Biochemical characterization of temperature stress response of *Cicer arietinum* L. and induction of thermotolerance

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TO WHOM IT MAY CONCERN

This is to certify that Ms. Cyaria Tongden has carried out her research work under my supervision. Her thesis entitled '**Biochemical characterization of temperature stress response of *Cicer arietinum* L. and induction of thermotolerance**', is based on her original work and is being submitted to the University of North Bengal for the award of Doctor of Philosophy (Science) degree in Botany, in accordance with the rules and regulations of the University of North Bengal.

Usha Chakraborty

(Usha Chakraborty)

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INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a major pulse crop of India, Middle-East and North-Africa and is an important source of dietary protein in the Third World countries. Globally, chickpea is cultivated on about 10.4 million ha area adding 8.57 million tonnes of grains to the global food basket, with an average productivity of 826 kg/ha. India grows chickpea on about 7.29 million ha, producing 5.77 million tonnes seed, which represents 30% and 38% of the national pulse acreage and production respectively (Ali and Kumar, 2005). It is an important self-pollinated pulse crop in the Indian subcontinent, and ranks third in production among pulses in the world (Plate I). It provides high quality protein, particularly for the vegetarian population and also plays a significant role in farming system as a substitute for fallow in cereal crop rotations, where it contributes towards the soil nitrogen thereby reducing the need for nitrogenous fertilizers through symbiotic fixation of atmospheric nitrogen (Singh *et al.*, 2002). However, the chickpea production has more or less stagnated for the past two decades. One major limiting factor has been the susceptibility of cultivars to various biotic and abiotic stresses that adversely affect the yield.

Stress is an integral component of the forces that drive the course of evolution. Changes in the physical environment generate stress, which in turn affects homeostasis. It is usually defined as an external factor that exerts a disadvantageous influence on plants. Abiotic stresses such as drought, high temperature, salinity etc are location specific, exhibiting internal variations in occurrence, intensity and duration and generally cause reduced crop productivity. There is a serious concern for food security in developing countries like India where the population is increasing exponentially with each passing day. Therefore, there is an urgent need to increase agricultural productivity and to expand productive areas of the world. This can be achieved only by making a conscious effort to improve the production by extending the cultivation of stress tolerant crops to areas commonly exposed to abiotic stresses such as high temperature, salinity, drought, chilling stress etc.

Plants are exposed to various environmental stresses both during the changes in season and more rapidly over the course of the individual days. The most typical kind of stress plants experience from the surrounding is the "temperature stress" and

Plate I: Cultivation of chickpea (*Cicer arietinum* L.) varieties in the field

the severity of this situation is increasing due to global warming. The temperature of an individual plant cell can change much more rapidly than other factors that cause stresses. Temperature stress exists in many forms both within and between the different climates of the world. In particular, plants that exist in regions of higher latitudes must endure dramatic seasonal temperature change, which can span from -40°C to + 40°C. At one extreme, the effects of freezing can induce dehydration, ice nucleation, protein inactivation and in extreme cases, cell death. Alternately, plants exposed to excessive heat can also succumb to dehydration, protein degradation and cell death. High temperature stress is detrimental to plant growth. It is also known to severely reduce seed germination, pollen viability and pollen germination in several crop species.

Levitt (1980) and Ho (1987) have suggested that there are three direct effect of heat stress on cells which could be the site(s) of perception of temperature: cell membrane, metabolic reaction and protein structure. Elevated temperatures will immediately alter the membrane fluidity and affect iron transport in the cell, at the same time, enzyme reaction rates will increase as a function of temperature and alter the concentration of metabolites in the cell, concomitantly, thermo-sensitive proteins will assume non-native configurations, again as a consequence of the heat stress. Adverse effects of high temperature stress leads to various metabolic changes deleterious to plant health and often leads to oxidative stress due to accumulation of toxic oxygen species (Inze and Van Montagu,1995; Dat *et al.*,1998a,1998b and Jiang and Huang, 2001) These toxic radicals can be removed by the scavenging enzyme systems comprising of peroxidase, ascorbate peroxidase, catalase, superoxide dismutase, glutathione reductase etc. However, the function of these antioxidative enzyme systems can be interrupted by heat stress, which results in increase in lipid peroxidation and consequent membrane damage (Jagtap and Bhargava,1995; Dat *et al.*,1998a,b and Jiang and Huang, 2001).

Plants have evolved various mechanisms to cope with stresses in a particular niche. Plants can resist high temperature stress either by avoidance or by tolerance mechanisms. Examples of heat avoidance mechanisms are insulation, decreased respiration, decreased absorption of radiant energy through reflectance or decreased chromophore content or transpirational cooling. Obviously, expression of some of

these types of avoidance mechanisms require the coordination of many cell and tissue types. Potential mechanisms of heat tolerance are the synthesis of protectants, increased thermostability of enzymes and increased saturation of fatty acids.

Ability of organisms to acquire thermotolerance to normally lethal temperatures is an ancient and conserved adaptive response (Hong *et al*, 2003). Thermotolerance represents a property of all living cells and refers to the capacity of the cells to survive or recover from normally lethal exposures to abrupt, severe heat shock if, before the lethal stress, the cells are exposed to milder or short period of heat stress condition. The various chemical pre-treatments and heat- acclimation treatments that induce the accumulation of heat shock proteins (HSPs) often result in thermotolerance of plants like *Arabidopsis*, Kentucky Bluegrass, *Zea maize* etc. The degree of thermotolerance of different plant species have been found to be directly related with the over and under expression of HSPs. Thermotolerance is characterized by halted plant growth in high temperature conditions. It is suspected that insensitivity to hormones such as auxin and cytokinin is responsible for the onset of thermotolerance, as these hormones normally induce growth responses (Michels, 2004).

One of the promising areas in increasing thermal stress resistance is the induction of thermotolerance by exposure to sub-lethal temperatures prior to sowing in high temperature soils. A preliminary treatment with a moderately elevated, non-lethal temperature can temporarily render plants more resistant to a subsequent potentially lethal heat shock- this phenomenon is known as heat-acclimation. Besides, in several studies, certain chemical treatments including salicylic acid (SA), calcium chloride ($CaCl_2$) and abscisic acid (ABA) have also been shown to induce thermotolerance to a certain degree (Jiang and Huang, 2001; Jiang and Zhang, 2001; Burke, 2001 Larkindale and Knight, 2002; and 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.

In an interesting study, Kloepper *et al.* (2004) have reported the role of Plant Growth Promoting Rhizobacteria (PGPR) in heat tolerance. PGPRs are the bacteria

that inhabit the rhizosphere-the soil immediately surrounding the roots and exert beneficial effects on plants. Rhizospheric microbe-plant interactions have a great influence on plant health and soil quality since these root assisted microorganisms are able to help the host plant to deal with drought, nutritional and soil-borne pathogen stress conditions. PGPRs are widely studied for their growth promoting nature and also for their role as resistance inducers in many crop species (Burdman *et al.*, 2000; Ramamoorthy *et al.*, 2001; Kloepper *et al.*, 2004).Though the research on PGPR- mediated resistance originated several decades ago, the putative mechanisms underlying such resistance mechanisms especially with regard to thermoprotection still remains to be fully understood. In this context, the present research will throw light on the possible mechanism of PGPR induced heat tolerance mechanism and will reinforce the existing knowledge in this area.

Although heat stress has become a subject of much interest owing to global warming and global climate change, not much research work on cool season legume, like chickpea have been carried out. The methods of screening the suitable genotypes for cultivation still eludes the growers because of the lack of information on the range in genetic diversity for heat tolerance and screening techniques. Hence, the present study was designed with the objectives to study the biochemical response of chickpea to heat stress and to induce thermotolerance by heat acclimation, treatment with salicylic acid (SA), abscisic acid (ABA), $CaCl_2$ and Plant Growth Promoting Rhizobacterial (PGPR) strain -*Bacillus megaterium*. The main objectives of the present study are:

(i) Screening of different genotypes of chickpea (*Cicer arietinum* L.) for heat tolerance; (ii) characterization of biochemical responses of different genotypes to elevated temperatures in terms of changes in the cellular constituents including proteins, carbohydrates, proline, phenols, chlorophylls, carotenoids etc; (iii) determination of the effect of elevated temperatures on enzyme activities-specially antioxidative enzymes like peroxidase, ascorbate peroxidase, catalase, superoxide dismutase and glutathione redutase; (iv) determination of the effect of heat stress on cell membrane stability and lipid peroxidation of membranes; (v) induction of thermotolerance by heat acclimation and various chemical pre-treatments before exposure to lethal temperature; (vi) determination of the specific expression of new

protein(s) or enzymes during heat shock and to determine their involvement in thermotolerance; (vii) analysis of the effect of elevated temperatures on *in vitro* callus formation and that of pre-treatments on thermoprotection and (viii) development of biochemical markers related to acquired thermotolerance in *Cicer arietinum* L.

In order to achieve the above-mentioned objectives, standard methods have been used which are described in the following pages. Besides, a brief review of literature in the line of work has also been presented.

LITERATURE REVIEW

2.1 Biochemical responses of plants to elevated temperature

Elevated temperatures are defined as the temperatures above the optimal growth temperature of plants and animals. The exposure of plants to elevated temperatures leads to heat stress that is often defined as the situation where temperatures are hot enough for sufficient time to cause irreversible damage to plant function or development. Different plant species and cultivars differ in their sensitivity to high temperatures and may be damaged to different extent by either high day or high night temperatures and by either high air or soil temperature. Plant responses to stressful environmental factors can be part of the mechanisms that permit the plant to withstand the stress. The response depends on the severity and duration of the stress, the developmental stage of the affected plant, the tissue type, and the interactions of multiple stresses. Progress in understanding plant responses to stress has been impressive. Many workers have worked on various crop plants to elucidate the biochemical and physiological responses of plants to elevated temperature stress. A brief review of literature in the line of investigation is presented below.

Blumenthal *et al.* (1995) designed experiments to identify wheat genotypes that might be tolerant to the effects of heat stress on grain quality and to further assess the molecular basis of these changes. Diverse set of 45 wheat genotypes was exposed to10h of 40°C on each of three consecutive days in a phytotron. Mean values of all the genotypes tested showed highly significant changes in 1000 kernel weight (-17%) and difference for heat resistance breakdown (17%). The general weakening of dough due to heat stress and decrease in protein content was accompanied by a decrease in glutenin and gliadin ratio and in the percentage of very large glutenin polymers. Bound lipid content increased, and there was a general reduction in the proportion of small starch granules. For all these attributes, reactions for individual genotype range from little change (tolerance to heat stress) to considerable change (susceptible to heat stress). They thus identified groups of genotypes that should be useful in breeding attempts to stabilize wheat against heat related variations in grain quality. Markers identified as potentially useful in

breeding for tolerance include the presence of Glu- Dld allele, and increase in glutenin to gliadin ratio and in the percentage of very large glutamine polymers.

Lafta and Lorenzen (1995) determined the role of sucrose-metabolizing enzymes in altered carbohydrate partitioning caused by heat stress. Potato (*Solanum tuberosum* L.) genotypes characterized as susceptible and tolerant to heat stress were grown at 19/17°C, and a subset was transferred to 31/29°C. Data were obtained for plant growth and photosynthesis. Enzyme activity was determined for sucrose-6-phosphate synthase (SPS) in mature leaves and for sucrose synthase, ADP-glucose pyrophosphorylase, and UDP-glucose pyrophosphorylase in developing tubers of plants. High temperatures reduced growth of tubers more than of shoots. Photosynthetic rates were unaffected or increased slightly at the higher temperature. Heat stress increased accumulation of foliar sucrose and decreased starch accumulation in mature leaves but did not affect glucose. SPS activity increased significantly in mature leaves of plants subjected to high temperature. Changes in SPS activity were probably not due to altered enzyme kinetics. The activity of sucrose synthase and ADP-glucose pyrophosphorylase was reduced in tubers, albeit less quickly than leaf SPS activity. There was no interaction of temperature and genotype with regard to the enzymes examined; therefore, observed differences do not account for differences between genotypes in heat susceptibility.

According to Stone and Nicolas (1995) short periods of very high temperature (>35°C) are common in many of the world's wheat growing areas and can be a significant factor in reducing yield and quality of wheat. A study was conducted by them to determine the stage at which the grain growth was most sensitive to a short period of high temperature and to examine whether varietal difference in heat tolerance were expresses in whole grain filling period. Two varieties of wheat differing in heat tolerance (cvv. Egret and Oxley) were exposed to a short (5 days) period of very high temperature (40°C max. for 6h each day) at 5 days interval throughout grain filling, starting from 15 days after anthesis (DAA) and concluding at 50 DAA. Response of grain dry matter accumulation and water content to high temperature was monitored throughout grain filling, and the result compared with the control maintained at 21/16°C day/night. Varietal difference was expressed throughout the grain filling period. Mature individual kernel mass was

most sensitive to heat stress applied early in grain filling and became progressively less sensitive throughout grain filling, for both varieties Reduction in mature kernel mass resulted primarily from reductions in duration rather than the rate of grain filling. To study the fractional protein accumulation in same experimental conditions, grain samples were taken through grain growth and analysed for protein content and composition (albumin/globulin, monomer, SDS soluble polymer and SDS insoluble polymer) using size exclusion high performance liquid chromatography (Stone and Nicholas, 1996). The timing of heat stress exerts a significant influence on the accumulation of total wheat protein and its fractions, and protein fractions differed in their responses to the timing of heat stress. Furthermore wheat genotypes influenced both the sensitivity of fractional protein accumulation to heat stress and the stage during grain filling at which maximum sensitivity to heat stress occurred.

Experiments were carried out with two wheat cultivars Marzak and Oum-rabia, which were subjected to three temperature regimes (20/15, 28/21and 36/29°C) beginning 10 days after anthesis to maturity. High temperature resulted in low values of seed yield and physical traits of seed quality. The effect of temperature on seed germination was not consistent between the two cultivars. High temperature during seed development and maturity had no effect on seed germination of Oum-rabia, whereas it decreased seed germination of Marzak. In contrast to seed germination, seed vigour was adversely affected by heat stress. This decline in seed germination vigor was reflected in reduced shoot and root dry weight, in increased shoot/root ratio, reduced root length, low root number per seedling, and high seed conductivity. Excised embryo culture showed marked differences in embryo growth potential. Although embryo from all treatments had germinated, a delay of 24-48 h was observed in the germination of embryos excised from seeds grown under high temperature conditions. Also their shoot and radical development over time lagged behind that of embryos isolated from seeds grown under cool temperature conditions. Exposing seeds to high temperature during development and maturity also resulted in low oxygen uptake. They also determined the effect of heat stress incase of nucleotide level and respiratory activity of mitochondria. Embryos from low temperature treatment showed rapid accumulation of ATP and higher levels and

rate of oxygen uptake then embryos from high temperature treatment. Embryos from medium temperature treatment exhibited intermediate values. Mitochondria from low temperature regimes were well developed with visible membranes and cristae; those from high temperature regimes were degenerating (Grass and Burris, 1995a & b).

High temperature tolerance of the pollen of P*etunia hybrida* L. and *Nicotiana sylvestis* L. was investigated by Rao *et al.* (1995) by treating dry pollen to temperature up to 75°C for 6-48 hrs and by studying their viability (by fluorochromatic reaction test), vigor and their ability to set fruits and seeds. In Petunia, temperature upto 60°C for 48 hrs did not affect pollen viability, vigour and their fruit and seed setting ability. A temperature of 75°C for 24 hrs reduced the pollen viability and vigour, but fruit and seed-setting ability existed. However, at 75°C exposure for 48 hrs proved lethal for *Petunia* pollen. In *Nicotiana*, pollen exposed to temperature of up to 75°C for 6-12 hrs were able to set seed. With a longer exposure the majority of pollen was FCR-positive, but they were unable to set seed. This result showed that pollen grains of *Petunia* and *Nicotiana* could withstand exposures of temperatures as high as 75°C and retain pollen function. This study also indicated that FCR test might not reflect true viability in pollen subjected to extreme stresses.

A system for the controlled expression of a foreign gene in the cultured tobacco cells (*Nicotiana tabacum*, BY2) by temperature shift was constructed by Yoshida *et al*, (1995). A 925 base pair DNA fragment containing the 5′ flanking region of a low-molecular mass heat shock protein gene (HSP 18.2) of *Arabidopsis thaliana* was inserted upstream of the β-glucuronidase reporter gene (GUS). The resulting HSP 18.2 GUS construct was introduced into BY2 cells by electroporation or *Agrobacterium* mediated transformation. Transient expression of HSP 18.2 promoter in protoplast was very low regardless of the heat shock. Although expression of the HSP 18.2 GUS chimeric gene in the stable transformants of BY2 was hardly detected in culture at 25°C, the expression increased rapidly on the transcriptional level when the incubation temperature was shifted to 35-37°C. After 2 hrs incubation at 37°C, GUS activity was about 1000 fold greater than that before

heat shock. The amount of GUS mRNA was maximum 2 hrs after heat shock, and then decreased gradually.

The responses of the photochemical apparatus of photosynthesis to low and high temperarures were compared by Verlag (1995) in leaves of the frost-sensitive *Solanum tuberosum* (cv. Haig) and of a frost-tolerant Andean potato, *Solanum* x *juzepczukii (*cv. Lucki). The main observations and conclusions of this study are that: (i) Photosystem II (PS II) is noticeably more heat-resistant in *S.* x *juzetczukii* than in *S. tuberosum*, indicating an enhanced generalized stress tolerance of the former genotype to extremes of temperature. (ii) T he higher thermostability of PS II in *S. x juzetczukii* leaves is not associated with any enhancement of the sensitivity of PS II photochemistry to chilling temperature. In both species, the chilling-induced inhibition of electron transport through PS II is closely correlated with the inhibition of the PSII-to-PSI electron flow, the rate of which is determined by the reoxidation of reduced plastoquinone. A slowdown of the latter reaction at low temperature can be attributed to the accumulation of protons in the thylakoid lumen associated with the inhibition of the Calvin cycle activity in chilled leaves, as suggested by the strong non-photochemical quenching of chlorophyll fluorescence. (iii) The photochemical activities of both species are similarly impaired by chilling treatments in the light, indicating that frost resistance does not preclude susceptibility to photoinhibition damage at temperature. (iv) A striking difference between *S. tuberosum* and *S. x juzeiczukii* is the high plasticity of the PS II thermotolerance in the latter species, with low (8°C) and high (35°C) temperature treatments respectively decreasing and increasing the heat-tolerance of PS II. These changes are not observed or are very limited in the Haig variety of S. tuberosum. (v) In contrast to the constitutive thermotolerance of PSII (measured in 23°C-grown plants), 35°C-induced thermotolerance has a dramatic effect on the photochemical activity at chilling temperature. When placed at 5°C, the intersystem electron flow of 35°C-treated leaves is dramatically inhibited as compared with non-treated leaves whereas triangle pH-related quenching of chlorophyll fluorescence is unchanged. These findings indicate independent control of non-acclimated heat-tolerance and thermally induced heat-tolerance of the photosynthetic membranes. Taken together, the presented data show that the photosynthetic apparatus of the cultivated Andean

hybrid, *S.* x *juzetczukii* though sensitive to chilling injury in the light, is adapted to the changing temperature conditions prevailing in the natural habitat of its wild progenitor where night frosts are associated with warm and sunny days.

Based on partial or complete sequence of 14 plant heat shock transcription factors from tomato, soybean, *Arabidopsis* and maize, Nover *et al.* (1996) proposed a general nomenclature with two basic classes, i.e. classes A and B containing two or more types of Hsfs (HsfA1and HsfA2). Despite some plants' specific peculiarities, essential functional domains and modules of these proteins are conserved among plants, yeast, *Drosophila* and vertebrates. Similar to the situation with the small heat shock proteins, the complexity of the hsf gene family in plants appears to be higher than in other eukaryotic organisms.

The expression of the heat shock protein (HSP) genes in the developing pollen and in the mature male gametophyte was surveyed by Mascarenhas and Crone (1996). In general, mature pollen lacks a normal heat shock response. In mature pollen of several species either no heat shock proteins are synthesized in response to heat stress, or if synthesized, only a subset are made and the response is weak both at transcriptional and translational level, compared with the response in the vegetative tissues. In developing pollen however, a subset of hsp is induced in response to heat stress. In addition, certain hsp genes or heat shock cognate genes are activated during normal pollen development in the absence of heat stress, indicating that these genes are likely to have important developmental functions.

Lipid composition of microsomes of heat stresses suspension culture was studied by Stryer *et al.* (1996). Heat stressed (30°C) cell suspension cultures of carrot attained a lower maximum cell density and showed browning earlier when compared with control cultures (22°C) over a 16 day growth period. Phospholipid class profile did not differ between cell grown at 30°C and 22°C. The fatty acid of phosphotidylcholine (PC) and phosphotidylethanolamine (PE) from microsomes of heat stressed cells were much less saturated than those of PC and PE from microsomes of control cells. In particular, there was a marked increase in the proportion of oleate [18:1 (9)] at the expense of linoleate [18:2 (9,12)] at the higher growth temperature. This difference could result from inhibition or loss of

microsomal lipid linked desaturase that inserts the double bond between carbon 12 and 13 of oleate anesterified to the glycerol moiety of PC and PE.

Heat tolerance in 23 tropical and one temperate fruit crop was evaluated by Yamada *et al.*(1996) by determining chlorophyll fluorescence [the ratio of the vriable fluorescence to the maximum fluorescence (F-v/ F-m), and the basal fluorescence (F-O)]. The ratio [R(v)] of F-v/F-m in leaves exposed to high temperature (45°C for 20 min.) to F-v/F-m to control temperature (25°C for 20 min.) was found to be highly and negatively correlated to the ratio [R(O)] of F-O exposed to the high temperature to F-O exposed to the control temperature. Leaves (3.5 months old) sampled in mid July were slightly but significantly more tolerant to heat than 2.5 months old leaves sampled in early to mid June. The ratio of the genetic variance to the total variance in the measurements was 0.90 for R(v), and 0.89 R (O). Pineapple, coconut palm and *Annona* species were heat tolerant, java apple rose apple, longan, and peach was sensitive.

De *et al.* (1996) showed the accumulation of proline in the seedlings as well as cultured cells of tomato as a consequence of short time heat shock (45°C for 4 and 8 hrs) and cold shock (4°C for 4 and 8 hrs) treatment. The involvement of calcium ion in the proline accumulation was demonstrated by using specific calcium chelator. EDTA and channel blockers. $CaCl_3$ and $CaCl_2$ pretreatment stimulated the accumulation of proline both in high and low temperature treated cultured cells and seedlings of tomato.

According to Bacci *et al.* (1996) in the leaves of herbaceous plants, sub-optimal temperatures influence the content and efficiency of the photosynthetic pigments and in more severe cases, alter mesophyll thickness. They examined the possibility of detecting the degree of alternation in sorghum leaf characteristics by indices of stress calculated from remotely sense data. Reflectants, colorimetric and ecophysiological measurements were performed on two cultivars of sorghum [*Sorghum bicolor* (L.) Moench.], grown at 15°C, 21°C and 32°C. Compared to plants growing at 21°C, the other two temperatures reduced the chlorophyll content and PS II efficiency in the leaves, which were less at 15°C than at 32°C.Slight differences, in these responses to temperature was also observed between the two

cultivars. Colorimetric coefficients detected a significant discoloration at 32°C and a marked reddening at 15°C. The indices calculated from the colorimetric data were able to to distinguish the differences between treatments, but they did not show a strict relationship with the trend of ecophysiological parameters.

The effect of heat stress on subcellular localization of Ca^{2+} in tomato fruits was studied by Garbaczewska *et al.*(1998). The tomato plants Robin cv., relatively tolerant to heat stress, were grown under uncontrolled greenhouse conditions to the stage of fruiting. The plants were placed for 20 hrs in two temperature regimes: 23°C (optimal temperature) and 40°C (heat stress) in darkness, under water vapour saturated atmosphere. Immediately after heat stress the fruits were harvested to estimate the water soluble and insoluble calcium contents and sub-cellular localization of Ca^{2+}. After heating, the concentration of calcium in tomato fruits increased about twice. In both temperature treatments the water-soluble fractions were lower than insoluble ones how ever, smaller differences between soluble and insoluble fractions were obtained after heat stress. The shapes and localization of Ca^{2+} detected with the use of potassium antimonate method showed that in fruits of control plants the precipitates were numerous, small and oval shape. They were dispersed in cytosol or adjoined to endoplasmic reticulum or to external membrane of chloroplast. In the fruits of heated plants the precipitates were irregular in shape, amorphous and singly dispersed in cytosol. They also observed some cytosolic changes in the structure of membranes and organelles of the plants of both experimental treatments. The heat induced increase of calcium content and changes in sub-cellular localization of Ca^{2+}under heat stress showed that calcium ions may be involved in avoiding heat injury.

Reproductive processes and pod yield in cowpea [(*Vigna unguiculata* (L.) Walp] an important crop grown in semi-arid sub-Saharan Africa were reported to be adversely affected by high temperature. Genotypic differences in heat tolerances have been identified under hot, long days, but it was not known if this tolerance was also exhibited in hot, short day environments typical of sub-Saharan Africa. The authors conducted the study to determine whether heat tolerance identified under hot, long days were expressed at the same stages of development under hot, short days, and whether responsiveness to temperature was additive and quantitative. A

heat tolerant (Prima), and heat susceptible (IT 84S-2246) cultivar of cowpea were grown in controlled environments under short days (12 h/day) initially at 30°C/24°C (Mod-T), where they remained for 0,10,20,30,40 days after emergence (DAE) to 36°C/27°C (High-T), where they remained for 5,10, or 20 days duration before returning to moderate temperature (Mod-T). Control plants were examined at Mod-T or High-T for 50 d when the first pods were mature and the experiments were terminated. There were significant effects of duration (D) and timing (T), and interactions between DxT, Tx genotypes (G) and DxTxG on pod weight plant (-1). Prima was significantly more tolerant to heat stress during flowering than IT 84S-2246 confirming that heat tolerance was expressed under hot, short days. The greater heat tolerance of Prima was associated with an ability to maintain peduncle and flower production at High-T and with greater podset. The sensitive period in IT 84S-2246 started at floral bud initiation (15-20 DAE), and effects of High-T thereafter were additive and quantitative (Craufurd *et al.*,1998).

Storozhenko *et al.* (1998) carried out experiments to better understand the role of ascorbate peroxidases in oxidative stress tolerance in which, the transcriptional regulation of the apx 1 gene from *Arabidopsis* was studied. The apx1 gene was expressed in all the tested organs of *Arabidopsis*; mRNA level were low in roots, leaves and stems and high in flowers. Steady state mRNA levels in leaves or cell suspensions increased after treatment with methyl viologen, etherphone, high temperature and illumination of etiolated seedlings. A putative heat shock element found in the apx1 promoter, was shown to be recognized by the tomato heat shock factor *in vitro* and to be responsible for the *in vivo* induction of the gene. The heat shock cis element also contributed partially to the induction of the gene by oxidative stress. By using *in vivo* dimethyl sulphate footprinting, they showed that protein interacted with the G/C rich element found in apx1 promoter.

Pareek *et al.*(1998) reported that while the rice 87 KDa protein was transiently synthesized within initial two hours of heat shock, high steady state level of the protein was retained even under prolonged high temperature stress condition or recovery following 4 hrs of heat shock. It was further shown that, fifteen different wild rice accumulated different levels of these proteins in response to heat shock treatment.

Presence of a high molecular weight protein in pea (*Pisum sativum* L.) seedlings was detected by means of Western Blotting by Chen and Su (1998).The protein consisted of an α (60.4 KDa) and a β (65.5 KDa) subunit. The protein had low ATPase activity. Its expression could be enhanced by 3 to 4 fold by under heat shock stress, but was not affected by exogenous application of ABA. The result of localization and 35 S-met labeling showed that it was a cytoplasmic protein and its synthesis was not inhibited by chloromphenicol.

Chen *et al.*(1998) also investigated the temperature, heat shock proteins and fertility changes in sorghum. They reported that sorghum sterile 3A line was induced to be fertile when it was heat shocked. By comparing mitochondria heat shock proteins of 3A line with 3B line they found that HSPs were encoded by nuclear DNA and were transported into mitochondria after being synthesized in the cytoplasm. When heat shocked for 2 hrs, 3A line produced 5 protein bands which weighed 70 KDa, 31 KDa, 24 KDa, 18 KDa and 16 KDa respectively whereasin 3B line, additional 94 KDa and 96 KDa bands appeared and the amount of HSP 70 was greater than in 3A line. When heat shock was given for 4 hrs, 94 KDa and 96 KDa HSPs in 3B line disappeared and 3B tended to be identical with 3A in HSPs.After heat shock treatment, the amount of mitochondrial total proteins increased greatly in both 3A and 3B. Then there was a sudden drop of HSPs. On the 8th hour 3B line had only four bands weighing 70 KDa, 32 KDa, 24 KDa and 16 KDa respectively and 70 KDa HSP was especially obvious while in 3A line, all HSPs disappeared. This indicates that HSPs are stable in 3B line but unstable in 3A line. Perhaps the difference is relevant to the stability of fertility of 3B line as well as infertility of 3A line.

Schraf *et al.* (1998) used Hsf knock out strains of yeast and transient reporter assays in tomato protoplast for functional analysis of HSF- coding cDNA clones and mutants derived from them. Hsf A2, which in tomato cell cultures was expressed after heat shock induction, tended to form large cytoplasmic aggregates together with other Hsps. In the transient expression assay its relatively low activator potential was evidently due to the inefficient nuclear import. However, the intermolecular shielding of the NLS could be released either by deletion of short

C-terminal fragment or by co expression with HsfA1, which form hetero-oligomers with HsfA2.

High temperature is a major determinant of wheat (*Triticum aestivum* L.) development and growth, decreasing yields by 3 to 5% per 1°C increase above 15°C in plants under controlled conditions. Even greater yield differences have been reported between favorable and unfavorable temperature conditions in the field. Gibson *et al.* (1999) studied the yield components of the hard red winter wheat cultivar Karl 92 that are affected by controlled high temperature during maturation of intact plants under simulated field populations. Day/night temperatures of 20/20, 25/20, 30/20, and 35/20°C were imposed from 10 and 15 d after anthesis until ripeness in two experiments, and temperatures of 25/20, 30/20, and 35/20°C were applied from 20 d after anthesis until ripeness in a third experiment. Grain yield was reduced by 78%, kernel number was reduced by 63%, and kernel weight was reduced by 29% at 35/20°C compared with 20/20°C from 10 d after anthesis until ripeness. The yield loss from high temperature applied during this period was much greater than for previous controlled-environment studies. Kernel numbers in treatments applied during early reproductive growth in our study were as sensitive to high temperature as wheat plants in previous field studies. High temperature applied 15 d after anthesis until ripening reduced grain yield 18%. Since kernel number was set by this time, the loss was exclusively due to decreased kernel weight. High temperature imposed from 20 d after anthesis decreased kernel weight by 18%.

The impact of simultaneous environmental stresses on plants and how they respond to combined stresses compared with single stresses is largely unclear. By using a transgene (*RD29A-LUC*) consisting of the firefly luciferase coding sequence (*LUC*) driven by the stress-responsive *RD29A* promoter, Xiong *et al.* (1999) investigated the interactive effects of temperature, osmotic stress, and the phytohormone abscisic acid (ABA) in the regulation of gene expression in *Arabidopsis* seedlings. Results indicated that both positive and negative interactions exist among the studied stress factors in regulating gene expression. At a normal growth temperature (22°C), osmotic stress and ABA act synergistically to induce the transgene expression. Low temperature inhibits the response to osmotic stress or to combined treatment of osmotic stress and ABA, whereas low temperature and ABA

treatments are additive in inducing transgene expression. Although high temperature alone does not activate the transgene, it significantly amplifies the effects of ABA and osmotic stress.

David and Steven (1999) carried out experiments by increasing the leaf temperature of intact cotton (*Gossypium hirsutum* L.) and wheat (*Triticum aestivum* L.) plants, which caused a progressive decline in the light, saturated CO_2 exchange rate (CER). CER was more sensitive to increased leaf temperature in wheat than in cotton and both species demonstrated photosynthetic acclimation when leaf temperature was increased gradually. Inhibition of CER was not a consequence of stomatal closure, as indicated by the positive correlation between leaf temperature and transpiration. The activation state of Ribulose bis-phosphate oxygenase/carboxylase, which is regulated by Rubisco activase, was closely correlated with temperature-induced changes in CER. Non-photochemical chlorophyll fluorescence quenching increased with leaf temperature in a manner consistent with inhibited CER and Rubisco activation. Both non-photochemical fluorescence quenching and Rubisco activation were more sensitive to heat stress than the maximum quantum yield of photochemistry of photosystem II. Heat stress led to decreased 3- phosphoglyceric acid content and increased Ribulose 1,5 bis-phosphate content which is indicative of inhibited metabolic flow through Rubisco. They concluded that heat stress inhibited CER primarily by decreasing the activation state of RUBISCO via inhibition of Rubisco activase. Although Rubisco activation was more closely correlated with CER than the maximum quantum yield of photochemistry of photosystem II, both processes could be acclimated to heat stress by gradually increasing the leaf temperature.

A band of heat shock proteins of 45 KD in leaf tissue of drought and heat tolerant maize line, ZPBL 1304 was reported by Ristic *et al.* (1999). This band has not been previously described in maize line and did not appear to be common in higher plants. It is not known how many polypeptides comprised this 45 KD band. For heat shock polypeptide study, plants were exposed to two environmental stress conditions, soil drying and high temperature (45°C) and high temperature (45°C) alone. Generally the pattern of heat shock polypeptide synthesis in both conditions was same. 2D electrophoresis revealed 3 heat shock polypeptides of 45 KD with

isoelectric points ranging from 5 to 5.5 and 2 heat shock polypeptides of 46 KD slightly above 5.5. Drought alone did not induce the synthesis of protein of 45 KD.

Two cDNAs, Ta HSP 23-5 and Ta HSP 23-6, encoding proteins with homology to mitochondrion localized (MT) small heat shock proteins (sHSPs) were isolated from heat shock cDNA library from *Triticum aestivum*. Ta HSP 23-5 specified a 214 amino acid protein and Ta HSP 23-6 specified a 216 amino acid protein. Amino acid sequence identity was only 45.7% between the two proteins. However, both proteins showed greater identity to MT sHSPs of other plant species than to any other sHSPs from wheat. Amino acid sequence alignments with other MT sHSPs identified the putative amino terminus of the mature proteins and consensus regions specific to this class of sHSPs. Transcripts of both genes were absent from control tissue, but strongly induced by heat stresses. Phylogenetic analysis indicated that these two wheat genes arose by duplication after the divergence of monocot and dicots (Basha *et al.*, 1999).

Heat shock protein 101 (HSP 101) cDNA and genomic clones were isolated by Nieto-Sotelo *et al.* (1999) from maize. The structure of maize HSP 101 revealed the presence of exons interrupted by 5 introns. Maize HSP 101 contained a predicted open reading frame that translated into a 912 amino acid sequence with a mass of 101 KD. Initiation of transcription was mapped 146 bases upstream of the AUG codon. Five HS element boxes were found. A protein sequence comparision showed that maize HSP 101 belonged to the heat shock 100 KD and caseno lytic protease B protein family that plays an important role in bacteria and yeast in survival to extremely high temperature and control of proteolysis. Accumulation of HSP 101 mRNA was strong under heat shock conditions, but was not detectable after cold or osmotic stress treatment or by application of ABA.

Lin *et al.* (1999) observed that 70 stress molecular chaperones are found in all the major sub-cellular compartments in plant cells and a multigene family encodes them. Twelve members of this family have been identified in spinach. The expression of the stress 70 molecular chaperones in response to heat shock is well known and it appears that low temperature exposure can also stimulate their expression. However, it was difficult to determine which members of the family are

specifically responsible to low temperature. This study concluded the levels of expression of the stress 70 family members and other selected chaperones in response to high and low temperature exposure. During heat shock of spinach, of the ten stress 70 family members that were examined all ten showed increased RNA levels after 1h, and all showed down regulation at longer duration of high temperature exposure.

Degradation of ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) due to elevation in atmospheric temperature and extent of enzyme damage due to varietal differences were studied in two rice cultivars by Bose *et al.*(1999) using 35 methionine pulse- chase.The cultivars N-22 and IR-8 were certified by the authors as thermotolerant and thermosensitive respectively. Differential response was observed in both cultivars with the N-22 showing greater thermostability of Rubisco protein upto 45°C, while IR-8 was found to be thermolabile.Elevation of temperature to 50°C favoured degradation of proteins in both the cultivars. Protease activity as measured by Western Blot analysis using purified Rubisco revealed the thermosensitive nature of IR-8 and this could be correlated with the protein turn over by 35 S.methionine and Northern blot analysis. The results indicate that genetic differences exist in two cultivars and that heat tolerant cultivars have a protective mechanism against thermal degradation of Rubisco.

Timmusk *et al.* (1999) addressed changes in plant gene expression induced by inoculation with plant-growth-promoting rhizobacteria (PGPR). They established gnotobiotic system with *Arabidopsis thaliana* as model plant, and isolates of *Paenibacillus polymyxa* as PGPR. Subsequent challenge by either the pathogen *Erwinia carotovora* or induction of drought (abiotic stress) indicated that inoculated plants were more resistant than control plants. With RNA differential display on parallel RNA preparations from *P. polymyxa* treated or untreated plants changes in gene expression were investigated. From a small number of candidate sequences obtained by this approach, one mRNA segment showed a strong inoculation dependent increase in abundance. The corresponding gene was identified as *ERD15*, previously identified to be drought stress responsive. Quantification of mRNA levels of several stress-responsive genes indicated that *P. polymyxa* induced mild biotic

stress. This suggests that genes and/or gene classes associated with plant defenses against abiotic and biotic stress may be co-regulated.

Proline accumulation in response to drought and heat stress in cotton (*Gossypium hirsutum*) in six different cultivars was studied by De Ronde *et al.*(2000). They induced drought and a combination of drought and heat stress in three-week-old seedlings in the greenhouse. Results revealed that with decreasing water content there was a progressive increase in free proline in all six cultivars, as well as differences in the proline level between the different cultivars. Maximum accumulation of free proline in drought stressed cotton occurred at 11 days without water. The combination of heat and drought stress exhibited an increase in proline concentrations in five cultivars. Different proline profiles were observed for the different treatments and different mechanisms for heat and drought. Therefore, they proposed that proline may be used as an index for heat and drought tolerance.

Jiang and Huang (2000) conducted a study to determine physiological responses of Kentucky bluegrass (*Poa pratensis* L.) to drought and heat alone or together, and the effects of drought preconditioning on plant responses to subsequent heat stress. Kentucky bluegrass (cv. Mystic) was subjected to drought and/or heat stress (35°C/30°C, day/night) in growth chambers for 40 days. Canopy photosynthetic rate (P_n) and leaf photochemical efficiency (Fv/Fm) decreased under drought and heat stress. The decline in P_n was more severe under heat than under drought stress during the first 12 days of treatment. The reduction in Fv/Fm ratio was more severe under drought stress than under heat stress after 20 days of treatment. The combined heat and drought stresses (H+D) caused more dramatic reductions in P_n and Fv/Fm than either heat or drought alone, starting at 3 and 9 days after treatment, respectively.Drought or heat alone, or H+D, significantly reduced root dry weight. However, reduction was more severe under heat alone than under drought stress, particularly in the top 20 cm of soil. Drought preconditioning enhanced plant tolerance to subsequent heat stress but had no influence on plant tolerance to H+D. Drought-preconditioned plants maintained higher water status, stomatal conductance, and transpiration rate, and had significantly higher P_n and root dry weight than non-preconditioned plants during subsequent heat stress. No significant difference in Fv/Fm was observed between drought-preconditioned and non-preconditioned plants

under either heat alone or H+D. The results indicated that simultaneous drought and heat stresses were more detrimental than either stress alone. Drought preconditioning could improve Kentucky bluegrass tolerance to subsequent heat stress.

The effect of high temperature stress on wild and spring wheats has been studied by Waines (2000) in wild wheats that includes species in the genera *Aegilops* L. and *Triticum* L. Species exist in a polyploid series, diploid, tetraploid and hexaploid, based on the genome formula, n = x = 7 chromosomes. Commercial durum wheat is tetraploid with the genome formula BBAA, while bread wheat is hexaploid (BBAADD).Wheats grown at Riverside, California, from June to October exhibit heat stress at the vegetative and reproductive stages. Under high temperatures (28/15°C day/night) during the vegetative stage, many diploid species do not grow well. Wild diploid *T. urartu* (AA) and *T. monococcum* ssp. *boeoticum* (AA) exhibited more effects of heat stress than the goat grasses *A. speltoides* (SS = BB?) or *A. tauschii* (DD). Wild tetraploid *T. turgidum* L. ssp. *dicoccoides* Korn (BBAA) exhibited more vegetative-phase stress tolerance than the diploid wheats. Modern Mexican cultivars of durum and bread wheats showed good establishment under high field temperatures, but often tiller number was reduced, and the developmental stages were reduced in time. All the spring durum and bread wheats tested flowered and set seed. They produced anthers with fertile pollen, and they had reproductive heat tolerance. Many wild *Aegilops* and *Triticum* accessions did not boot for lack of vernalisation, or they showed reproductive heat stress. Ten wild accessions, including *A. speltoides*, *A. longissima* and *A. searsii*, showed normal vegetative and reproductive development and were considered heat tolerant. They came from the same geographic area in Palestine, which should be searched for landraces of wheats that show heat tolerance.

Changes in photosystem II (PSII) thermotolerance during drought and recovery were studied under controlled conditions in three Mediterranean cedar species (*Cedrus brevifolia* Henry, *C. libani* Loudon and *C. atlantica* Manetti) by Ladjal *et al.* (2001). The temperature at which the quantum yield of PSII photochemistry was reduced by 15% of its value at 25°C was 3 to 4°C higher in drought-treated plants than in well-watered plants. The drought-induced increase in PSII thermotolerance was already evident 8 days after water had been withheld from

the seedlings, when net CO_2 assimilation was still at 80% of its initial value, and was visible for up to 12 days after re-watering. When seedlings of the three species were exposed to temperatures above 45°C for 5 h, both maximal quantum yield of PSII photochemistry and net CO_2 assimilation rate were significantly reduced in unconditioned seedlings, whereas drought-preconditioned seedlings were almost unaffected by the heat treatment. Drought-preconditioned seedlings still exhibited a higher tolerance to heat stress than unconditioned seedlings 60 days after re-watering, although the transient, drought-induced osmotic adjustment had fully disappeared. Among species, *C. atlantica* was the most heat sensitive, whereas the heat treatment had no significant effect on the parameters measured in *C. brevifolia*.

In *Nicotiana attenuata*, systemic induction of heat-shock proteins (Hsps) was detected by Hamilton and Coleman (2001) in response to the treatment of single leaves by heat shock, mechanical damage, or exogenous application of methyl jasmonate (MJ). All treatments increased the abundance of members of the 70-kD Hsp (Hsp70) family and induced synthesis of one or more of the small Hsps (sHsp) (16–23 kDa) in both treated and untreated leaves. These results provide the first evidence that Hsps can be systemically induced in plants and suggest that systemic induction of Hsps may be important in pre-adapting leaves to stress.

