24h at 5 °C was also monitored every two hours. Results revealed that both catalase and ascorbate peroxidase showed an initial decline in activity for 4-6h 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. Zhang *et al.* (2010) also reported that antioxidative enzymes were induced in rice during chilling stress and they found that the activities of superoxide dismutase , ascorbate peroxidase and glutathione reductase were higher in the super-hybrid *Liangyoupeijjiu* (LYPJ). They concluded that tolerance to chilling stress in LYPJ might be adopted from maternal cultivar.

In case of elevated temperature stress in lentil, results of the present study revealed that two out of the five enzymes tested- ascorbate peroxidase and superoxide dismutase showed an increase in activities until 40-45 °C in four varieties and at 30°C in the other two. Most significant increase was obtained in case of SOD and APOX. It is clear that increase in temperature led to an increased expression of these antioxidative enzymes till a particular temperature after which they decline. The temperature till 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 significant increase in activity of SOD in all tested genotypes of wheat, though it was greater in tolerant genotypes. 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 though 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 though in Sehore and Lv there was an initial increase in POX activity before declining. Similar results have also been reported by previous workers (Jiang and Huang, 2001). CAT activities increased to some extent but declined either after 35°C or 30°C.

There are also certain contrasting results from previous workers. Gulen and Eris (2004) reported that peroxidation activity increased in strawberry plants subjected to high temperature. Gur *et al.* (2010) also reported that there was a decline in the activity of superoxide dismutase in cotton plant exposed to high temperature. In the contrary catalase activity increased at 45°C, peroxidase increased at 38°C and ascorbate peroxidase activity increased at 38°C and 45°C. The author also reported increased accumulation of hydrogen peroxide with increase in temperature. In case of H_2O_2 accumulation in present study it was found that in four of the varieties IPL 81, IPL 406, Asha and Subrata there was an initial decrease in accumulation which was followed by an increase at 40°C onwards; however, in the other two varieties Sehore and Lv increase accumulation of H_2O_2 was obtained at all higher temperatures .Thus it is quite clear that in tolerant varieties the initial increase of catalase activities indicate the initial ability to scavenge H_2O_2 which lead to decrease in accumulation of H_2O_2 and its detoxification. Previous studies have also reported a decrease in catalase activity along with an increase of H_2O_2 (Blokhina *et al.* 2003, Babu and Devaraj 2008).

Decline in activity of catalase and ascorbate peroxidase in cold stressed soybean seedlings could be correlated in the present study with an increase in accumulation of H_2O_2 detected during the early period of oxidative stress. H_2O_2 besides being ROS is also involved in signalling (Chakraborty 2005). It is now clear that protein and DNA are also involved in signalling, for guard cell functioning, photo protection, pathogenesis and development. With increase in duration of cold stress, it is observed that catalase and ascorbate peroxidase increase resulting in breakdown of H_2O_2; prolonged period of stress or increasing the stress intensity however led to decline

activity indicating that the plant succumbs to oxidative stress after initial resistance. Analysis of isozymes of different enzymes also revealed that temperature stress could alter the expression of isozymes of peroxidase, catalase and superoxide dismutase. Anderson *et al* (1995) reported changes in isozymes profile of catalase, peroxidase and glutathione reductase during acclimatising of chilling in monocotyledons of maize seedlings.

It is clear that the activity of antioxidative enzymes changed following both elevated and low temperature stress. Other than antioxidative enzymes, accumulation of small antioxidant molecules such as ascorbate and carotenoid were also involved in the plants response to temperature stress. Both ascorbate and carotenoid showed increase accumulation initially during stress periods followed by a decline in tolerant varieties. However in the susceptible variety accumulation of both ascorbate and carotenoid showed a decline at al high temperatures. This is in the conformity with the results of previous works (Mahan and Mauget, 2005). Similar results were also observed in case of low temperature stress where initially there was an increased in accumulation followed by a decline. Radyuk *et al.* (2009) reported that at temperature close to zero (2°C) the contents of both total and reduced ascorbate in the leaves of barley increased, but during the post stress period the ascorbate content declined. They suggested that total ascorbate accumulation under stress conditions indicates that the increment in reduced ascorbate could occur not only at expense of reduction of fixed oxidised form but also due to *de novo* molecule synthesis. In their study Radyuk *et al.*, (2009) also reported that the amount of carotenoid increased by 20% during the first 8h of stress factor which however reduced after 8h to a level lower than that of control. In the present study also it was observed that carotenoid accumulation in all varieties increased initially (until 8h for susceptible varieties and 16-20h for Rossio and NRC 37) after which there was a decline. Total antioxidant activity was also increased initially before declining. In case of high temperature stress it was observed that the DPPH scavenging activities at 40°C were also double in comparison to control in the four tolerant varieties of lentil. Significant correlation of total antioxidant activity was obtained with accumulation of antioxidants and activity of certain antioxidative enzymes. Antioxidant activities were more pronounced in elevated temperature stress than in chilling stresses. Kang and Saltveit (2002) reported that heat shocked rice seedlings had greater DPPH scavenging activity than control.