Sharkova (2001) analyzed the effect of heat shock on the capacity of wheat plants to restore their photosynthetic electron transport after photoinhibition or repeated heating. The shoots of 16-day-old spring wheat plants (*Triticum aestivum* L., cv. Albidum 29) were subjected to heat shock (HS) at 40, 41, or 43°C for 10 min. The activity of the Hill reaction in chloroplasts isolated immediately after HS was 83, 61, and 30% of the initial value, respectively. The activity of the Hill reaction was also estimated after plant return to the initial growth conditions for one day. It was completely restored after heating at 40°C and achieved 82 and 30-33% of the initial level after heating at 41 and 43°C, respectively. Thereafter, the shoots were heated repeatedly at 42, 43, or 43.5°C for 10 min, and the activity of the Hill reaction was measured immediately or one day after this heating. Immediately after the second heating, the activity decreased again as compared to its value before heating. The percent of inhibition of the Hill reaction was similar in the control plants not subjected to preliminary HS and HS-treated plants independently of the temperature

used. However, after one-day growth under normal conditions, the activity of the Hill reaction was restored almost completely in HS-treated plants but not more than by 10% in the control plants. The conclusion is that different mechanisms underlie the development of the tolerance to HS and recovery. Some plants were tested for the effect of HS (40°C) on their tolerance to photoinhibition. The degree of the Hill reaction inhibition after plant exposure to the light of 65-75 klx for 3 hrs was essentially similar in detached leaves from the HS-treated and unheated plants and comprised about 40% of the activity before light stress. After the leaves were returned to the low-light conditions for 3 hrs, the Hill reaction was restored and attained about 75% of that before photoinhibition in both HS-treated and untreated plants. The lack of the HS-induced stimulation of the Hill reaction recovery after photoinhibition is evidently related to the fact that heating and excess light damage different sites of photosystem II, which implies the different pathways for the recovery of its functional activity.

Dalal and Khannna–Chopra (2001) investigated the activities of the antioxidant enzymes in the leaves of necrotic wheat hybrids, Kalyansona×C306 (K×C) and WL711×C306 (WL×C) and their parents at different developmental stages. The K×C hybrid exhibited more severe necrosis than WL×C. In K×C, superoxide dismutase (SOD) activity showed no increase over the parents, while WL×C showed an early increase, but it was possibly insufficient to scavenge increased superoxide. Activities of guaiacol peroxidase, ascorbate peroxidase and glutathione reductase were enhanced, while catalase exhibited a decrease in activity, with the appearance of visible necrosis in both the hybrids. The isozyme profile of the antioxidant enzymes was similar in the hybrids and their parents. One existing isoform of guaiacol peroxidase showed an early appearance in the hybrid and increased in intensity with the progression of necrosis. The results reveal a differential response of antioxidant enzymes in necrotic wheat hybrids as compared to their parents. The response differed in magnitude at developmental stages of the leaves, which might be related to the intensity of necrosis expressed by the hybrids.

The genetic control of heat tolerance through diallel analysis of selected wheat (*Triticum aestivum* L.) germplasm was determined by Ibrahim and Quick (2001). Heat-induced damage of plant membranes was assayed by the membrane thermal stability (MTS) assay, which measures electrolyte leakage from leaf tissue after exposure to high temperature. Six wheat genotypes ('TAM 107', 'TAM 108', 'Arlin',' Kauz', 'Glennson 82', and 'Siete Cerros') were hybridized in a complete diallel, and MTS was measured on 12 days old F_1 seedlings. The mean square for general combining ability (GCA) was four times that of specific combining ability (SCA), indicating the importance of additive gene effects in acquired thermal tolerance. Maternal effects accounted for 67% of reciprocal variation, suggesting that maternal seed-source effects may be important in hybrid seed. These results suggest that heat tolerance based on MTS can be improved using the existing genetic variability available within the germplasm.

Pressman *et al.* (2002) stated that continuous exposure of tomato 'Trust' to high temperatures (day/night temperatures of 32/26°C) markedly reduced the number of pollen grains per flower and decreased viability. The effect of heat stress on pollen viability was associated with alterations in carbohydrate metabolism in various parts of the anther during its development. Under control, favourable temperature conditions (28/22°C), starch accumulated in the pollen grains, where it reached a maximum value 3 days before anthesis; it then diminished towards anthesis. During anther development, the concentration of total soluble sugars gradually increased in the anther walls and in the pollen grains (but not in the locular fluid), reaching a maximum at anthesis. Continuous exposure of the plants to high temperatures (32/26°C) prevented the transient increase in starch concentration and led to decreases in the concentrations of soluble sugars in the anther walls and the pollen grains. In the locular fluid, however, a higher soluble sugar concentration was detected under the high-temperature regime throughout anther development. These results suggest that a major effect of heat stress on pollen development is a decrease in starch concentration 3 days before anthesis, which results in a decreased sugar concentration in the mature pollen grains. These events possibly contribute to the decreased pollen viability in tomato.

The molecular mechanisms by which plants acclimate to oxidative stress are poorly understood. To identify the processes involved in acclimation, Vranova *et al.*(2002) performed a comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. Combining mRNA differential display and cDNA array analysis, they estimated that at least 95 genes alter their expression in tobacco leaves acclimated to oxidative stress, of which 83% are induced and 17% repressed. Sequence analysis of 53 sequence tags revealed that, in addition to antioxidant genes, genes implicated in abiotic and biotic stress defenses, cellular protection and detoxification, energy and carbohydrate metabolism, *de novo* protein synthesis, and signal transduction showed altered expression. Expression of most of the genes was enhanced, except for genes associated with photosynthesis and light-regulated processes that were repressed. During acclimation, two distinct groups of coregulated genes ("early" and "late-response" gene regulons) were observed, indicating the presence of at least two different gene induction pathways. These two gene regulons also showed differential expression patterns on an oxidative stress challenge. Expression of "late-response" genes was augmented in the acclimated leaf tissues, whereas expression of "early-response" genes was not. Together, these data suggest that acclimation to oxidative stress is a highly complex process associated with broad gene expression adjustments. Moreover, the datas indicate that in addition to defense gene induction, sensitization of plants for potentiated gene expression might be an important factor in oxidative stress acclimation.

Plants respond to temperature stress by synthesizing a set of heat shock proteins (HSPs), which may be responsible for the acquisition of thermotolerance. The induction of small HSPs (sHSPs) in eight common bean varieties was evaluated by Simoes-Araujo *et al.* (2003) using Northern blot analysis and W HSP 16.9 cDNA as heterologous probe. Cowpea was used, as a positive control since this plant, as opposed to common bean, is known to grow well under high temperature regimes such as that found in the Brazilian semi-arid region. After the growth period, the plants were submitted to two h of heat shock at 40°C. All varieties tested were able to induce sHSP mRNAs that hybridized with W HSP 16.9 probe. However, significant kinetic differences were found when comparing different varieties. SHSP mRNA levels induced after heat shock in cowpea was higher than the levels

observed on the bean varieties displaying the highest expression of these proteins. Besides, the sHSP expression was also assessed at the protein accumulation level by Western-blot analysis for cowpea and both IPA 7 and Negro Argel varieties of bean plants. The revealed protein pattern confirmed that sHSPs are differentially expressed in distinct varieties of common bean according their heat stress tolerance.

Heat stress can detrimentally affect the reproductive capacity of many plants. The effect of a 7 or 14 days heat stress on flowering, seed set, pollen viability and germinability of flax (*Linum usistatissimum* L.) was assessed under growth chamber conditions by Cross *et al.* (2003). An incremental (2°C/h), cyclical (daytime high 40°C and night-time low 18°C) heat stress was applied 12 days after the initiation of flowering. Although flower formation in flax was not affected by heat stress, boll formation and seed set were reduced with onset of the heat stress. On removal of heat stress the stressed plants showed a compensatory response, flowering and producing bolls at a greater rate than the control plants. Heat stress significantly prolonged flowering by 17 days. Boll weight and seed weight were reduced with heat stress and the number of malformed, sterile seed increased three-fold after 14 days of heat stress. Pollen viability and appearance were negatively affected after 6 and 10 days of heat stress, respectively. Pollen germinability decreased by the sixth day of heat stress, with no pollen germinating by the tenth day. Effects of heat stress on pollen viability and germinability alone, which did not occur until after the sixth day of the stress, could not account for the decreased boll formation due to heat stress in flax. These observations suggest that a combined effect of heat stress on both pollen and ovules contributes to decreased boll formation and seed set in flax.

The accumulation of hydrogen peroxide (H_2O_2) in plants is typically associated with biotic or abiotic stresses. However, H_2O_2 is continuously produced in cells during normal metabolism. Yet, little is known about how H_2O_2 accumulation will affect plant metabolism in the absence of pathogens or abiotic stress. It has been reported that a deficiency in the H_2O_2-scavenging enzyme, cytosolic ascorbate peroxidase (APX1), results in the accumulation of H_2O_2 in *Arabidopsis* plants grown under optimal conditions. Knockout-Apx1 plants were characterized by suppressed growth and development, altered stomatal responses, and augmented induction of heat shock proteins during light stress. The inactivation of Apx1 resulted in the

induction of several transcripts encoding signal transduction proteins. These were not previously linked to H_2O_2 signaling during stress and may belong to a signal transduction pathway specifically involved in H_2O_2 sensing during normal metabolism. Surprisingly, the expression of transcripts encoding H_2O_2 scavenging enzymes, such as catalase or glutathione peroxidase, was not elevated in knockout-Apx1 plants. The expression of catalase, two typical plant peroxidases, and several different heat shock proteins was however elevated in knockout-Apx1 plants during light stress. The results demonstrate that in plants accumulation of H_2O_2 can suppress plant growth and development, interfere with different physiological processes, and enhance the response of plants to abiotic stress conditions. These findings also suggest that at least part of the induction of heat shock proteins during light stress in *Arabidopsis* is mediated by H_2O_2 that is scavenged by APX1 (Pnueli *et al.*, 2003).

Srivalli *et al.*(2003) have reported antioxidative defense system in an upland rice cultivar subjected to increasing intensity of water stress followed by recovery. They subjected rice (*Oryza sativa* L.) cv. Tulsi to three cycles of water stress of increasing stress intensity. Rewatering the plants for 48-hrs period terminated each stress cycle. The level of stress was measured by quantification of H_2O_2. The response of antioxidant metabolites such as ascorbate and glutathione, and enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2) and guaiacol peroxidase (POX, EC 1.11.1.7) was analysed in terms of activity and isozyme pattern for each cycle of stress and recovery. The differential response of the antioxidant enzymes with increasing stress intensity followed by recovery, highlight the different role of each in the drought acclimation process of upland rice. SOD and POX activity in stressed plants was higher than the controls in all the three cycles. The second level of stress saw an increase in all the enzymes with APX and GR showing its maximum activity and there was a better management of H_2O_2 levels. There was an induction of a new CAT isoform in stressed plants in the third cycle of water stress. The co-ordinated defense helped the plants to recover in terms of growth on rewatering after stress cycles.

Young *et al.* (2003) used table ADC-225-MK3 CO2 gas analyzer and PAM-2000 portable fluorescence measurement system to measure the net photosynthetic rate (Pn), initial fluorescence (Fo), maximal photochemical efficiency of PS II (Fv/Fm) and electron transport rate (ETR) of satsuma mandarin (*Citrus unshiu* Marc.) and navel orange (*C. sinensis* Osbeck) leaves. The results showed that exposure of citrus plants to high temperature (38-40° C) led to a lowering of Pn, Fv/Fm, and ETR, whereas Fo increased. After exposure to high temperature for 25 days, compared with normal temperature (25°C), the Pn of satsuma mandarin and navel orange leaves decreased by 55.6% and 39.8%, Fv/Fm decreased by 22.0% and 6.7% and ETR reduced by 55.0% and 41.5%, respectively. On the other hand, Fo increased 113.8% and 14.9%, respectively. With subsequent transfer to the 25°C culture room for 10 days, Pn,Fv/Fm, Fo and ETR recovered significantly. These results demonstrated that the reduction of Pn in *Citrus* was related to the inactivation of PS II reaction center.

Although the catalytic activity of Rubisco increases with temperature, the low affinity of the enzyme for CO_2 and its dual nature as an oxygenase limit the possible increase in net photosynthesis with temperature. For cotton, comparisons of measured rates of net photosynthesis with predicted rates that take into account limitations imposed by the kinetic properties of Rubisco indicate that direct inhibition of photosynthesis occurs at temperatures higher than about 30°C. Inhibition of photosynthesis by moderate heat stress (i.e.30–42°C) is generally attributed to reduced rates of RuBP regeneration caused by disruption of electron transport activity, and specifically inactivation of the oxygen evolving enzymes of photosystem II. However, measurements of chlorophyll fluorescence and metabolite levels at air-levels of CO_2 indicate that electron transport activity is not limiting at temperatures that inhibit CO_2 fixation. Instead, recent evidence shows that inhibition of net photosynthesis correlates with a decrease in the activation state of Rubisco in both C3 and C4 plants and that this decrease in the amount of active Rubisco can fully account for the temperature response of net photosynthesis. Biochemically, the decrease in Rubisco activation can be attributed to: (1) more rapid de-activation of Rubisco caused by a faster rate of dead-end product formation; and (2) slower re-activation of Rubisco by activase. The net result is that as temperature increases

activase becomes less effective in keeping Rubisco catalytically competent (Salvuccia *et al.*, 2004).

High temperature stress (HTS), during flowering decreases seed production in many plants. Young *et al.* (2004) determined the effect of a moderate HTS on flowering, fruit and seed set in *Brassica napus*. They exposed plants to a HTS (8/16 h dark/light, 18°C night, ramped at 2°C h^{-1}, over 6 hrs, to 35°C for 4 hrs, ramped at 2°C h^{-1} back to 23°C for 6 hrs) for 1 or 2 weeks after the initiation of flowering. Although flowering on the HTS-treated plants, during both the 1-week and 2-week HTS treatments, was equal to that of control-grown plants, fruit and seed development, as well as seed weight, were significantly reduced. Under HTS, flowers either developed into seedless, parthenocarpic fruit or aborted on the stem. At the cessation of the HTS, plants compensated for the lack of fruit and seed production by increasing the number of lateral inflorescences produced. During the HTS, pollen viability and germinability were slightly reduced. *In vitro* pollen tube growth at 35°C, from both control pollen and pollen developed under a HTS, appeared abnormal; however, *in vivo* tube growth to the micropyle appeared normal. Reciprocal pollination of HTS or control pistils with HTS or control pollen indicated that the combined effects of HTS on both micro-and megagametophytes were required to knock out fruit and seed development. Expression profiles for a subset of heat shock proteins (HSP101, HSP70, HSP17.6) showed that both micro- and megagametophytes were thermosensitive despite HTS-induced expression from these genes.

The effects of water deficit and high temperature on the production of α-amylase inhibitor 1 (α-AI-1) were studied in transgenic peas (*Pisum sativum* L.) that were developed by Majer *et al.* (2004) to control the seed-feeding pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae). Transgenic and non-transgenic plants were subjected to water-deficit and high-temperature treatments under controlled conditions in the glasshouse and growth cabinet, beginning 1 week after the first pods were formed. In the water-deficit treatments, the peas were either adequately watered (control) or water was withheld after first pod formation. The high-temperature experiments were performed in two growth cabinets, one maintained at 27/22°C (control) and one at 32/27°C day/night temperatures, with the vapour

pressure deficit maintained at 1.3 kPa. The plants exposure to high temperatures and water deficit produced 27% and 79% fewer seeds, respectively, than the controls. In the transgenic peas the level of α-AI-1 as a percentage of total protein was not influenced by water stress, but was reduced on average by 36.3% (the range in two experiments was 11–50%) in the high-temperature treatment. Transgenic and non-transgenic pods of plants grown at 27/22°C and 32/27°C were inoculated with pea weevil eggs to evaluate whether the reduction in level of α-AI-1 in the transgenic pea seeds affected pea weevil development and survival. At the higher temperatures, 39% of adult pea weevil emerged, compared to 1.2% in the transgenic peas grown at the lower temperatures, indicating that high temperature reduced the protective capacity of the transgenic peas.

Ali *et al.* (2005) studied the effects of temperature on oxidative stress defense systems, lipid peroxidation and lipoxygenase activity in *Phalaenopsis*. The thermal dependencies of the activities of protective enzymes, photosynthetic efficiency (Fv/Fm), protein, non-protein thiol (NP-SH), cysteine content, lipoxygenase (LOX) activity (EC 1.13.11.12) and malondialdehyde (MDA) content at 25-40°C were determined for 4, 24 and 48 hrs in leaf and root segments of *Phalaenopsis*. Temperature-stress induced not only activities of active oxygen species (AOS) scavenging enzymes but also protein, NP-SH and cysteine content in both leaf and root segments at 30°C for 4 and 24 hrs (except for 48 h in some cases) compared to 25°C and greenhouse-grown leaf and root segments indicating that antioxidants enzymes played an important role in protecting plant from temperature-stress. However, activities of dehydroascorbate reductase (DHAR, EC 1.8.5.1), glutathione peroxidase (GPX, EC 1.11.1.9) and glutathione-*S*-transferase (GST, EC 2.5.1.18) in leaf and root, glutathione reductase (GR, EC 1.6.4.2) in leaf and guaiacol peroxidase (G-POD, 1.11.1.7) in root segments were induced significantly at 40°C compared to 25°C and greenhouse-grown plants suggesting that these enzymes play protective roles at high temperature. In contrast, activities of superoxide dismutase (SOD, EC 1.15.1.1) and monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) in leaf and root, catalase (CAT, EC 1.11.1.6) in root, GR in root, and protein, cysteine, NP-SH content in both root and leaf and Fv/Fm ratio were diminished significantly at 40°C compared to 25°C and greenhouse-grown plants.

The close relation between activities of enzymes with their metabolites at 30°C than 40°C indicated that the antioxidants enzymes and metabolites both may play an important role in protecting cells against the temperature-stress.

Photosynthesis is particularly sensitive to heat stress and recent results provide important new insights into the mechanisms by which moderate heat stress reduces photosynthetic capacity. Perhaps most surprising is that there is little or no damage to photosystem II as a result of moderate heat stress even though moderate heat stress can reduce the photosynthetic rate to near zero. Moderate heat stress can stimulate dark reduction of plastoquinone and cyclic electron flow in the light. In addition, moderate heat stress may increase thylakoid leakiness. At the same time, rubisco deactivates at moderately high temperature. Relationships between effects of moderate heat on rubisco activation and thylakoid reactions are not yet clear. Reactive oxygen species such as H_2O_2 may also be important during moderate heat stress. Rubisco can make hydrogen peroxide as a result of oxygenase side reactions and H_2O_2 production by rubisco was recently shown to increase substantially with temperature. The ability to withstand moderately high temperature can be improved by altering thylakoid lipid composition or by supplying isoprene. Sharkey (2005) opined that this indicates that thylakoid reactions are important during moderate heat stress. The deactivation of rubisco at moderately high temperature could be a parallel deleterious effect or a regulatory response to limit damage to thylakoid reactions.

Net photosynthesis (Pn) is reversibly inhibited at moderately high temperature. To investigate this further, Kim and Portis (2005) examined the effects of heat stress on *Arabidopsis* plants in which Rubisco activase or thylakoid membrane fluidity had been modified. During heating leaves from 25 to 40°C at 250 ppm CO_2 and 1% O_2, the wild-type (WT), plants expressing the 43 kDa isoform only (rwt43), and plants accumulating activase 40% of WT (R100) exhibited similar inhibitions in the Pn and Rubisco activation state. Despite better membrane integrity than WT, plants having less polyunsaturation of thylakoid lipids (*fad7/8* double mutant) failed to maintain greater Pn than the WT. Plants expressing the 46 kDa isoform only (rwt46) exhibited the most inhibition, but plants expressing a 46 kDa isoform incapable of redox regulation (C411A) were similar to the WT. The null mutant (*rca*) exhibited a continuous decline in Pn. As measured by fluorescence,

electron transport activity decreased concomitantly with Pn but PSII was not damaged. Following a quick recovery to 25 from 40°C, whereas most lines recovered 90% Pn, the rwt46 and *rca* lines recovered only to 59 and <10%, respectively. As measured by NADP-malate dehydrogenase activation, after an initial increase at 30°C, stromal oxidation in the WT and rwt46 plants did not increase further as Pn decreased. These results provide additional insight into the role of Rubisco activation and activase in the reversible heat inhibition of Pn.

Gupta and Gupta (2005) studied the high temperature induced antioxidative defense mechanism in seedlings of contrasting wheat genotypes. Leaf discs of 15 d old seedling of wheat genotypes C-306(temperature tolerant) and HD 2329(widely adapted) were incubated at 25, 35 and 45°C to analyze the extent of membrane injury and antioxidative defense mechanisms. It is suggested that the tolerant genotype C-306 exhibited lower accumulation of MDA and H_2O_2 content owing to increased activities of superoxide dismutase, peroxidase and catalase under high temperature conditions. The higher water retention capacity and lower membrane injury in C-306 further helped in impairing high temperature tolerance. The HD 2329 was also able to resist high temperature stress to some extent via above adjustments.

Yang *et al.* (2005) genetically engineered tobacco (*Nicotiana tabacum*) with the ability to synthesis glycinebetaine by introducing the BADH gene for betaine aldehyde dehydrogenase from spinach (*Spinacia oleracea*). The genetic engineering enabled the plants to accumulate glycinebetaine mainly in chloroplasts and resulted in enhanced tolerance to high temperature stress during growth of young seedlings. Moreover, CO_2 assimilation of transgenic plants was significantly more tolerant to high temperatures than that of wild-type plants. The analyses of chlorophyll fluorescence and the activation of Rubisco indicated that the enhancement of photosynthesis to high temperatures was not related to the function of photosystem II but to the Rubisco activase-mediated activation of Rubisco. Western-blotting analyses showed that high temperature stress led to the association of Rubisco activase with the thylakoid membranes from the stroma fractions. However, such an association was much more pronounced in wild-type plants than in transgenic plants. The results in this study suggest that under high temperature stress, glycinebetaine maintains the activation of Rubisco by preventing the sequestration of Rubisco

activase to the thylakoid membranes from the soluble stroma fractions and thus enhances the tolerance of CO_2 assimilation to high temperature stress. The results seem to suggest that engineering of the biosynthesis of glycinebetaine by transformation with the BADH gene might be an effective method for enhancing high temperature tolerance of plants.

Seedlings of two tomato genotypes, *Lycopersicon esculentum* Mill.var.amalia and the wild thermotolerant type Nagcarlang, were grown under a photoperiod of 16 hrs light at 25°C and 8 hrs dark at 20°C. At the fourth true leaf stage, a group of plants were exposed to a heat-shock temperature of 45°C for 3 hrs, and measurements of chlorophyll fluorescence, gas-exchange characteristics, dark respiration and oxidative and antioxidative parameters were made after releasing the stress. The heat shock induced severe alterations in the photosynthesis of Amalia that seem to mitigate the damaging impact of high temperatures by lowering the leaf temperature and maintaining stomatal conductance and more efficient maintenance of antioxidant capacity, including ascorbate and glutathione levels. These effects were not evident in Nagcarlang. In Amalia plants, a larger increase in dark respiration also occurred in response to heat shock and the rates of the oxidative processes were higher than in Nagcarlang. This suggests that heat injury in Amalia may involve chlorophyll photooxidation mediated by activated oxygen species (AOS) and more severe alterations in the photosynthetic apparatus. All these changes could be related to the more dramatic effect of heat shock seen in Amalia than in Nagcarlang plants (Camejo *et al.*, 2006).

Hikosaka *et al.* (2006) reported that growth temperature alters temperature dependence of the photosynthetic rate (temperature acclimation). In many species, the optimal temperature that maximizes the photosynthetic rate increases with increasing growth temperature. Based on the biochemical model of photosynthesis, change in the photosynthesis–temperature curve was found to be attributable to four factors: intercellular CO_2 concentration, activation energy of the maximum rate of RuBP (ribulose-1,5-*bis*phosphate) carboxylation ($V_{c\ max}$), activation energy of the rate of RuBP regeneration (J_{max}), and the ratio of J_{max} to $V_{c\ max}$. In the survey, every species increased the activation energy of $V_{c\ max}$ with increasing growth temperature. Other factors changed with growth temperature, but their responses were different

among species. Among these factors, activation energy of $V_{c\ max}$ may be the most important for the shift of optimal temperature of photosynthesis at ambient CO_2 concentrations.

The impact of heat stress on the functioning of the photosynthetic apparatus in pea (*Pisum sativum* L.) plants grown at control (25°C; 25°C-plants) or moderately elevated temperature (35°C; 35°C-plants) was analyzed by Haldimann and Feller (2006). In both types of plants net photosynthesis (Pn) decreased with increasing leaf temperature (LT) and was more than 80% reduced at 45°C as compared to 25°C. In the 25°C-plants, LTs higher than 40°C could result in a complete suppression of Pn. Short-term acclimation to heat stress did not alter the temperature response of Pn. Chlorophyll a fluorescence measurements revealed that photosynthetic electron transport (PET) started to decrease when LT increased above 35°C and that growth at 35°C improved the thermal stability of the thylakoid membranes. In the 25°C-plants, but not in the 35°C-plants, the maximum quantum yield of the photosystem II primary photochemistry, as judged by measuring the Fv/Fm ratio, decreased significantly at LTs higher than 38°C. A post-illumination heat-induced reduction of the plastoquinone pool was observed in the 25°C-plants, but not in the 35°C-plants. Inhibition of Pn by heat stress correlated with a reduction of the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Western-blot analysis of Rubisco activase showed that heat stress resulted in a redistribution of activase polypeptides from the soluble to the insoluble fraction of extracts. Heat-dependent inhibition of Pn and PET could be reduced by increasing the intercellular CO_2 concentration, but much more effectively so in the 35°C-plants than in the 25°C-plants. The 35°C-plants recovered more efficiently from heat-dependent inhibition of Pn than the 25°C-plants. The results show that growth at moderately high temperature hardly diminished inhibition of Pn by heat stress that originated from a reversible heat-dependent reduction of the Rubisco activation state. However, by improving the thermal stability of the thylakoid membranes it allowed the photosynthetic apparatus to preserve its functional potential at high LTs, thus minimizing the after-effects of heat stress.

Borjigidai *et al.* (2006) studied the effect of elevated CO_2 on temperature dependence of photosynthetic rates in rice (*Oryza sativa*) grown in a paddy field, in

relation to seasons in two years. Photosynthetic rates were determined monthly for rice grown under free-air CO_2 enrichment (FACE) compared to the normal atmosphere (570 vs 370 μmol mol^{-1}). Temperature dependence of the maximum rate of RuBP (ribulose-1,5-bisphosphate) carboxylation (V_{cmax}) and the maximum rate of electron transport (J_{max}) were analysed with the Arrhenius equation. The photosynthesis–temperature response was reconstructed to determine the optimal temperature (T_{opt}) that maximizes the photosynthetic rate. There was both an increase in the absolute value of the light-saturated photosynthetic rate at growth CO_2 (P_{growth}) and an increase in T_{opt} for P_{growth} caused by elevated CO_2 in FACE conditions. Seasonal decrease in P_{growth} was associated with a decrease in nitrogen content per unit leaf area (N_{area}) and thus in the maximum rate of electron transport (J_{max}) and the maximum rate of RuBP carboxylation (V_{cmax}). At ambient CO_2, T_{opt} increased with increasing growth temperature due mainly to increasing activation energy of V_{cmax}. At elevated CO_2, T_{opt} did not show a clear seasonal trend. Temperature dependence of photosynthesis was changed by seasonal climate and plant nitrogen status, which differed between ambient and elevated CO_2.

Heat-shock proteins (HSPs) protect cells from abiotic stresses. However, most work on HSPs in plants has been carried out in laboratory-grown crop or model species. Few studies have examined field expression of HSPs or HSP expression in response to multiple stresses that often occur simultaneously in nature. Heat stress in nature is frequently accompanied by high light, and photoinhibition is a major limitation for photosynthesis. Barua and Heckathorn (2006) reported that light induction of HSPs may help ameliorate damage from excess light. They analyzed whether accumulation of representative HSPs differed in naturally occurring *Solidago altissima* (goldenrod) in contrasting light microclimates (open sun vs. shade) and on cool vs. warm days. Their results show that HSP content in field-grown plants, undergoing natural temperature stress, was greater in open sun than shaded environments. Supporting these results, both light and temperature significantly affected accumulation of HSPs in the laboratory. This was the first study to show that the interaction of light microclimate and temperature can significantly influence HSP accumulation in field-grown plants.

Porch (2006) screened 14 genotypes of common bean for heat tolerance in both the greenhouse and field in Puerto Rico using previously developed stress indices. A total of three sets of paired trials were conducted in the field and in the greenhouse under high temperature (stress) and lower temperature (low-stress) conditions. The geometric mean (GM), stress tolerance index (STI) and stress susceptibility index (SSI) were used to evaluate the genotypic performance under stress and low-stress conditions. The results indicate that it was possible to identify superior genotypes for heat tolerance based on their stress indices. Porch (2006) also suggested that in this evaluation of heat tolerance indices, STI and GM, although correlated, were found to be effective stress indices for the selection of genotypes with good yield potential under stress and low-stress conditions.

Oxidative damage resulting from temperature extremes was studied by Mahan and Mauget. (2006) in cotton (*Gossypium hirsutum* L.) cultivar Fibermax 958. Cultivars were planted at Lubbock, TX, in 2003 and 2004 to investigate the effect of low and high temperatures on oxidative stress and antioxidant metabolism in seedlings exposed to normal thermal variation. Early and late plantings in 2003 provided seedlings of different ages for comparisons. Malondialdehyde was slightly increased in response to low temperatures indicating some oxidative damage in the seedlings. The activities of ascorbate peroxidase and glutathione reductase were not altered in response to low or high temperatures. The glutathione pool was predominately reduced in all plantings in both years indicating sufficient reduced glutathione. It was therefore concluded that antioxidant metabolism in the seedlings was sufficient to mitigate oxidative damage with only minor alterations.

Wang *et al.* (2006) determined the effect of high temperature stress during reproductive development on pod fertility, seed set, and seed yield of chickpea (*Cicer arietinum* L). They grew 'Myles' desi and 'Xena' kabuli chickpea in a controlled environment under 20/16°C day/night air temperatures (control). High (35/16°C) and moderate (28/16°C) temperature stresses were imposed for 10 days during early flowering and pod development. Compared to the control, the early flower high temperature stress decreased ($P < 0.01$) pod production by 34% for Myles and 22% for Xena, whereas high temperature stress during pod development decreased ($P < 0.05$) seeds per plant by 33% for Myles and 39% for Xena.

Consequently, the high temperature stress during pod development decreased ($P < 0.01$) seed yield by 59% for Myles and 53% for Xena. Yield reduction was greater due to the stress during pod development compared to the stress during early flowering. Plants recovered to a greater degree from the early flower stress compared to the pod development stress. The Myles desi produced 40 seeds per plant and the Xena kabuli produced 15 seeds per plant, whereas the Myles had smaller individual seed size than the Xena. Consequently, the Myles desi produced 26% greater seed yield than the Xena kabuli under the same conditions. Minimizing the exposure of chickpea to high temperature stress during pod development will increase pod fertility, seed set, and seed yield of the crop.

2.2 Metabolic changes associated with induction of thermotolerance

Heat stress responses are widely conserved among different organisms. Thermotolerance can be developed as plants acclimate to a non lethal high temperature. During heat stress in plants, as in other organisms, gene expression patterns, including transcription and translation, are altered to promote the accumulation of HSPs. The induced expression of HSPs is correlated with development of a thermotolerant state. Thermotolerance represents a property of all living cells and refers to the capacity of cells to survive or recover from normally lethal exposures to abrupt, severe heat shock, if, before the lethal stress, the cells are exposed to milder or short period of heat stress condition. Previous studies have shown that plants acquire thermotolerance under conditions that induce the accumulation of HSPs. Timed temperature treatment which induced a thermotolerant state also induced the expression of HSPs. In a study with *Arabidopsis thaliana* plants containing antisense DNA sequence that reduces HSP 70 synthesis Lee and Schoeffl (1996) showed that the high temperature extreme at which the plants could survive was reduced by 2°C compared with controls, although the mutant plants grew normally at optimum temperature. Presumably failure to synthesize the entire range of HSPs that are usually induced in plants would lead to a much more dramatic loss of thermotolerance. Other studies with both *Arabidopsis* mutants and transgenic plants demonstrate that at least HSP 101 is a critical component of both induced and constitutive thermotolerance in plants.

Rao *et al.* (1997) investigated how salicylic acid (SA) enhances H_2O_2 and the relative significance of SA enhanced H_2O_2 in *Arabidopsis thaliana*. SA treatments enhanced H_2O_2 production, lipid peroxidation, and oxidative damage to proteins, and resulted in the formation of chlorophyll and carotene isomers. SA-enhanced H_2O_2 levels were related to increased activities of Cu, Zn-superoxide dismutase and were independent of changes in catalase and ascorbate peroxidase activities. Prolonging SA treatments inactivated catalase and ascorbate peroxidase and resulted in phytotoxic symptoms, suggesting that inactivation of H_2O_2 degrading enzymes serves as an indicator of hypersensitive cell death. Treatment of leaves with H_2O_2 alone failed to invoke SA-mediated events. Although leaves treated with H_2O_2 accumulated *in vivo* H_2O_2 by 2 fold compared with leaves treated with SA, the damage to membranes and proteins was significantly less, indicating that SA can cause greater damage than H_2O_2. However, pretreatment of leaves with dimethylthiourea, a trap for H_2O_2, reduced SA-induced lipid peroxidation, indicating that SA requires H_2O_2 to initiate oxidative damage.

Hormones govern all aspects of plant metabolism. When plants are subjected to heat stress during vegetative growth stage, among other things, it alters hormone homeostasis, including hormone stability, content, biosynthesis and compartmentalization. Abscisic acid (ABA) is implicated in induction of HSPs, plant osmotic stress response and mediates one of the intracellular dehydration signaling pathways. In the field, where heat and drought stresses frequently occur simultaneously, ABA induction can be an important component of thermotolerance. Indeed, in maize (*Zea mays*), exogenously applied ABA has been reported by Gong *et al.* (1998a) to mimic water stress in increasing thermotolerance of photosystem II; additional application of calcium in maize also acted synergistically with ABA (Gong *et al.*, 1998a).

Exposure of plants to elevated temperatures results in a complex set of changes in gene expression that induce thermotolerance and improve cellular survival to subsequent stress. Gong *et al.* (1998b) reported that pretreatment of young tobacco (*Nicotiana plumbaginifolia*) seedlings with Ca^{2+} or ethylene glycol-bis (aminoethylether)-N,N,N,N-tetraacetic acid enhanced or diminished subsequent thermotolerance, respectively, compared with untreated seedlings, suggesting a

possible involvement of cytosolic $^{Ca2+}$ in heat-shock (HS) signal transduction. Using tobacco seedlings transformed with the Ca^{2+}-sensitive, luminescent protein aequorin, they observed that HS temperatures induced prolonged but transient increases in cytoplasmic but not chloroplastic Ca^{2+}. A single HS initiated a refractory period in which additional HS signals failed to increase cytosolic Ca^{2+}. However, throughout this refractory period, seedlings responded to mechanical stimulation or cold shock with cytosolic Ca^{2+} increases similar to untreated controls. These observations suggest that there may be specific pools of cytosolic Ca^{2+} mobilized by heat treatments or that the refractory period results from a temporary block in HS perception or transduction. Use of inhibitors suggests that HS mobilizes cytosolic Ca^{2+} from both intracellular and extracellular sources.

Lopez-Delgado *et al.* (1998) reported the induction of thermotolerance in potato microplants by acetylsalicylic acid and H_2O_2. They subjected potato microplants propagated as nodal explants to heat treatments *in vitro* similar to those employed in the thermotherapy step of virus eradication procedures.Low concentrations (10^{-6}-10^{-5} M) of acetyl salicylic acid (ASA) in the culture medium improved (by 3-7 fold) tolerance of a 5 week high temperature (35°C) treatment. Furthermore, tissues subcultured on ASA-free medium following several weeks of growth on ASA were more thermotolerant (by 3-8 folds) to a 7 week 35°C treatment, and (by 3-8 folds) to 15h 42°C heat-shock. Stems of microplants grown on ASA contained significantly less catalase activity and higher levels of H_2O_2 than controls. Explanting and heat treatment, however, reduced catalase activity to similar levels in ASA-treated and control microplant tissues. To confirm the role of H_2O_2 in induction of thermotolerance, nodal explants were incubated for 1hr in H_2O_2 (0.1-50 mM), and then cultured under standard conditions. The microplants that grew from H_2O_2-treated explants showed concentration dependent decreases in stem height, but were significantly more thermotolerant than control, more than 1 month after the H_2O_2 treatment further confirming the direct roles of ASA and H_2O_2 in thermotolerance.

Heat acclimation and salicylic acid treatment were earlier shown to induce thermotolerance in mustard (*Sinapsis alba* L.) by Dat *et al.*(1998a). In seedlings subjected to 1 hour of heat acclimation treatment glucosylated SA increased 5.5 fold

and then declined during the next 6 hrs. Increases in SA were smaller (2 fold) but significant. Changes in antioxidants revealed that the reduced to oxidized ascorbate ratio was 5 fold lower than in controls after 1 hr of treatment. Glutathione reductase (GR) activity in treated samples was found to be more than 50% higher during the first 2 hrs of treatment. Activities of dehydroascorbate reductase decreased by at least 25% during the first 2 hrs but were 20% to 60% higher in treated samples than in control leaves after 3-6 hrs. Ascorbate peroxidase (APOX) activity was found to be 30% higher after one hour of heat treatment. Young leaves appeared to be better protected by antioxidant enzymes following heat acclimation than the cotyledons or stem. Changes in endogenous SA and antioxidants may be involved in heat acclimation.

Dat *et al.* (1998b) also reported that spraying mustard (*Sinapis alba* L.) seedlings with salicylic acid (SA) solutions between 10 and 500 µm significantly improved their tolerance to a subsequent heat shock at 55°C for 1.5 hr. The effects of SA were concentration dependent, with higher concentrations failing to induce thermotolerance. The time course of thermotolerance induced by 100 µm SA was similar to that obtained with seedlings acclimated at 45°C for 1 hr. Heat shock at 55°C caused a significant increase in endogenous H_2O_2 and reduced catalase activity. A peak in H_2O_2 content was observed within 5 min of either SA treatment or transfer to the 45°C acclimation temperature. Between 2 and 3 hrs after SA treatment or heat acclimation, both H_2O_2 and catalase activity significantly decreased below control levels. The lowered H_2O_2 content and catalase activity occurred in the period of maximum thermoprotection. It is suggested that thermoprotection obtained either by spraying SA or by heat acclimation may be achieved by a common signal transduction pathway involving an early increase in H_2O_2.

Trofimova *et al.* (1999) studied the effect of Ca^{2+} on heat shock induced changes in the cell protein synthesis incorporation of 35 S methionine into protein in cultured sugarbeet (*Beta vulgaris* L.) cells incubated in the media containing different Ca^{2+} concentrations. Heat shock inhibited the synthesis of non-heat shock proteins and promoted the synthesis of set of HSPs, typical of plants. The synthesis of non-HSPs was found to be greatly inhibited by external Ca^{2+} removal by treatment of the cells with ethylene glycol-bis (beta aminoethylether)–N, N, N', N' tetra acetic

acid. In contrast, extracellular Ca^{2+} appeared to be not strictly required for the de novo synthesis of HSPs. Cell injury increased if the cells were exposed simultaneously to high temperature and Ca^{2+} deficent medium. Recovery of HSP synthesis and reduced cell injury were observed after addition of exogenous Ca^{2+} to Ca^{2+} depleted cells. These findings were consistent with the findings of the other workers who also mentioned the requirement of Ca^{2+} for the survival of cells under heat shock and the involvement of Ca^{2+} ions in the development of thermotolerance.

It has been demonstrated that treatment with 24-epibrassinolide, a brassinosteroid increases the basic thermotolerance of *Brassica napus* and tomato seedlings. Recent studies have shown that brassinosteroids are essential for proper plant development. Several lines of evidences suggest that in addition to their role in plant development brassinosteroids exert anti stress effects on plants. However the mechanisms by which they modulate plants' stress responses are not known. *Brassica napus* and tomato seedlings grown in presence of 24-epibrassinolide (EBR) were found to be more tolerant to lethal temperature treatment than control plants grown in absence of EBR. Since a pre- conditioning treatment with this compound was not required to observe the effect, it was concluded that EBR treatment incrases the basic thermotolerance of seedlings. Analysis of HSPs in seedlings by Western Blot analysis indicated that HSPs did not accumulate preferentially in EBR treated seedlings at control temperature. However, after heat stress, HSP accumulation was higher in EBR treated seedlings than in untreated seedlings. The results of present study provide the first direct evidence for EBR induced accumulation of HSPs. The higher accumulation of HSPs in EBR treated seedlings raises the possibility that HSPs contribute at least in part to thermotolerance in EBR treated seedlings. A search for factors other than HSps, which may directly or indirectly contribute to brassinosteroid mediated increase in thermotolerance is underway (Dhaubadel *et al.*,1999).

Hardin *et al.*(1999) demonstrated root membrane thermostability in *Cornus florida* L. using flowering dogwood seeds from different heat zones. The unsuberised, current season, fine root tissues were subjected to temperature treatment ranging from 20°C to 60°C for 30 minutes and analyzed for cellular electrolyte leakage. Electrolyte leakage from root tissue exhibited a sigmoidal

response to temperatures for trees from each location. Critical mid point temperature (T_m) was found to be greater for seedlings native to USDA hardiness zone 6b (AHS heat zone 7, 52.4 ± 0.6°C) than T_m for seedlings originating from USDA zone 7a (AHS heat zone 51.2 ± 0.5°C). However seedlings from USDA zone 8a (AHS 8 zone) at 51.5 ± 0.4°C were smaller to those collected in USDA zones 6b (AHS zone 7). The results of this study found little genetic variability across this part of the native range of flowering dogwood regarding root thermotolerance.

Queitsch *et al.* (2000) reported that transgenic *Arabidopsis* plants expressing less than usual amounts of HSP101, a result of either antisense inhibition or cosuppression, grew at normal rates but had a severely diminished capacity to acquire heat tolerance after mild conditioning pretreatments. The naturally high tolerance of germinating seeds, which express HSP101 as a result of developmental regulation, was also profoundly decreased. Conversely, plants constitutively expressing HSP101 tolerated sudden shifts to extreme temperatures better than did vector controls. Therefore, it was concluded that HSP101 plays a pivotal role in heat tolerance in *Arabidopsis*.

A screening method, based on hypocotyl elongation, for mutants of *Arabidopsis thaliana* that were unable to acquire thermotolerance to high-temperature stress was developed by Hong *et al.* (2000). They have defined four separate genetic loci, *hot1-4*, required for this process *hot1* was found to have a mutation in the heat shock protein 101 (Hsp101) gene, converting a conserved Glu residue in the second ATP-binding domain to a Lys residue, a mutation that is predicted to compromise Hsp101 ATPase activity. In addition to exhibiting a thermotolerance defect as assayed by hypocotyl elongation, 10-day-old *hot1* seedlings were also unable to acquire thermotolerance, and *hot1* seeds had greatly reduced basal thermotolerance. Complementation of *hot1* plants by transformation with wild-type Hsp101 genomic DNA restored *hot1* plants to the wild-type phenotype. The *hot* mutants are the first mutants defective in thermotolerance that have been isolated in a higher eukaryote, and *hot1* represents the first mutation in an Hsp in any higher plant. The phenotype of *hot1* also provides direct evidence that Hsp101, which is required for thermotolerance in bacteria and yeast, is also essential for thermotolerance in a complex eukaryote.

Jiang and Huang (2001) suggested that calcium (Ca^{2+}) may be involved in plant tolerance to heat stress by regulating antioxidant metabolism or/and water relations. They examined whether external Ca^{2+} treatment would improve heat tolerance in two C(3), cool-season grass species, tall fescue (*Festuca arundinacea* L.) and Kentucky bluegrass *(Poa pratensis* L.), and determined the physiological mechanisms of Ca^{2+} effects on grass tolerance to heat stress. Grasses were treated with $CaCl_2$ (10 mM) or H_2O by foliar application and then exposed to heat stress (35/30°C) in growth chambers. Some of the Ca^{2+} untreated plants were maintained at 20/15°C as the temperature control. Heat stress reduced grass quality, relative water content (RWC), and chlorophyll (Chl) content of leaves in both species, but Ca^{2+} treatment increased all three factors under heat stress. The Ca^{2+} concentration in cell saps increased with heat stress and with external Ca^{2+} treatment in both species. Osmotic potential increased with heat stress, but external Ca^{2+} treatment had no effect. Osmotic adjustment increased during short-term heat stress, but then decreased with a prolonged period of stress; it was not influenced by Ca^{2+} treatment. The activity of superoxide dismutase (SOD) in both species increased transiently at 12 days of heat stress and then remained at a level similar to that of the control. External Ca^{2+} treatment had no effect on SOD activity. The activities of catalase (CAT), ascorbate peroxidase (AP), and glutathione reductase (GR) of both species decreased during heat stress. Plants treated with Ca^{2+} under heat stress had higher CAT, GR and AP activities than untreated plants. Lesser amounts of malondialdehyde (MDA) accumulated in Ca^{2+} treated plants than in untreated plants during extended periods of heat stress. The results suggested that exogenous Ca_2, treatment enhanced heat tolerance in both tall fescue and Kentucky bluegrass. This enhancement was related to the maintenance of antioxidant activities and a decrease in membrane lipid peroxidation, but not to the regulation of osmotic potential and osmotic adjustment.

Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage were studied in leaves of maize (*Zea mays* L.) seedlings by Jiang and Zhang (2001). Seedlings were supplied with different concentrations of abscisic acid (ABA) and its effects on the levels of superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the content of catalytic Fe, the activities

of several antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), the contents of several non-enzymatic antioxidants such as ascorbate (ASC), reduced glutathione (GSH), alpha-tocopherol (α-TOC) and carotenoid (CAR), and the degrees of the oxidative damage to the membrane lipids and proteins were examined. Treatment with 10 and 100 μM ABA significantly increased the levels of O^{2-} and H_2O_2 followed by an increase in activities of SOD, CAT, APX and GR, and the contents of ASC, GSH, α-TOC and CAR in a dose- and time-dependent pattern in leaves of maize seedlings. An oxidative damage expressed as lipid peroxidation, protein oxidation, and plasma membrane leakage did not occur except for a slight increase with 100μM ABA treatment for 24 hrs. Treatment with 1,000μM ABA led to a more abundant generation of O^{2-} and H_2O_2 and a significant increase in the content of catalytic Fe, which is critical for H_2O_2 dependent hydroxyl radical production. The activities of these antioxidative enzymes and the contents of α-TOC and CAR were still maintained at a higher level, but no longer further enhanced when compared with the treatment of 100 μ M ABA. The contents of ASC and GSH had no changes in leaves treated with 1,000 μM ABA. These results indicate that treatment with low concentrations of ABA (10 to 100 μM) induced an antioxidative defence response against oxidative damage, but a high concentration of ABA (1,000 μM) induced an excessive generation of AOS and led to an oxidative damage in plant cells.

Roles of abscissic acid (ABA) in water stress-induced oxidative stress were further investigated by Jiang and Zhang (2002) in leaves of maize (*Zea mays* L.) seedlings exposed to water stress induced by polyethylene glycol (PEG 6000). Treatment with PEG at -0.7 MPa for 12 and 24 hrs led to a reduction in leaf relative water content (RWC) by 7.8 and 14.1%, respectively. The mild water stress caused significant increases in the generation of superoxide radical (O2-) and hydrogen peroxide (H_2O_2), the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) and the contents of ascorbate (ASC), reduced glutathione (GSH). The moderate water stress failed to further enhance the capacity of antioxidant defense systems, as compared to the mild water stress. The contents of catalytic Fe, which is critical for H_2O_2-dependent hydroxyl radical (OH) production, and the oxidized forms of ascorbate and

glutathione pools, dehydroascorbate (DHA) and oxidized glutathione (GSSG), markedly increased a significant oxidative damage to lipids and proteins took place under the moderate water stress. Pretreatment with ABA caused an obvious reduction in the content of catalytic Fe and significant increases in the activities of antioxidant enzymes and the contents of non-enzymatic antioxidants, and then significantly reduced the contents of DHA and GSSG and the degrees of oxidative damage in leaves exposed to the moderate water stress. Pretreatment with an ABA biosynthesis inhibitor, tungstate, significantly suppressed the accumulation of ABA induced by water stress, reduced the enhancement in the capacity of antioxidant defense systems, and resulted in an increase in catalytic Fe, DHA and GSSG, and oxidative damage in the water-stressed leaves. These effects were completely prevented by addition of ABA, which raised the internal ABA content. These data indicate that ABA plays an important role in water stress-induced antioxidant defense against oxidative stress.

Protection against heat stress-induced oxidative damage in *Arabidopsis* involving calcium, abscisic acid, ethylene and salicylic acid were reported by Larkindale and Knight (2002). Heat caused increased thiobarbituric acid reactive substance levels and reduced survival. Both effects required light and were reduced in plants that had acquired thermotolerance through a mild heat pre-treatment. Calcium channel blockers and calmodulin inhibitors increased the effects of heating and added calcium reversed them, implying that protection against heat-induced oxidative damage in *Arabidopsis* requires calcium and calmodulin. Similar to calcium, SA, 1- aminocyclopropane-1-carboxylic acid and ABA added to plants protected them from heat-induced oxidative damage. The ethylene-insensitive mutant etr-1, the ABA-insensitive mutant abi, and a transgenic line expressing nahG (consequently inhibited SA production) showed increased susceptibility to heat. These data suggest that protection against heat-induced oxidative damage also involves ethylene, ABA and SA. Measurement of cytosolic calcium level during heating revealed that calcium peaks were greater in thermotolerant plants, implying that these calcium signals might play a role in mediating the effects of acquired thermotolerance.

Results of growth responses to cytokinin indicate that heat stress injury in creeping bentgrass (*Agrostis palustris* L.) can be alleviated to some extent by injection of cytokinin to the root zone (Liu and Huang,,2002). Creeping bentgrass was exposed to three air/soil temperature regimes for 56 days in growth chambers: (i) low air and soil temperature control (20/20°C); (ii) high soil temperature (20/35°C); and (iii) high air/soil temperatures (35/35°C). Four different concentrations (0.01, 0.1, 1, and 10 μmol) of zeatin riboside (ZR) or water (control) were injected into the root zone (0 to 5 cm depth) of plants on the day before heat stress was imposed (0 day) and 14 days after. Leaf electrolyte leakage (EL) and the content of a lipid peroxidation product, malondialdehyde (MDA), increased, whereas leaf chlorophyll content and activities of superoxide dismutase (SOD) and catalase (CAT) decreased at 20/35°C or 35/35°C for ZR-untreated plants. Exogenous ZR significantly suppressed these responses under both high temperature regimes. Application of 10 μmol ZR was most effective in slowing leaf senescence and alleviating heat-induced lipid peroxidation of cell membranes, followed by 1 μmol at 35/35°C. Applying 0.1 and 0.01 μmol ZR had no effects on creeping bentgrass responses to 20/35 or 35/35°C. The results therefore, suggested the alleviating effects of ZR at 1and 10 μmol in heat injury to creeping bentgrass was related to the inhibition of lipid peroxidation and slowing leaf senescence.

Mitogen-activated protein kinases (MAPKs) appear to be ubiquitously involved in signal transduction during eukaryotic responses to extracellular stimuli. In plants, no heat shock-activated MAPK has so far been reported. Also, whereas cold activates specific plant MAPKs such as alfalfa SAMK, mechanisms of such activation are unknown. Sangwan *et al.* (2002) reported the presence of heat shock-activated MAPK (HAMK) immunologically related to ERK (Extracellular signal-Regulated Kinase) superfamily of protein kinases. Molecular mechanisms of heat-activation of HAMK and cold-activation of SAMK were investigated. It was found that cold-activation of SAMK requires membrane rigidification, whereas heat-activation of HAMK occurs through membrane fluidization. The temperature stress and membrane structure-dependent activation of both SAMK and HAMK is mimicked at 25°C by destabilizers of microfilaments and microtubules, latrunculin B and oryzalin, respectively; but is blocked by jasplakinolide, a stabilizer of actin

microfilaments. Activation of SAMK or HAMK by temperature, chemically modulated membrane fluidity, or by cytoskeleton destabilizers is inhibited by blocking the influx of extracellular calcium. Activation of SAMKor HAMK is also prevented by an antagonist of calcium-dependent protein kinases (CDPKs). In summary, the datas indicate that cold and heat are sensed by structural changes in the plasma membrane that translates the signal via cytoskeleton, Ca^{2+} fluxes and CDPKs into the activation of distinct MAPK cascades.

Bowen *et al.* (2002) demonstrated that the heat shock response is also involved in thermotolerance in suspension- cultures cells of apple fruit. When cultured apple cells (*Malus domestica*) were heat-treated at temperatures from 24 to 42°C , an increase in expression of heat shock protein mRNA transcripts was detected within 5°C of the culture growth temperature. An increase in the expression of HSP transcripts was also associated with a 1hr 38°C heat pre-treatment that made the apple cells tolerant to a subsequent 1h 42°C lethal heat treatment.

Panchuk *et al.* (2002) studied the effects of elevated growth temperatures and heat stress on the activity and expression of ascorbate peroxidase (APX). They compared wild-type Arabidopsis with transgenic plants over expressing heat shock transcription factor 3 (HSF3), which synthesize heat shock proteins and are improved in basal thermotolerance. Following heat stress, APX activity was positively affected in transgenic plants and correlated with a new thermostable isoform, APX^S. This enzyme was present in addition to thermolabile cytosolic APX1, the prevalent isoform in unstressed cells. In HSP3-transgenic plants, APX^S activity was detectable at normal temperature and persisted after severe heat stress at 44°C. In nontransgenic plants, APX^S was undetectable at normal temperature, but could be induced by moderate heat stress. The mRNA expression profiles of known and three new Apx genes were determined using real-time PCR. Apx1 and Apx2 genes encoding cytosolic APX were heat stress and HSF dependently expressed but only the representations of Apx2 mRNA met the criteria that suggest identity between APX^S and APX2: not expressed at normal temperature in wild type, strong induction by heat stress, and HSF3-dependent expression in transgenic plants. Their data suggest that Apx2 is a novel heat shock gene involved in thermoprotection and

that the enzymatic activity of APX2/APX^{s} is required to compensate heat stress dependent decline of APX1 activity in the cytosol.

Hu and Kloepper (2003) tested the hypothesis that some Plant growth-promoting rhizobacteria (PGPR) strains can activate heat stress tolerance. Two-week old tomato plants with or without PGPR treatment were subjected to heat stress with or without heat pre-treatment, which can activate thermotolerance. Some PGPR strains increased the tomato seedling survival rate and enhanced the shoot weight under heat stress conditions. These results suggest that the response of PGPR-treated plants subjected to heat stress mimicked the classic heat- shock response.

Salicylic acid (SA) is reported to protect plants from heat shock (HS), but insufficient is known about its role in thermotolerance or how this relates to SA signaling in pathogen resistance. Clarke *et al.* (2004) tested thermotolerance and expression of pathogenesis-related (PR) and HS proteins (HSPs) in *Arabidopsis thaliana* genotypes with modified SA signaling: plants with the SA hydroxylase NahG transgene, the non expresser of PR proteins (npr1) mutant, and the constitutive expressers of PR proteins (cpr1 and cpr5) mutants. At all growth stages from seeds to 3-week-old plants, role of SA-dependent signaling in basal thermotolerance (i.e. tolerance of HS without prior heat acclimation) was evident. Endogenous SA correlated with basal thermotolerance, with the SA-deficient NahG and SA-accumulating cpr5 genotypes having lowest and highest thermotolerance, respectively. SA promoted thermotolerance during the HS itself and subsequent recovery. Recovery from HS apparently involved an NPR1-dependent pathway but thermotolerance during HS did not. SA reduced electrolyte leakage, indicating that it induced membrane thermoprotection. PR-1 and Hsp17.6 were induced by SA or HS, indicating common factors in pathogen and HS responses. SA-induced Hsp17.6 expression had a different dose–response to PR-1 expression. HS-induced Hsp17.6 protein appeared more slowly in NahG. However, SA only partially induced HSPs. Hsp17.6 induction by HS was more substantial than by SA, and we found no SA effect on Hsp101 expression. All genotypes, including NahG and npr1, were capable of expression of HSPs and acquisition of HS tolerance by prior heat acclimation. Although SA promotes basal thermotolerance, it is not essential for acquired thermotolerance. Salicylic acid

(SA) is reported to protect plants from heat shock (HS), but insufficient is known about its role in thermotolerance or how this relates to SA signaling in pathogen resistance. Clarke *et al.* (2004) tested thermotolerance and expression of pathogenesis-related (PR) and HS proteins (HSPs) in *Arabidopsis thaliana* genotypes with modified SA signaling: plants with the SA hydroxylase *NahG* transgene, the nonexpresser of PR proteins (*npr1*) mutant, and the constitutive expressers of PR proteins (*cpr1* and *cpr5*) mutants. At all growth stages from seeds to 3-week-old plants, for SA-dependent signaling in basal thermotolerance (i.e. tolerance of HS without prior heat acclimation) was evident. Endogenous SA correlated with basal thermotolerance, with the SA-deficient *NahG* and SA-accumulating *cpr5* genotypes having lowest and highest thermotolerance, respectively. SA promoted thermotolerance during the HS itself and subsequent recovery. Recovery from HS apparently involved an NPR1-dependent pathway but thermotolerance during HS did not. SA reduced electrolyte leakage, indicating that it induced membrane thermoprotection. PR-1 and Hsp17.6 were induced by SA or HS, indicating common factors in pathogen and HS responses. SA-induced Hsp17.6 expression had a different dose–response to PR-1 expression. HS-induced Hsp17.6 protein appeared more slowly in *NahG*. However, SA only partially induced HSPs. Hsp17.6 induction by HS was more substantial than by SA, and we found no SA effect on Hsp101 expression. All genotypes, including *NahG* and *npr1*, were capable of expression of HSPs and acquisition of HS tolerance by prior heat acclimation. Although SA promotes basal thermotolerance, it is not essential for acquired thermotolerance.

Larkindale and Huang (2004) investigated whether pre-treating plants with specific putative signaling components and heat acclimation would induce tolerance of a cool-season grass, creeping bentgrass (*Agrostis stolonifera* var. palustris), to subsequent heat stress and whether thermotolerance induction of those pretreatments was associated with the regulation of antioxidant regenerating enzymes. The treatments included foliar application of salicylic acid (SA), abscisic acid (ABA), calcium chloride ($CaCl_2$), hydrogen peroxide (H_2O_2), 1-aminocyclopropane-1-carboxylic acid (ACC, a precursor of ethylene prior to the exposure of plants to heat stress (35°C) in a growth chamber. Physiological

measurements including turf quality, leaf photosynthetic rate, and levels of oxidative damage demonstrated that all treatments increased heat tolerance. The better heat tolerance for pre-treated plants as compared to controls was related to the protection of oxidative damage under heat stress. APX activity increased over the first 2 days and 5 days of heating for ACC and $CaCl_2$ respectively, but for only 12 hrs for H_2O_2. SA and ABA pre-treatments had no effects on APX activity earlier, but maintained APX activity at a significantly higher level than in controls after 24 hrs of heating. SA and ABA pre-treatments had no effects on POX activity. ACC treatment significantly increased POX activity. Pre-treatment with $CaCl_2$, H_2O_2, and HA reduced POX activity, particularly during the later phase of heating. Plants treated with SA, $CaCl_2$, H_2O_2 and HA had lower CAT activity than their control plants prior to heating and within 48 hrs of heat stress. ABA and ACC pre-treatments maintained higher CAT activity than the controls after 48 hrs of heating. ACC, $CaCl_2$, or HA pre-treatments increased SOD activity only before 5 days of heat stress. SA and ABA pre-treatments had less effect on APX activity earlier under heat stress. These results suggest that specific groups of potential signaling molecules may induce tolerance of creeping bentgrass to heat stress by reducing oxidative damage.

Larkindale and Huang (2005) suggested that signaling molecules like abscisic acid (ABA), salicylic acid (SA), ethylene, and hydrogen peroxide (H_2O_2) are involved in the regulation of plant responses to heat stress in creeping bentgrass (*Agrostis stolonifera*). The effects of treatment with ABA, SA, H_2O_2, and ACC (an ethylene precursor) on physiological damage occurring in creeping bentgrass during heat stress (35°C for 1 month) and the effects of chemical application and the induction of thermotolerance using moderate heat stress (30°C for 24 hrs) were compared by the authors. All of the pre-treatments (heat or chemical) resulted in increased tolerance to prolonged heat stress (1 month) compared to control plants. All treated samples showed more green leaves, decreased membrane leakage and reduced oxidative damage compared to control plants. An oxidative burst was detected 5 min after the initiation of heat treatment, with the increase in H_2O_2 being detected primarily in the apoplast of the cells in both leaf and root tissues. Free SA was detected only an hour after the initiation of heat stress, and concentration

remained low subsequently. Neither ABA nor ethylene concentrations rose during heat stress, but the concentration of both increased during subsequent cooling. These results suggest that the signaling components of interest are involved in thermotolerance in creeping bentgrass, but that the different chemicals are likely to be involved in separate signaling pathways. An oxidative burst and SA may be bona fide heat stress signals, but ABA and ethylene appear to be involved in signaling pathways in response to recovery from heat stress in this species.

Larkindale *et al.* (2005) also further investigated the importance of different processes to heat stress tolerance, in 45 *Arabidopsis thaliana* mutants and one transgenic line for basal and acquired thermotolerance at different stages of growth. Plants tested were defective in signaling pathways (abscisic acid, salicylic acid, ethylene, and oxidative burst signaling) and in reactive oxygen metabolism (ascorbic acid or glutathione production, catalase) or had previously been found to have temperature-related phenotypes (e.g. fatty acid desaturase mutants, uvh6). Mutants were assessed for thermotolerance defects in seed germination, hypocotyl elongation, root growth, and seedling survival. Fifteen mutants showed significant phenotypes. Abscisic acid (ABA) signaling mutants (abi1 and abi2) and the UV-sensitive mutant, uvh6, showed the strongest defects in acquired thermotolerance of root growth and seedling survival. Mutations in nicotinamide adenine dinucleotide phosphate oxidase homolog genes (atrbohB and D), ABA biosynthesis mutants (aba1, aba2, and aba3), and NahG transgenic lines (salicylic acid deficient) showed weaker defects. Ethylene signaling mutants (ein2 and etr1) and reactive oxygen metabolism mutants (vtc1, vtc2, npq1, and cad2) were more defective in basal than acquired thermotolerance, especially under high light. All mutants accumulated wild-type levels of heat shock protein 101 and small heat shock proteins. These data indicate that, separate from heat shock protein induction, ABA, active oxygen species, and salicylic acid pathways are involved in acquired thermotolerance and that UVH6 plays a significant role in temperature responses in addition to its role in UV stress.

He *et al.* (2005) reported that application of salicylic acid (SA) to the shoots and soil could improve heat tolerance of Kentucky bluegrass (*Poa pratensis* L.). Effects of SA at different concentrations (0, 0.1, 0.25, 0.5, 1, and 1.5 mmol) on heat

tolerance were examined in Kentucky bluegrass exposed to 46°C for 72 hrs in a growth chamber. Influences of SA on the production of active oxygen species (AOS), superoxide anion ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2), and activities of antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT), were also examined. Among SA concentrations, 0.25 mmol was found to be most effective in enhancing heat tolerance in Kentucky bluegrass, which was manifested by improved regrowth potential following heat stress of 72 hrs and maintenance of leaf water content at 77% during the 12 hrs stress period similar to that under normal temperature conditions. The $O_2^{\cdot-}$ generating rate increased significantly at 6 hrs of heat stress, and SOD activity increased significantly at 2 h but decreased to the control level at 6 hrs of heat stress in SA-untreated plants. The SA application suppressed the increase of $O_2^{\cdot-}$ generating rate and enhanced SOD activity significantly at 2 and 6 hrs of heat stress, respectively. The SA application decreased H_2O_2 level significantly at 2 and 12 hrs of heat stress, and increased CAT activity significantly within 12 hrs of heat stress. The results suggest that SA application enhanced heat tolerance in Kentucky bluegrass and SA could be involved in the scavenging of AOS by increasing SOD and CAT activities under heat stress. The effect of abscisic acid (ABA) on *Stylosanthes guianensis* (Aublet) Sw. and its relation to antioxidant systems under chilling stress was studied by Zhou *et al.* (2005). *Stylosanthes guianensis* seedlings were sprayed with 10 mg L^{-1} ABA or water. One day later, the plants were transferred to a 10°C growth chamber and grown for 7 days with a 12 hrs photoperiod at 160 μmol m^{-2} s^{-1} photosynthetic photon flux density. The chilling treated plants were then rewarmed to 28°C for 2 days. During the 9-days treatment, a series of enzyme activities, relative water content (RWC), and electrolyte leakage were measured on sampled leaflets. The results showed that chilling increased electrolyte leakage of both water- and ABA-treated plants, while RWC decreased under chilling conditions. ABA-treated plants had lower electrolyte leakage and higher RWC than those of water-treated plants. Activities of ascorbate peroxidase (APX) and catalase (CAT) and contents of reduced glutathione (GSH) and ascorbic acid (AsA) were transiently enhanced by ABA treatment before the plants were subject to chilling. ABA-treated *S. guianensis* retained higher levels of superoxide dismutase (SOD), APX, GSH, and AsA than

water-treated ones under chilling conditions. The results suggested that ABA-increased chilling resistance in *S. guianensis* is partially associated with enhanced scavenging systems.

Thermotolerance induced by isoprene has been assessed by Velikova and Loreto (2005), during heat bursts but there is little information on the ability of endogenous isoprene to confer thermotolerance under naturally elevated temperature, on the interaction between isoprene-induced thermotolerance and light stress, and on the persistence of this protection in leaves recovering at lower temperatures. Moderately high temperature treatment (38°C for 1.5 hrs) reduced photosynthesis, stomatal conductance, and photochemical efficiency of photosystem II in isoprene-emitting, but to a significantly lower extent than in isoprene-inhibited *Phragmites australis* leaves. Isoprene inhibition and high temperature independently, as well as together, induced lipid peroxidation, increased level of H_2O_2, and increased catalase and peroxidase activities. However, leaves in which isoprene emission was previously inhibited developed stronger oxidative stress under high temperature with respect to isoprene-emitting leaves. The heaviest photosynthetic stress was observed in isoprene-inhibited leaves exposed to the brightest illumination (1500 μmol m^2 s^{-1}) and, in general, there was also a clear additive effect of light excess on the formation of reactive oxygen species, antioxidant enzymes, and membrane damage. The increased thermotolerance capability of isoprene-emitting leaves may be due to isoprene ability to stabilize membranes or to scavenge reactive oxygen species. Irrespective of the mechanism by which isoprene reduces thermal stress, isoprene-emitting leaves are able to quickly recover after the stress. This may be an important feature for plants coping with frequent and transient temperature changes in nature.

Methionine biosynthesis has taken different evolutionary pathways in bacteria, fungi and plants. To gain insight into these differences and to search for new ways of manipulating methionine biosynthesis in plants, the yeast (*Saccharomyces cerevisiae*) Met2 gene and the bacteria (*Leptospira meyeri)* MetX gene, both encoding homoserine O-acetyltransferase, were expressed in tobacco plants by Gamrasni *et al.* (2005). They found protein aggregates in extracts of these transgenic plants, whose levels were much higher in plants grown at 35°C than at 25°C. It appears that the yeast and the

bacterial proteins are heat labile and tend to change their intracellular conformation. These conformational changes of the transgenic proteins were more prominent at high temperature and most probably triggered aggregation of the yeast and the bacterial proteins. Moreover, plants expressing the yeast gene that grew at 35°C over-accumulated stress-associated metabolites, such as phenolic compounds, including tannins, as well as the amino acid arginine. In addition, the transgenic plants expressing high levels of the foreign genes showed growth retardation, which further suggests that, these plants suffer from internal stress. The changes in protein conformation and the consequent triggering of stress response may account for the ability of these transgenic plants to tolerate more extreme heat stress (60°C) than the wild-type plants.

A primary economic concern of sod producers is loss of sod quality during the transportation and storage phases of a sale. Previous research and field experience indicate that soil and plant respiration rates, and thus the rate of pallet heating, may be reduced by harvesting in the morning, lowering mowing heights and removing clippings, and minimizing tissue nitrogen and soil moisture before harvest. However, even when proper cultural guidelines are followed, excessive sod heating and tissue damage often occurs. Various pre-and post-harvest chemical treatments aimed at protecting leaf tissue integrity during and after supraoptimal heating have shown promise for increasing transplant success. One of these compounds is the natural plant growth regulator salicylic acid (SA). This study was conducted by Ervin *et al.* (2005) to investigate the influence of pre-harvest foliar application of SA on transplant injury and root strength of tall fescue (TF; *Festuca arundinacea* Schreb.) and Kentucky bluegrass (KBG; *Poa pratensis* L.) sod following supraoptimal heating. Salicylic acid was applied at 0.5 kg ha^{-1} to the turfgrass 10 days before harvest and canopy photochemical efficiency was measured 1 day before harvest. Harvested and rolled sod was subjected to high temperature stress (38–40°C for 72 or 96 hrs), transplanted into the field, and injury and root strength were determined. Application of SA enhanced the pre-harvest canopy photochemical efficiency of KBG and TF sod in both years. Averaged over years and heat duration, SA increased canopy photochemical efficiency by 12% for KBG and 14% for TF. Salicylic acid reduced visual injury and enhanced post-harvest root strength in both

years. Averaged over years and heat duration, SA increased transplant root strength by 26% for KBG and 9% for TF. These data suggest that pre-harvest foliar SA application may improve shelf life and transplant success of supraoptimally heated cool-season sod.

Lakindale *et al.*,2005 investigated the importance of different processes to heat stress tolerance in 45 *Arabidopsis* (*Arabidopsis thaliana*) mutants and one transgenic line. Plants tested were defective in signaling pathways (abscisic acid, salicylic acid, ethylene, and oxidative burst signaling) and in reactive oxygen metabolism (ascorbic acid or glutathione production, catalase) or had previously been found to have temperature-related phenotypes (e.g. fatty acid desaturase mutants, *uvh*6). Mutants were assessed for thermotolerance defects in seed germination, hypocotyls elongation, root growth, and seedling survival. Fifteen mutants showed significant phenotypes. Abscisic acid (ABA) signaling mutants (*abi 1 and abi 2*) and the UV-sensitive mutant, *uvh6*, showed the strongest defects in acquired thermotolerance of root growth and seedling survival. Ethylene signaling mutants (*ein2 and etr1*) and reactive oxygen metabolism mutants (*vtc1, vtc2, npq1, and cad2*) were more defective in basal than acquired thermotolerance, especially under high light. All mutants accumulated wild-type levels of heat shock protein 101 and small heat shock proteins. Their data therefore, indicate that, separate from heat shock protein induction, ABA, active oxygen species, and salicylic acid pathways are involved in acquired thermotolerance and that UVH6 plays a significant role in temperature responses in addition to its role in UV stress.

The relationship between the accumulation in endogenous free salicylic acid (SA) induced by heat acclimation (37°C) and the activity of PIP2-phospholipase C (PIP2-PLC; EC 3.1.4.3) in the plasma membrane fraction was investigated in pea (*Pisum sativum* L.) leaves by Liu *et al.* (2006). Heat acclimation induced an abrupt elevation of free SA preceding the activation of PLC toward PIP2. Immunoblotting indicated a molecular mass with 66.5 kDa PLC plays key role in the development of thermotolerance in pea leaves. In addition, some characterizations of PLC toward PIP2 isolated from pea leaves with two-phase purification containing calcium concentration, pH and a protein concentration were also studied. Neomycin sulfate, a well-known PIP2-PLC inhibitor, was employed to access the involvement of PIP2-

PLC in the acquisition of heat acclimation induced-thermotolerance. They were able to identify a PIP2-PLC, which was similar to a conventional PIP2-PLC in higher plants was identified from pea leaves suggesting that PIP2-PLC was involved in the signal pathway that leads to the acquisition of heat acclimation induced-thermotolerance. On the basis of these results, it was concluded that the free SA may function as the upstream event in the stimulation of PIP2-PLC in response to heat acclimation treatment.

Plants and animals share similar mechanisms in the heat shock (HS) response, such as synthesis of the conserved HS proteins (Hsps). However, because plants are confined to a growing environment, in general they require unique features to cope with heat stress. Charng *et al.* (2006) analyzed the function of a novel Hsp, heat-stress-associated 32-kD protein (Hsa32), which is highly conserved in land plants but absent in most other organisms. The gene responds to HS at the transcriptional level in moss (*Physcomitrella patens*), *Arabidopsis* (*Arabidopsis thaliana*), and rice (*Oryza sativa*). Like other Hsps, Hsa32 protein accumulates greatly in *Arabidopsis* seedlings after HS treatment. Disruption of Hsa32 by T-DNA insertion does not affect growth and development under normal conditions. However, the acquired thermotolerance in the knockout line was compromised following a long recovery period (>24 hrs) after acclimation HS treatment, when a severe HS challenge killed the mutant but not the wild-type plants, but no significant difference was observed if they were challenged within a short recovery period. Quantitative hypocotyl elongation assay also revealed that thermotolerance decayed faster in the absence of Hsa32 after a long recovery. Similar results were obtained in *Arabidopsis* transgenic plants with Hsa32 expression suppressed by RNA interference. Microarray analysis of the knockout mutant indicates that only the expression of Hsa32 was significantly altered in HS response. Taken together, the results suggest that Hsa32 is required not for induction but rather maintenance of acquired thermotolerance, a feature that could be important to plants.

The heat shock protein ClpB is a member of the Clp family and functions as molecular chaperones. ClpB is related to the acquired thermotolerance in organisms. A cDNA of 3144 bp was screened out of a tomato cDNA library. The polypeptide deduced from the longest ORF contains 980 amino acid residues, and was classified

into HSP100/ClpB family based on the result of molecular phylogenesis analysis. Thus it was named as LeHSP110/ClpB according to its calculated molecular weight. LeHSP110/ClpB was characteristic of heat-inducibility but no constitutive expression, and was demonstrated to locate in chloroplastic stroma. An antisense cDNA fragment of LeHsp110/ClpB under the control of CaMV 35S promoter was introduced into tomato by *Agrobacterium tumefactions*-mediated method. At high temperature, the mRNA levels of LeHsp110/ClpB in antisense transgenic plants were lower than those in control plants. The PS II of transgenic plants is more sensitive to high temperature than that of control plants according to data of Fv/Fm. These results of Gong and Bao (2006) clearly showed that HSP110/ClpB plays an important role in thermotolerance of high plants.

Chen *et al.* (2006) revealed an important role of galactolipids in thermotolerance To identify the various mechanisms that plants have evolved to cope with high temperature stress, they isolated a series of *Arabidopsis* mutants that are defective in the acquisition of thermotolerance after an exposure to 38°C, a treatment that induces acquired thermotolerance in wild-type plants. One of these mutants, *atts 02*, was not only defective in acquiring thermotolerance after the treatment, but also displayed a reduced level of basal thermotolerance in a 30°C growth assay. The affected gene in *atts 02* was identified by positional cloning and encodes digalactosyldiacylglycerol synthase 1 (DGD1) (the *atts02* mutant was, at that point, renamed *dgd1-2*). An additional dgd1 allele, *dgd1-3*, was identified in two other mutant lines displaying altered acquired thermotolerance, *atts100* and *atts104*. Expression patterns of several heat shock proteins (HSPs) in heat-treated *dgd1-2* homozygous plants were similar to those from identically treated wild-type plants, suggesting that the thermosensitivity in the *dgd1-2* mutant was not caused by a defect in HSP induction. Lipid analysis of wild type and mutant plants indicated a close correlation between the ability to acquire thermotolerance and the increases in digalactosyldiacylglycerol (DGDG) level and in the ratio of DGDG to monogalactosyldiacylglycerol (MGDG). These results suggest that the DGDG level and/or the ratio of DGDG to MGDG may play an important role in basal as well as acquired thermotolerance in *Arabidopsis*.

MATERIAL AND METHODS

3.1 Plant material

3.1.1. Collection

The seeds of different genotypes of *Cicer arietinum* L. (Plate II) required for the experimental purpose were obtained from the seed germplasm bank of International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Seedlings of different genotypes were then raised from this seed stock in the experimental garden, Department of Botany, University of North Bengal.

3.1.2. Propagation

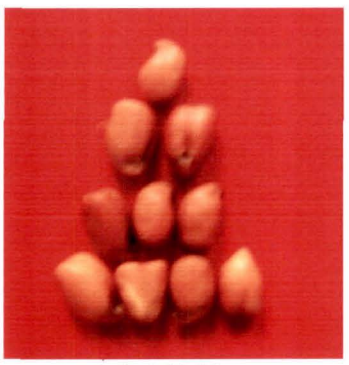
The propagation of the crop was done from the fresh seeds obtained from ICRISAT initially and then from the fresh seeds harvested (after 120-150 days of sowing) from the plants grown in the experimental garden. The seeds harvested were labeled and preserved carefully in air tight containers or sealed paper packets for future use.

3.1.3. Plantation

Chickpea seeds were sown in sandy-loam soil during middle of October to first week of November. Seeds were sown in lines at a distance of 25-30 cm between the rows and at a depth of 7-10 cm for better grain yield in the field. For pot culture, the seedlings were grown in 10 inch size clay pots containing steam sterilized soils. The soil used was thoroughly powdered and mixed with sand and farmyard manure in the proportion of 2:2:1 by volume (Plate III).

3.1.4. Maintenance

Because of slow initial growth the crop is often badly infested by weeds which suppress the crop growth and results into poor yield, therefore, one or two weedings or spraying of a herbicide was practiced for increasing the yield. Further Endosulfan (0.7% solution) was used once/twice during fruiting stage to prevent the crop from the attack of pod borer at pod filling stage. Plants were regularly watered



ICC 14340

ICC 10035

ICC 5003

ICC 4918

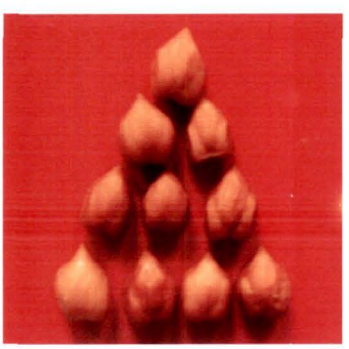
ICC 4969

ICC 16359

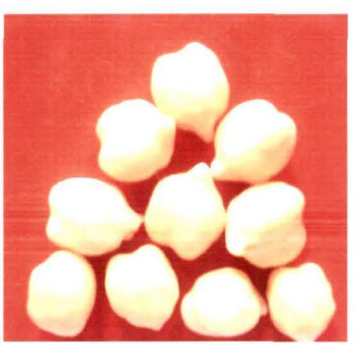
ICC 5319

ICCV 2

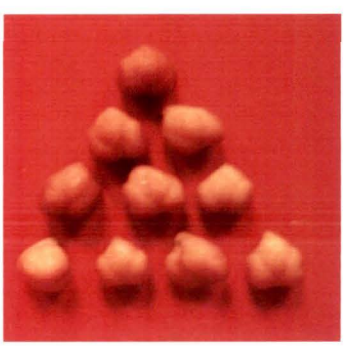
ICCC 37 (Kranthi)



ICCV 10 (Bharathi)



ICC 7344



ICC 1852

Plate II: Seeds of different genotypes of *Cicer arietinum* L.

Plate III (A-D): Growth of chickpea seedlings of different genotypes in pots
A: ICC V10 **B**: ICC C37 **C**: Different genotypes and **D**: ICC 5003

and maintained at normal temperatures of 25 ± 2°C. Plants were also fed regularly with Hoagland solution at 15days interval.

3.1.5. Selection of genotypes

On the basis of germination percentage and field performance the following 15 genotypes were selected for the purpose of study from the 37 genotypes obtained from the seed germplasm bank of ICRISAT, Patancheru, Andhra Pradesh, India. Selected genotypes included the common desi types, kabuli, green gram and a pod mutant genotype (Plate IV).

ICC 1852, ICC 2042, ICC 4918, ICC 4969, ICC 5003, ICC 5319, ICC 6119, ICC 7344, ICC 10035, ICC 14340, ICC 16359, ICCV 10 (Bharathi), ICCV 1, ICC C 37 (Kranthi) and ICCV 2 (Swetha).

3.2. Seed treatment

3.2.1. Chemical treatment of seeds

Selected seeds of various genotypes were imbibed in 100 µM salicylic acid (SA), 50 µM abscisic acid (ABA) and 10mM solution of $CaCl_2$ overnight. The seeds imbibed in different solutions were blotted dry on filter paper and were allowed to germinate at room temperature. The effect of various chemical treatments on seed germination was analyzed by recording the percentage of germination of treated seeds with respect to untreated control seeds imbibed in distilled water.

3.2.2. Seed bacterization with *Bacillus megaterium*

Chickpea seeds were washed gently with distilled water and surface sterilized before bacterization. Seeds of different genotypes were separately inoculated with PGPR strain *Bacillus megaterium* having a concentration of $2x10^6$cfu/ml suspended in 50 ml of 0.2% carboxy methyl cellulose (CMC) solution. The seeds soaked in sterile distilled water served as control. The seeds were imbibed for 24 hrs in bacterial suspension on a shaker to ensure uniform coating of bacterial cells on seed surface. After 24 hrs the seeds were blotted dry on filter paper (Whatman no.1), and incubated for 10 days for growth. The effect of seed bacterization on germination

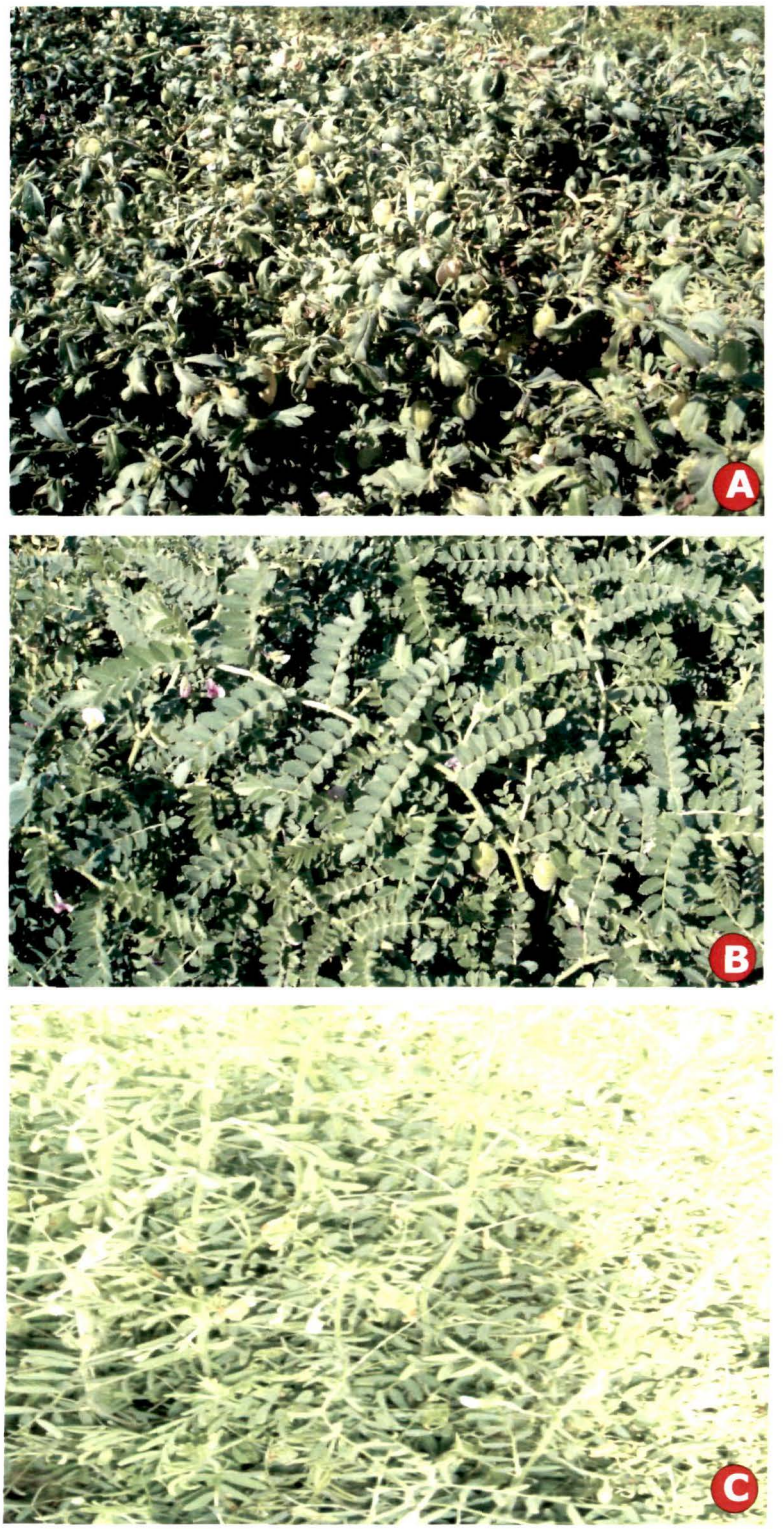


Plate IV (A-C): Chickpea plants bearing flowers and fruits **A**: ICC 7344 (Kabuli);**B:** ICC 1852 (Desi black gram) and **C:** ICC 14340 (Pod mutant)

was tested by recording the percentage of germination of bacterized seeds with respect to untreated control seeds after 24, 48 and 72 hrs.