Proteins are one of the most important metabolites in plants and would thus be involved during a plant's response to stress. In the present study it was observed that while elevated temperature stress led to an initial increase upto 35°C or 40°C followed by a decline, low temperature stress resulted in a decline in protein contents. Analysis of protein profile by SDS-also revealed that certain protein was inhibited by the different whereas other protein showed increased expression.

Phenols are also important in plants as they act as natural antioxidants which help to neutralize free radicals. Similar to other metabolites accumulations of phenols were also found to increase at the early periods of stress followed by decline this was evident in both high and low temperature stresses. Chakraborty *et al.* (2001) obtained increased levels of phenols in tea leaves subjected to temperature stress up to 45°C, and a decline thereafter. Analysis of phenols by HPLC in elevated temperature stress revealed that salicylic acid seems to be an important component which was elevated during high temperature stress.

One of the most common stress responses in plants is over production of different types of compatible organic solutes such as proline and betaine (Serraj and Sinclair, 2002) which are low molecular weight highly soluble compounds that are usually non toxic at high cellular concentration. Generally they protect plants from stress through various mechanisms including contribution to cellular osmotic adjustment, detoxification of ROS, protection of membrane integrity and stabilization enzymes or protein (Ashraf and Foolad, 2007). However in many crop plants a natural accumulation of such osmolites is lower than sufficient to ameliorate the adverse affect of various environment stress (Subbarao *et al.* 2001). Proline being a stress metabolite is known to increase under various stress (Kramarova *et al.*1999; Chakraborty *et al.* 2002; Agarwal and Pandey, 2003).Result of present study also showed that proline accumulation increased initially as a response to both high and low temperature stresses but declined later. Ability to maintain high levels of proline was also dependent on the varieties, with tolerant varieties having the ability to accumulate increased amount during prolonged periods of stress. In a study by Kumar and Yadav (2009), they reported that proline and betaine provide protection to cold stress in tea by regulating methylglyoxal and lipid peroxidation formation as well as activity of some of antioxidants and glyoxalase enzyme pathways.

Temperature stress is also known to have significant effects on photosynthetic apparatus which may be due to their effect on membranes or the direct effect of stress on chlorophylls. Keeping this in mind, in the present study besides the effect of temperature stress on membranes, their influence on chlorophyll contents was also determined. It was observed that in soybean subjected to low temperature stress chlorophyll content directly declined whereas in lentil an initial increase in accumulation was observed till 40°C after which there was a significant decline in five varieties . However in Sehore which was a susceptible variety chlorophyll contents recorded continuous decline. Higher thermotolerance of cell membranes and photosynthesis has been reported to contribute to adaptation at high temperature in several crops (Shannan *et al.*, 1990; Ibrahim and Quick, 2001; Talwar *et al.* 2002). Photosynthesis is one of the physiological processes that is most sensitive to high temperature stress (Yang *et al.*2005). Inhibition of photosynthesis by high temperature stress is of common occurrence for plants in tropical and sub-tropical regions and the temperate zones where the plants are exposed periodically to high temperatures (Larcher, 1995). However, Aarti *et al.* (2006) reported that oxidative stress showed greater impact on chlorophyll biosynthesis than on photosystem II in *Cucumis sativus* (cucumber). The authors also suggested that oxidative stress impedes key steps in chlorophyll biosynthesis by either directly or indirectly inhibiting the activity of Mg-chelatase, Fe-chelatase and protoporphyrinogen IX oxidase. Haldimann (1997) reported that during chilling stress there was a decrease in chlorophyll-a content which was more significant in the chilling sensitive genotype of maize. Similarly, Bertamine *et al.* (2007) also reported that the contents photosynthetic pigments decreased significantly in one of the genotypes during low temperature stress but remain unchanged in another genotype.