3.3. Temperature treatment

3.3.1. Seeds: Chickpea seeds of different genotypes soaked overnight in sterile distilled water after surface sterilization with 0.1% $HgCl_2$ were treated at elevated temperatures of 30, 35, 40, 45, 50 and 55°C for 2 hrs duration in moist petri plates following which they were allowed to germinate at room temperature.

3.3.2. Seedlings: 20 days old seedlings were exposed to elevated temperatures of 30, 35, 40, 42, 44 and 46°C for 2 hrs duration which was followed by a recovery time of 96 hrs. The seedlings exposed to elevated temperatures were allowed to recover at room temperature of 25°C ± 2°C and relative humidity (RH) of 60-70% under 11-12 hrs of photoperiod.

3.4. Heat acclimation treatment

For heat–acclimation (HA) treatments of seedlings, the seedlings were pre-exposed to elevated but sub-lethal temperatures of 35, 40, 42 and 44°C for 2 hrs duration prior to lethal temperature treatment. Best heat acclimation was achieved with the exposure of seedlings to 42°C for 2 hrs. Hence this treatment of 42°C for 2 hrs was considered as heat acclimation treatment in all experiments.

3.5. Foliar application of chemicals

3.5.1. Salicylic acid (SA)

Seedlings were sprayed twice a day with 100 µM solution of salicylic acid (SA). The spray treatment was carried out for five consecutive days and finally just prior to exposure to heat stress. The pre-treated seedlings and distilled water treated (control) seedlings dipped in respective solutions in conical flasks were then subjected to heat stress (46°C for 2hrs) and sampled for experimental purposes.

3.5.2. Abscisic acid (ABA)

Abscisic acid (ABA) solution of strength 50 µM was used as a foliar spray on seedlings. The same volume (50 mL.) of distilled water was sprayed on control seedlings. The spraying treatment was carried out exactly in the same manner as mentioned in case of SA.

3.5.3. Calcium chloride ($CaCl_2$)

Calcium chloride solution of 10mM strength was sprayed on test seedlings in the form of foliar spray. Controls consisted of plants on which the same volume of distilled water was sprayed and treated in the same manner as test plants. The seedlings were immersed in respective solutions in conical flasks for heat treatments. After heat treatments, plants were returned to the room conditions and allowed to recover for up to 96 hrs.

3.6. Determination of tolerance index (TI) of seedlings

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

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

3.7. Determination of plant growth promoting activity of *Bacillus megaterium*

The experiment was conducted under greenhouse conditions (in potted plants) and in the open field to assess the efficacy of *Bacillus megaterium* on plant growth promotion. The growth promotion was assessed in seedlings and field grown plants on the basis of vigour index (VI) by comparing the increase in root and shoot length of the treated plants with the untreated control plants under the same environmental conditions (temperature of 25± 2°C, 60-70% RH and 10-11hrs photoperiod (Baki and

Anderson, 1973). The experiment consisted of ten replicates with 10 seeds in each treatment in completely randomized design. The vigour index of seedlings and mature plants was calculated by applying the following formula :

Vigour index = (mean shoot length + root length) × % of germination

3.8. Protein analysis

3.8.1. Extraction of soluble proteins

Soluble proteins were extracted from seeds, leaves, stem and roots of different genotypes of *Cicer arietinum* L. following the method of Chakraborty *et al.* (1995) with modifications. Plant tissues were frozen in liquid nitrogen and ground in 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$ and 2 mM PMSF added at the time of crushing and centrifuged at 4°C for 20 min at 10000 g. The supernatant was used as crude protein extract.

3.8.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1% $CuSO_4$ and 1 ml of 2% sodium potassium tartarate, dissolved in 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 15-20 min following which absorbance was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.8.3. SDS-PAGE analysis

Total soluble proteins extracted in 0.05 M sodium phosphate buffer were used as crude protein extract for analysis of protein pattern. Analysis was carried out on 10% SDS- PAGE gels following the method of Sambrook *et al.* (1989). Protein samples were loaded on the gel and separated for 3 hours at 200 V and 15-20 mA

current. Following electrophoresis the gel was fixed, stained in Coomassie Brilliant Blue (R-250) solution and finally destained in a solution of methanol, glacial acetic acid and water (4.5: 4.5: 1).

3.8.3.1. Preparation of stock solution

For the preparation of gel the following stock solutions were prepared:

(A) Acrylamide and N' N'- methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in water. As both of these amides are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below 7.0. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4°C and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

(C) Tris Buffer

i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for use.

(D) Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10 % SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was prepared by dissolving 0.5 ml of 1M

Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue,1 ml glycerol in 6.8 ml of distilled water.

3.8.3.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by Pasture pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

Composition of solutions

10% resolving gel

Name of the compound	Amount (ml)
Distilled water	2.85
30 % acrylamide	2.55
1.5M Tris (pH 8.8)	1.95
10 % SDS	0.075
10 % APS	0.075
TEMED	0.003

5 % stacking gel

Name of the compound	Amount (ml)
Distilled water	2.10
30 % acrylamide	0.50
1.5M Tris (pH 6.8)	0.38
10 % SDS	0.030
10 % APS	0.030
TEMED	0.003

After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 2 hrs. After polymerization of the

resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and overlayered with water. Finally the gel was kept for polymerization for 30-45 min. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris- Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

3.8.3.3. Sample Preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 3 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of eleven proteins ranging from high to low molecular masses (Phosphorylase b-97; Fructose-6- phosphate kinase- 84; Bovine Serum Albumin-66; Glutamic dehydrogenase-55; Ovalbumin-45; Glyceraldehyde-3-phosphate dehydrogenase-35; Carbonic anhydrase- 29; Trypsinogen-24; Trypsin inhibitor-20; α Lactalbumin- 14.2 and Aprotin 8.5 kDa) was treated as the other sample and loaded in a separate well.

3.8.3.4. Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

3.8.3.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The gel was removed from the fixer and stained in Coomassie blue stain for 4 hrs at 37°C with constant shaking at low speed. The staining solution was prepared by dissolving 250 mg of Coomassie

brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.9. Extraction of enzymes from seedlings

3.9.1. Peroxidase (POX; EC.1.11.1.7)

For the extraction of POX the plant tissues were ground to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM β mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay or stored at -20°C (Chakraborty *et al.*, 1993).

3.9.2. Ascorbate peroxidase (APOX; EC. 1.11.1.11)

APOX enzyme extract was prepared by powdering the tissues in liquid nitrogen and extracting in 0.05 M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.9) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethanesulphonylfluoride (PMSF) and 2mM polyvinylpyrrolidone (PVP) following the method of Asada (1984). The homogenate was centrifuged at 4°C for 20 min at 15000 g. The supernatant obtained was used for enzyme assay and estimation of total soluble protein content.

3.9.3. Catalase (CAT; EC 1.11.1.6)

The green tissues were ground to powder in liquid nitrogen and extracted with 2 ml of 0.05 M sodium phosphate buffer (pH 6.8) following the method of Chance and Machly (1955). The homogenate was centrifuged at 4°C for 20 min at 15000 g. The supernatant was used for the assay of CAT and total soluble proteins.

3.9.4. Superoxide dismutase (SOD; EC 1.15.1.1)

The SOD enzyme extract was prepared by grinding the plant tissue in liquid nitrogen to powder form and extracting in 0.1 M Potassium phosphate buffer (pH 7.8) containing 1% (W/V) insoluble Polyvinylpyrrolidone (PVPP), 1mM EDTA and 1mM PMSF following the method of Dhindsa *et al.* (1981). Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C.

3.9.5. Glutathione reductase (GR; EC 1.6.4.2)

The enzyme extract for GR was prepared by crushing the plant tissue in liquid nitrogen and extracting with 0.1 M Sodium Phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA),1mM phenylmethanesulphonylfluoride (PMSF) and 20 mM polyvinylpyrrolidone (PVP) and activity was determined following the method of Lee and Lee (2000).

3.10. Assay of enzyme activities

3.10.1. Peroxidase

For determination of peroxidase activity, 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2 M Sodium phosphate buffer (pH 5.4), 100 µl of 4mM 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 (DIGISPEC-200GL) 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 the increase in absorbance at 460 nm/g tissue/min.

3.10.2. Ascorbate peroxidase

Ascorbate peroxidase activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada *et al.* (1984) with some modification. The reaction mixture consisted of 0.1 ml of

enzyme extract, 0.1 ml of 0.5 mM ascorbic acid, 0.1 ml of H_2O_2 and 2.7 ml of sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as change (decrease) in absorbance (ΔA_{290}) mg protein $^{-1}$ min $^{-1}$.

3.10.3. Catalase

Catalase activity was measured according to Chance and Machly (1955). Enzyme extract (20 µl) was added to 3 ml of H_2O_2- phosphate buffer (0.16 ml of 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 enzyme units mg protein^{-1} where one enzyme unit was defined as a change of 0.01 absorbance min^{-1} caused by the enzyme aliquot.

3.10.4. Superoxide dismutase

Superoxide dismutase 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 (0.1 M, pH 7.8), 0.1 ml Na_2CO_3 (1.5 M), 0.1 ml NBT (2.25 mM), 0.2 ml methionine (200 mM), 0.1 ml EDTA (3 mM), 0.1 ml riboflavin (60 µM) 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 absorbances of samples were 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.

3.10.5. Glutathione reductase

Glutathione reductase activity was determined by the oxidation of NADPH at 340 nm with extinction coefficient of 6.2 mMcm^{-1} as described by Lee and Lee

(2000).The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH at 25°C. Enzyme activity was finally expressed as μM min^{-1} mg $protein^{-1}$.

3.11. Isozymes analysis by polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) using 8% resolving gel and 5% stacking gel in Tris-glycine buffer (pH 8.3) was performed for isozyme analysis of different enzymes following the method of Davis (1964). The various solutions required for the analysis were prepared as follows:

Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N' N' methelene bisacrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of bis- acrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with conc. HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with conc. HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammoniumpersulphate solution (APS)

Fresh soution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g Glycine in 1 L of distilled water.

Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: distilled water in the ratio of 1:1: 4:1 by pasture pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hrs. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2:1:1:4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 min in strong sunlight. After polymerization of the

stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus. Tris-Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixer in ice. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3-4 hrs at 4°C until the dye font reached the bottom of the gel.

Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained using suitable staining dye.

3.11.1. Peroxidase

Extract for peroxidase isozyme analysis was prepared by grinding 1 g of plant tissue in liquid nitrogen in pre-chilled mortar and pestle and finally extracting in 0.1 M Sodium phosphate buffer (pH 7.0) as described by Davis (1964). Peroxidase isozyme pattern was estimated by staining the gel in Benzidine dye in acetic acid-water mixture consisting of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 min followed by 15 min incubation in 0.1mM H_2O_2 with gentle shaking (Reddy and Gasber.1973). The reaction was finally stopped with 7% acetic acid after

the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.11.2. Catalase

Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a pre-chilled mortar and pestle in 0.1 M potassium phosphate buffer (pH 7.0) on ice as described by Woodbury *et al.* (1971) and immediately used for the isozyme analysis.

After electrophoresis the gel was soaked in 3.3 mM H_2O_2 for about 20 min. The gel was then rinsed in water and incubated in a freshly mixed solution consisting equal volumes of 1% potassium ferricyanide and 1% ferric chloride for about 20 min. Analysis of the isozymes was done immediately and R_m values for different isozymes were calculated.

3.12. Extraction and quantification of non-enzymatic antioxidants

3.12.1. Ascorbate

Quantification of ascorbate was done following the method of Mukherjee and Choudhuri (1983). The seedlings were homogenized in a cold mortar placed on ice using 10 ml of 6% trichloroacetic acid. To 4.0 ml of the extract, 2.0 ml of 2% dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% thiourea (in 70% ethanol) were added. The mixture was then kept in a boiling water bath for 15 min and after cooling at room temperature 5 ml of 80% (v/v) H_2SO_4 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.

3.12.2. Carotenoids

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). 1g of the plant sample was homogenized in methanol for the extraction of carotenoids. After extraction the O.D. value was observed at 480 nm in a UV-VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})\ \mu g\ g^{-1}$ fresh weight

3.13. Determination of peroxidation of membrane lipids

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa *et al.*(1981).1 ml of supernatant of leaf extracts was mixed with 4mL of 20% (v/v) trichloroacetic acid containing 0.5% (v/v) thiobarbituric acid. The mixture was heated at 100°C for 30 min, quickly cooled, and then centrifuged at 10000 g 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 $mM^{-1}cm^{-1}$ (Heath and Packer 1968).

3.14. 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).Leaf tissues were washed with 3-4 changes of distilled water and placed in test tubes (150×25 mm) containing 2 ml of pre-heated (to the treatment temperature) water. Tubes were covered with plastic wrap and placed in a water bath at the desired temperature for 15 min, while the control tubes were kept at 25°C. After cooling to room temperature, distilled water was added to make up the final volume of 10 ml. Samples were incubated at 10°C for 16 hrs and conductivity measured with a conductance meter (EI Model 181 E). The tubes were covered with aluminum foil and autoclaved at 120°C for 15 min. to release all electrolytes. After cooling tubes to 25°C. the contents were mixed and final conductance measured. The injury was determined as follows:

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

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

3.15. Extraction and estimation of chlorophyll

Chlorophyll was extracted according to the method of Harbone (1973) by homogenizing 1 g of leaf sample in 80% acetone and filtering through Whatman No.1 filter paper 80% acetone was repeatedly added from the top till the residue

became colourless. The filtrate was collected and the total volume was made up to 25 ml. The chlorophyll content was estimated by observing the O.D. values at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done using the following formulae (Arnon,1949).

Total chlorophyll: $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} fresh weight

Chlorophyll a: $(12.7\ A_{663} - 2.69\ A_{645})$ mg g^{-1} fresh weight

Chlorophyll b: $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} fresh weight

3.16. Determination of Hill activity

3.16.1. Extraction

1 g of fresh weight of pinnules kept in a tray filled with ice water were macerated by mortar and pestle with 5 ml of chilled suspension medium (pH 7.6) consisting of 20 ml (1 M) Sorbitol, 15 ml (0.1 M) K_2HPO_4, 12 ml (25 mM) sodium EDTA, 12 ml (10 m M) $NaHCO_3$ and 1 ml distilled water (Trebst, 1972). Extract was filtered through muslin cloth in an ice-cold beaker. After centrifuging the filtrate at 3000 rpm for 10 min, the supernatant was decanted and pellet was resuspended in 3 ml suspension medium and stored at 10°C.

3.16.2. Assay of chloroplast activity by Hill reation

2.8 ml of assay medium and 0.1 ml of chloroplast suspension was taken into cuvette of spectrophotometer. 0.1 ml of DCPIP (2,6, dichlorophenol indophenol) dye was added. The cuvette was inverted once for mixing. Absorbance was determined immediately after mixing in dark at 600 nm representing the starting point. Next the cuvette was exposed to light source for 5 min. The contents were mixed by inverting the tube once and absorbance was taken. Hill activity was calculated in terms of μM of DCPIP reduced per minute per mg of chloroplast (μM of DCPIP min^{-1} mg^{-1} chloroplast) following the method of Trebst (1972).

3.17. Extraction and estimation of free proline

3.17.1. Extraction

For the extraction of free proline the method of Bates *et al.*(1973) was followed. 1 g of plant tissue was crushed with 5ml of 3% sulfosalicylic acid in mortar with pestle.The slurry was filtered through Whatman No.1 filter paper at room temperature in dark condition. The supernatant was collected and stored at 4°C for further analysis.

3.17.2. Estimation

Proline content of the extract was estimated as described by Bates *et al.* (1973) with some modification. To 1ml of extract, 3 ml of distilled water and 1 ml of Ninhydrin solution (2 g in 50 ml of acetone and water mixture) were added. Then the mixtures were kept on a boiling water bath for 15 min. After cooling, the reaction mixture was poured in a separating funnel and 5 ml of toluene was added and mixed vigorously. Lower colored layer was taken and O.D.values were measured at 520 nm.Quantification was done from a standard curve of proline.

3.18. Extraction and estimation of total and reducing sugar

3.18.1. Extraction

For extraction of sugar, method of Harbone (1973) was followed. Fresh tissues were crushed with 95% ethanol and filtered. Then the alcoholic fractions of the filtrate were evaporated off on a boiling water bath. The aqueous fractions were centrifuged in a table centrifuge and the supernatants were collected. Finally the volume was made up with distilled water.

3.18.2. Estimation

Estimation of total sugar was done following Anthrone's method as described by Plummer (1978). To 1 ml of test solution 4 ml of Anthrone's reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for

10 min. The reaction mixture was then cooled under running tap water. Absorbance was measured at 570 nm in a colorimeter. Total sugar content was then calculated from the standard curve of dextrose solution

Somogyi's method as described by Plummer (1978) was followed for the estimation of reducing sugar. 1ml alkaline Cu-tartrate solution (prepared by dissolving 4 g $CuSO_4 .5H_2O$, 24 g of anhydrous Na_2CO_3, 16 g of sodium potassium tartrate and 180 g of anhydrous Na_2SO_4 in distilled water and making up the final volume to 1L) was added to 1 ml of test solution. Reaction mixture was then allowed to boil in a water bath for 15 min. The mixture was then cooled under running tap water and to each reaction mixture 1ml of Nelson's arseno molybdate reagent was added. Reaction mixtures were diluted to 5 ml by adding 2 ml more distilled water and O.D. values were measured in a colorimeter at 540 nm. The concentration of reducing sugar was determined by plotting the O.D. values on the standard curve of dextrose solution.

3.19. Extraction of phenolic compounds from seedlings and HPLC analysis

Phenolic compounds from freshly harvested plant tissues of different genotypes were extracted in ethanol-water following the method of Sarma *et al.* (2002).1 g of tissue was macerated using a pestle and mortar followed by suspension of finely crushed samples in 5 ml of ethanol-water (80:20 v/v). Samples were collected in screw-capped tubes and the suspension was subjected to ultrasonication (Sonics Vibra cell) at 60% duty cycles for 15 min at 40°C followed by centrifugation at 7500 rpm. for 15 min. The clear greenish supernatant was then subjected to charcoal treatment to remove pigments in each sample and transferred to glass tubes after filtering through Whatman filter paer No.1. The residue was re-extracted twice and the supernatant was pooled prior to evaporation under vacuum. Dried samples were re-suspended in 1 ml HPLC grade methanol by vortexing and stored at 4°C for further analysis.

High performance liquid chromatography (HPLC) of the samples were performed according to Sarma *et al.* (2002). The HPLC system (Shimadzu Advanced VP Binary Gradient) used for sample analysis was equipped with C-18 hypersil column. Reverse phase chromatographic analysis was carried out using this C-18

reverse phase HPLC column at 25°C under isocratic conditions where the concentration of mobile phase was constant throughout the run. Running conditions included an injection volume of 20 µl, mobile phase methanol- 0.4% acetic acid (80:20 v/v), flow rate 1ml min^{-1}, attenuation 0.02 and detection at 290 nm. Samples were filtered through a membrane filter (pore size 0.45 µm) prior to injection in the sample loop. Phenolic compounds and salicylic acid present in the samples were identified by comparing retention time (Rt) of standards and coinjection.

3.20. *In vitro* callus formation

For *in vitro* callus formation, seeds were washed with teepole and were surface sterilized with 0.1% mercuric chloride for 10 min and soaked overnight in sterilized distilled water. Seeds were cultured asceptically (Plate V) on germination medium having sucrose (2.0%) and agar (0.8%) as suggested by Batra *et al.* (2004).

Ten-day old *in vitro* grown seedlings were used for excision of explants i.e. shoot tip, cotyledonary node, internode and hypocotyls for callus initiation (Plate VI). A number of medium having MS salts (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 8 g/L agar and B_5 vitamins (Gamborg *et al.*, 1968) with varying concentrations and combinations of cytokinins, auxins and growth regulators (NAA+BAP, NAA+Kinetin and IAA+BAP) were used for initiation of callus from different explants. MS media used were supplemented with 1 mg/L and 1.5 mg/L of NAA, 0.5 mg/L of BAP and 1.5 and 2.0 mg/L of kinetin. The pH of the medium was adjusted to 5.8 and autoclaved at 15 psi for 15 min. The cultures were incubated under 70 µE $m^{-2}s^{-1}$ incandescent light in a photoperiod cycle of 16 hrs light/8 hrs dark at 25 ± 1°C (Roy *et al.*, 2001). The callus was subcultured three times at 15 days interval in MS medium with different combinations of growth regulators for regeneration.

3.21. Treatment of calli

Calli (20 days old) raised from different plant explants were subjected to two different pre-treatments followed by lethal temperature treatment of 40°C for 2 hrs. First pre-treatment involved heat-acclimation treatment of 35°C for 2 hrs followed

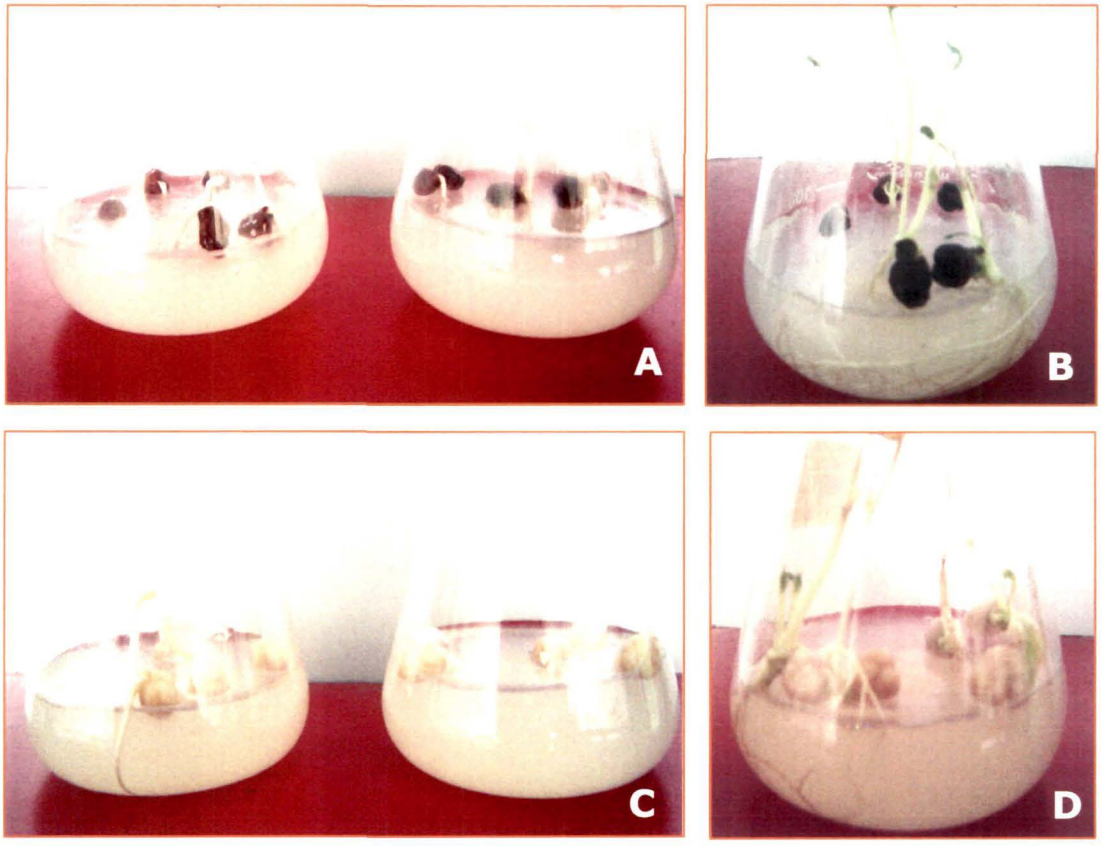


Plate V (A-D) : Aseptic germination of chickpea seeds of two different genotypes for callus culture **A & B:** ICC C37 (Kranthi). **C& D:** ICC 7344

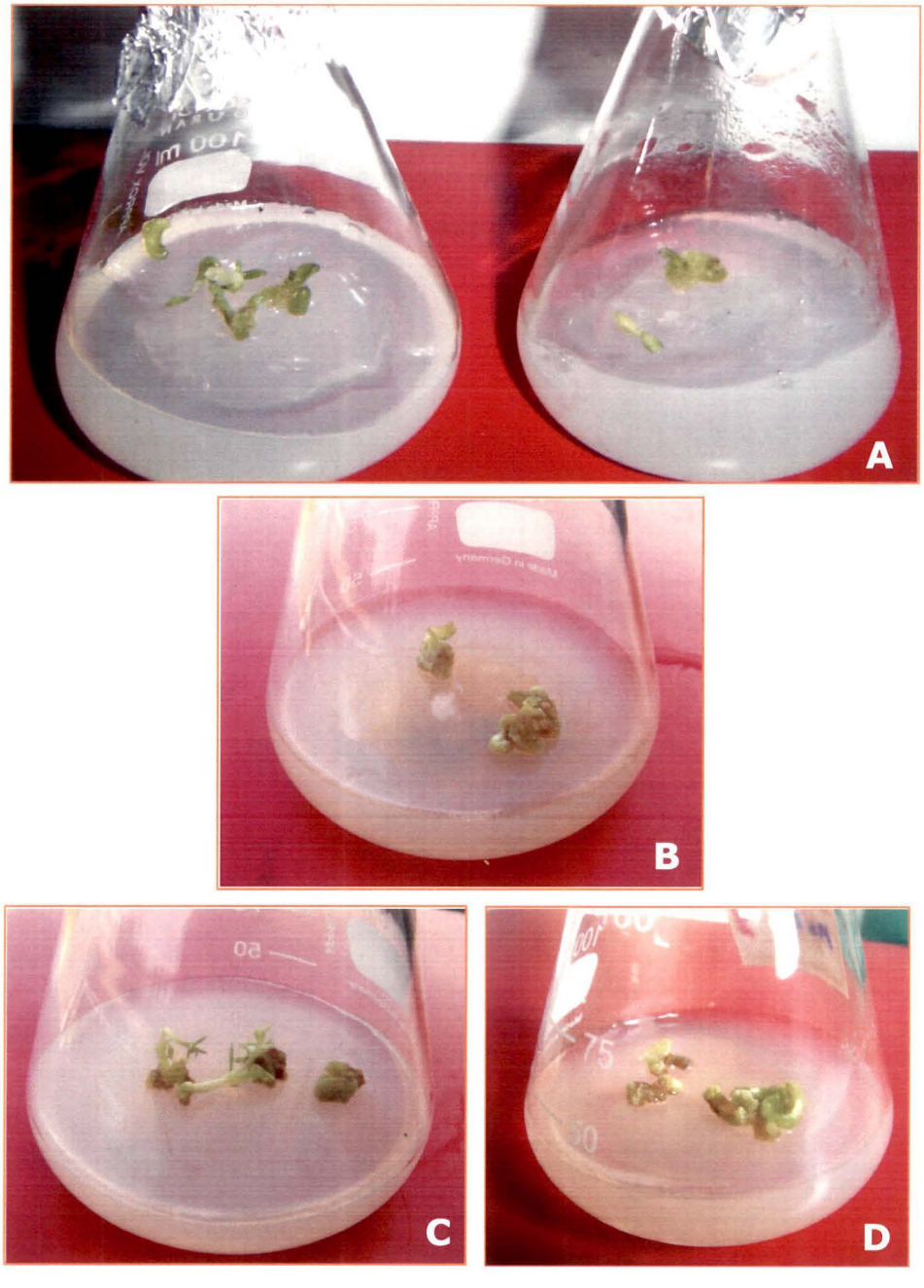


Plate VI (A-D): Induction of callus formation from different plant parts. **A:** shoot tip; **B & D:** internode and **C:** shoot tip with a portion of leaf

by challenge with a lethal temperature for the same duration. The second pre-treatment involved growing of calli in MS media supplemented with 10^{-5} and 10^{-6} M salicylic acid (SA) and subjecting the calli to lethal temperature treatment. The calli subjected to two different pre-treatments following exposure to lethal temperature were returned to normal conditions and allowed to grow for 60 days. The changes in growth of the calli following lethal temperature treatment were recorded regularly till 60 days at 10 days time interval by checking the weights of calli. Experiments were carried out in multiple sets and calli once used were discarded after weighing.


EXPERIMENTAL

4.1. Screening of thermotolerant and susceptible genotypes

4.1.1. Tolerance index (TI)

Tolerance index (TI) of fifteen genotypes chosen for this study was calculated by directly exposing the seedlings to lethal temperature of 46°C for 2 hrs. Six of the genotypes out of 15 recorded negative tolerance index vaues of less than -10 and were therefore categorized as tolerant (ICC 4918, ICC 2042, ICC 1852, ICC C37, ICC V2 and ICC V10) while 5 genotypes which exhibited values ranging from - 23 to -32 were categorized as susceptible (ICC 5319, ICC 10035, ICC 16359, ICC 7344 and ICC V1). The remaining genotypes (ICC 6119, ICC 14340, ICC 5003 and ICC 4969) which showed moderate TI values were categorized as moderately tolerant (Table 1).

Table 1. Tolerance index (TI) values of various genotypes

Genotypes (%)	Tolerance index (TI) values in percentage
ICC 4918	- 07.86 ± 1.06
ICC 4969	- 15.23 ± 2.02
ICC 7344	- 32.60 ± 1.89
ICC 1852	- 08.23 ± 0.76
ICC 10035	- 28.09 ± 1.82
ICC 6119	- 18.72 ± 2.18
ICC 5003	- 12.67 ± 1.62
ICC 14340	- 15.96 ± 0.96
ICC 5319	- 27.39 ± 3.28
ICC 2042	- 08.97 ± 0.79
ICC 16359	- 25.38 ± 3.26
ICC C37	- 05.76 ± 0.08
ICC V10	- 07.79 ± 0.11
ICC V1	- 23.76 ± 0.32
ICC V2	- 06.33 ± 0.05

Values represent mean ± SE (n =5).Values are mean of five replicates.

4.1.2. Cell membrane thermostability

Cell membrane stability of various genotypes to elevated temperature stress was tested by calculating the percentage of relative injury (RI) of the membranes in seedlings exposed to lethal temperature treatment for 2 hrs with respect to untreated control seedlings. Cell membrane stability (CMS) test revealed significantly high values of RI in some genotypes (ICC 7344, ICC 5319, ICC 10035, ICC 16359 and ICC V1) moderate values in (ICC 14340, ICC 4969, ICC 6119 and ICC 5003) and relatively much lower in others (ICC 4918, ICC 1852, ICC C37, ICC V10, ICC 2042 and ICC V2) with respect to control (Table 2).

Table 2. Effect of elevated temperature stress on cell membrane stability

Genotypes	Relative Injury (RI) in percentage (%)	
	Control	Lethal
ICC 4918	29.83 ±1.98	40.59 ±2.35
ICC 4969	42.20 ±2.10	58.89 ±2.24
ICC 7344	50.86 ±1.35	86.32 ±1.19
ICC 1852	35.03 ±1.36	45.65 ±2.53
ICC 10035	60.64 ±1.57	82.04 ±1.71
ICC 6119	33.60 ±1.80	52.79 ±1.76
ICC 5003	39.06 ±1.98	55.06 ±2.21
ICC 14340	45.39 ±2.26	60.87 ±1.90
ICC 5319	52.97 ±1.51	84.02±2.51
ICC 2042	38.02 ±2.46	48.37 ±1.79
ICC 16359	58.92 ±1.98	80.69 ±1.84
ICC C37	30.08 ±3.45	43.70 ±2.30
ICC V10	29.18 ±1.68	42.46 ±2.65
ICC V1	55.89 ±1.38	80.08 ±2.20
ICC V2	40.06 ±1.84	52.02 ±2.28

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 2A. Analysis of variance for the data presented in table 2

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	7641.516	545.823	95.6262	2.28913
TREATMENT	1	1481.515	1481.515	259.5561	-
ERROR	14	79.910	5.708	-	-
TOTAL	29	9202.940			

Screening of thermotolerant, moderately tolerant and susceptible genotypes of chickpea was carried out on the basis of the above two criteria and also on the comparative analysis of the test genotypes with the known tolerant genotypes like ICC C37 (Kranthi) and ICC V10. The genotypes were thus categorized into the following types initially (Table 3). Further, a large number of biochemical analyses were carried out to determine correlation of tolerance to biochemical parameters.

Table 3. Categorization of chickpea genotypes on the basis of their tolerance to elevated temperatures

Thermotolerant	Moderately tolerant	Susceptible
ICC 4918, ICC 2042	ICC 6119, ICC 14340,	ICC 5319, ICC 10035,
ICC 1852, ICC V2	ICC 5003 and ICC 4969	ICC 16359, ICC 7344,
ICC C37 and ICC V10		and ICC V1

Values are mean of five replicates.

4.2. Analysis of the effects of temperature and pre-treatments on seed germination and seedling growth of *Cicer arietinum* and determination of lethal temperature

4.2.1. Lethal temperature determination

Exposure of seeds to a wide range of elevated temperatures ranging from 35-55°C for 2 hrs duration revealed that approximately 100% germination occurred in control, 35, 40 and 45°C treatments, after which there was a reduction in germination percentage at higher temperatures. Delayed germination was observed at 50°C and no germination was recorded at 55°C treatments. Therefore, for all further

experiments this temperature of 55°C was considered as the lethal temperature for seed germination.

Similarly the treatment of seedlings at elevated temperatures (35, 40, 42, 44 and 46°C) for 2 hrs each revealed that most genotypes could not tolerate a high temperature of 46°C in which only 10-15% recovery was recorded compared to 100% recovery in 35°C and 80-90% recovery in 40°C treatments. Seedlings treated to 46°C for 2 hrs showed no recovery during the recovery period of 96 hrs when returned to normal growing conditions. Hence this temperature of 46°C was considered as the lethal temperature for the seedling growth in all future experiments.

Lethal temperature treatments were carried out by directly exposing the seeds and seedlings to lethal temperatures of 55°C and 46°C respectively for 2 hrs duration in each case without any pre-treatments.

4.2.2. Seed germination

Seeds of different genotypes were subjected to different temperature regimes ranging from 35°C-55°C for the period of 2 hrs in moist petri plates following which they were allowed to germinate. Results (Table 4) revealed that 100% germination occurred in controls and 35°C treatments in most of the genotypes, after which there was a reduction in germination percentage at elevated temperatures. Significant differences were observed between germination at 30°C (control) 40, 45, 50 and 55°C treatments. Delayed germination was observed in case of thermotolerant and moderately tolerant genotypes while a sharp decline in germination percentage was recorded at 50°C treatments in case of susceptible genotypes. In the susceptible genotypes. no germination was observed at 55°C, while only a few seeds (1-10%) of thermotolerant genotypes germinated at 55°C treatments for 2 hrs.

Effect of chemical pre-treatments prior to lethal temperature on seed germination was varied. Salicylic acid and calcium chloride pre-treatments and seed bacterization with *Bacillus megaterium* enhanced the rate of germination in most genotypes (Plate VII). Abscisic acid on the other hand exhibited inhibitory effects on

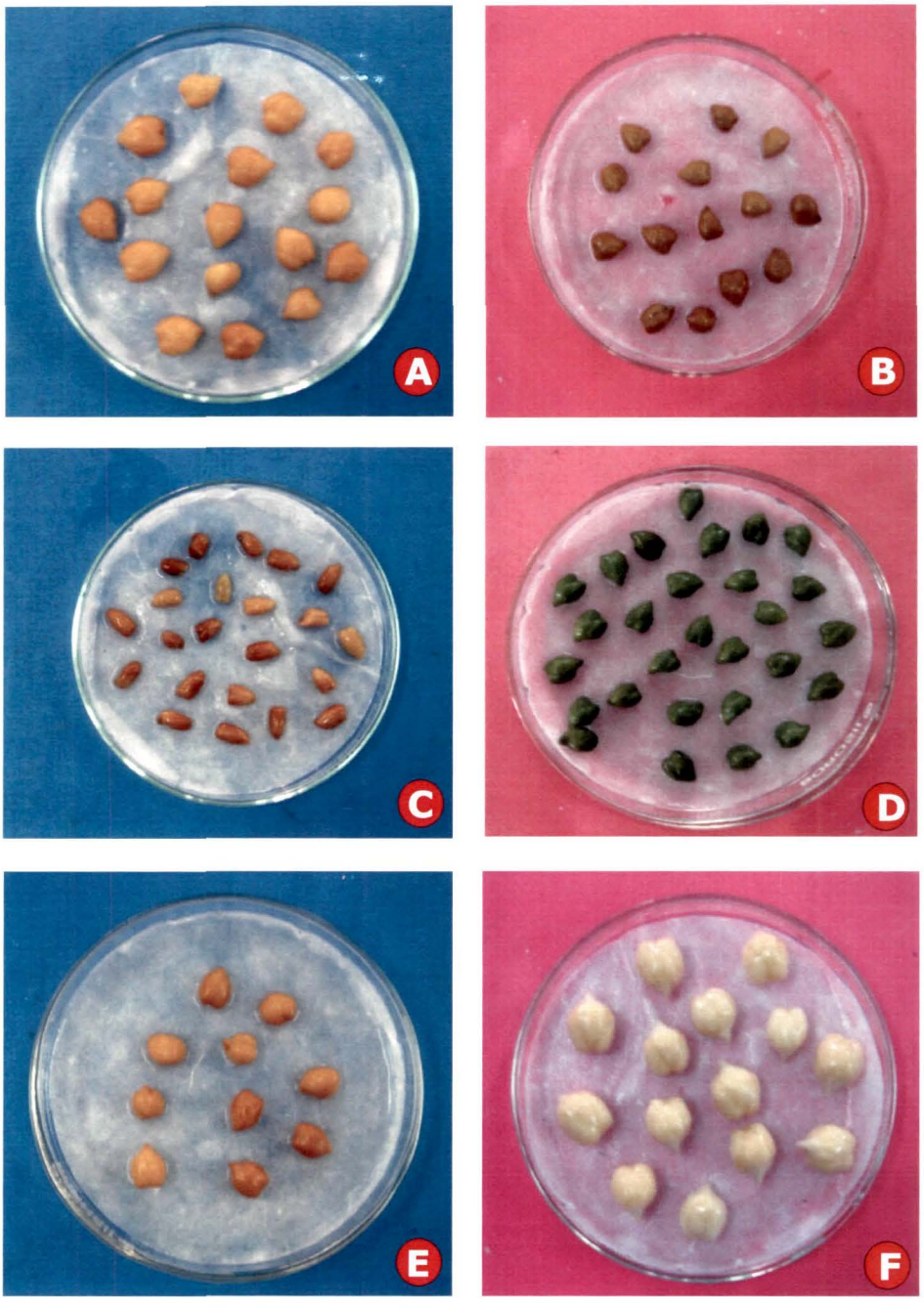


Plate VII (A-F): Effect of pre-treatments on seed germination of different genotypes. **A:** $CaCl_2$ treated ICC 5003; **B:** untreated control ICC 1852; **C:** ABA treated ICC 14340; **D:** SA treated ICC 4969; **E:** Heat acclimatized ICC 2042; **F:** *Bacillus megaterium* coated seeds of ICC 7344.

germination in early stages, which declined gradually with the onset of germination (Tables 5 and 6). Reduction of germination by ABA pre-treatment was not statistically significant, while enhancement by SA, $CaCl_2$ and bacterization were significant. Germination after lethal temperature treatment was almost nil or very low. Better germination percentage was observed in case of seeds pre-treated with $CaCl_2$ solution and seeds bacterized (Plate VIII) with *Bacillus megaterium* (Figs.1and 2).

Table 4. Effect of elevated temperature treatment (2 hrs) on seed germination - 96 hrs

	Germination percentage					
Genotypes	**30°C**	**35°C**	**40°C**	**45°C**	**50°C**	**55°C**
ICC 4918	100 ± 14.8	100 ± 18.2	95 ± 19.2	95 ± 12.3	90 ± 10.5	10* ± 0.6
ICC 4969	100 ± 18.3	100 ± 16.7	80 ± 9.5	70 ± 20.4	65* ± 12.4	05* ± 0.6
ICC 7344	100 ± 18.7	95 ± 13.2	70 ± 18.7	60 ± 11.8	35* ± 5.4	-
ICC 1852	100 ± 17.6	100 ± 16.4	95 ± 13.2	95 ± 17.8	90 ± 14.6	09* ± 0.3
ICC 10035	100 ± 13.9	90 ± 17.3	85 ± 13.2	60* ± 11.7	30 ± 07.8	-
ICC 6119	100 ± 21.1	95 ± 16.4	95* ± 13.9	80* ± 16.7	70* ± 18.7	05* ± 0.4
ICC 5003	100 ± 19.7	90 ± 17.8	90* ± 17.2	70* ± 14.8	65* ± 12.1	04* ± 0.3
ICC 14340	95 ± 18.8	90 ± 17.2	90* ± 11.9	70 ± 13.8	55 ± 09.7	02* ± 0.7
ICC 5319	100 ± 21.7	90* ± 19.4	85* ± 14.6	50* ± 12.4	30* ± 18.1	-
ICC 2042	100 ± 14.7	95 ± 17.9	95 ± 19.4	95 ± 16.4	90 ± 14.9	05* ± 0.2
ICC 16359	95 ± 16.6	95 ± 49.6	90* ± 22.1	55* ± 15.9	30* ± 09.7	-
ICC C37	100 ± 19.9	100 ± 23.4	100 ± 17.2	95 ± 18.9	90 ± 14.6	10* ± 0.6
ICC V10	100 ± 21.2	100 ± 24.1	100 ± 19.4	95 ± 11.2	90 ± 20.4	12* ± 3.2
ICC V1	100 ± 18.7	95 ± 17.3	90* ± 13.7	55* ± 13.9	30* ± 12.8	-
ICC V2	100 ± 17.5	90 ± 18.6	85 ± 16.8	85 ± 17.8	85* ± 19.6	08* ± 1.2

Values are mean of three replicates. * Delayed germination

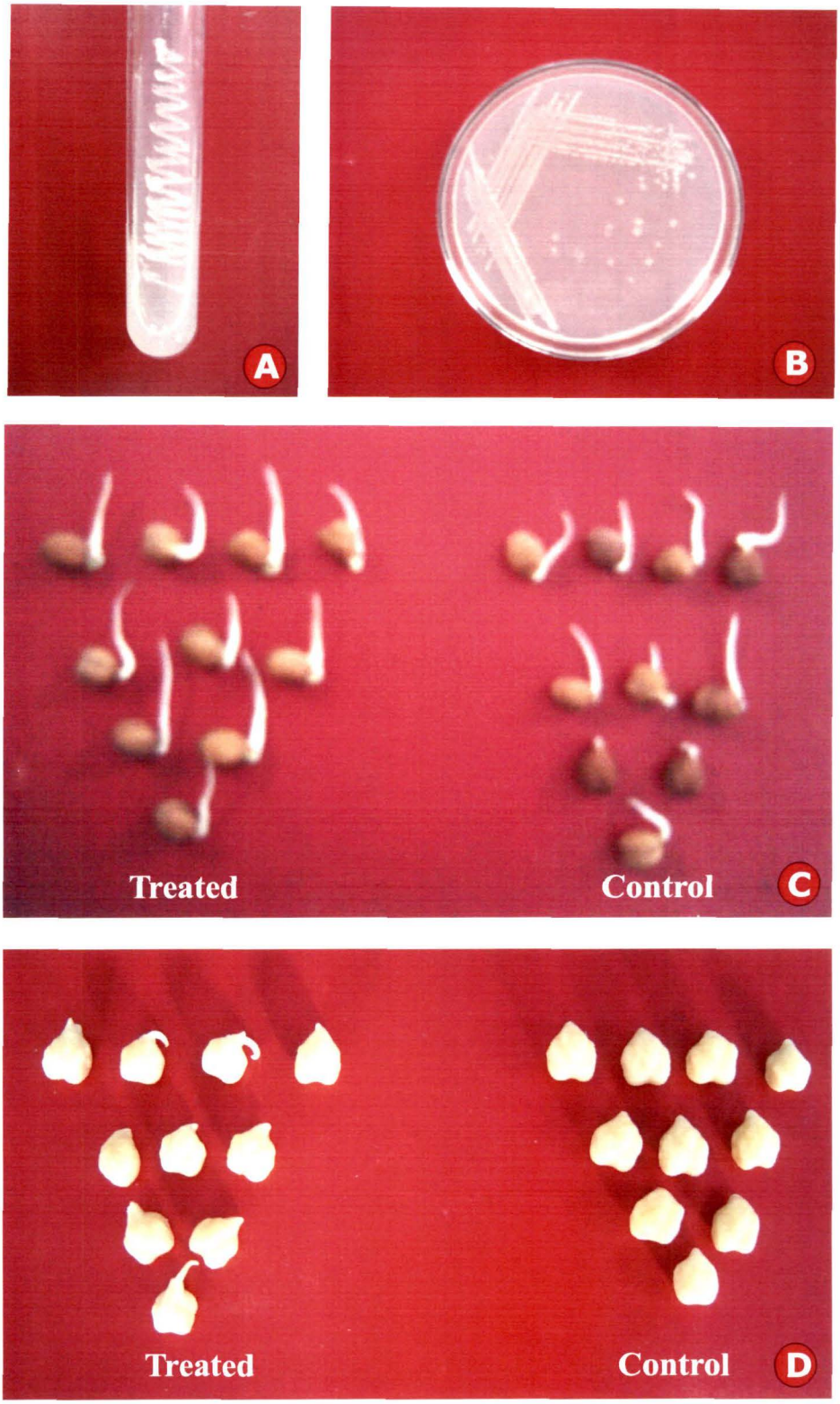


Plate VIII(A-D): Effect of $CaCl_2$ treatment and seed bacterization on seed germination. *Bacillus megaterium* in Nutrient Agar slant **(A)** and Nutrient Agar plate **(B);(C):** control and $CaCl_2$ treated seeds of ICC 1852; **(D):** control and *B. megaterium* treated seeds of ICC 7344.

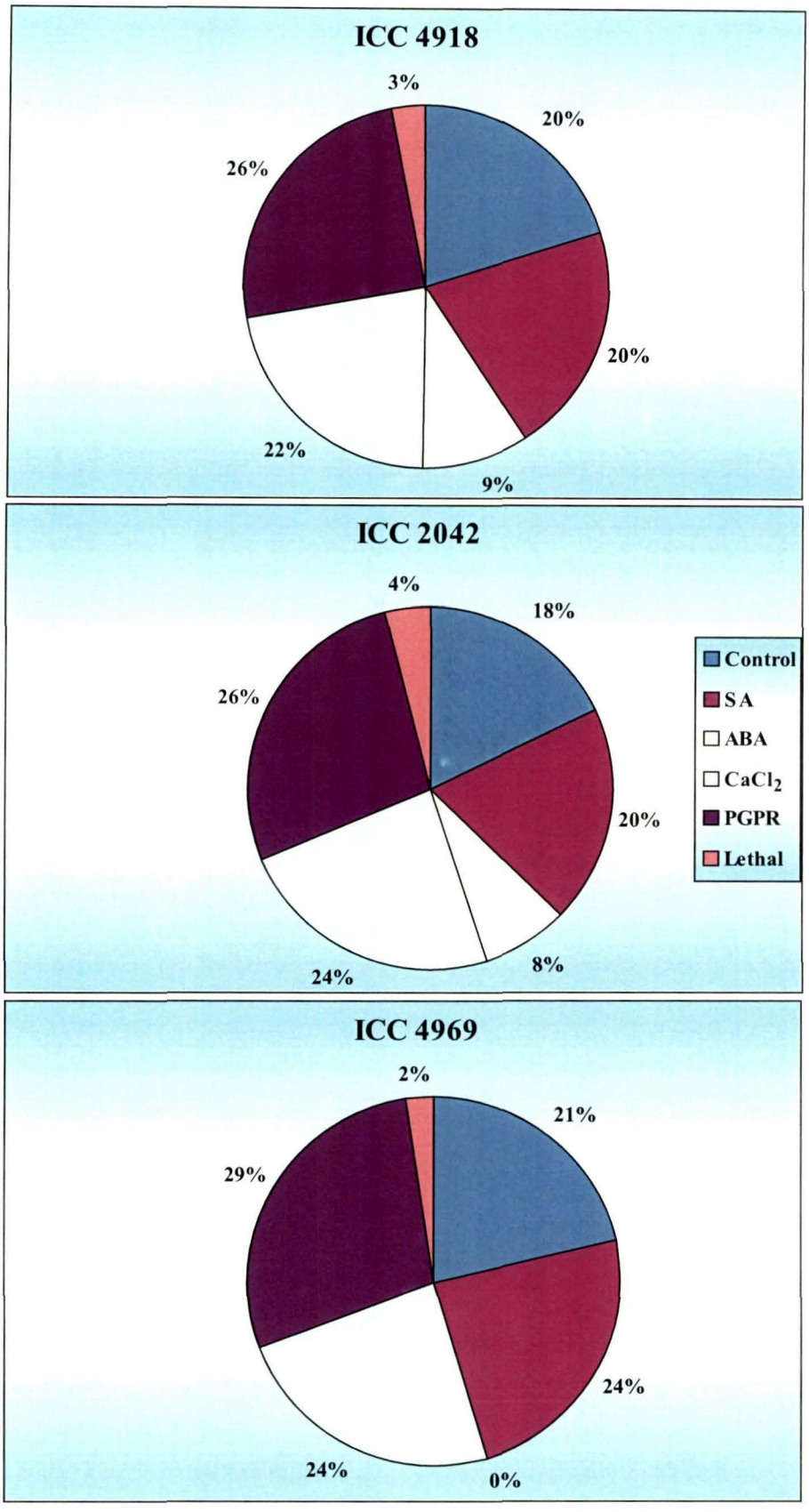


Fig. 1: Germination of three varieties of *Cicer arietinum* L. seeds subjected to different pre-treatments followed by lethal temperature.

Table 4 A Analysis of variance for the data presented in Table 4

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	7055.927	503.995	4.4803	9.19408
TREATMENT	5	92857.330	18571.460	165.0934	-
ERROR	70	7874.346	112.491	-	-
TOTAL	89	107787.600			

Table 5. Effect of various pre-treatments on seed germination after 24 hrs

	Germination percentage					
Genotypes	**Control**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	45 ± 11.1	65 ± 13.2	45 ± 9.8	65 ± 15.5	60 ± 15.1	10 ± 2.1
ICC 4969	30 ± 09.8	45 ± 14.2	30 ± 12.8	45 ± 12.2	50 ± 13.1	05 ± 0.3
ICC 7344	-	20 ± 05.6	-	30 ± 12.4	40 ± 15.5	_
ICC 1852	45 ± 14.2	65 ± 13.7	50 ± 16.7	60 ± 14.7	55 ± 06.4	09 ± 1.2
ICC 10035	20 ± 05.2	30 ± 06.3	10 ± 03.9	40 ± 09.5	40 ± 06.7	-
ICC 6119	30 ± 04.1	40 ± 04.9	35 ± 11.1	45 ± 13.4	45 ± 09.7	06 ± 0.7
ICC 5003	30 ± 06.8	45 ± 07.9	45 ± 15.4	45 ± 12.7	45 ± 10.7	06 ± 1.0
ICC 14340	20 ± 02.3	35 ± 06.2	25 ± 08.1	40 ± 09.9	40 ± 16.4	02 ± 0.5
ICC 5319	20 ± 05.1	40 ± 14.7	15 ± 09.1	40 ± 06.9	45 ± 07.3	-
ICC 2042	30 ± 08.9	50 ± 13.7	40 ± 14.1	40 ± 09.6	45 ± 09.1	08 ± 0.9
ICC 16359	20 ± 03.6	30 ± 07.1	15 ± 09.4	45 ± 10.8	40 ± 11.4	01 ± 0.1
ICC C37	50 ± 16.9	60 ± 14.6	55 ± 13.7	50 ± 08.7	60 ± 09.8	10 ± 0.7
ICC V10	40 ± 04.9	50 ± 08.1	50 ± 02.8	55 ± 13.1	60 ± 09.1	08 ± 0.4
ICC V1	-	-	-	20 ± 02.3	30 ± 04.1	-
ICC V2	-	20 ± 04.2	10 ± 03.6	20 ± 06.1	30 ± 04.1	08 ± 0.5

Values represent mean ± SE (n = 3). Values are mean of three replicates.

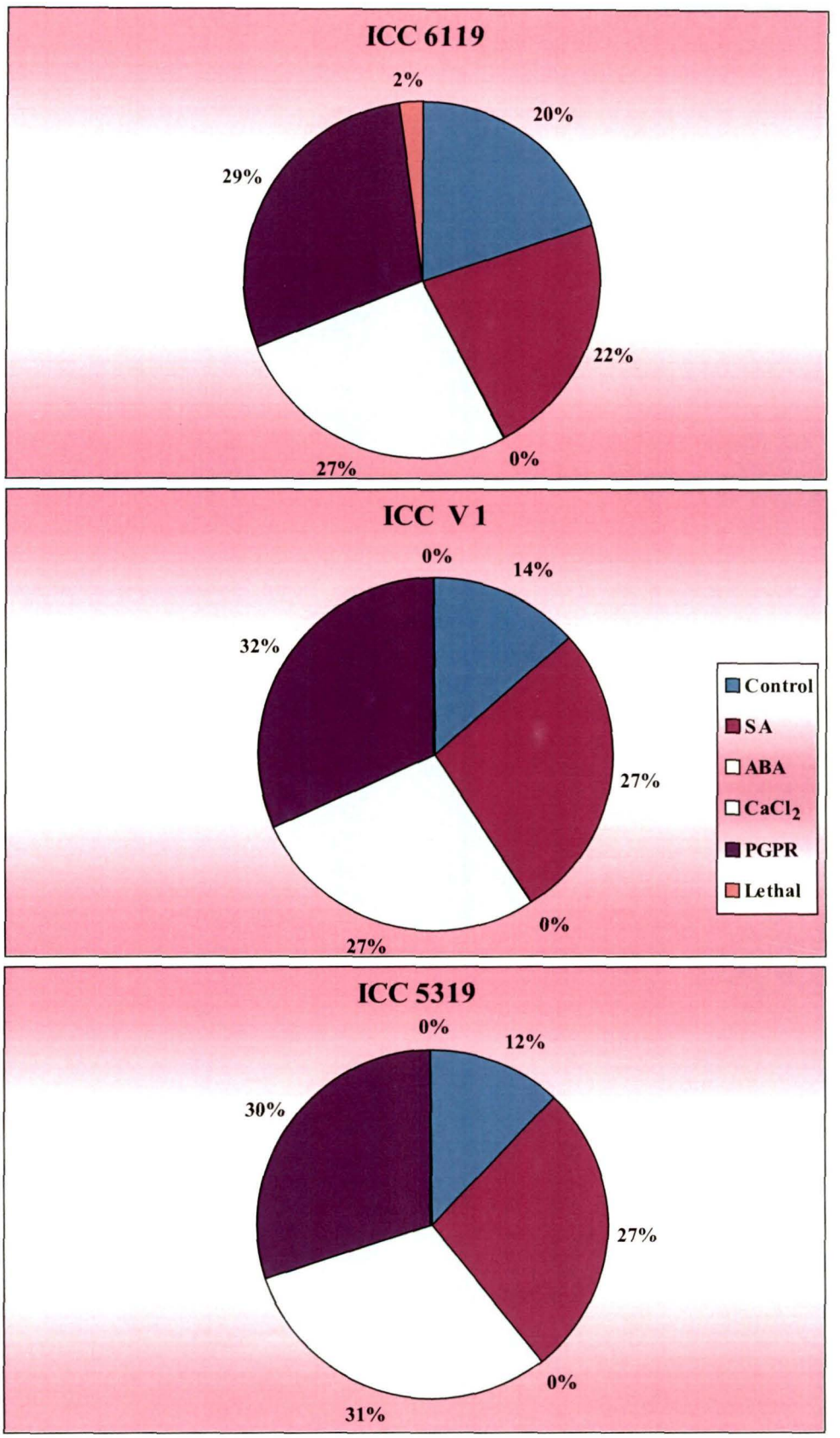


Fig. 2: Germination of three varieties of *Cicer arietinum* L. seeds subjected to different pre-treatments followed by lethal temperature.

Table 5A. Analysis of variance for the data presented in Table

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	11141.290	795.806	17.3805	5.86574
TREATMENT	5	15464.890	3092.978	67.5510	-
ERROR	70	3205.111	45.787	-	-
TOTAL	89	29811.290			

Table 6. Effect of various pre-treatments on seed germination after 72 hrs

	Germination percentage					
Genotypes	**Control**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	80 ± 06.7	90 ± 10.5	70 ± 14.1	95 ± 09.7	95 ± 11.8	10 ± 04.1
ICC 4969	60 ± 07.8	70 ± 08.7	60 ± 15.1	70 ± 17.1	80 ± 09.9	05 ± 01.1
ICC 7344	40 ± 08.9	40 ± 05.4	30 ± 05.2	60 ± 14.2	70 ± 08.6	_
ICC 1852	85 ± 11.5	95 ± 13.3	70 ± 11.9	90 ± 14.2	85 ± 14.1	09 ± 01.7
ICC 10035	50 ± 16.8	70 ± 14.2	30 ± 07.5	70 ± 13.2	75 ± 08.4	-
ICC 6119	60 ± 13.4	65 ± 12.3	50 ± 06.7	70 ± 08.4	70 ± 14.2	06 ± 02.1
ICC 5003	60 ± 18.9	85 ± 8.9	70 ± 10.4	80 ± 11.2	85 ± 15.4	06 ± 03.d1
ICC 14340	60 ± 12.3	65 ± 15.6	60 ± 13.7	80 ± 17.8	85 ± 19.4	02 ± 0.09
ICC 5319	50 ± 06.9	60 ± 14.2	40 ± 07.1	70 ± 09.3	75 ± 12.4	-
ICC 2042	60 ± 09.7	80 ± 09.8	70 ± 07.9	90 ± 14.3	85 ± 09.5	08 ± 02.3
ICC 16359	50 ± 08.8	60 ± 08.9	60 ± 11.7	75 ± 18.9	70 ± 09.4	01 ± 00.0
ICC C37	90 ± 16.8	100 ± 07.9	100 ± 12.3	100 ± 15.9	100 ± 13.9	10 ± 04.6
ICC V10	80 ± 08.9	100 ± 22.2	80 ± 17.5	95 ± 16.4	90 ± 09.7	08 ± 02.8
ICC V1	30 ± 05.6	60 ± 09.7	50 ± 07.5	80 ± 09.7	90 ± 13.0	-
ICC V2	30 ± 06.8	70 ± 16.6	45 ± 09.6	80 ± 13.4	90 ± 16.7	08 ± 03.4

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 6A. Analysis of variance for the data presented in Table 6

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	10534.070	752.434	9.7077	7.63180
TREATMENT	5	62055.170	12411.030	160.1231	-
ERROR	70	5425.653	77.509	-	-
TOTAL	89	78014.900			

4.2.3. Seedling growth

Chickpea seedlings (20 days old) used in the present study were found to grow best at temperatures ranging from 18°C-25°C after which there was a gradual decline in growth rate with the increase in temperature. The ability of the seedlings to tolerate high temperature exposure was found to be directly related to the age of the seedlings and plant vigour. However, no seedlings could tolerate temperatures higher than 46°C for 2 hrs or more.

Pre-treatment of seedlings with various chemicals like SA, ABA and $CaCl_2$ had no major influence in the growth rate and developmental process. But seed bacterization and application of *Bacillus megaterium* suspension as a soil drench had a remarkable influence on plant growth and development both in pot cultures and in the field (Plate IX). The growth promotion was assessed in seedlings of both desi black gram and kabuli genotypes (two each of thermotolerant and susceptible) on the basis of increase in germination percentage and vigour index (VI) (Table 7).Vigour index was calculated by comparing the increase in root and shoot length of the treated samples with the untreated control plants under the same environmental and physical conditions (temperature 25 ± 2°C and RH of 60-70%).

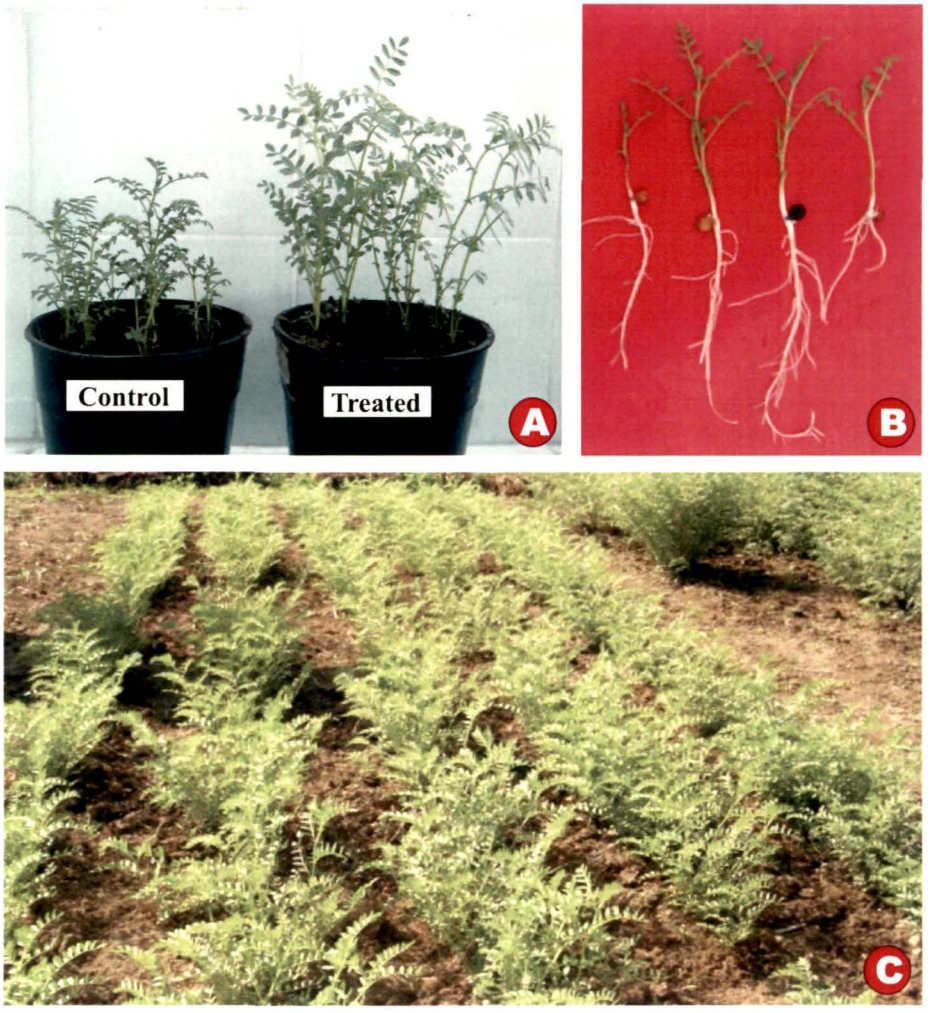


Plate IX (A-C): Effect of *Bacillus megaterium* treatment on growth of chickpea seedlings.

Table 7. Effect of *Bacillus megaterium* on seed germination and vigour index.

Genotypes	Germination percentage		Mean root + shoot length (cm)		Vigour index (VI)	
	Control	*B.megaterium*	Control	*B.megaterium*	Control	*B.megaterium*
ICC C37	90.0 ± 10.8	100.0 ± 08.0	4.4 ± 0.9	6.3 ± 1.6	409.2 ± 22.1	630.0 ± 32.1
ICC V2	30.0 ± 05.2	90.0 ± 12.1	2.6 ± 1.1	4.0 ± 0.9	78.0 ± 12.5	360.0 ± 16.9
ICC 5319	50.0 ± 09.2	75.0 ± 09.4	3.1 ± 0.8	4.2 ± 1.3	155.0 ± 14.6	315.0 ± 13.8
ICC 7344	60.0 ± 11.7	70.0 ± 06.9	3.8 ± 1.3	4.9 ± 1.8	220.0 ± 14.8	343.0 ± 21.5

Table 7A Analysis of variance for the data presented in Table 7

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	3	117186.800	39062.250	16.0142	158.58490
TREATMENT	1	77185.250	77185.250	31.6434	-
ERROR	3	7317.656	2449.219	-	-
TOTAL	7	201689.700			

4.3. Induction of thermotolerance in *Cicer arietinum* by pre-treatments

Although the ability of organisms to acquire thermotolerance to normally lethal temperatures is an inherent and conserved adaptive response, it can be manipulated in various ways to make plants more tolerant to elevated temperatures. In this study, an attempt was made to induce thermotolerance by applying various chemicals in the form of foliar spray, soil drench and by pre-exposing the seedlings to sub-lethal temperature (heat-acclimation) treatment.

Assessment of induced heat tolerance was done by recording the survival percentage. Survival was determined by the capacity of the seedlings to grow after lethal temperature treatment. Thermotolerance was assessed from the percentage survival in each sample of 20 plants 24, 48, 72 and 96 hrs after imposition of heat stress. Seedling death was characterized by permanent wilting and collapse of seedling.

Seedlings pre-treated with SA, ABA, $CaCl_2$ or subjected to heat acclimation treatment before lethal temperature exposure were found to be more tolerant in comparison to the untreated control samples (Plate X). Most remarkable enhancement in the level of thermotolerance due to chemical pre-treatments was exhibited by the genotype ICC 5003. Heat susceptible genotypes like ICC 5319, ICC 7344, ICC 10035 and ICC V1 also showed a remarkable improvement in the degree of thermotolerance in pre-treated samples. Induced level of heat tolerance was also exhibited by seedlings raised from bacterized seeds and treated with bacterial suspension (2×10^6 cfu/ml) overnight (Plate XI). Increase in the level of thermotolerance due to *Bacillus megaterium* was found to be primarily associated with increased growth and vigour.

4.4. Effect of pre-treatments and elevated temperature treatments of seeds and seedlings on proteins

4.4.1. Protein content

Protein content of control, heat pre-treated and challenged and heat- shocked seeds, different plant parts grown from normal and heat treated seeds and whole seedlings were determined as described earlier. Protein content of seeds varied with the type of genotype and interestingly maximum protein contents were recorded in kabuli genotypes like ICC 7344, ICC V2 and ICC 10035. Protein contents increased significantly due to pre-treatment at sub-lethal temperature (45°C-2hrs), while it declined at lethal temperature (Table 8).

The increase in protein content of the pre-treated seeds was maximum in the genotypes ICC C37 and ICC V10 and minimum in ICC 7344 and ICC 5319, whereas the decrease in protein content of the seeds exposed to lethal temperature was maximum in the genotype ICC 7344 followed by ICC 10035 and minimum in ICC V2 and ICC 2042. Analysis of variance revealed significant increase in SA and $CaCl_2$ pre-treatments with respect to control.

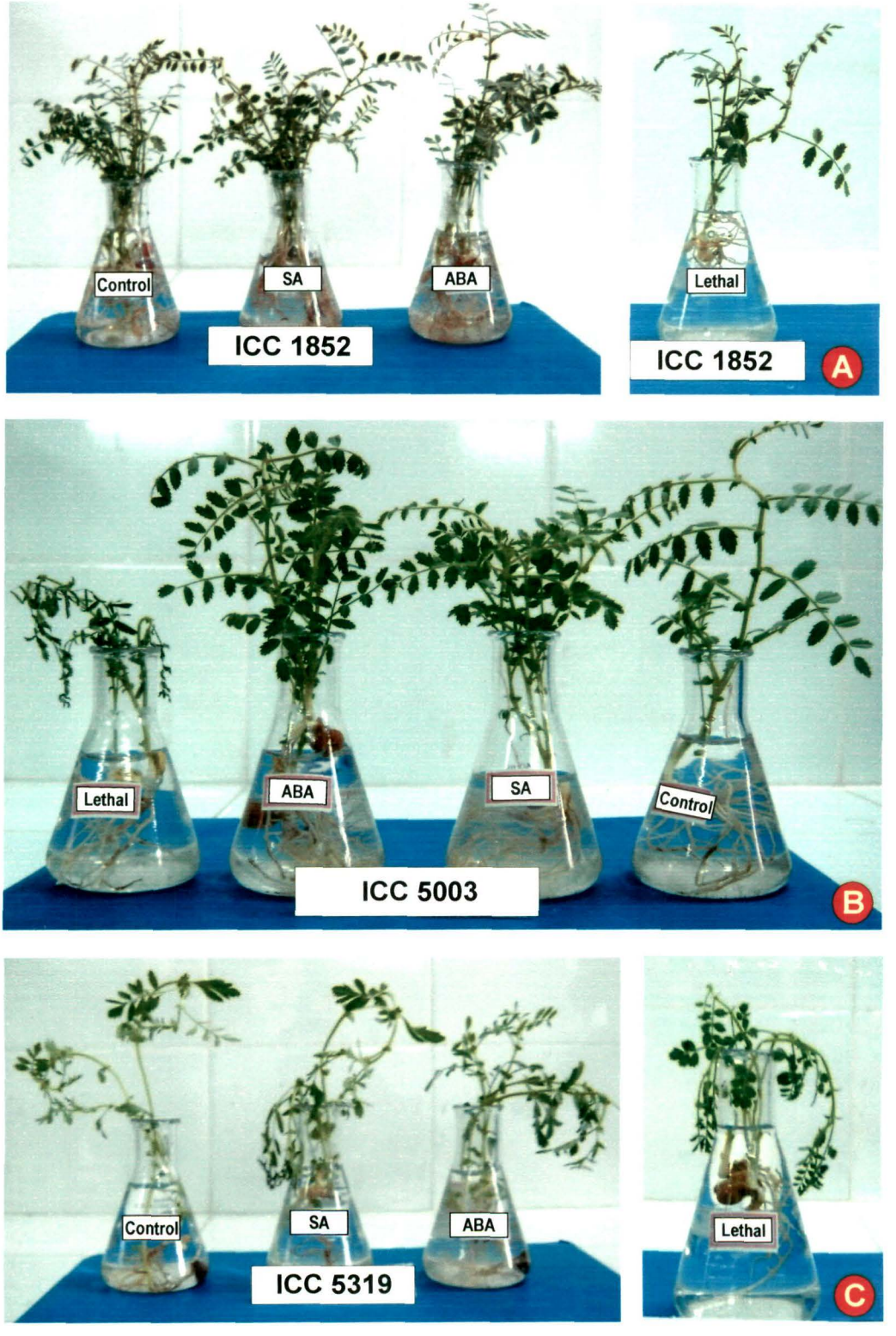


Plate X (A-C): Treatment of chickpea seedlings of different genotypes in chemical solutions prior to lethal temperature treatment as well as direct lethal temperature treatment.

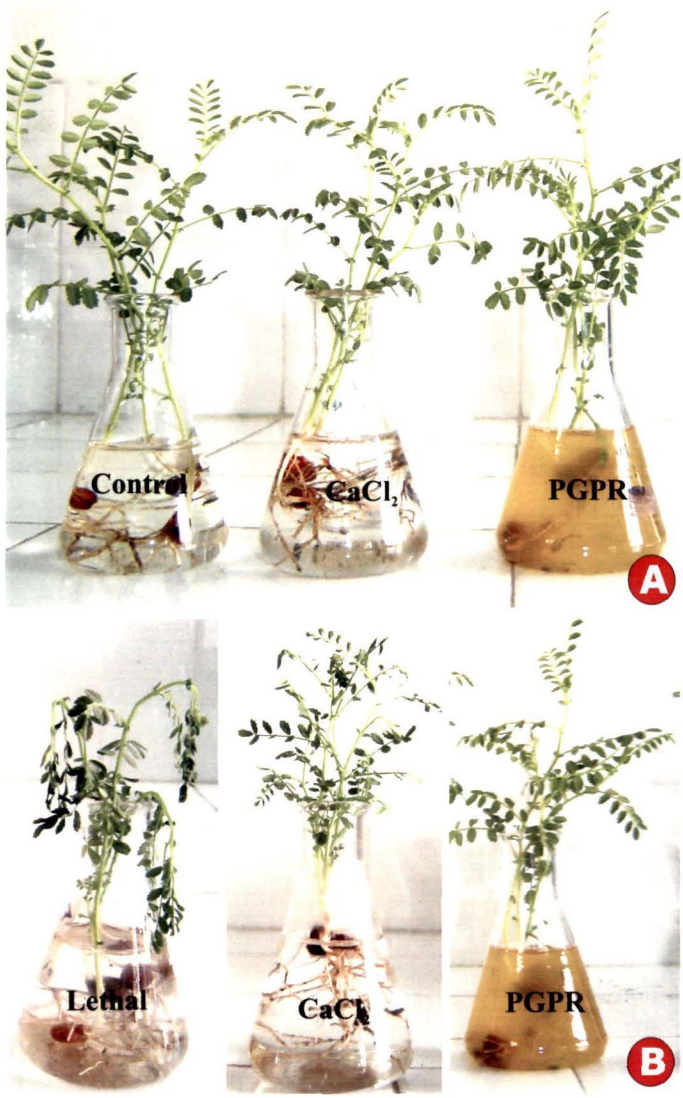


Plate XI (A-B): Treatment of chickpea seedlings (ICC 2042) in $CaCl_2$ solution and *Bacillus megaterium* suspension. **A:** Before heat treatment; **B:** After lethal temperature treatment

Table 8. Protein content of chickpea seeds of different genotypes pre-treated at 45°C for 2 hrs before exposure to 55°C for the same duration

Genotypes	Protein content (mg g^{-1} tissue)		
	Control	Pre- treated	Direct Lethal
ICC 4918	39.25±0.38	46.73±1.14	35.18±1.72
ICC 4969	37.22±0.98	41.98±0.59	29.86±1.24
ICC 7344	48.91±1.17	51.08±0.63	32.18±1.17
ICC 1852	36.83±1.21	42.66±1.44	31.20±1.71
ICC 10035	43.93±2.36	46.73±1.15	30.01±1.15
ICC 6119	38.08±0.46	42.62±0.61	30.12±1.16
ICC 5003	33.76±1.73	38.43±1.15	26.20±1.13
ICC 14340	33.61±1.75	36.02±0.59	25.59±1.74
ICC 5319	32.43±1.14	34.60±0.60	23.08±1.78
ICC 2042	38.54±1.15	44.33±0.58	35.37±1.29
ICC 16359	36.00±1.15	39.64±1.74	30.92±1.14
ICC C37	37.50±0.76	47.08±0.08	33.31±1.73
ICC V10	32.33±1.05	41.24±1.14	27.08±1.72
ICC V1	31.40±0.58	33.87±1.76	23.62±1.73
ICC V2	40.19±1.16	46.92±1.13	38.16±2.29

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 8A Analysis of variance for the data presented in Table 8

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	795.926	56.852	11.5849	1.99556
TREATMENT	2	1117.717	558.859	113.8805	-
ERROR	28	137.408	4.907	-	-
TOTAL	44	2051.051			

Chickpea plants being cultivated worldwide for their protein content, these were studied in greater detail with respect to protein metabolism. Protein content of different plant parts (Table 9) obtained from the plants raised from heat treated seeds revealed an increase at elevated temperature treatment registering maximum

value at 45°C treatment in all genotypes tested. Maximum protein content was recorded in leaves while the minimum was recorded in roots. Protein content increased gradually with increase in temperature in some genotypes, while in some genotypes there was a slight reduction in 35°C treatment followed by a maximum increment at 45°C treatment (Fig.3).

Table 9. Protein content of different plant parts raised from seeds treated at various elevated temperatures

Genotypes	Protein content (mg g^{-1} tissue)					
	Parts	Control	35°C	40°C	45°C	Lethal
4969	Leaf	35.03±1.89	36.09±1.09	36.91±2.90	38.02±1.58	30.01±0.86
	Stem	22.20±1.05	23.19±2.23	24.06±3.02	25.19±1.76	18.96±3.56
	Root	15.86±1.93	16.17±1.64	16.89±0.49	17.01±4.67	11.07±2.75
7344	Leaf	41.12±5.81	40.98±4.38	42.68±3.85	43.89±8.12	30.13±3.05
	Stem	36.23±5.04	36.92±4.33	37.81±3.90	38.17±1.90	29.07±2.78
	Root	19.06±1.68	20.01±2.36	21.37±0.66	22.49±2.29	11.07±1.70
1852	Leaf	31.11±3.28	33.31±2.30	36.39±1.87	36.96±6.14	28.85±2.58
	Stem	26.21±2.69	28.46±4.24	30.91±3.15	31.61±2.71	23.17±3.30
	Root	17.31±1.77	19.57±0.39	21.66±2.38	22.00±3.26	14.97±1.04
6119	Leaf	35.07±5.01	36.50±2.66	37.00±1.73	38.26±2.31	30.01±0.99
	Stem	27.39±1.50	29.12±2.35	30.37±3.19	32.02±5.24	21.73±4.69
	Root	18.96±1.92	19.19±0.62	20.18±2.37	21.35±6.01	10.58±2.90
14340	Leaf	30.44±3.21	28.38±2.46	30.06±3.87	33.91±3.49	24.33±4.28
	Stem	19.09±3.25	18.00±1.82	20.01±2.36	22.83±4.37	14.50±1.88
	Root	12.50±2.38	10.07±1.78	11.98±1.89	12.07±2.18	06.50±0.67
5319	Leaf	32.12±5.76	30.00±3.28	32.83±1.99	34.02±3.22	23.42±2.36
	Stem	22.01±2.09	22.01±1.85	22.95±4.52	23.03±3.47	14.18±1.92
	Root	12.06±1.05	10.98±0.58	11.31±0.55	14.13±2.11	06.13±0.63

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 9A Analysis of variance for the data presented in Table 9 (leaf protein)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	351.436	70.287	35.6300	2.04997
TREATMENT	4	328.898	82.225	41.6813	-
ERROR	20	39.454	1.973	-	-
TOTAL	29	719.788			

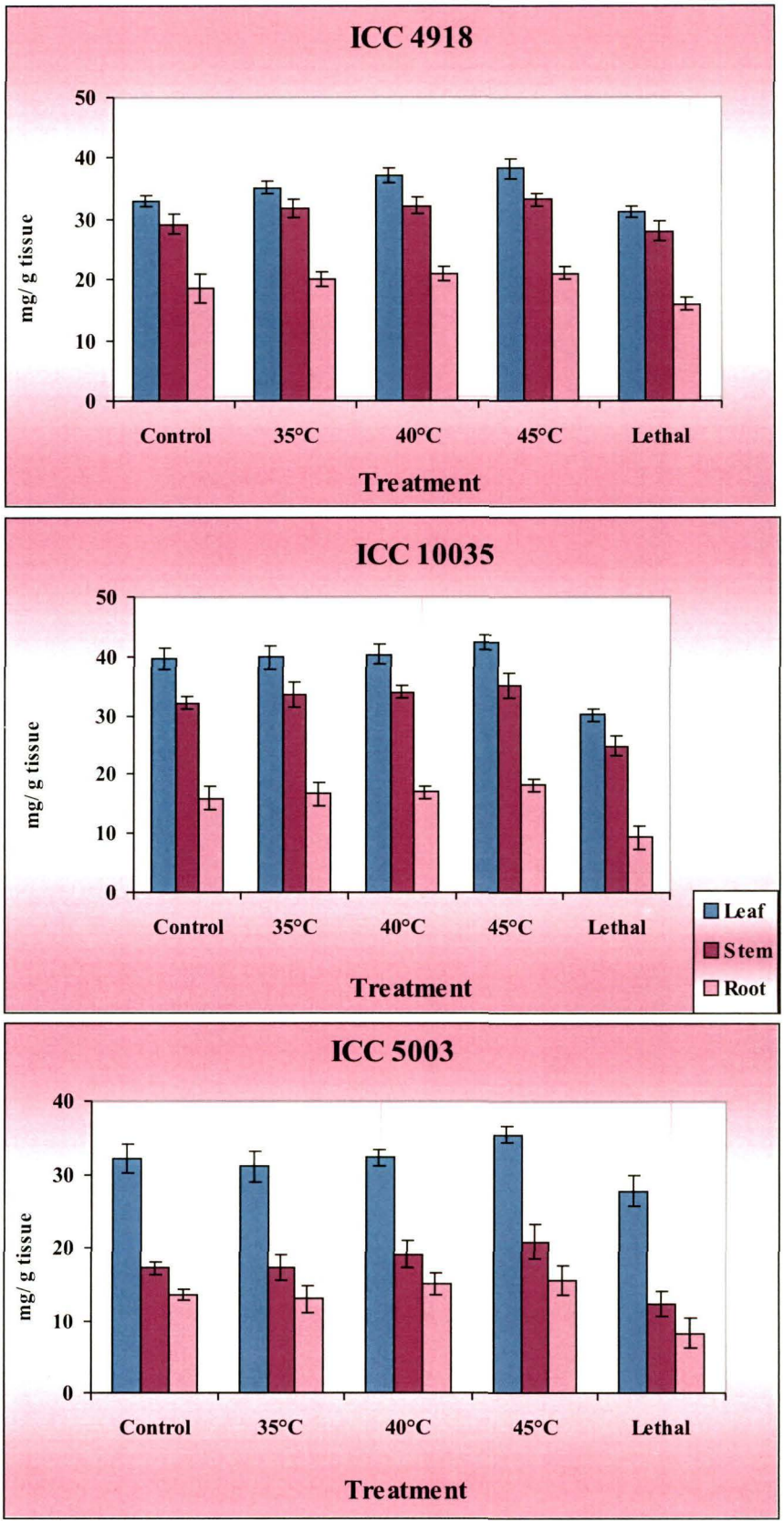


Fig. 3: Protein contents of root, stem and leaves of chickpea plants grown from seeds pre-treated at various elevated temperatures.

Table 9B Analysis of variance for the data presented in Table 9 (stem protein)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	946.417	189.283	171.9654	1.53127
TREATMENT	4	260.857	65.214	59.2476	-
ERROR	20	22.014	1.101	-	-
TOTAL	29	1229.288			

Table 9C Analysis of variance for the data presented in Table 9 (root protein)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	460.056	92.011	13.0330	3.87806
TREATMENT	4	272.676	68.169	9.6559	-
ERROR	20	141.197	7.060	-	-
TOTAL	29	873.930			

Protein contents in seedlings increased following moderate heat treatment (heat acclimation) but showed a rapid decline at lethal temperature in all genotypes. Protein degradation following lethal temperature treatment was lower in tolerant genotypes like ICC 4918, ICC 1852, ICC 2042, ICC C37 (Kranthi), ICC V2 (Swetha) and ICC V10; moderate in genotypes like in ICC 4969, ICC 14340, ICC 6119 and ICC 5003, and highest in susceptible genotypes like ICC 7344, ICC 10035 and ICC V1 and ICC 5319.(Table 10). Pre-treatments with SA, ABA, $CaCl_2$ or heat-acclimation not only lowered the extent of decrease in total protein content but also led to increased protein synthesis following lethal temperature exposure (Fig. 4). Maximum protein contents were recorded in case of SA and $CaCl_2$ pre-treated seedlings following lethal temperature treatment.

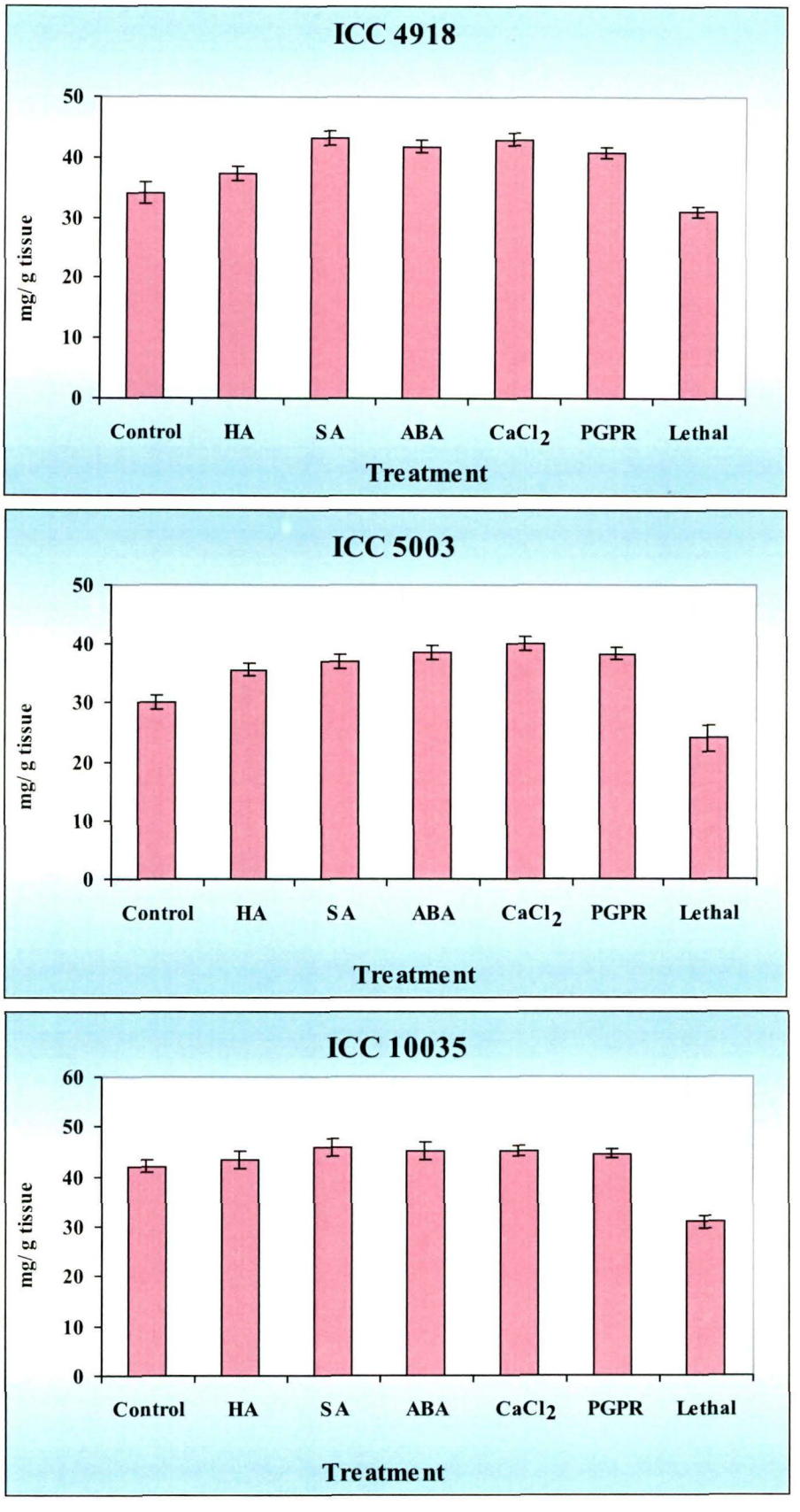


Fig. 4 : Effect of pre-treatments and lethal temperature on protein content of seedlings.