Zhang *et al.* (2010) however reported that chlorophyll content of all genotypes of rice gradually declined with chilling stress. Decrease in chlorophyll content due to high temperature has also been reported earlier by earlier workers (Bhullar and Jenner 1985). Significant reduction in chlorophyll content under late and very late planting was observed in all genotypes and at all stages of plant growth in wheat by Almeselmani *et al.* (2006). However, tolerant genotype HDR 77 and HD 2815 maintained comparatively higher chlorophyll content and showed less reduction compared to other genotypes under increasing temperature of late and very late

planting. Premature loss of chlorophyll due to heat sensitivity in wheat crop has been reported earlier (Reynolds *et al.* 1994).

Results of the present study indicated that at a temperature of 50°C all metabolic activities had been reduced to very low levels. Since there are reports that effects of temperature can be ameliorated to certain extent by pre treatment with chemicals, a similar approach was adopted in the present study. Three chemicals known to have a protective effect against high temperature stress were selected i.e., salicylic acid, abscisic acid and calcium chloride. Plants were initially pre-treated with the chemicals and then subjected to high temperatures of 50 °C. Results revealed that the activities of an antioxidant enzymes catalase, peroxidase, ascorbate peroxidase, superoxide dismutase and glutathione reductase which had decreased to very low level at 50°C were enhanced in those cases were seedlings were subjected to pre treatments with chemical. Jiang and Zhang (2002) also obtained increased activities of APOX in leaves of maize seedlings following ABA treatment. Larkindale and Huang (2004) reported that SA and ABA pre-treatments for thermotolerance induction had no effects on SOD activity under heat stress while pre-treatment with ACC, $CaCl_2$ or heat acclimation (HA) increased activity to some extent. It has a higher affinity to H_2O_2 than CAT and POX and it may have a more crucial role in the management of reactive oxygen species (ROS) during stress or it may be responsible for fine modulation of ROS for signalling (Srivalli *et al.*, 2003). Dat *et al.* (1998) working with induction of SA or heat acclimation (HA) in mustard seedlings reported a parallel decrease of both H_2O_2 and CAT during the initial period of thermoprotection. They suggested that the metabolic and molecular mechanisms associated with the observed decline in H_2O_2 content and CAT activity during this period may be relevant to thermotolerance. The decline in H_2O_2 content may be indicative of the enhanced antioxidant potential in the tissue which could contribute to enhanced thermotolerance. Similar result was observed in the present study. The accumulation of H_2O_2 increased to very high level at 50°C was decreased in those cases where seedlings were subjected to pre treatments with chemical. Besides antioxidative enzymes other high temperature related inhibition of normal metabolic processes was also protected by these three treatments Chakraborty and Tongden, (2005) reported that SA could provide theromotolerance to *Cicer arietinum* seedlings. In the present study the increased effect of lethal temperature was partially overcome by the pre-treatment where lipid peroxidation though higher than the untreated controls were

lesser than the lethal temperature treatment. Similar results were also obtained by Larkindale and Huang (2004) in creeping bent grass where lipid peroxidation increased during heat stress but was lowered by pre-treatment with chemicals and sub-lethal temperature. Saleh *et al.*(2007), also reported that high temperature induced lipid peroxidation in mungbean could be reduced by SA treatments. He *et al.*(2005) suggested that SA induced heat tolerance could be related to higher O^{-2} and H_2O_2 scavenging potential due to higher catalase activities and heat stress. Further, results obtained in Kentucky blue grass agree with the reult in Arabidopsis and creeping bent grass that SA is involved in protection against heat stress induced oxidative damage (Larkindale and Knight , 2002 , Larkindale and Huang , 2004).

All the results of the present study taken together indicated that plants respond to both high and low temperature more or less similar fashion. Initial imposition of stress results in the acclimation of antioxidation and other defense mechanisms which however are maintained up to a certain level after which these decline when plants succumb to the stress. Tolerant and susceptibility are related to a great degree to the ability of the particular variety to maintain antioxidant responses for greater period of time whereas in susceptible varieties antioxidant decreased faster. Results also indicate that it is possible to ameliorate temperature stress induced disorders by protective mechanisms like pre treatment with chemicals such as SA, ABA, $CaCl_2$.

In conclusion, it can be stated that loss of cell membrane stability, peroxidation of lipid membranes could be considered as markers of susceptibility, while maintenance of high total antioxidative activity at higher temperatures was correlated to tolerance.