Table 10. Changes in protein contents of seedlings subjected to various pre-treatments before exposure to lethal temperature

	Protein content (mg g^{-1} tissue)						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	34.05 ± 1.76	37.26 ± 1.13	43.13 ± 1.15	41.81 ± 0.58	42.91 ± 0.59	40.86 ± 0.19	31.03 ± 0.04
ICC 4969	36.84 ± 1.14	38.21 ± 1.15	40.85 ± 1.15	41.09 ± 0.47	41.26 ± 1.07	38.72 ± 0.64	30.16 ± 1.18
ICC 7344	46.08 ± 0.29	48.32 ± 1.16	49.35 ± 1.17	50.19 ± 0.57	52.18 ± 0.36	39.06 ± 0.48	32.63 ± 1.14
ICC 1852	33.65 ± 0.57	38.55 ± 1.17	43.56 ± 1.18	43.02 ± 0.03	43.32 ± 0.99	40.11 ± 1.14	30.06 ± 1.14
ICC 10035	42.11 ± 1.16	43.29 ± 1.73	45.98 ± 1.37	45.06 ± 1.70	45.14 ± 1.14	44.60 ± 0.28	30.89 ± 1.14
ICC 6119	36.70 ± 1.16	38.16 ± 1.14	40.97 ± 1.15	41.30 ± 0.56	42.18 ± 1.09	39.56 ± 1.17	30.85 ± 1.15
ICC 5003	30.15 ± 1.14	35.68 ± 1.18	37.14 ± 1.15	38.62 ± 1.16	40.01 ± 1.15	38.40 ± 0.69	24.02 ± 2.31
ICC 14340	30.65 ± 0.32	33.55 ± 1.74	37.56 ± 1.73	36.02 ± 1.01	37.44 ± 1.03	35.46 ± 0.68	24.06 ± 2.31
ICC 5319	28.23 ± 1.14	31.18 ± 0.60	32.71 ± 1.13	32.02 ± 0.69	34.56 ± 0.80	32.33 ± 0.41	18.09 ± 0.49
ICC 2042	34.56 ± 2.30	38.95 ± 1.16	42.85 ± 1.15	43.62 ± 1.73	44.77 ± 0.64	41.25 ± 0.99	32.56 ± 1.17
ICC 16359	32.50 ± 1.15	34.06 ± 0.11	36.08 ± 1.15	37.81 ± 1.20	38.62 ± 0.93	35.51 ± 0.89	22.98 ± 0.51
ICC C37	32.21 ± 1.15	35.91 ± 1.73	40.08 ± 1.18	39.76 ± 1.73	40.21 ± 1.09	38.88 ± 1.23	28.94 ± 1.16
ICC V10	29.04 ± 1.18	31.92 ± 3.38	37.18 ± 1.18	36.29 ± 0.28	39.06 ± 0.91	36.80 ± 0.92	24.86 ± 2.31
ICC V1	23.98 ± 1.74	23.01 ± 0.02	28.07 ± 1.16	27.37 ± 1.15	29.92 ± 0.71	26.63 ± 1.16	14.83 ± 1.16
ICC V2	37.84 ± 1.15	40.08 ± 1.16	43.16 ± 1.73	44.19 ± 2.32	44.70 ± 0.43	42.16 ± 0.53	32.75 ± 1.16

Table 10A. Analysis of variance for the data presented in Table 10

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	2332.482	166.606	21.1953	2.43039
TREATMENT	6	1856.833	309.472	39.3705	-
ERROR	84	660.282	7.861	-	-
TOTAL	104	4849.598			

4.4.2. Protein profile

SDS–PAGE analysis revealed changes in protein profile of control, pre-treated and challenged and heat shocked seeds and seedlings. Pre-treated seeds of all varieties following heat shock showed an increase in synthesis of some proteins. New proteins having molecular masses of 18, 25, 26, and 82 kDa were observed in case of seeds subjected to heat-acclimation prior to lethal temperature treatment. SA and ABA pre-treatments also led to the expression of some new proteins having molecular masses of 13.3, 18, 20, 25, 26, 26.5, 44, 82, 84 and 96 kDa in SA and 13.3, 18, 25, 26, 27.5, 82 and 96 kDa in ABA pre-treatments following exposure to lethal temperature (Tables 11-14, Plate XII). Seeds and seedlings when exposed to lethal temperature showed considerable reduction in the number of protein bands and also revealed significant genotypic difference in protein profile (Tables 15-17, Plate XIII).

Table 11. SDS-PAGE analysis of chickpea seed proteins

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
Mol. wt. marker	1	11	8.5, 14.2, 20, 24, 29, 35, 45, 55, 66, 84 and 97
ICC 4918 C	2	18	8.5,12, 14.2, 18, 19, 20, 21, 22, 24, 27, 29, 34, 35, 40, 42, 50, 55 and 66
ICC 4918 SA	3	19	8.5,12, 14.2, 18, 19, 20, 21, 22, 24, 27, 29, 34, 35, 40, 42, 50, 55, 66 and 96*
ICC 4918 ABA	4	19	8.5, 12, 14.2, 18, 19, 20, 21, 22, 24, 27, 29, 34, 35, 40, 42, 50, 55, 66 and 96*
ICC 4918 $CaCl_2$	5	19	8.5, 12, 14.2, 18, 19, 20, 21, 22, 24, 27,29, 34, 35, 40, 42, 48*, 50, 55 and 66
ICC 4918 PGPR	6	18	8.5, 12, 14.2, 18, 19, 20, 21, 22, 24, 27,29, 34, 35, 40, 42, 50, 55 and 66
ICC 4918 L	7	14	8.5, 12, 19, 20, 21, 22, 24, 27, 35, 40,50, 55, 62and 66

* newly synthesized proteins

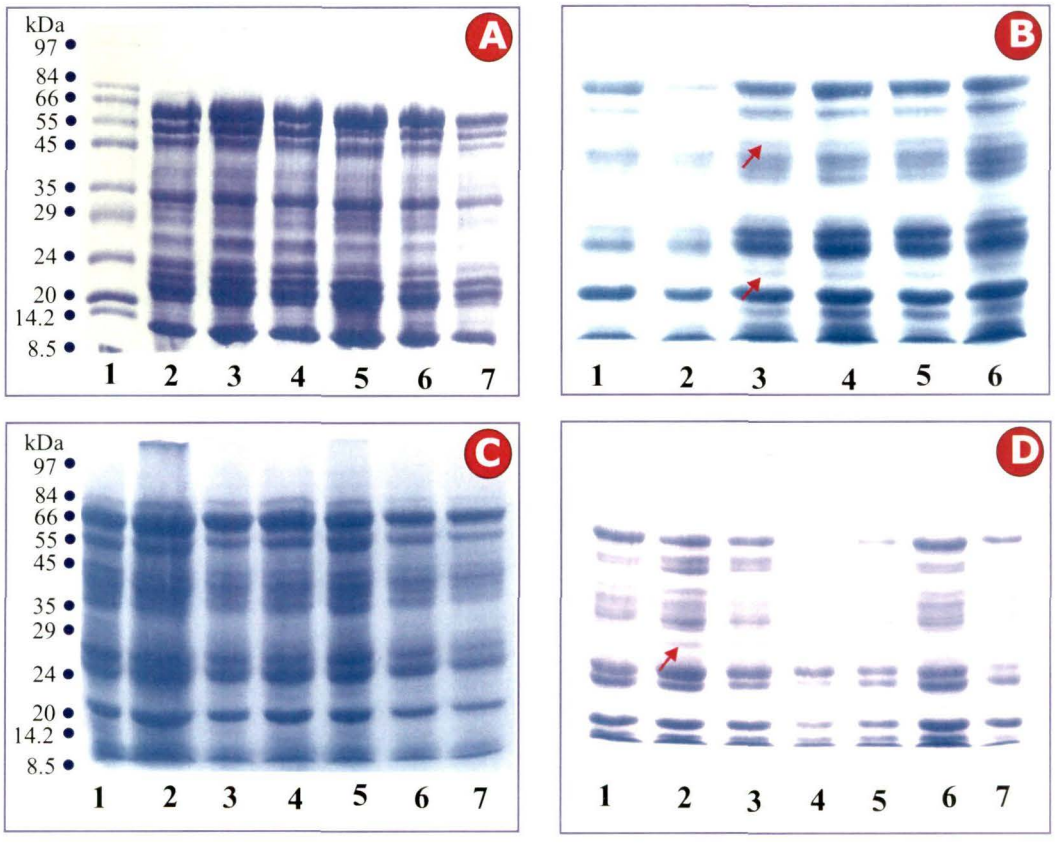


Plate XII (A-D) :SDS-PAGE analysis of seed proteins of different genotypes subjected to pre-treatments and lethal temperature treatment. **A:** Lane 1: Marker; 2: ICC 4918C; 3: 4918SA; 4: 4918 ABA; 5: 4918 $CaCl_2$; 6: 4918 PGPR & 7: 4918L. **B:** Lane1 : ICC 4969 C; 2: 4969L; 3: 4969SA;4: 4969ABA; 5: 4969 HA & 6: 4969$CaCl_2$. **C:** Lane 1: ICC 2042C; 2: 2042HA; 3: 2042L;4: 2042ABA; 5: 2042SA; 6: 2042L & 7: 2042L. **D:** Lane 1: ICC 5003C; 2: 5003SA; 3: 5003ABA; 4: 5003HA; 5: 5003HA; 6: 5003$CaCl_2$ & 7: 5003L.

Table 12. SDS-PAGE analysis of chickpea seed proteins

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC 4969C	1	18	8.5, 9.0, 12, 14.2, 20, 24, 25, 27, 29, 30, 30.5,32,35, 40, 42, 45, 55 and 66
ICC 4969L	2	16	8.5, 9.0, 12, 14.2, 18*, 24, 25, 26*, 29,30,30,5,32, 33.5*, 35, 40 and 55
ICC 4969 SA	3	18	8.5, 9.0, 12, 14.2, 18*, 24, 26*, 26.5*, 27,29,30,30.5, 32, 33*, 35, 45, 55 and 66
ICC 4969 ABA	4	19	8.5, 9.0, 12,14.2,18*, 24, 26*, 27,27.5* 29,30,31, 32, 35, 40, 42,45,55 and 66
ICC 4969 HA	5	18	8.5, 9.0, 12, 14.2, 18*, 24, 26*, 27, 29, 30,31,32,35, 40, 42, 45, 55 and 66
ICC 4969 $CaCl_2$	6	17	8.5,9.0,12,14.2, 18*, 24, 26*, 27,27.5* 27.5*,29,30,31, 32, 35, 40, 42 and 55

* newly synthesized proteins

Table 13. SDS-PAGE analysis of chickpea seed proteins

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC 2042 C	1	16	8.5, 9.5, 11, 12, 14.2, 22, 24, 27,29,34 35,39, 45, 55,56 and 66
ICC 2042 HA	2	16	8.5, 9.5,11, 14.2, 22, 24, 25*, 29,34,35, 39, 45, 55, 56, 66 and 82*
ICC 2042 L	3	12	8.5, 9.5, 14.2, 22, 24, 29, 35, 39*,45,55 56 and 66
ICC 2042 ABA	4	16	8.5, 9.5,11, 14.2, 22, 24, 25*, 29,34,35, 39, 45, 55, 56, 66 and 82*
ICC 2042 SA	5	19	8.5, 9.5, 11, 14.2,20*,22, 24, 25*,29,34, 35,39,44*,45,55,56,66,82*and 84*
ICC 2042 L	6	12	8.5, 9.5, 14.2, 22, 24, 29, 35, 39*,45,55 56 and 66
ICC 2042 L	7	12	8.5, 9.5, 14.2, 22, 24, 29, 35, 39*,45,55, 56 and 66

* newly synthesized proteins

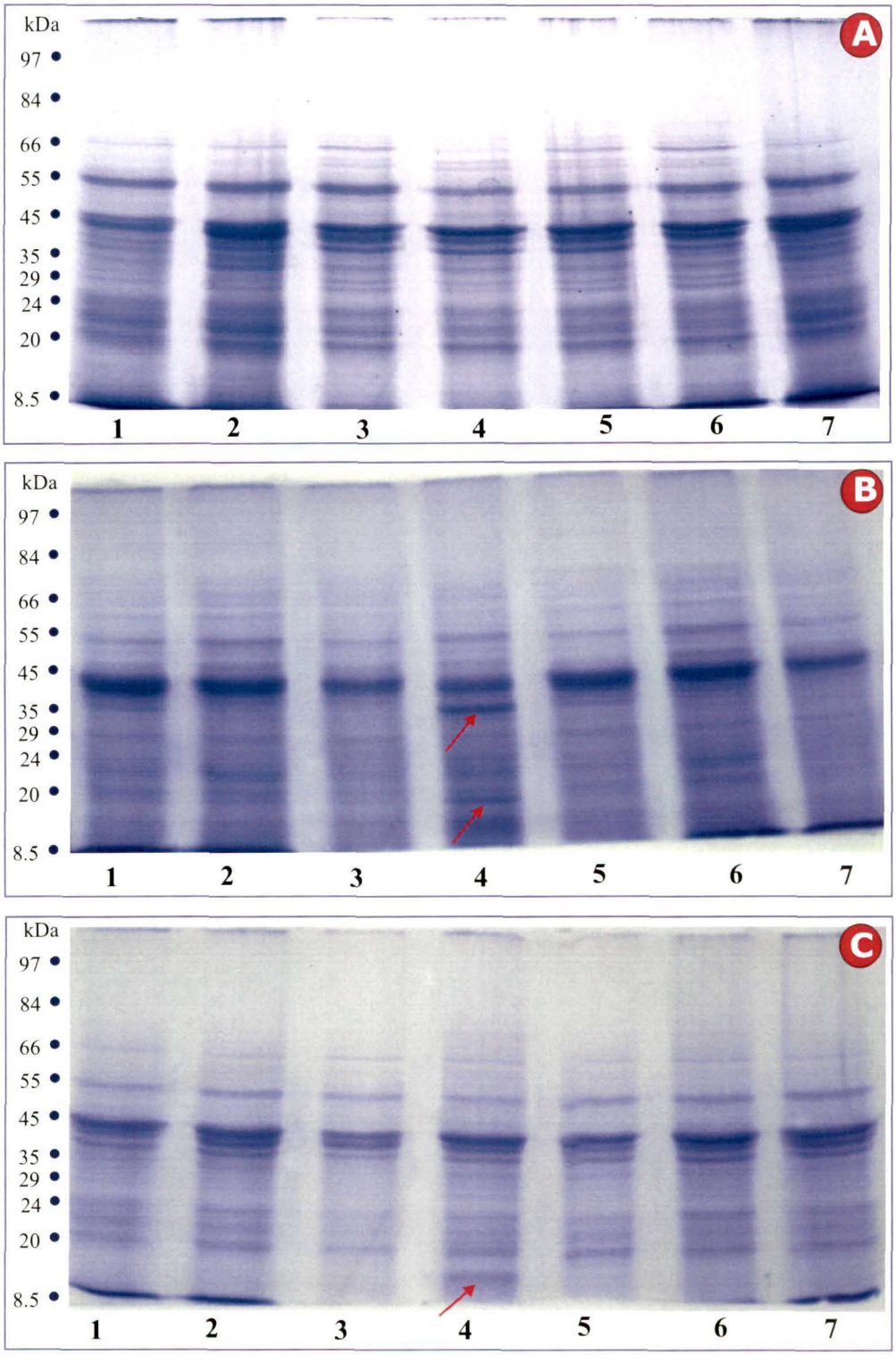


Plate XIII (A-C): SDS-PAGE analysis of proteins from chickpea seedlings of different genotypes subjected to pre-treatments and lethal temperature treatment. **A:** Lane 1: ICC 35 C; 2:10035 ABA; 3: 10035L; 4: 10035 SA; 5: 10035 HA; 6: 10035 CaCl2; 7: 10035 PGPR. **B:** Lane 1: ICC C37 C; 2: ICC C37 SA, 3: ICC 37 L; 4: ICC V2C; 5:ICC C37 ABA; 6: ICC C37 SA; 7: ICC C37 L.**C:** Lane 1: ICC 14340 C; 2: 14340 SA; 3: 14340 L; 4: 14340 ABA; 5: 14340 HA, 6: 14340 SA

Table 14. SDS-PAGE analysis of chickpea seed proteins

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC5003 C	1	21	9.4, 10.1, 11.2, 14.2, 15.4, 16.7, 17.8,19.9 22.8, 23.8, 25.1, 28.1, 29.8, 33.4,34.4,44.6, 47.3, 53, 56.2, 57.8 and 66.8
ICC 5003 SA	2	22	9.4,10.1,11.2,13.3*,15.4, 16.7 17.8, 19.9, 22.8,23.8,25.1,28.1,29.8, 33.4,34.4, 39.8*, 44.6, 47.3, 53, 56.2, 57.8 and 66.8
ICC 5003 ABA	3	22	9.4,10.1,11.2,13.3*,15.4, 16.7 17.8, 19.9, 22.8,23.8,25.1,28.1,29.8, 33.4,34.4, 39.8*, 44.6, 47.3, 53, 56.2, 57.8 and 66.8
ICC 5003 HA	4	19	9.4, 10.1, 11.2, 15.4, 16.7, 17.8, 19.9, 22.8, 23.8, 25.1,28.1, 29.8, 33.4, 34.4, 44.6, 47.3, 53, 56.2 and 57.8
ICC 5003 HA		5	19 9.4, 10.1, 11.2, 15.4, 16.7, 17.8, 19.9, 22.8, 23.8, 25.1, 28.1, 29.8, 33.4, 34.4,44.6, 47.3, 53, 56.2 and 57.8
ICC 5003 $CaCl_2$	6	21	9.4, 10.1, 11.2, 13.3*, 14.2, 14.9*,15.4, 16.7 17.8,19.9, 22.8, 22.9*,23.8, 25.1, 28.1,29.8 33.4, 34.4, 53, 56.2, and 66.8
ICC 5003 L	7	16	9.4, 10.1,11.2,15.4,16.7,17.8,19.9,28.1,29.8, 29.8,33.4, 34.4,44.6,47.3 53 56.2 and 57.8

* newly synthesized proteins

Pre-treated seedlings following exposure to lethal temperature also revealed the expression of some new proteins (Tables 18-20, Plate XIV). Low molecular masses proteins of 15.6 and 17.3 and other proteins having molecular masses of 21.2, 22.3, 25.1, 39.8, 42.1, 44.6, 55, 66 and 70.7 k Da. (approx.) were observed in SA pre-treated seedlings challenged with lethal temperature. ABA and $CaCl_2$ pre-treatments also led to the expression of new proteins of molecular masses 10.6, 21.1, 22.3, 29.4, 39.8, 45.3 and 55 kDa and 11.2, 22.3, 33.5, 35.4, 39.8, 44.6, 45.3, 55 and 66 kDa respectively. Exposure of seedlings directly to lethal temperature without any pre-treatments however led to loss of some protein bands.

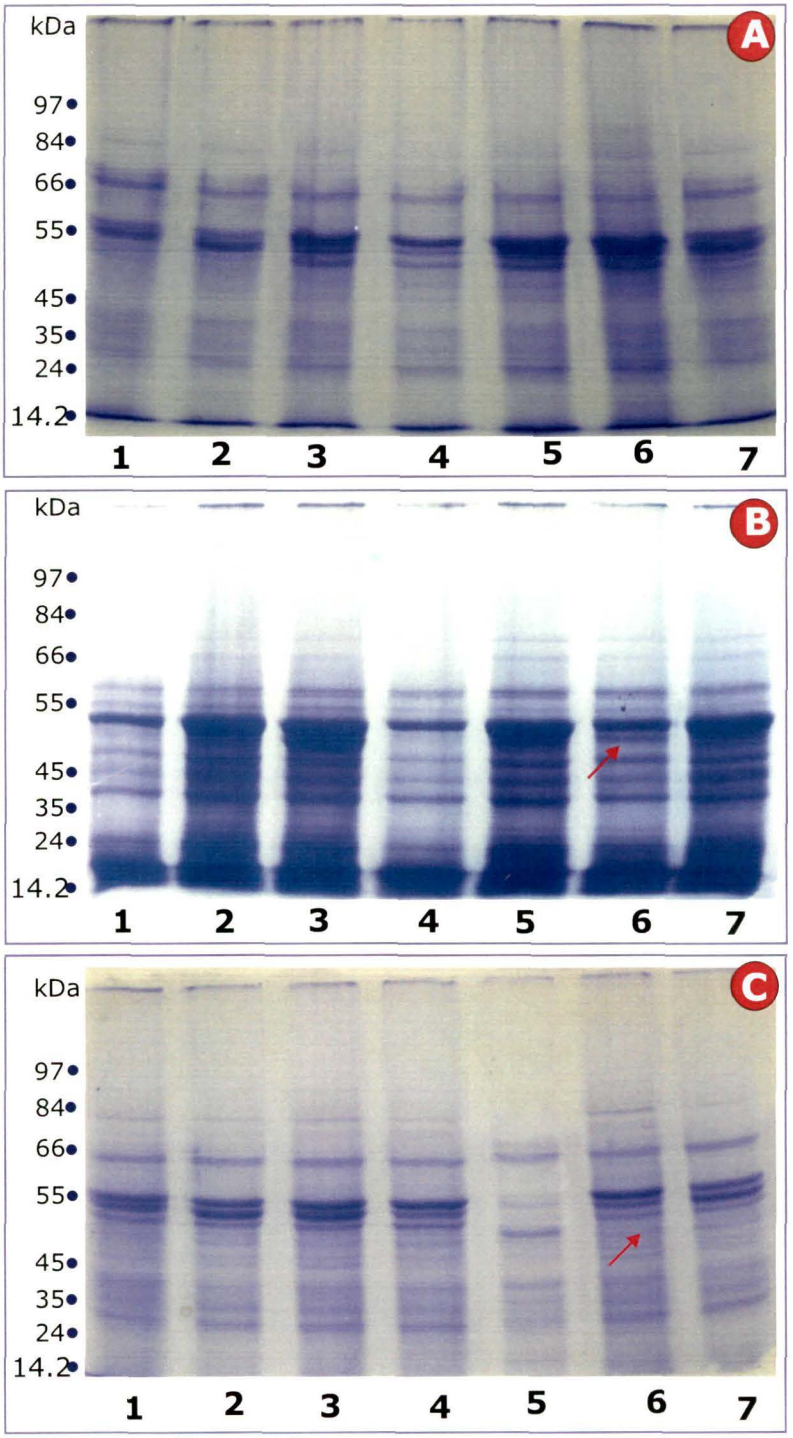


Plate XIV(A-C): SDS-PAGE analysis of proteins from chickpea seedlings of three different genotypes subjected to pre-treatments and lethal temperature treatment. **A:** Lane 1: ICC 1852 C; 2: 1852 HA; 3: 1852 SA; 4: 1852L; 5: 1852 ABA; 6: 1852 $CaCl_2$, 7: 1852 HA.**B:** Lane 1: ICC 7344 C; 2:7344 SA; 3: 7344 $CaCl_2$; 4: 7344L; 5: 7344SA, 6: 7344 ABA; 7: 7344 $CaCl_2$.**C:** Lane 1: ICC 2042 C; 2:2042 ABA; 3: 2042 SA, 4:2042 HA; 5: 2042 L;6: 2042 $CaCl_2$; 7: 2042 HA

Table 15. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC 10035 C 23.7,	1	14	8.4, 13.3, 14.9, 15.8, 16.7, 19.9,22.3, 25.1, 26.6, 27.3, 29.8, 39.8 and 66.8
ICC 10035 ABA	2	13	8.4, 13.3, 14.9, 15.8, 16.7, 19.9, 23.7, 25.1,26.6, 27.3, 29.8, 39.8, and 66.8
ICC10035 L	3	13	8.4, 13.3, 14.9, 16.7, 19.9, 22.3, 23.7, 26.6, 27.3, 29.8, 39.8, 44.6 and 66.8
ICC 10035 SA	4	15	8.4, 13.3, 14.9, 16.7, 19.9, 22.3 23.7, 26.6, 27.3, 29.8, 39.8,42.1*,44.6,66.8 and 70.7*
ICC 10035 HA	5	13	8.4, 13.3, 14.9, 16.7, 19.9, 22.3, 23.7, 26.6, 27.3, 29.8, 35.4*, 39.8 and 66.8
ICC 10035 $CaCl_2$	6	16	8.4, 13.3, 14.9, 16.7, 19.9, 22.3,23.7, 25.1, 26.6, 27.3, 29.8, 33.5*, 35.4*, 39.8, 44.6 and 66.8
ICC 10035 PGPR	7	13	8.4, 13.3, 14.9, 15.8,16.7,19.9,22.3, 23.7, 26.6, 27.3, 29.8, 39.8 and 66.8

* newly synthesized proteins

4.5. Studies on activities of antioxidative enzymes in pre-treated seedlings of chickpea following lethal temperature treatment

4.5.1. Changes in enzymatic activities

4.5.1.1. Peroxidase

Peroxidase activity assay of pre-treated seedlings revealed increase in activity in all genotypes over control following lethal temperature treatment (Table 21). However, quantum of increase was relatively much more significant in tolerant cultivars (ICC 4918, ICC 2042, ICC C37, ICC V10, ICC V2 and ICC 1852) than in susceptible ones (ICC 7344, ICC V1, ICC 5319, ICC 16359 and ICC 10035) (Fig. 5). Pre-treatment of seedlings with SA and $CaCl_2$ solution prior to heat treatment led to 2-3 fold increase in activity in tolerant genotypes. Moreover, the tolerant genotypes and green gram (ICC 4969) also exhibited constitutively higher peroxidase activity.

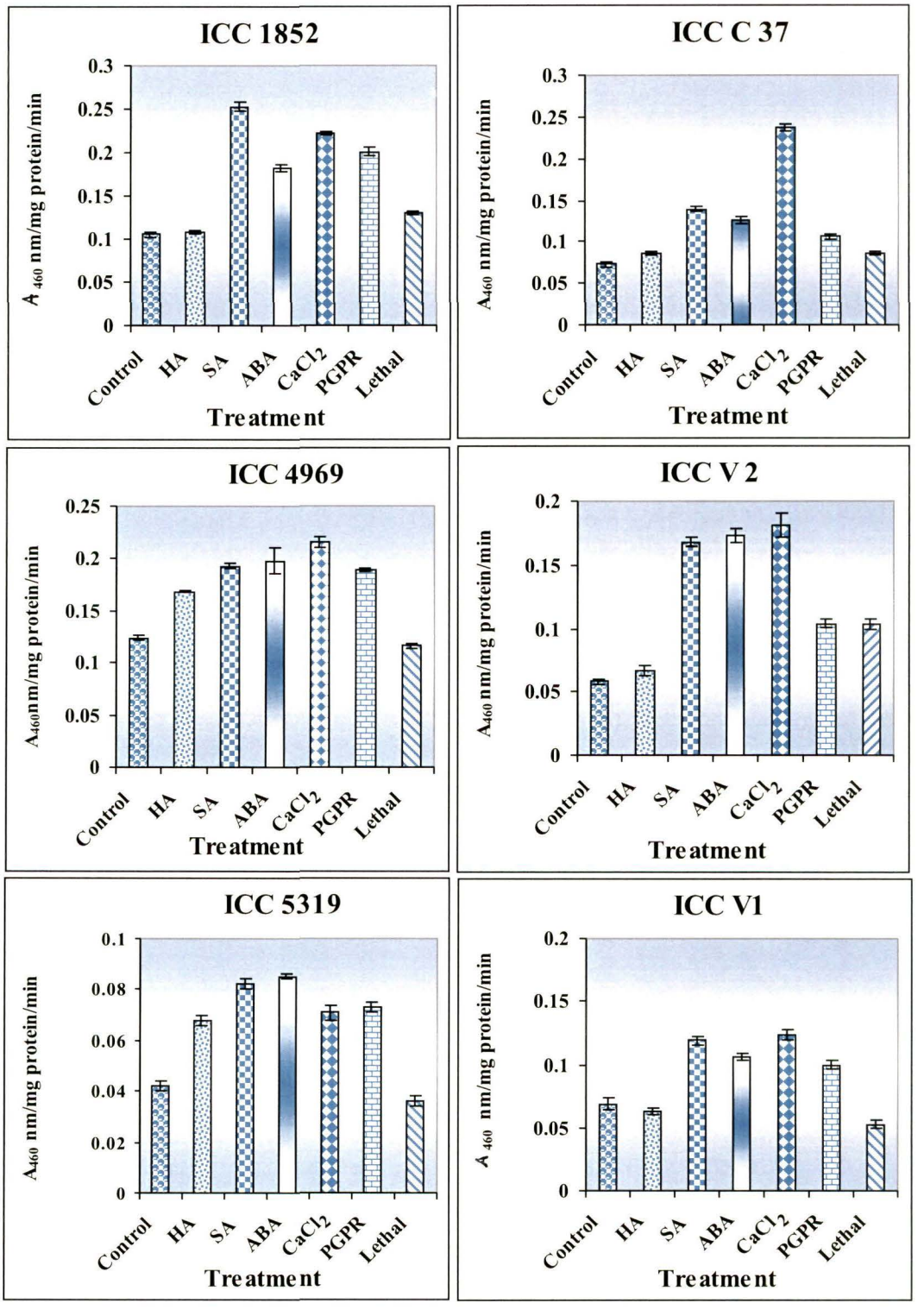


Fig. 5: Peroxidase activities in pre-treated seedlings of different *Cicer arietinum* L. genotypes subjected to lethal temperature.

Unlike in tolerant genotypes, the activity decreased in susceptible genotypes following exposure to lethal temperature.

Table 16. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC C37 C	1	19	8.5,9.4,10, 11.2, 14.7,15.1, 15.8, 16.7, 18.8, 23.7, 26.6, 33.4, 37.5, 39.2, 47.3, 53.0, 59.5,63 and 79.4
ICC C37 SA	2	20	8.5, 11.2, 14.7, 15.1, 15.8, 16.7, 17.3*, 18.8, 21.2*,23.7,25.1*,26.6 , 33.4 , 37.5,39.2,47.3, 53,59.5, 63 and 79.4
ICC C37 L	3	15	8.5, 11.2, 14.7, 15.1, 15.8, 18.8, 23.7, 25.1*, 26.6, 33.4, 39.2, 47.3, 53.0, 59.5 and 63
ICC V2 C	4	18	8.5, 10, 11.2,14.7, 15.1,15.8,16.7, 18.8, 21.2*, 23.7, 26.6, 33.4, 37.5,39.2, 47.3, 53.0, 59.5 and 63
ICC C37 ABA	5	15	8.5, 11.2, 15.1, 15.8, 16.7, 18.8, 23.7, 26.6,33.4,37.5,39.2,47.3,53.0, 59.5 and 63
ICC C37 SA	6	20	8.5, 11.2, 14.7, 15.1, 15.8, 16.7, 17.3*, 18.8,21.2*,23.7,25.1*,26.6,33.4,37.5,39.2,47.3, 53,59.5, 63 and 79.4.
ICC C37 L	7	15	8.5, 11.2, 14.7, 15.1, 15.8, 18.8, 23.7, 25.1*, 26.6, 33.4, 39.2, 47.3, 53.0, 59.5 and 63

* newly synthesized proteins

4.5.1. 2. Ascorbate peroxidase

Ascorbate peroxidase activity of seedlings registered a marginal decrease over control in heat-acclimation treatment in heat susceptible genotypes (Table 22). SA, ABA, $CaCl_2$ and PGPR pre-treated seedlings however, showed increased activity following lethal temperature treatment in all genotypes (Fig. 6).The magnitude of increase in activity due to pre-treatments was not as high as in the case of peroxidases in all tested genotypes. The activity decreased marginally in tolerant genotypes while the susceptible genotypes showed a sharp decrease in activity following lethal temperature treatment.

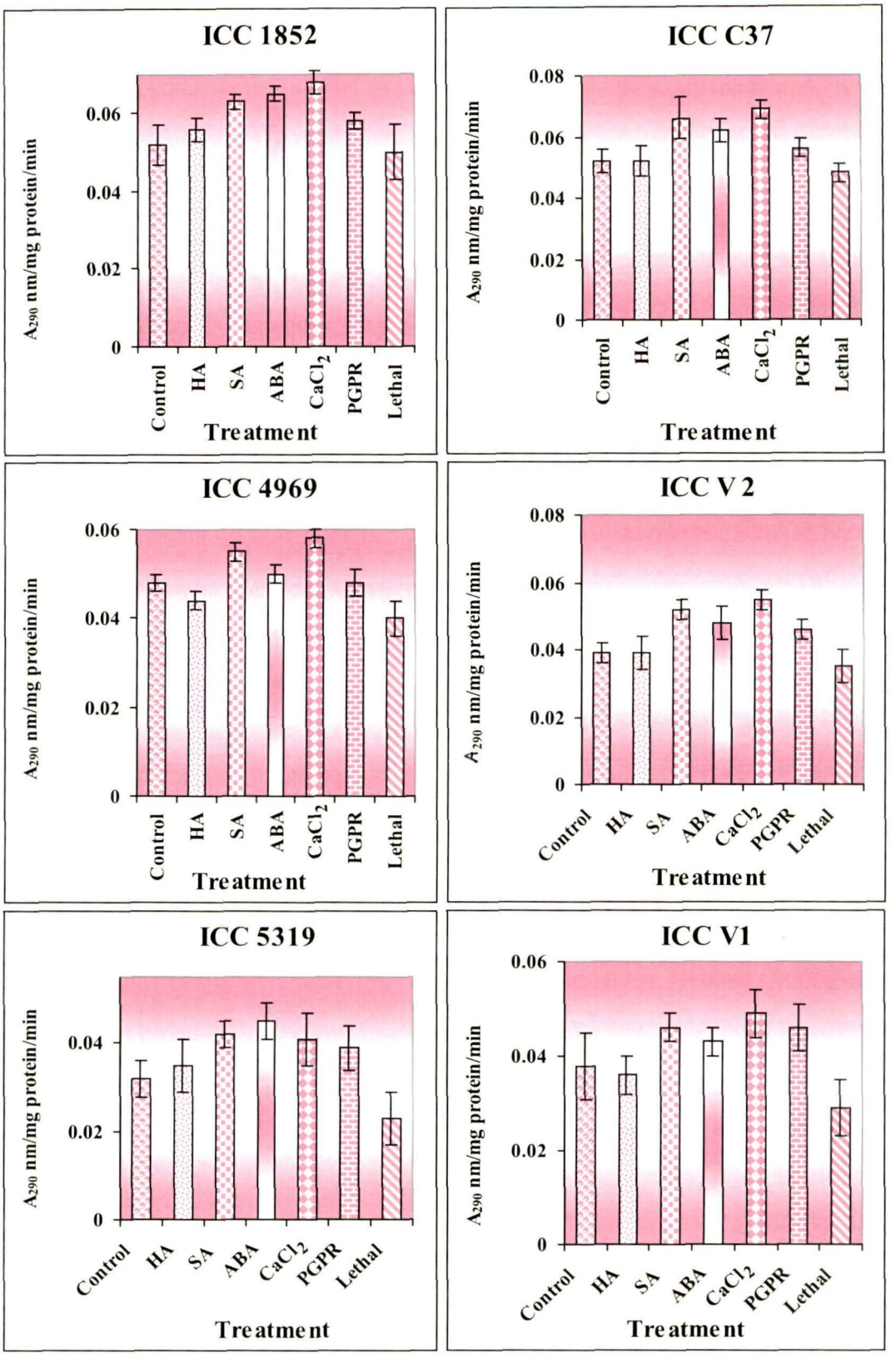


Fig. 6: Changes in ascorbate peroxidase activities in pre-treated seedlings subjected to lethal temperature treatment.

Table 17. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC14340 C	1	11	7.9, 12.5, 14.1, 15.8, 18.8, 25.1, 26.6, 28.1, 33.5, 50.1 and 59.5
ICC14340 SA	2	14	7.9,12.5,14.1,15.6*,15.8,18.8,21.1*, 22.3*, 25.1, 26.6, 28.1, 33.5, 50.1 and 59.5
ICC 14340 L	3	10	12.5, 14.1, 15.6*,15.8, 21.1*, 25.1, 26.6, 28.1, 33.5 and 50.1
ICC 14340 ABA	4	13	10.6*,12.5, 14.1, 15.6, 15.8, 18.8, 22.3*, 25.1, 26.6, 28.1, 33.5, 50.1 and 59.5
ICC 14340 HA	5	11	12.5, 14.1, 15.6, 15.8, 18.8, 22.3*25.1, 26.6, 28.1, 33.5 and 50
ICC14340 SA	6	14	7.9, 12.5,14.1,15.6*,15.8,18.8,21.1*, 22.3*,25.1, 26.6, 28.1, 33.5,50.1and 59.5

* newly synthesized proteins

Table 18. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC1852 C	1	12	8.4,12.5, 14.9, 15.8, 18.8, 26.6, 28.1, 29.8, 37.5, 39.8, 44.6 and 50.1
ICC1852 HA	2	12	8.4, 12.5,14.9,15.8,18.8, 22.3*, 26.6, 28.1, 29.8, 37.5, 39.8 and 50.1
ICC 1852 SA	3	13	8.4, 12.5, 14.9, 15.8, 18.8, 22.3*,26.6, 28.1, 29.8, 37.5, 39.8, 44.6 and 50.1
ICC 1852 L	4	09	8.4, 12.5, 14.9, 15.8, 22.3*, 26.6, 29.8, 37.5 and 39.8
ICC 1852 ABA	5	11	8.4, 12.5, 14.9, 15.8, 18.8, 22.3*,26.6, 28.1, 29.8, 37.5 and 50.1
ICC1852 $CaCl_2$	6	13	8.4, 11.2*,12.5, 14.9, 15.8, 18.8, 22.3*, 26.6, 28.1, 29.8, 37.5, 44.6 and 50.1
ICC 1852 HA	7	12	8.4, 12.5,14.9,15.8,18.8, 22.3*,26.6, 28.1, 29.8, 37.5, 39.8 and 50.1

* newly synthesized proteins

Table 19. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC 7344 C	1	11	8.5,12.0, 14.2, 20.0, 24.2, 27.0, 29.8, 32.0, 35.4, 37.0 and 39.8
ICC 7344 SA	2	15	8.5, 12.0, 14.2, 20.0, 22.3*, 24.2, 27.0,29.8, 32.0,35.4,37, 39.8*,45.3*, 55.0*,and 66.0*
ICC 7344 $CaCl_2$	3	15	8.5,12.0,14.2,20.0, 22.3*,24.2, 27.0,29.8, 32.0, 35.4 37.0, 39.8* 45.3*, 55.0*and 66*
ICC 7344 L	4	10	8.5,12.0, 14.2, 20.0, 24.2, 27.0, 29.8,35,4, 37.0 and 39.8
ICC 7344 SA	5	15	8.5,12.0,14.2,20.0,22.3*24.2,27.0,29.8, 32.0, 35.4,37.0,39.8* 45.3* 55.0*and 66*
ICC 7344 ABA	6	15	8.5,12.0,14.2,20.0,22.3*,24.2, 27.0, 29.4*, 29.8, 32.0,35.4,37.0,39.8*,45.3* and 55.0*
ICC 7344 $CaCl_2$	7	15	8.5, 12,14.2, 20 ,22.3*, 24.2, 27.0, 29.8, 32, 35.4, 37, 39.8*, 45.3*, 55.0*and 66.0*

* newly synthesized proteins

Table 20. SDS-PAGE analysis of proteins obtained from chickpea seedlings

Source of protein	Lane No.	No. of protein bands	Approx. molecular masses (kDa)
ICC 2042 C	1	13	8.4,12.5, 14.1, 16.7,17.7, 26.6, 22.3, 26.6, 28.1, 29.8, 31.6, 37.5 and 50.1
ICC 2042 ABA	2	13	8.4,12.5, 14.1, 16.7, 21.1*, 26.6, 22.3, 26.6, 28.1,29.8, 31.6, 37.5, and 50.1
ICC 2042 SA	3	13	8.4, 12.5, 14.1, 16.7, 21.1*, 26.6, 28.1,29.8, 31.6, 37.5, 44.6*, and 50.1
ICC 2042 HA	4	12	8.4, 12.5, 14.1, 16.7, 21.1*, 26.6, 26.6, 28.1,29.8, 37.5, 44.6* and 53.0*
ICC 2042 L	5	09	8.4, 12.5, 14.1, 16.7, 21.1*, 22.3, 29.8, 37.5 and 39.8
ICC 2042 $CaCl_2$	6	13	8.4, 14.1, 16.7, 17.7, 18.8, 21.1*,22.3, 26.6, 28.1, 29.8, 37.5, 44.6* and 53.0
ICC 2042 HA	7	12	8.4, 12.5, 14.1, 16.7, 21.1*, 26.6, 28.1, 29.8, 37.5, 44.6* and 53.0*

* newly synthesized proteins

Table 21. Effect of pre-treatments on peroxidase enzyme activity in seedlings following lethal temperature treatment

Genotypes	Peroxidase activity (ΔA_{460} mg protein^{-1} min^{-})						
	Control	HA	SA	ABA	$CaCl_2$	PGPR	Direct lethal
ICC 4918	0.082 ± 0.001	0.206 ± 0.005	0.311 ± 0.003	0.171 ± 0.002	0.289 ± 0.003	0.239 ± 0.001	0.175 ± 0.001
ICC 4969	0.124 ± 0.002	0.168 ± 0.000	0.193 ± 0.003	0.198 ± 0.012	0.216 ± 0.005	0.189 ± 0.002	0.116 ± 0.002
ICC 7344	0.048 ± 0.001	0.112 ± 0.002	0.126 ± 0.003	0.102 ± 0.003	0.132 ± 0.002	0.118 ± 0.003	0.036 ± 0.002
ICC 1852	0.106 ± 0.003	0.186 ± 0.002	0.253 ± 0.005	0.183 ± 0.001	0.223 ± 0.002	0.201 ± 0.005	0.130 ± 0.002
ICC 10035	0.038 ± 0.004	0.065 ± 0.005	0.095 ± 0.002	0.084 ± 0.001	0.076 ± 0.002	0.071 ± 0.002	0.024 ± 0.002
ICC 6119	0.052 ± 0.001	0.108 ± 0.003	0.189 ± 0.001	0.194 ± 0.002	0.186 ± 0.002	0.183 ± 0.004	0.102 ± 0.002
ICC 5003	0.062 ± 0.001	0.081 ± 0.002	0.192 ± 0.002	0.206 ± 0.002	0.218 ± 0.003	0.196 ± 0.001	0.067 ± 0.002
ICC 14340	0.057 ± 0.002	0.076 ± 0.002	0.093 ± 0.002	0.089 ± 0.002	0.085 ± 0.001	0.071 ± 0.002	0.050 ± 0.003
ICC 5319	0.042 ± 0.002	0.068 ± 0.082	0.082 ± 0.002	0.085 ± 0.001	0.071 ± 0.003	0.073 ± 0.002	0.036 ± 0.002
ICC 2042	0.086 ± 0.002	0.197 ± 0.001	0.287 ± 0.001	0.289 ± 0.002	0.320 ± 0.002	0.269 ± 0.002	0.271 ± 0.003
ICC 16359	0.045 ± 0.004	0.069 ± 0.002	0.087 ± 0.001	0.092 ± 0.002	0.090 ± 0.004	0.074 ± 0.002	0.042 ± 0.002
ICC C37	0.073 ± 0.003	0.086 ± 0.002	0.139 ± 0.003	0.126 ± 0.004	0.237 ± 0.005	0.d106 ± 0.004	0.086 ± 0.002
ICC V10	0.079 ± 0.003	0.113 ± 0.002	0.216 ± 0.001	0.201 ± 0.003	0.283 ± 0.004	0.176 ± 0.003	0.126 ± 0.004
ICC V1	0.069 ± 0.005	0.063 ± 0.003	0.119 ± 0.003	0.106 ± 0.003	0.123 ± 0.004	0.100 ± 0.009	0.053 ± 0.003
ICC V2	0.058 ± 0.002	0.067 ± 0.004	0.168 ± 0.004	0.173 ± 0.005	0.181 ± 0.054	0.104 ± 0.003	0.103 ± 0.004

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 21A. Analysis of variance for the data presented in Table 21

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	0.312	0.022	23.6002	0.02665
TREATMENT	6	0.152	0.025	26.8352	-
ERROR	84	0.079	0.001	-	-
TOTAL	104	0.544			

Table22. Changes in ascorbate peroxidase activities in pre-treated seedlings following lethal temperature treatment

	Ascorbate peroxidase activity (Δ A $_{290}$ mg protein^{-1} min^{-1})						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	0.058 ± 0.008	0.059 ± 0.003	0.067 ± 0.002	0.072 ± 0.003	0.076 ± 0.003	0.059 ± 0.002	0.055 ± 0.003
ICC 4969	0.048 ± 0.002	0.044 ± 0.002	0.055 ± 0.002	0.050 ± 0.002	0.058 ± 0.002	0.048 ± 0.003	0.040 ± 0.004
ICC 7344	0.037 ± 0.003	0.034 ± 0.003	0.042 ± 0.004	0.048 ± 0.003	0.046 ± 006	0.038 ± 0.003	0.029 ± 0.003
ICC 1852	0.052 ± 0.005	0.056 ± 0.003	0.063 ± 0.002	0.065 ± 0.002	0.068 ± 0.004	0.058 ± 0.002	0.050 ± 0.007
ICC 10035	0.039 ± 0.004	0.037 ± 0.003	0.048 ± 0.005	0.045 ± 0.004	0.046 ± 0.003	0.042 ± 0.002	0.029 ± 0.005
ICC 6119	0.050 ± 0.006	0.054 ± 0.001	0.067 ± 0.003	0.063 ± 0.003	0.066 ± 0.004	0.053 ± 0.002	0.043 ± 0.004
ICC 5003	0.048 ± 0.005	0.052 ± 0.004	0.058 ± 0.002	0.063 ± 0.003	0.066 ± 0.003	0.066 ± 0.004	0.043 ± 0.003
ICC 14340	0.043 ± 0.003	0.048 ± 0.003	0.055 ± 0.003	0.053 ± 0.005	0.052 ± 0.005	0.048 ± 0.004	0.038 ± 0.009
ICC 5319	0.032 ± 0.004	0.030 ± 0.006	0.042 ± 0.003	0.045 ± 0.004	0.041 ± 0.006	0.039 ± 0.005	0.023 ± 0.006
ICC 2042	0.047 ± 0.003	0.054 ± 0.002	0.063 ± 0.004	0.065 ± 0.007	0.066 ± 0.003	0.059 ± 0.003	0.042 ± 0.003
ICC 16359	0.040 ± 0.004	0.042 ± 0.004	0.046 ± 0.003	0.053 ± 0.055	0.051 ± 0.006	0.040 ± 0.004	0.034 ± 0.004
ICC C37	0.052 ± 0.004	0.052 ± 0.005	0.066 ± 0.006	0.062 ± 0.004	0.069 ± 0.003	0.056 ± 0.003	0.048 ± 0.003
ICC V10	0.049 ± 0.004	0.051 ± 0.005	0.059 ± 0.002	0.062 ± 0.002	0.063 ± 0.003	0.055 ± 0.002	0.045 ± 0.004
ICC V1	0.038 ± 0.007	0.036 ± 0.004	0.046 ± 0.003	0.043 ± 0.003	0.049 ± 0.005	0.046 ± 0.005	0.029 ± 0.006
ICC V2	0.039 ± 0.003	0.039 ± 0.005	0.052 ± 0.003	0.048 ± 0.005	0.055 ± 0.003	0.046 ± 0.003	0.035 ± 0.005

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 22A. Analysis of variance for the data presented in Table 22

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	0.007	0.001	68.7397	0.00235
TREATMENT	6	0.004	0.001	99.6178	-
ERROR	84	0.001	0.000	-	-
TOTAL	104	0.012			

4.5.1.3. Catalase

Catalase activity, in contrast to peroxidase and ascorbate peroxidase activities revealed a decreasing trend in all pre-treated seedlings over control samples (Table 23). The decrease in activity was more significant in pre-treated seedlings following exposure to lethal temperature than in direct lethal treatment (Fig.7). Significant differences were observed in the constitutive levels of catalase activity, with ICC 10035 showing lowest activity (11.15 enzyme units mg protein^{-1} min^{-1}) and ICC C37 showing the highest (30.13 enzyme units mg protein^{-1} min^{-1}).

Table 23. Changes in catalase activities in pre-treated seedlings following lethal temperature treatment

	Catalase activity (enzyme units mg protein^{-1} min^{-1})						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	27.26 ± 1.24	16.03 ± 1.70	10.23 ± 1.90	11.91 ± 0.58	10.06 ± 1.62	10.59 ± 0.75	20.26 ± 1.86
ICC 4969	29.34 ± 2.32	18.80 ± 1.20	15.87 ± 1.42	15.04 ± 0.94	16.23 ± 1.10	16.91 ± 2.32	24.98 ± 1.81
ICC 7344	14.96 ± 1.98	12.28 ± 0.66	10.19 ± 1.54	11.87 ± 0.75	10.64 ± 1.04	10.83 ± 1.14	10.98 ± 1.21
ICC 1852	24.28 ± 2.37	13.09 ± 1.58	09.01 ± 2.77	12.39 ± 1.05	10.68 ± 1.78	11.58 ± 0.75	18.87 ± 0.66
ICC 10035	11.15 ± 1.51	08.38 ± 1.28	06.32 ± 1.19	07.94 ± 0.53	08.46 ± 0.50	09.42 ± 0.37	08.83 ± 0.54
ICC 6119	24.61 ± 1.28	20.10 ± 2.21	16.73 ± 1.19	16.21 ± 2.39	18.16 ± 2.43	19.07 ± 2.77	22.43 ± 1.89
ICC 5003	22.42 ± 2.24	18.37 ± 3.18	12.38 ± 1.16	10.83 ± 2.30	10.66 ± 1.09	11.06 ± 1.53	18.96 ± 0.82
ICC 14340	18.33 ± 1.13	16.01 ± 1.03	12.07 ± 1.90	14.43 ± 1.18	12.52 ± 1.95	13.48 ± 1.95	16.95 ± 0.81
ICC 5319	19.94 ± 1.81	17.92 ± 1.19	15.64 ± 1.67	14.58 ± 2.11	14.41 ± 1.19	16.39 ± 1.18	17.58 ± 1.20
ICC 2042	26.36 ± 1.06	22.46 ± 1.68	10.61 ± 1.58	16.87 ± 1.84	16.66 ± 1.27	18.59 ± 2.60	20.53 ± 1.94
ICC 16359	20.06 ± 1.20	18.39 ± 1.08	16.01 ± 2.30	14.98 ± 0.77	14.51 ± 1.22	16.78 ± 0.98	14.97 ± 0.90
ICC C37	30.13 ± 1.89	20.05 ± 1.32	16.39 ± 1.17	18.75 ± 2.85	13.83 ± 1.11	18.01 ± 2.49	24.20 ± 1.52
ICC V10	28.32 ± 2.38	20.07 ± 1.78	13.71 ± 0.96	13.08 ± 1.84	12.46 ± 2.25	16.92 ± 0.85	21.07 ± 3.02
ICC V1	26.68 ± 1.37	23.34 ± 1.62	20.03 ± 2.63	22.81 ± 2.05	20.87 ± 2.32	24.59 ± 2.62	23.60 ± 1.06
ICC V2	25.80 ± 1.58	21.36 ± 2.34	18.11 ± 2.03	17.62 ± 1.29	16.85 ± 2.09	20.01 ± 2.28	21.16 ± 2.10

Values represent mean ± SE (n = 3). Values are mean of three replicates

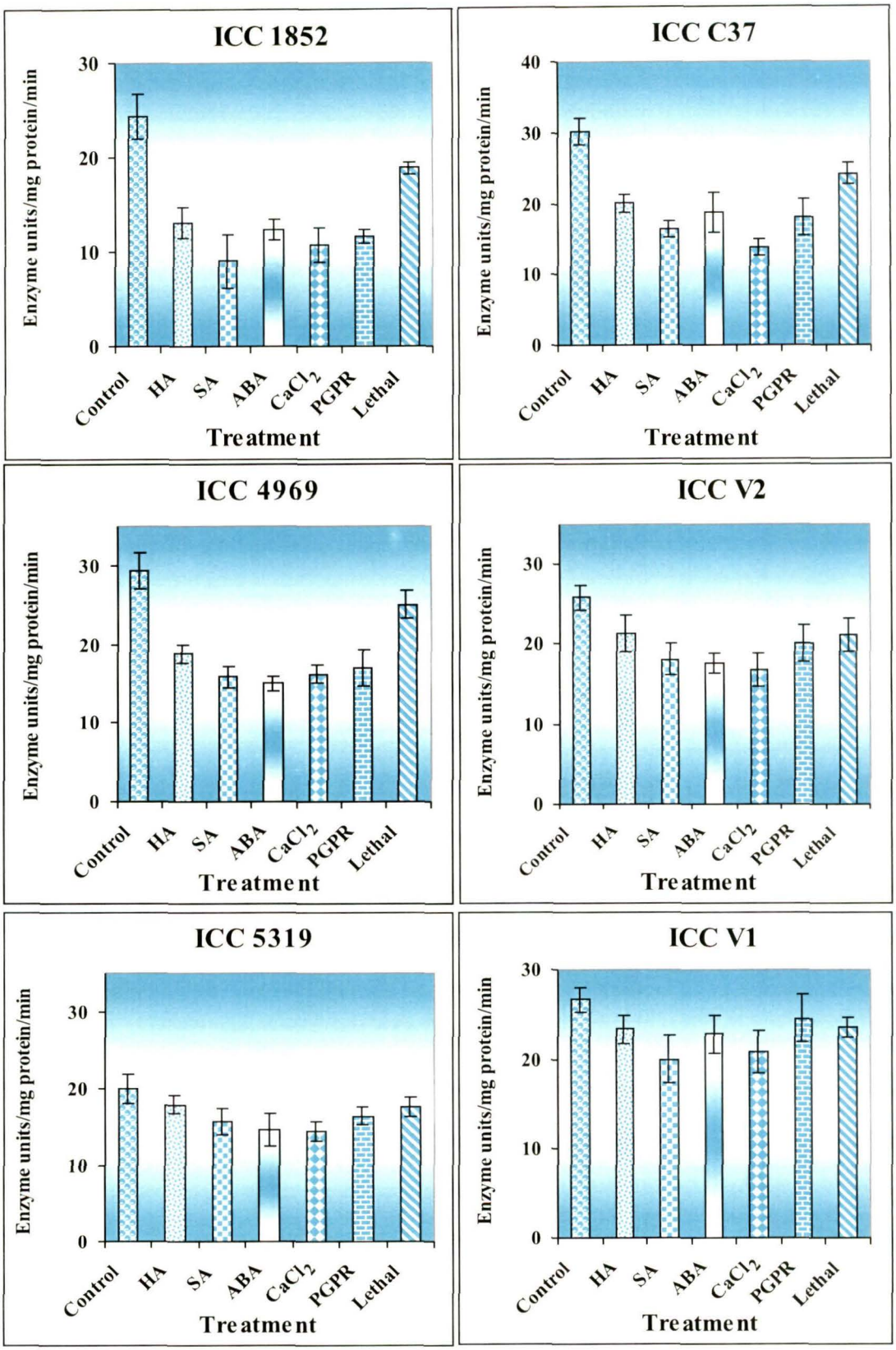


Fig. 7: Effect of various pre-treatments on catalase activities of seedlings subjected to lethal temperature treatment.

Table 23 Analysis of variance for the data presented in Table 23

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	1352.660	96.619	20.1146	1.89987
TREATMENT	6	1105.785	184.297	38.3681	-
ERROR	84	403.486	4.803		
TOTAL	104	2861.931			

4.5.1.4. Superoxide dismutase

Superoxide dismutase activity followed a pattern similar to that of peroxidases. The activity increased under heat stress in most genotypes tested (Fig. 8). However, direct exposure of seedlings to lethal temperature treatment exhibited inhibitory effect on the activity of enzymes in all tested genotypes (Table 24). The most remarkable increase in activity due to pre-treatments was recorded in genotype ICC C37 which recorded enzyme activities of 0.072, 0.069 and 0.068 enzyme units mg^{-1} $tissue^{-1}min^{-1}$ in seedlings subjected to SA, ABA and $CaCl_2$ pre-treatments respectively.

4.5.1.5. Glutathione reductase

Pre-treatment of seedlings before exposure to lethal temperature treatment in the present study led to an increase in the activity of glutathione reductase. Heat acclimation of seedlings prior to lethal temperature exposure did not significantly affect the activity of glutathione reductase in most genotypes. However, pre-treatment with SA, ABA, $CaCl_2$ or *Bacillus megaterium* increased the activity significantly. The GR activity in tolerant genotypes either showed no change or only a marginal decrease in the activity following lethal temperature treatment. In contrast, the susceptible genotypes showed relatively a much higher decrease in activity when directly exposed to lethal temperature (Table 25, Fig. 9).

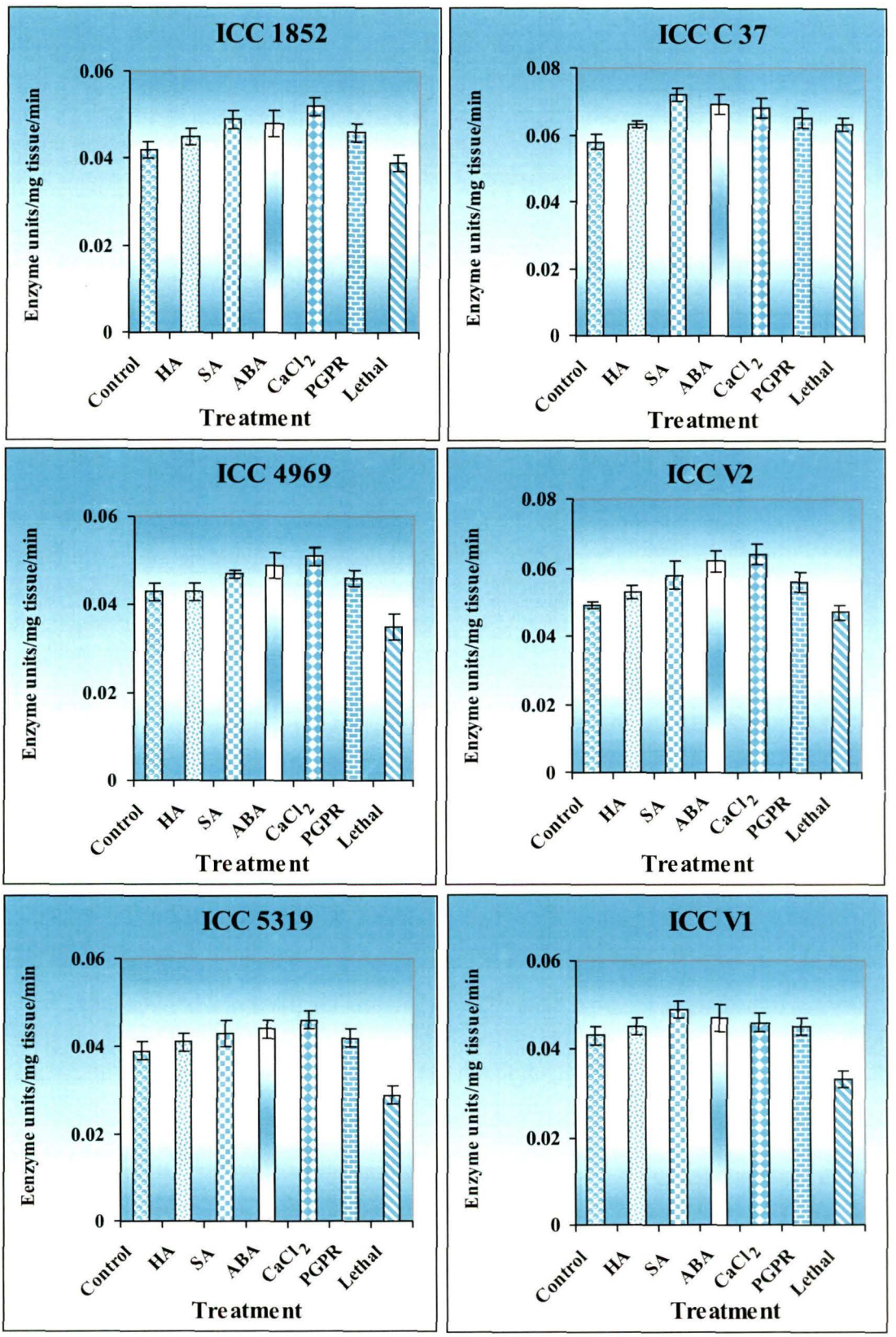


Fig. 8: Superoxide dismutase activities in pre-treated seedlings following a 2h lethal temperature treatment.

Table 24. Changes in activity of superoxide dismutase enzyme in pre-treated seedlings subjected to lethal temperature treatment

	Superoxide dismutase activity (enzyme units mg tissue^{-1} min^{-1})						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	0.040 ± 0.004	0.042 ± 0.002	0.048 ± 0.003	0.046 ± 0.001	0.051 ± 0.002	0.046 ± 0.002	0.038 ± 0.002
ICC 4969	0.043 ± 0.002	0.043 ± 0.001	0.047 ± 0.001	0.049 ± 0.002	0.051 ± 0.002	0.046 ± 0.002	0.035 ± 0.002
ICC 7344	0.040 ± 0.003	0.041 ± 0.002	0.044 ± 0.001	0.042 ± 0.002	0.045 ± 0.003	0.043 ± 0.002	0.030 ± 0.002
ICC 1852	0.042 ± 0.002	0.045 ± 0.002	0.049 ± 0.002	0.048 ± 0.003	0.052 ± 0.002	0,046 ± 0.002	0.039 ± 0.002
ICC 10035	0.031 ± 0.003	0.026 ± 0.003	0.033 ± 0.002	0.029 ± 0.003	0.032 ± 0.003	0.031 ± 0.002	0.020 ± 0.002
ICC 6119	0.038 ± 0.002	0.0389 ± 0.002	0.042 ± 0.002	0.040 ± 0.002	0.042 ± 0.002	0.039 ± 0.003	0.031 ± 0.003
ICC 5003	0.041 ± 0.002	0.043 ± 0.002	0.048 ± 0.003	0.047 ± 0.003	0.049 ± 0.003	0.046 ± 0.002	0.034 ± 0.002
ICC 14340	0.036 ± 0.001	0.034 ± 0.003	0.037 ± 0.002	0.036 ± 0.002	0.039 ± 0.002	0.037 ± 0.002	0.031 ± 0.001
ICC 5319	0.039 ± 0.002	0.041 ± 0.002	0.043 ± 0.003	0.044 ± 0.002	0.046 ± 0.002	0.042 ± 0.002	0.029 ± 0.002
ICC 2042	0.039 ± 0.002	0.039 ± 0.002	0.045 ± 0.001	0.043 ± 0.002	0.049 ± 0.002	0.041 ± 0.002	0.036 ± 0.002
ICC 16359	0.040 ± 0.001	0.036 ± 0.002	0.042 ± 0.002	0.040 ± 0.002	0.044 ± 0.002	0.041 ± 0.001	0.030 ± 0.001
ICC C37	0.050 ± 0.002	0.063 ± 0.001	0.072 ± 0.002	0.069 ± 0.003	0.068 ± 0.003	0.065 ± 0.003	0.063 ± 0.002
ICC V10	0.048 ± 0.002	0.050 ± 0.002	0.054 ± 0.002	0.056 ± 0.002	0.056 ± 0.003	0.052 ± 0.002	0.052 ± 0.002
ICC V1	0.043 ± 0.002	0.045 ± 0.002	0.049 ± 0.002	0.047 ± 0.003	0.046 ± 0.002	0.045 ± 0.002	0.032 ± 0.002
ICC V2	0.049 ± 0.001	0.053 ± 0.002	0.058 ± 0.004	0.062 ± 0.003	0.064 ± 0.03	0.056 ± 0.003	0.047 ± 0.002

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 24 A. Analysis of variance for the data presented in Table 24

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	0.008	0.001	88.8311	0.00215
TREATMENT	6	0.002	0.000	42.3368	-
ERROR	84	0.001	0.000	-	-
TOTAL	104	0.010			

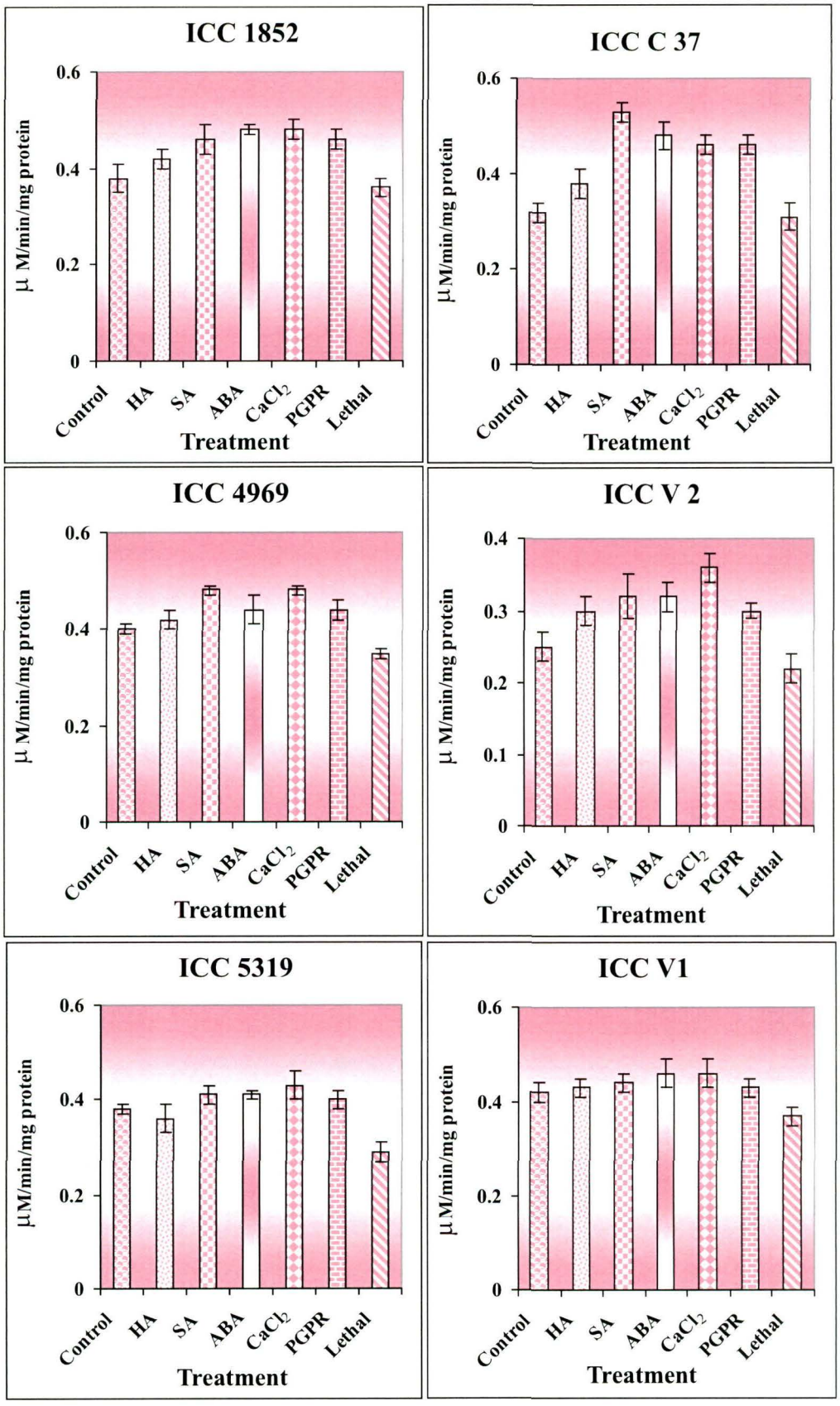


Fig. 9: Changes in glutathione reductase activities in pre-treated seedlings following lethal temperature treatment.

Table 25. Changes in activity of glutathione reductase in pre-treated seedlings subjected to lethal temperature treatment

Genotypes	Glutathione reductase activity (μM min^{-1} mg $protein^{-1}$)						
	Control	HA	SA	ABA	$CaCl_2$	PGPR	Direct lethal
ICC 4918	0.39 ± 0.02	0.042 ± 0.03	0.79 ± 0.02	0.68 ± 0.03	0.82 ± 0.02	0.64 ± 0.02	0.39 ± 0.02
ICC 4969	0.40 ± 0.03	0.42 ± 0.02	0.48 ± 0.03	0.44 ± 0.02	0.48 ± 0.02	0.44 ± 0.01	0.35 ± 0.01
ICC 7344	0.30 ± 0.02	0.30 ± 0.01	0.46 ± 0.03	0.44 ± 0.02	0.56 ± 0.02	0.42 ± 0.02	0.22 ± 0.02
ICC 1852	0.38 ± 0.03	0.42 ± 0.02	0.46 ± 0.03	0.48 ± 0.01	0.48 ± 0.02	0.46 ± 0.02	0.36 ± 0.02
ICC 10035	0.36 ± 0.01	0.32 ± 0.03	0.49 ± 0.01	0.47 ± 0.02	0.52 ± 0.02	0.47 ± 0.02	0.26 ± 0.02
ICC 6119	0.38 ± 0.02	0.38 ± 0.01	0.48 ± 0.02	0.46 ± 0.02	0.45 ± 0.02	0.43 ± 0.02	0.32 ± 0.02
ICC 5003	0.39 ± 0.02	0.42 ± 0.02	0.66 ± 0.02	0.58 ± 0.03	0.70 ± 0.03	0.53 ± 0.02	0.36 ± 0.03
ICC 14340	0.30 ± 0.02	0.28 ± 0.03	0.40 ± 0.03	0.36 ± 0.02	0.38 ± 0.02	0.34 ± 0.02	0.24 ± 0.02
ICC 5319	0.38 ± 0.01	0.36 ± 0.03	0.41 ± 0.02	0.41 ± 0.03	0.43 ± 0.02	0.40 ± 0.02	0.29 ± 0.03
ICC 2042	0.42 ± 0.02	0.45 ± 0.02	0.68 ± 0.02	0.59 ± 0.01	0.73 ± 0.01	0.56 ± 0.02	0.39 ± 0.02
ICC 16359	0.35 ± 0.02	0.32 ± 0.02	0.42 ± 0.02	0.38 ± 0.02	0.45 ± 0.03	0.40 ± 0.02	0.25 ± 0.02
ICC C37	0.32 ± 0.02	0.38 ± 0.03	0.53 ± 0.02	0.48 ± 0.03	0.46 ± 0.02	0.46 ± 0.02	0.31 ± 0.03
ICC V10	0.38 ± 0.02	0.45 ± 0.02	0.59 ± 0.03	0.56 ± 0.03	0.60 ± 0.03	0.48 ± 0.02	0.38 ± 0.03
ICC V1	0.42 ± 0.02	0.43 ± 0.02	0.44 ± 0.02	0.46 ± 0.03	0.46 ± 0.03	0.43 ± 0.02	0.37 ± 0.02
ICC V2	0.25 ± 0.02	0.30 ± 0.02	0.32 ± 0.03	0.32 ± 0.02	0.36 ± 0.02	0.30 ± 0.01	0.22 ± 0.02

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 25 A. Analysis of variance for the data presented in Table 25

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	0.290	0.021	50.2633	0.01760
TREATMENT	6	0.193	0.032	78.1279	-
ERROR	84	0.035	0.000	-	-
TOTAL	104	0.518			

4.5.2. Changes in isozyme profile

For studies on isozymes of enzymes, from the different genotypes, 3 were selected which were tolerant (black gram ICC C37), kabuli for tropics (ICC V2) and susceptible (ICC V1) to temperature stress

4.5.2.1. Peroxidase

Experiments were performed in three different genotypes to determine whether elevated temperature stress is responsible for induction of new isozymes. Analysis of isozymes was done on native PAGE gel and the bands were visualised using suitable chemical dyes.

Presence of five isozyme bands of R_m values 0.166, 0.300, 0.336, 0.500 and 0.566 were recorded in control samples and seedlings subjected to elevated temperature treatments in the range of 35-45°C for 2 hrs. (Table 26).Seedlings directly subjected to lethal temperature treatment without any pre-treatment however showed the loss of both isozymes 1, 2 and 4 in case of susceptible genotype (ICC V1). Induction of many new isoforms of peroxidase was observed in pre-treated seedlings subjected to lethal temperature treatment for the same duration. An isozyme 5 (R_m0.5) was found to occur commonly in all three genotypes. Intense isozyme bands (R_m 0.416 and 0.916) were recorded in SA pre-treatment while new isoforms having R_m values of 0.333, 0.875 and 0.983 were recorded in seedlings directly exposed to lethal treatment. $CaCl_2$ pre-treatment prior to exposure to lethal temperature also revealed new isozymes of R_m values 0.333, 0.633 and 0.875 with respect to control (Table 27, Plate XV) following exposure to lethal temperature.

4.5.2.2. Catalase

Induction of new isozymes of catalase if any, was tested on native-PAGE using 8% resolving and 5% stacking gel in three different genotypes ICC C37, ICC V2 and ICC V1. Isozyme analysis revealed the presence of only a single isozyme band of R_m value 0.466 (Plate XVI) in all three tested varieties in both control and pre-treated seedlings. However, the intensity and time of appearance of band varied greatly in all three genotypes. The solitary band in tolerant genotypes was much more intense and appeared much earlier than in the susceptible genotype (ICC V1).

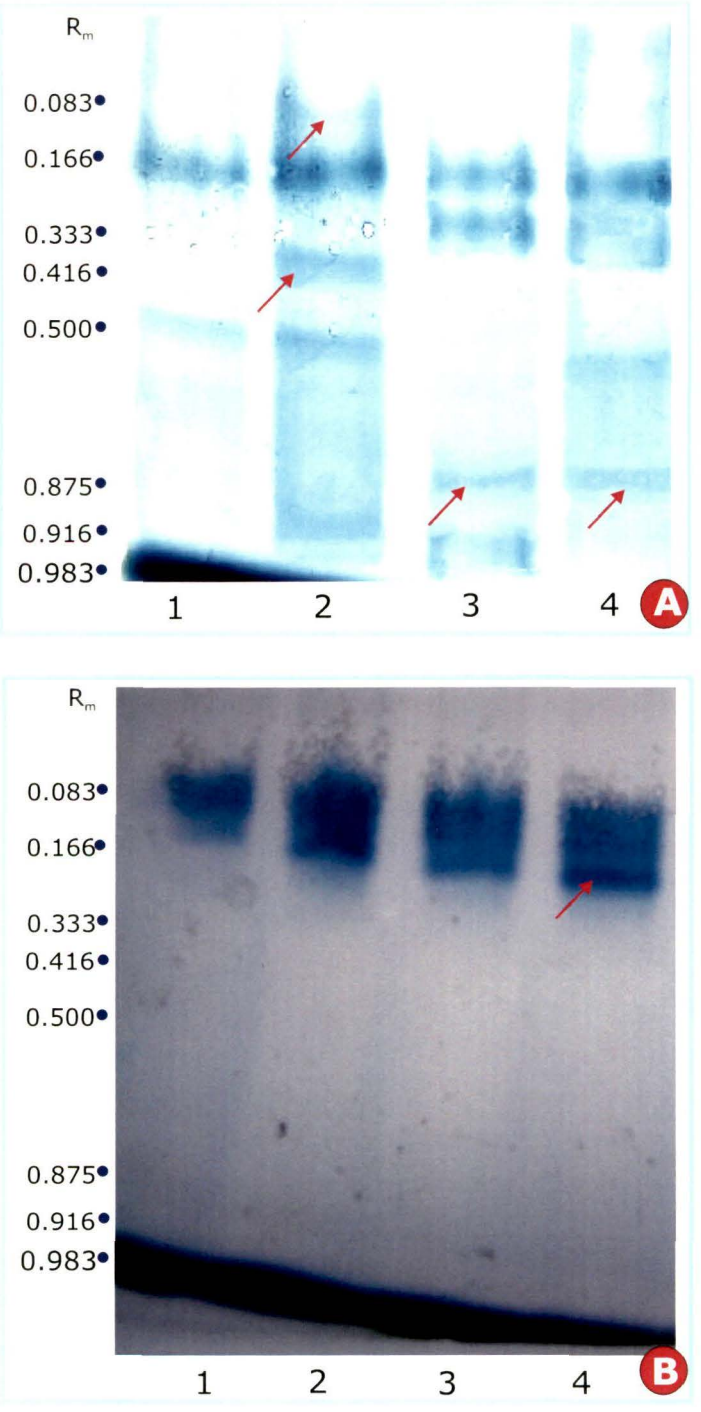


Plate XV(A-B): Peroxidase isozyme analysis by native PAGE. **A:** Lane 1: ICC C37 control;2: SA; 3: Lethal; 4:$CaCl_2$. **B:** Lane 1: ICC V1 control; 2: SA; 3: Lethal; 4: $CaCl_2$.

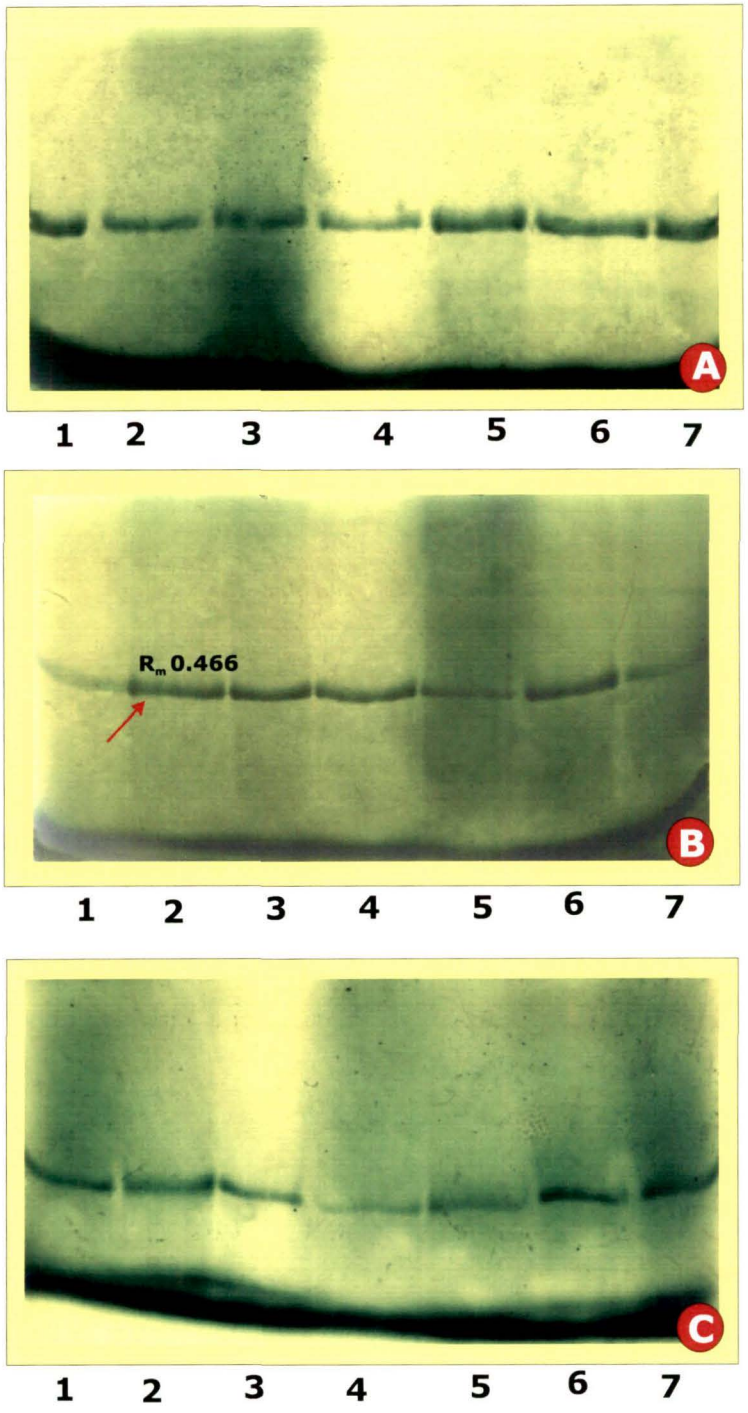


Plate XVI (A-C): Catalase isozyme analysis by native PAGE. **A**: ICC C37 Lane 1: control; 2:SA; 3:ABA; 4: heat acclimation; 5:$CaCl_2$; 6:lethal and 7: *Bacillus megaterium* treated. **B:** ICC V2 Lane 1: control; 2:SA; 3:ABA; 4: $CaCl_2$, 5: lethal; 6: *Bacillus megaterium* treated and 7: heat acclimation. **C**: ICC V1 Lane 1: control; 2:SA; 3: ABA; 4: lethal; 5: heat acclimation;6: $CaCl_2$ and 7: *Bacillus megaterium* treated

Table 26. Relative mobility (R_m) values of peroxidase isozymes of seedlings subjected to elevated temperature stress

Genotypes	Isozyme No.	R_m values	Different isozymes induced				
			Control	35°C	40°C	45°C	Lethal
ICC C37	1	0.166	+	+	+	+	+
	2	0.300	+	+	+	+	+
	3	0.336	+	+	+	+	-
	4	0.500	+	+	+	+	+
	5	0.566	-	-	-	-	-
ICC V2	1	0.166	+	+	+	+	+
	2	0.300	+	+	+	+	-
	3	0.336	+	+	+	+	-
	4	0.500	+	+	+	+	-
	5	0.566	-	-	-	-	-
ICC V1	1	0.166	+	+	+	-	-
	2	0.300	+	+	-	-	-
	3	0.336	+	+	+	-	-
	4	0.500	+	+	+	+	-
	5	0.566	-	-	-	-	+

4.6. Variations in levels of non-enzymatic antioxidants as a consequence of lethal temperature

Antioxidants like ascorbate and carotenoids are also important in ameliorating oxidative stress. In the present study, six genotypes showing different levels of thermotolerance were selected for studies on such antioxidants.

4.6.1. Ascorbate

The pre-treatments of seedlings affected the content of non-enzymatic antioxidants like ascorbate and carotenoid in different ways. ABA pre-treatment did not have a major impact on the water soluble antioxidant ascorbate. SA and $CaCl_2$ pre-treatments in contrast, led to a significant increase in ascorbate content even in susceptible genotypes. The reduction in ascorbate content in untreated samples directly exposed to lethal temperature was highly significant in susceptible genotypes (Table 28).

Table 27. Relative mobility (R_m) values of peroxidase isozymes of pre-treated seedlings subjected to lethal temperature treatment

Genotypes	Isozyme No.	R_m values	Different isozymes induced by pre-treatments					
			Control	SA	ABA	$CaCl_2$	PGPR	Lethal
ICC C37	1	0.083	-	+	-	-	-	-
	2	0.166	+	+	+	+	+	+
	3	0.333	-	-	+	+	+	+
	4	0.416	-	+	-	+	+	-
	5	0.500	+	+	-	-	-	-
	6	0.633	+	+	+	+	+	-
	7	0.875	-	-	-	+	+	+
	8	0.916	-	+	-	-	-	-
	9	0.983	-	-	+	-	-	+
ICC V2	1	0.083	-	+	-	-	+	-
	2	0.166	+	+	+	+	+	+
	3	0.333	+	+	+	+	+	+
	4	0.416	-	-	+	-	-	-
	5	0.500	+	+	+	+	+	+
ICC V1	1	0.083	+	-	+	+	+	-
	2	0.133	-	+	+	-	+	-
	3	0.166	+	+	+	+	-	-
	4	0.216	-	-	-	+	-	-
	5	0.500	+	+	+	+	+	-

4.6.2. Carotenoids

Carotenoids are known to play a significant role in photo-protection of chlorophyll molecules by functioning as non enzymatic antioxidant. Analysis of carotenoid content in this study, showed a remarkable variation in pre-treated and untreated control samples. Carotenoid content of pre-treated seedlings of tolerant genotypes (ICC 4918, 1852 and 2042) was found to be significantly increased following lethal temperature treatment (Table 29). The susceptible genotypes in contrast showed a rapid decline in carotenoid content following heat stress (Fig. 10).

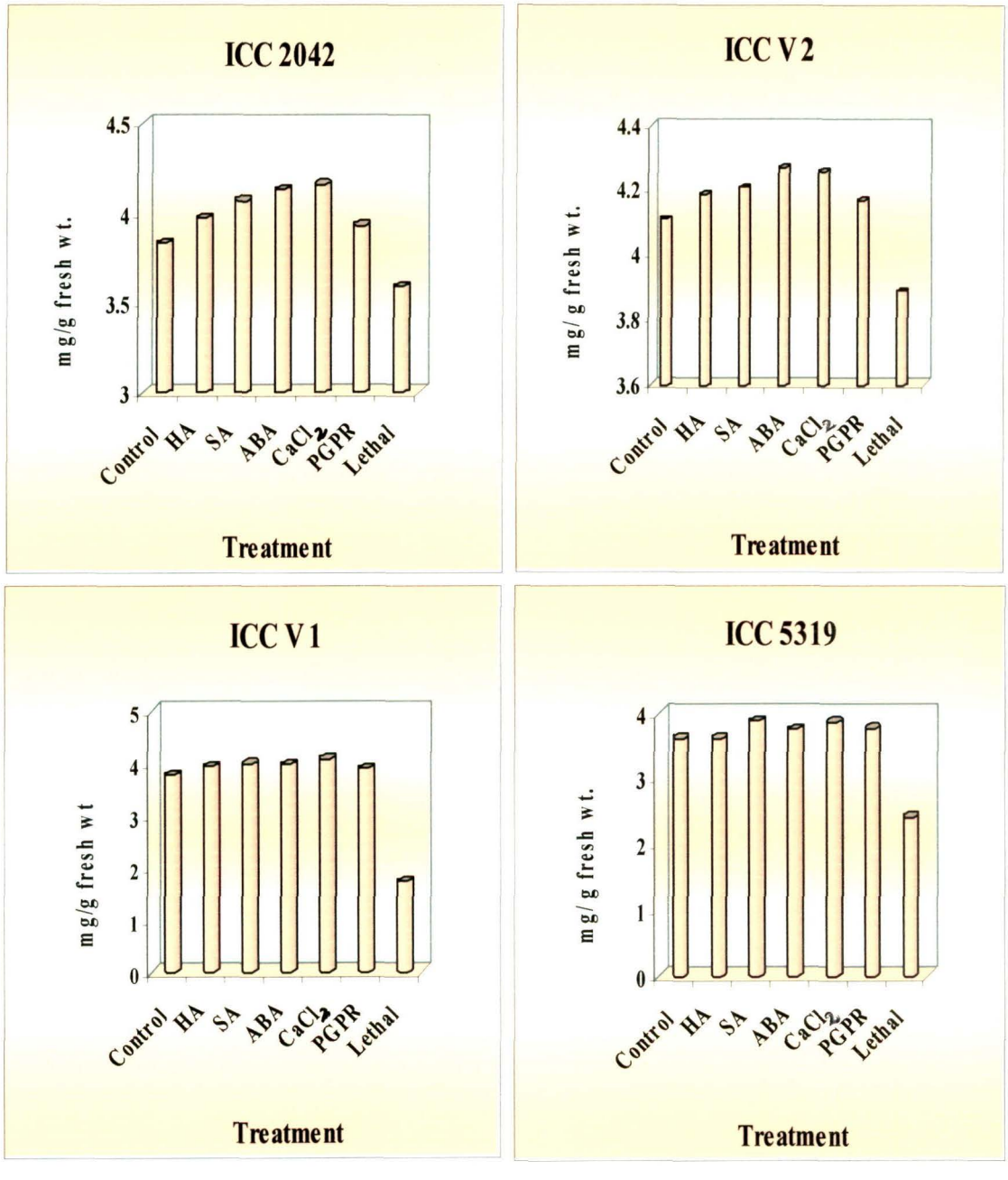


Fig.10 : Changes in carotenoid contents of pre-treated seedlings exposed to lethal temperature.

Table 28. Effect of various pre-treatments on ascorbate content of seedlings exposed to lethal temperature

	Ascorbate content (µM g^{-1} fresh wt.)					
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**Direct lethal**
ICC 4918	2.42 ± 0.1	2.52 ± 0.6	3.59 ± 0.05	2.49 ± 0.1	3.90 ± 0.1	2.37 ± 0.1
ICC 4969	1.86 ± 0.3	2.01 ± 0.1	2.25 ± 0.1	1.88 ± 0.1	2.39 ± 0.1	1.77 ± 0.2
ICC 7344	1.65 ± 0.1	1.80 ± 0.0	2.78 ± 0.1	1.62 ± 0.1	2.93 ± 0.1	1.05 ± 0.2
ICC 1852	1.96 ± 0.2	2.95 ± 0.1	3.50 ± 0.1	2.06 ± 0.1	3.13 ± 0.1	1.92 ± 0.0
ICC 10035	1.73 ± 0.9	1.76 ± 0.1	2.81 ± 0.2	1.75 ± 0.3	3.17 ± 0.1	0.96 ± 0.1
ICC 5003	2.18 ± 0.2	2.32 ± 0.1	3.09 ± 0.3	2.28 ± 0.1	3.31 ± 0.2	2.01 ± 0.1

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 28 A. Analysis of variance for the data presented in Table 28

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	2.812	0.562	1129.3420	0.03201
TREATMENT	5	0.173	0.035	69.4338	-
ERROR	25	0.012	0.000	-	-
TOTAL	35	2.997			

Table 29. Effect of pre-treatments on carotenoid contents in seedlings exposed to lethal temperature

	Carotenoid content (µg g^{-1} fresh wt.)						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	3.63 ± 0.23	3.77 ± 0.08	4.62 ± 0.10	4.01 ± 0.12	5.06 ± 0.10	3.98 ± 0.05	3.51 ± 0.12
ICC 4969	6.00 ± 0.18	6.31 ± 0.10	7.20 ± 0.10	6.41 ± 0.07	7.19 ± 0.09	6.23 ± 0.07	4.91 ± 0.13
ICC 7344	4.65 ± 0.09	4.76 ± 0.06	5.90 ± 0.21	4.58 ± 0.05	5. 68 ± 0.03	4.88 ± 0.07	3.23 ± 0.09
ICC 1852	4.26 ± 0.07	4.42 ± 0.08	5.58 ± 0.08	4. 94 ± 0.08	4.52 ± 0.05	5.45 ± 0.08	4.09 ± 0.05
ICC 10035	4.32 ± 0.08	4.32 ± 0.05	4.76 ± 0.07	4.48 ± 0.07	5.36 ± 0.09	4.93 ± 0.07	3.02 ± 0.13
ICC 5003	4.73 ± 0.05	4.98 ± 0.05	5.84 ± 0.04	4.97 ± 0.11	5.86 ± 0.07	4.91 ± 0.04	4.11 ± 0.06

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 29A. Analysis of variance for the data presented in Table 29

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	18.434	3.687	104.5261	0.26642
TREATMENT	6	3.925	0.654	18.5471	-
ERROR	30	1.058	0.035	-	-
TOTAL	41	23.418			

4.7. Changes in lipid peroxidation of membranes following lethal temperature

Levels of heat induced oxidative damage were measured in terms of lipid peroxidation as reflected by malondialdehyde (MDA) content. The synthesis of MDA is considered as a useful index of general lipid peroxidation and was thus analyzed in different genotypes. The peroxidation of membranes was found to be significantly reduced in SA, ABA, $CaCl_2$ and PGPR pre-treated seedlings in comparison to untreated control samples directly exposed to lethal temperature treatment (Table 30, Fig.11). Differences in lipid peroxidation between SA, ABA and $CaCl_2$ were not significantly different.

The peroxidation of membranes at direct lethal temperature was maximum in the genotypes ICC 10035 followed by ICC V1 and minimum in the genotypes ICC 4918 and ICC C37. Analysis of variance reveals significant differences between SA, $CaCl_2$ and PGPR pre-treatments of seedlings with respect to control samples at 0.05 level of significance.

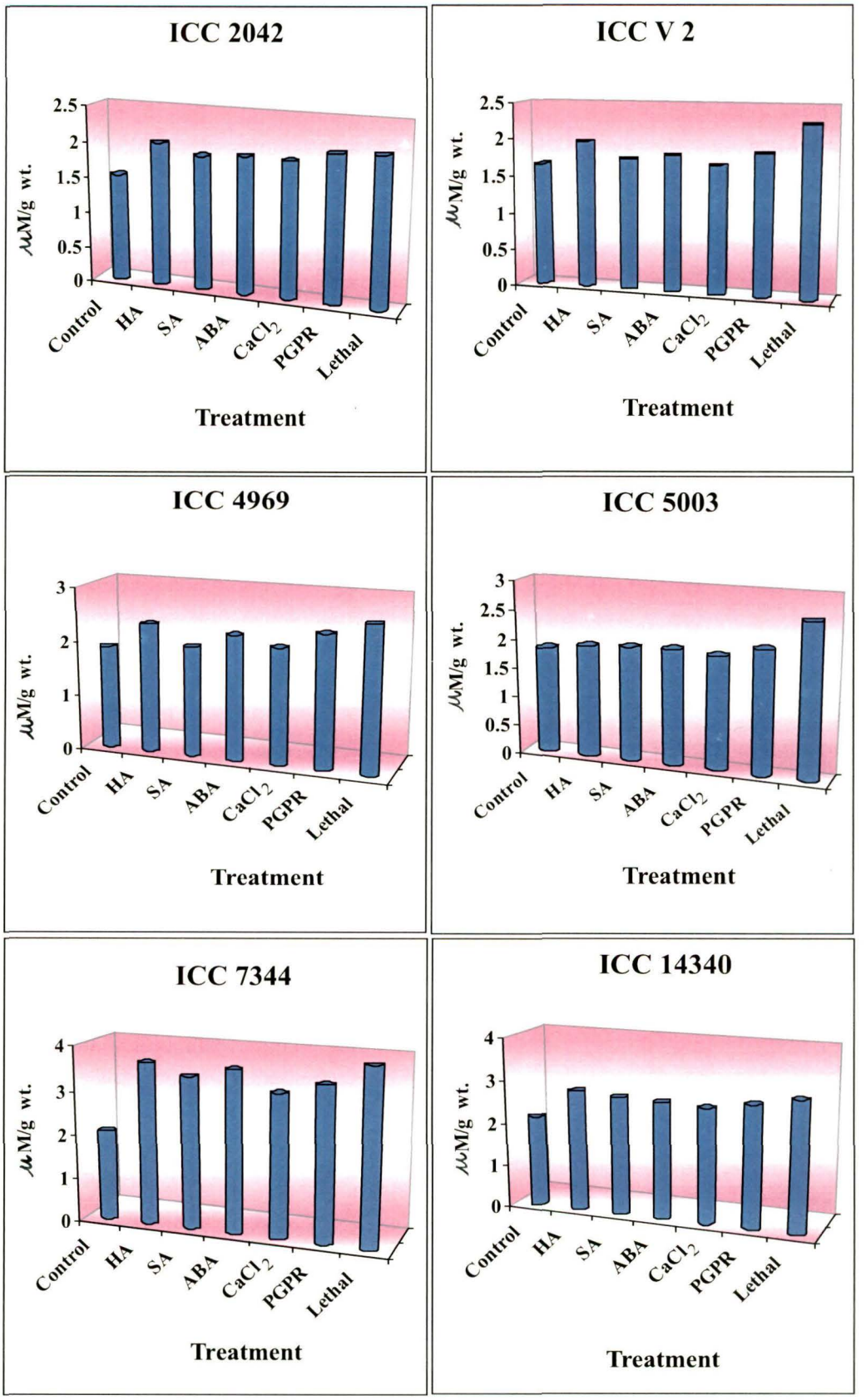


Fig. 11: Effect of pre-treatments on malondialdehyde (MDA) content of membranes in seedlings exposed to lethal temperature.

Table 30. Effect pre-treatments on MDA content of membranes in seedlings exposed to lethal temperature

Genotypes	MDA content (μM g^{-1}fresh wt.)						
	Control	HA	SA	ABA	$CaCl_2$	PGPR	Direct lethal
ICC 4918	2.23 ± 0.07	2.39 ± 0.08	2.10 ± 0.09	2.21 ± 0.07	2.16 ± 0.07	2.46 ± 0.06	2.60 ± 0.07
ICC 4969	1.89 ± 0.02	2.37 ± 0.04	2.01 ± 0.03	2.28 ± 0.05	2.12 ± 0.03	2.42 ± 0.06	2.65 ± 0.07
ICC 7344	2.08 ± 0.05	3.69 ± 0.12	3.45 ± 0.14	3.67 ± 0.16	3.24 ± 0.10	3.40 ± 0.16	3.97 ± 0.09
ICC 1852	1.16 ± 0.02	1.62 ± 0.09	1.12 ± 0.04	1.18 ± 0.09	1.01 ± 0.04	1.29 ± 0.04	1.73 ± 0.06
ICC 10035	1.99 ± 0.06	2.81 ± 0.07	2.53 ± 0.07	2.64 ± 0.07	2.51 ± 0.04	2.75 ± 0.06	4.39 ± 0.12
ICC 6119	1.71 ± 0.04	2.48 ± 0.11	2.06 ± 0.05	2.12 ± 0.06	2.01 ± 0.09	2.26 ± 0.07	2.91 ± 0.09
ICC 5003	1.83 ± 0.09	1.92 ± 0.05	1.96 ± 0.06	1.98 ± 0.06	1.93 ± 0.05	2.10 ± 0.05	2.61 ± 0.06
ICC 14340	2.14 ± 0.06	2.83 ± 0.06	2.76 ± 0.08	2.73 ± 0.05	2.69 ± 0.05	2.85 ± 0.08	3.03 ± 0.10
ICC 5319	1.99 ± 0.061	2.80 ± 0.05	2.08 ± 0.05	2.23 ± 0.06	2.11 ± 0.09	2.29 ± 0.07	3.99 ± 0.15
ICC 2042	1.52 ± 0.04	2.01 ± 0.04	1.88 ± 0.05	1.92 ± 0.05	1.91 ± 0.04	2.05 ± 0.07	2.08 ± 0.06
ICC 16359	2.17 ± 0.06	2.77 ± 0.13	2.11 ± 0.07	2.21 ± 0.06	2.23 ± 0.06	2.71 ± 0.07	3.99 ± 0.11
ICC C37	2.09 ± 0.04	2.13 ± 0.11	2.23 ± 0.07	2.22 ± 0.08	2.20 ± 0.07	2.35 ± 0.08	2.47 ± 0.14
ICC V10	1.74 ± 0.08	1.97 ± 0.07	1.79 ± 0.010	1.93 ± 0.06	2.00 ± 0.07	2.09 ± 0.05	2.21 ± 0.05
ICC V1	1.69 ± 0.04	2.61 ± 0.10	2.46 ± 0.01	2.56 ± 0.07	2.36 ± 0.10	2.46 ± 0.07	3.54 ± 0.09
ICC V2	1.66 ± 0.08	1.98 ± 0.06	1.76 ± 0.05	1.83 ± 0.06	1.72 ± 0.06	1.89 ± 0.06	2.28 ± 0.06

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 30A Analysis of variance for the data presented in Table 30

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	19.862	1.419	34.6468	0.17542
TREATMENT	6	07.393	1.232	30.0895	-
ERROR	84	03.440	0.041	-	-
TOTAL	104	30.695			

4.8. Effect of elevated temperatures and pre-treatments on cell membrane thermostability

Membranes are the first organelles to be affected by temperature stress and membrane thermostability is important for adaptation to higher temperatures. Membrane stability of different genotypes was tested in terms of electrolyte leakage. Membrane stability index or % of relative injury (RI) of membranes was significantly high with heat stressed plants compared to control (Table 31, Fig.12). Lower injury was recorded with plants pre-treated with SA, ABA, $CaCl_2$ and PGPR than heat-acclimatized plants. Lowest injury was recorded in genotype ICC 4918 closely followed by ICC 1852 and maximum in ICC 7344.

4.9. Effect of pre-treatment of seedlings and lethal temperature on chlorophylls and Hill activity

4.9.1. Chlorophyll content

Analysis of the photosynthetic pigments of all genotypes (total chlorophyll, chlorophyll a and chlorophyll b) showed a decline at lethal temperature. However, pre-treatments of seedlings were found to minimize the effects of heat stress on pigment contents. A decrease in the major photosynthetic pigment chlorophyll a clearly indicated the toxic and lethal effect of heat stress on plants. (Table 32, Fig.13). Significant differences were observed in the constitutive levels of total chlorophyll contents among the genotypes, with ICC 5003 recording the highest content (7.35 mg g^{-1} fresh weight) and ICC 1852 exhibiting the lowest (3.23 mg g^{-1} fresh weight).

4.9.2. Hill activity

Assay of Hill activity in six different genotypes consisting of two each of tolerant, moderately tolerant and susceptible genotypes revealed that lethal temperature led to a significant decrease in Hill activity. The activity was found to be marginally enhanced with pre-treatment of seedlings. However, direct exposure of seedlings led to a remarkable decrease in activity, especially in susceptible genotypes like ICC 7344 and ICC 10035 (Table 33).

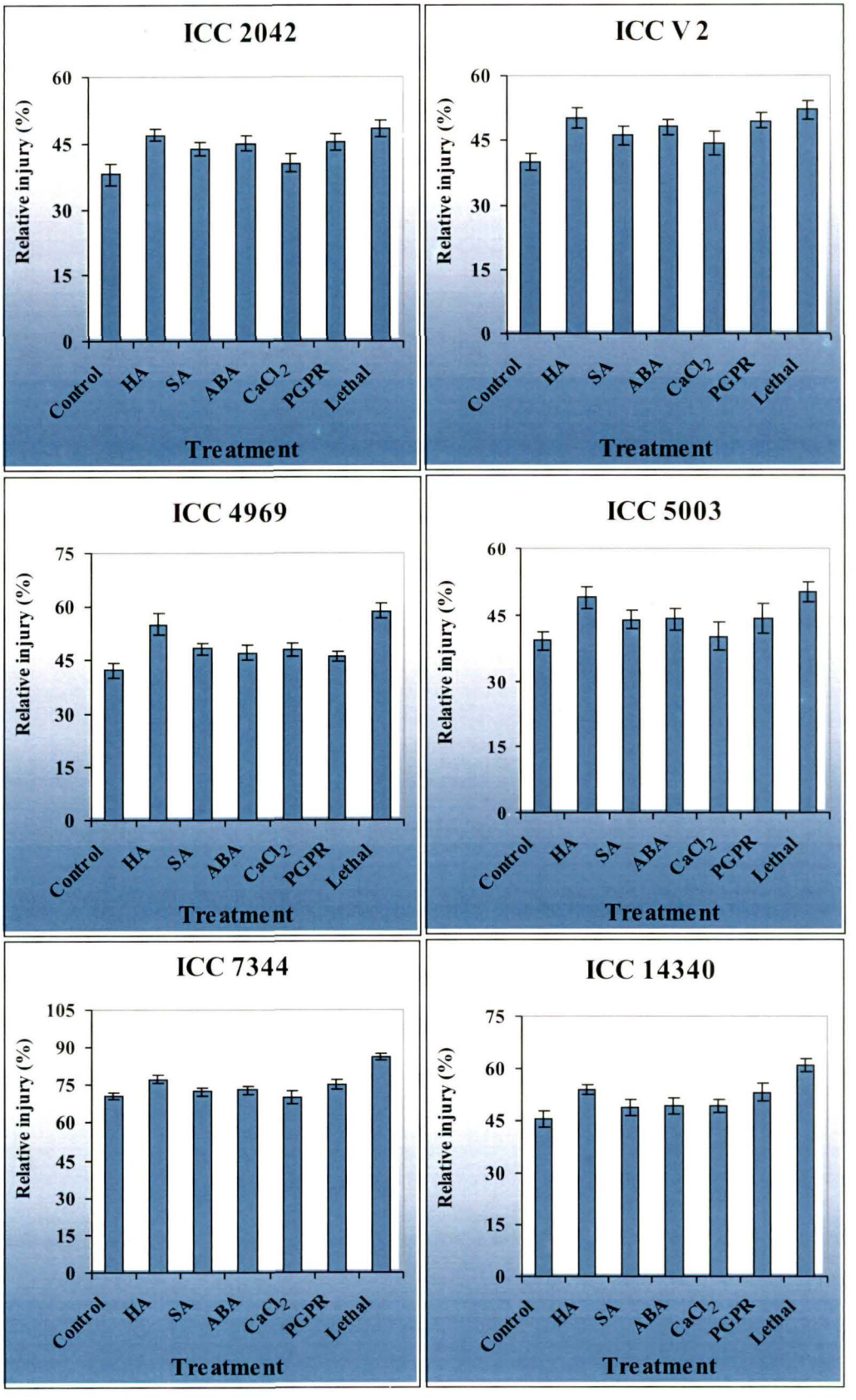


Fig.12: Percentage relative injury of chickpea seedlings following different pre-treatments and lethal temperature.

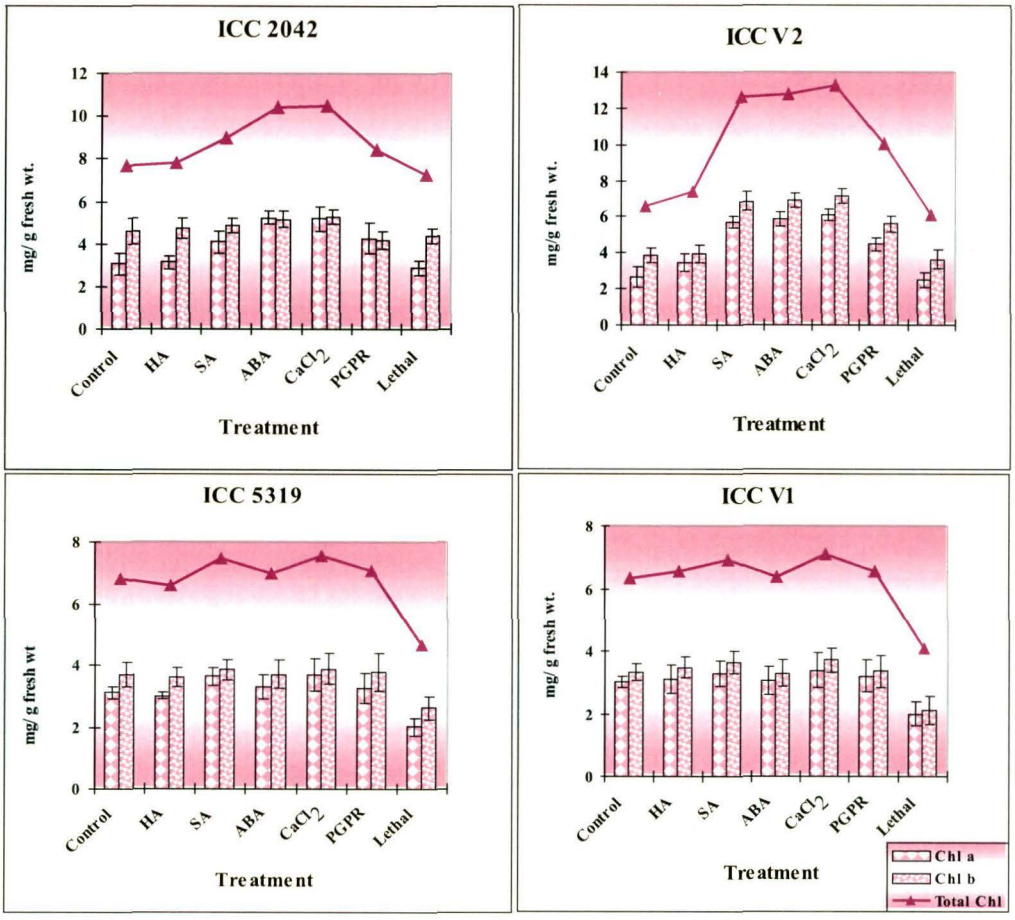


Fig.13: Changes in total chlorophyll, chlorophyll a and chlorophyll b contents of pre-treated seedlings subjected to lethal temperature.

Table 31. Effect of pre-treatments on cell membrane stability of seedlings exposed to lethal temperature

Genotypes	Relative Injury (RI) in percentage (%)						
	Control	HA	SA	ABA	$CaCl_2$	PGPR	Direct lethal
ICC 4918	29.83 ± 1.98	36.32 ± 2.21	30.63 ± 1.54	34.92 ± 2.10	28.14 ± 2.25	34.12 ± 1.27	40.59 ± 2.35
ICC 4969	42.20 ± 2.10	55.18] ± 3.00	48.23 ± 1.83	47.09 ± 2.09	48.01 ± 1.96	46.18 ± 1.34	58.89 ± 2.24
ICC 7344	70.86 ± 1.35	77.32 ± 1.64	72.38 ± 1.56	73.07 ± 1.65	70.10 ± 2.65	75.13 ± 1.70	86.32 ± 1.19
ICC 1852	35.03 ± 1.36	42.02 ± 2.10	32.93 ± 4.98	38.06 ± 2.33	35.08 ± 1.79	40.24 ± 1.93	45.65 ± 2.53
ICC 10035	60.64 ± 1.57	79.63 ± 2.00	65.84 ± 1.48	73.39 ± 1.68	64.92 ± 2.28	76.14 ± 2.55	82.04 ± 1.71
ICC 6119	33.60 ± 1.80	50.03 ± 1.98	45.69 ± 2.26	47.39 ± 1.95	42.76 ± 2.02	48.13 ± 1.54	59.79 ± 1.76
ICC 5003	39.06 ± 1.98	48.96 ± 2.51	43.95 ± 2.18	44.07 ± 2.47	40.04 ± 3.14	44.08 ± 3.47	52.06 ± 2.21
ICC 14340	45.39 ± 2.26	53.98 ± 1.51	48.94 ± 2.32	49.06 ± 2.33	49.01 ± 1.85	53.12 ± 2.52	60.87 ± 1.90
ICC 5319	52.97 ± 1.51	82.71 ± 1.53	75.59 ± 2.08	78.07 ± 2.40	70.33 ± 2.54	78.72 ± 1.58	84.02 ± 2.51
ICC 2042	38.02 ± 2.46	46.96 ± 1.22	43.72 ± 1.51	45.06 ± 1.71	40.52 ± 1.98	45.16 ± 1.89	48.37 ± 1.79
ICC 16359	58.92 ± 1.98	75.08 ± 2.18	68.79 ± 2.08	726.73 ± 1.59	63.30 ± 2.02	70.45 ± 3.44	80.69 ± 1.84
ICC C37	30.08 ± 3.45	40.13 ± 1.92	40.34 ± 1.43	40.56 ± 1.87	36.81 ± 2.45	38.03 ± 1.88	43.70 ± 2.30
ICC V10	29.18 ± 1.68	38.11 ± 2.44	34.18 ± 2.45	39.32 ± 2.02	36.43 ± 2.50	38.50 ± 2.36	42.46 ± 2.65
ICC V1	55.89 ± 1.38	75.44 ± 2.01	65.39 ± 1.97	70.45 ± 1.94	68.88 ± 2.19	73.27 ± 2.21	80.08 ± 2.20
ICC V2	40.06 ± 1.84	50.36 ± 2.38	46.18 ± 2.24	48.22 ± 1.77	44/31 ± 2.61	49.55 ± 1.81	52.02 ± 2.28

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 31A. Analysis of variance for the data presented in Table 31

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	25024.560	1787.469	404.6644	1.82189
TREATMENT	6	1730.113	288.352	65.2799	-
ERROR	84	371.042	4.417	-	-
TOTAL	104	27125.720			

Table 32. Effect of various pre-treatments on chlorophyll content in seedlings subjected to lethal temperature treatment

Genotypes	Treatment	Chlorophyll content (mg g^{-1} fresh wt.) Chlorophyll a	Chlorophyll b	Total chlorophyll
	Control	2.05±0.02	3.24±0.03	5.29±0.05
	HA	2.06±0.03	3.28±0.02	5.34±0.05
	SA	2.31±0.05	3.84±0.04	6.15±0.09
ICC 4918	ABA	2.39±0.03	3.86±0.03	6.25±0.06
	$CaCl_2$	2.91±0.06	4.19±0.05	7.10±0.11
	PGPR	2.45±0.17	3.97±0.06	6.42±0.23
	Lethal	1.98±0.06	3.20±0.04	5.18±0.10
	Control	1.90±0.03	2.33±0.04	4.23±0.07
	HA	1.98±0.05	2.36±0.05	4.34±0.10
	SA	3.49±0.03	3.38±0.04	6.87±0.07
ICC 4969	ABA	2.89±0.01	2.96±0.02	5.85±0.03
	$CaCl_2$	3.63±0.03	3.64±0.05	7.27±0.08
	PGPR	2.02±0.02	2.49±0.03	4.51±0.05
	Lethal	1.61±0.03	2.03±0.03	3.64±0.06
	Control	1.94±0.04	2.32±0.04	4.26±0.08
	HA	1.81±0.03	2.23±0.03	4.04±0.06
	SA	2.21±0.02	2.46±0.03	4.67±0.05
ICC 7344	ABA	2.10±0.04	2.39±0.04	4.49±0.08
	$CaCl_2$	2.26±0.03	2.53±0.04	4.79±0.07
	PGPR	2.09±0.03	2.38±0.03	4.47±0.06
	Lethal	1.04±0.02	2.01±0.02	3.05±0.04
	Control	1.29±0.02	1.94±0.04	3.23±0.06
	HA	1.43±0.03	2.06±0.03	3.49±0.06
	SA	1.72±0.04	2.58±0.04	4.30±0.08
ICC1852	ABA	1.67±0.03	2.55±0.03	4.22±0.06
	$CaCl_2$	1.73±0.03	2.62±0.04	4.35±0.07
	PGPR	1.40±0.03	2.26±0.04	3.66±0.07
	Lethal	1.18±0.03	1.85±0.03	3.03±0.06
	Control	2.28±0.03	2.65±0.02	4.93±0.05
	HA	2.20±0.03	2.63±0.03	4.83±0.06
	SA	2.54±0.03	2.97±0.05	5.51±0.08
ICC 10035	ABA	2.43±0.03	2.78±0.03	5.21±0.06
	$CaCl_2$	2.63±0.02	2.87±0.03	5.50±0.05
	PGPR	2.12±0.03	2.76±0.03	4.88±0.06
	Lethal	1.18±0.03	2.01±0.03	3.19±0.06
	Control	3.03±0.03	4.32±0.02	7.35±0.05
	HA	3.12±0.04	4.41±0.03	7.53±0.07
	SA	5.16±0.07	6.60±0.04	11.76±0.11
ICC 5003	ABA	5.19±0.04	6.18±0.03	11.37±0.08
	$CaCl_2$	5.62±0.04	6.19±0.05	11.81±0.09
	PGPR	4.31±0.04	5.07±0.04	9.38±0.08
	Lethal	2.01±0.03	3.94±0.04	6.65±0.07

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 32A. Analysis of variance for the data presented in Table 32 (Chl a)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	26.806	5.361	19.3092	0.74747
TREATMENT	6	9.127	1.521	5.4785	-
ERROR	30	8.329	0.278	-	-
TOTAL	41	44.262			

Table 32B. Analysis of variance for the data presented in Table 32 (Chl b)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	45.447	9.089	69.1620	0.51426
TREATMENT	6	7.465	1.244	9.4674	-
ERROR	30	3.943	0.131	-	-
TOTAL	41	56.855			

Table 32C. Analysis of variance for the data presented in Table 32 (Total Chl)

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	143.584	28.717	48.0818	1.09628
TREATMENT	6	35.305	5.884	9.8520	-
ERROR	30	17.917	0.597	-	-
TOTAL	41	196.806			

4.10. Changes in free proline in pre-treated seedlings of *Cicer arietinum* in response to lethal temperature treatment

Accumulation of an amino acid like proline which acts a compatible solute in plants exposed to various stress conditions is a well documented phenomenon. The results of the present study also revealed a remarkable increase in proline content in pre-treated seedlings with respect to untreated control samples following lethal

temperature treatment (Table 34, Fig.14). Increase in proline content was higher in all genotypes, irrespective of their tolerance. Even exposure to lethal temperature led to an increase in proline content over control. However, the quantum of increase was more in tolerant genotypes (ICC 4918, ICC 1852, ICC 2042, ICC C37, ICC V10 and ICC V2) than in susceptible ones (ICC 5319, ICC 7344, ICC 10035, ICC V1 and ICC 16359).

Table 33. Effect of pre-treatments on Hill activity of seedlings exposed to lethal temperature

	Hill activity (μM DCPIP min^{-1} mg^{-1} chloroplast)					
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**Direct lethal**
ICC 4918	23.64 ± 0.8	23.96 ± 0.1	30.66 ± 1.5	26.21 ± 1.6	32.89 ± 1.3	18.70 ± 1.8
ICC 4969	20.12 ± 1.3	21.36 ± 1.8	28.68 ± 1.8	22.78 ± 1.3	28.45 ± 2.2	12.02 ± 1.9
ICC 7344	18.44 ± 1.7	18.40 ± 2.3	20.05 ± 2.2	20.32 ± 1.9	23.56 ± 1.9	08.28 ± 2.6
ICC 1852	22.22 ± 1.9	23.18 ± 2.5	24.90 ± 2.5	23.26 ± 2.2	23.99 ± 1.4	20.76 ± 2.7
ICC 10035	20.48 ± 2.5	20.65 ± 1.8	23.83 ± 1.7	20.56 ± 3.0	25.59 ± 1.8	10.04 ± 2.9
ICC 5003	23.86 ± 1.9	26.06 ± 2.3	32.42 ± 1.7	28.44 ± 2.5	30.98 ± 1.7	15.08 ± 2.3

Values represent mean ± SE (n = 3). Values are mean of three replicates.

Table 33 A. Analysis of variance for the data presented in Table 33

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	5	238.231	47.646	33.1919	1.71895
TREATMENT	5	188.791	37.758	26.3036	-
ERROR	25	35.887	1.435	-	-
TOTAL	35	462.909			

4.11. Effect of pre-treatment of seedlings and lethal temperature treatment on carbohydrates of chickpea

4.11.1. Total sugar

Carbohydartes like total and reducing sugars are the building blocks of cells and highly responsive to stress environments. The total sugar content of seedlings

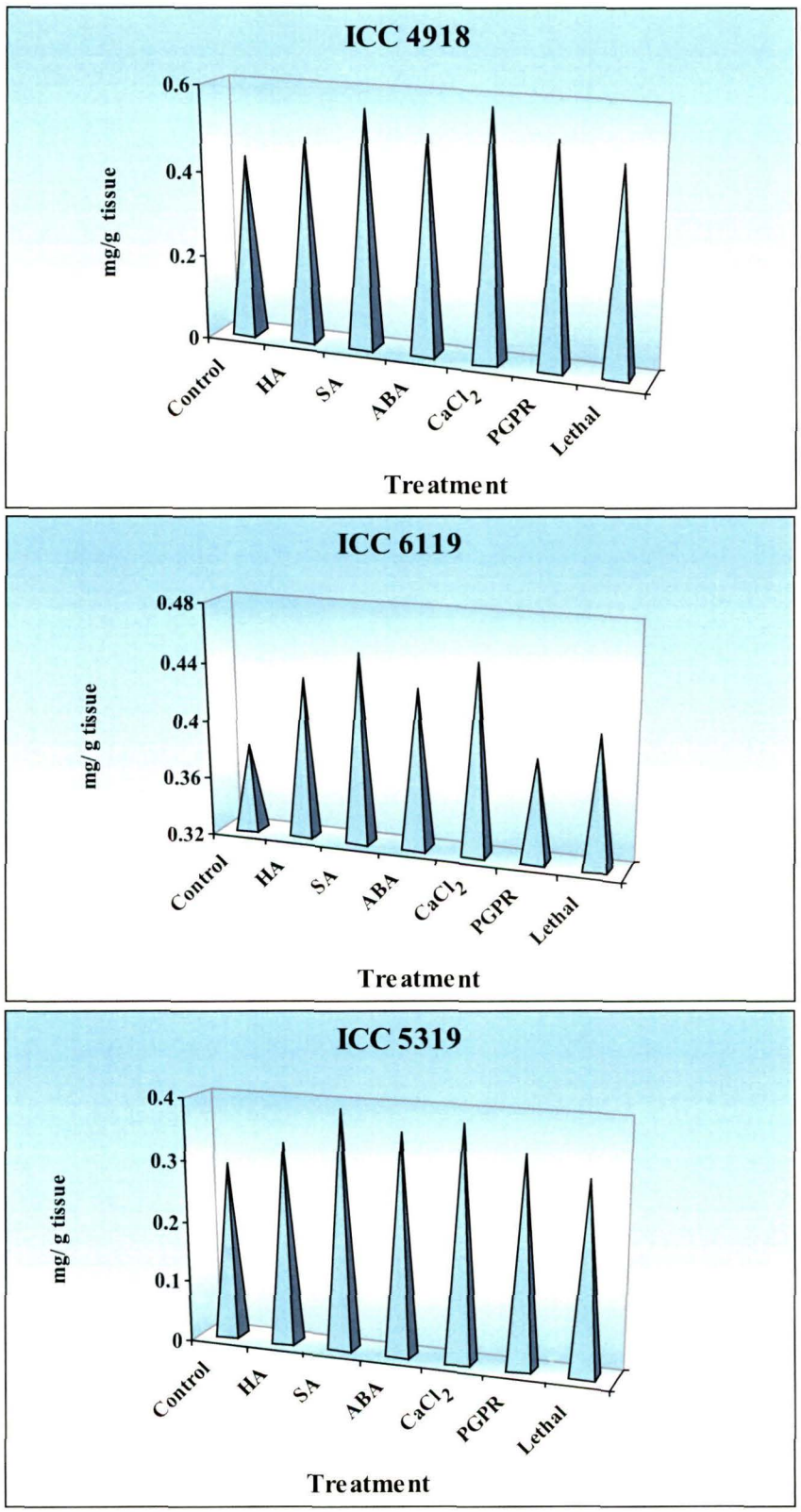


Fig. 14. Proline content of three different genotypes of chickpea seedlings subjected to pre-treatments and lethal temperature.

directly exposed to lethal temperature without any pre-treatment showed a significant decrease. Pre-treatments on the other hand were found to be quite effective in reducing the impact of heat stress. In cases where seedlings were pre-treated, the total sugar content was higher than that of lethal temperature treatment (Table 35, Fig. 15).

Table 34 Effect of pre-treatments followed by lethal temperature treatment on proline contents of seedlings

	Proline content (mg g^{-1} tissue)						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	0.43 ± 0.02	0.48 ± 0.02	0.56 ± 0.03	0.051 ± 0.02	0.58 ± 0.02	0.52 ± 0.05	0.48 ± 0.03
ICC 4969	0.36 ± 0.03	0.42 ± 0.04	0.47 ± 0.02	0.48 ± 0.02	0.50 ± 0.02	0.45 ± 0.02	0.39 ± 0.04
ICC 7344	0.29 ± 0.05	0.34 ± 0.02	0.37 ± 0.03	0.36 ± 0.01	0.38 ± 0.03	0.36 ± 0.02	0.33 ± 0.03
ICC 1852	0.48 ± 0.05	0.56 ± 0.04	0.62 ± 0.02	0.65 ± 0.002	0.64 ± 0.02	0.60 ± 0.03	0.53 ± 0.03
ICC 10035	0.27 ± 0.03	0.32 ± 0.02	0.35 ± 0.02	0.33 ± 0.02	0.36 ± 0.02	0.32 ± 0.03	0.30 ± 0.03
ICC 6119	0.38 ± 0.03	0.43 ± 0.02	0.45 ± 0.02	0.43 ± 0.02	0.45 ± 0.02	0.39 ± 0.02	0.41 ± 0.02
ICC 5003	0.28 ± 0.03	0.36 ± 0.02	0.39 ± 0.01	0.41 ± 0.02	0.42 ± 0.03	0.38 ± 0.02	0.31 ± 0.02
ICC 14340	0.48 ± 0.03	0.53 ± 0.03	0.55 ± 0.03	0.56 ± 0.02	0.56 ± 0.03	0.52 ± 0.02	0.51 ± 0.02
ICC 5319	0.29 ± 0.02	0.33 ± 0.02	0.39 ± 0.02	0.36 ± 0.02	0.39 ± 0.02	0.34 ± 0.02	0.31 ± 0.01
ICC 2042	0.46 ± 0.05	0.52 ± 0.02	0.56 ± 0.02	0.54 ± 0.02	0.55 ± 0.02	0.51 ± 0.03	0.51 ± 0.02
ICC 16359	0.30 ± 0.03	0.36 ± 0.03	0.38 ± 0.02	0.39 ± 0.02	0.38 ± 0.03	0.36 ± 0.04	0.34 ± 0.02
ICC C37	0.44 ± 0.01	0.49 ± 0.01	0.56 ± 0.02	0.52 ± 0.02	0.56 ± 0.02	0.53 ± 0.02	0.48 ± 0.01
ICC V10	0.44 ± 0.02	0.49 ± 0.03	0.54 ± 0.03	0.52 ± 0.02	0.54 ± 0.02	0.53 ± 0.03	0.48 ± 0.04
ICC V1	0.38 ± 0.02	0.42 ± 0.02	0.44 ± 0.02	0.43 ± 0.02	0.45 ± 0.02	0.42 ± 0.02	0.42 ± 0.02
ICC V2	0.37 ± 0.02	0.42 ± 0.02	0.45 ± 0.02	0.46 ± 0.02	0.46 ± 0.03	0.43 ± 0.03	0.41 ± 0.02

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 34A. Analysis of variance for the data presented in Table 34

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	14	0.661	0.047	187.9069	0.01374
TREATMENT	6	0.121	0.020	80.5135	-
ERROR	84	0.021	0.000	-	-
TOTAL	104	0.803			

Table 35. Changes in total sugar content of seedlings following lethal temperature treatment

	Total sugar content (mg g^{-1} fresh wt.)						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	28.13 ± 1.66	24.74 ± 1.44	27.31 ± 1.29	26.32 ± 1.21	28.16 ± 1.82	26.21 ± 1.59	20.19 ± 0.88
ICC 4969	18.01 ± 1.07	18.38 ± 0.89	20.15 ± 1.21	22.37 ± 1.33	24.16 ± 1.18	20.36 ± 1.10	13.91 ± 0.81
ICC 7344	18.52 ± 0.94	18.40 ± 0.99	19.21 ± 0.83	19.15 ± 1.25	21.44 ± 0.80	18.78 ± 1.24	07.65 + 0.54
ICC 1852	22.68 ± 1.06	23.16 ± 1.12	25.87 ± 0.86	24.69 ± 0.68	27.63 ± 1.13	24.62 ± 1.26	18.02 ± 1.44
ICC 10035	14.81 ± 1.29	14.00 ± 1.13	15.96 ± 0.84	15.04 ± 1.11	16.08 ± 0.84	14.73 ± 1.18	09.60 ± 0.36
ICC 6119	14.32 ± 0.89	15.64 ± 0.77	16.50 ± 0.80	15.91 ± 0.69	18.71 ± 1.19	15.37 ± 0.67	8.18 ± 0.84
ICC 5003	24.10 ± 0.88	25.03 ± 1.48	29.32 ± 1.10	29.19 ± 1.17	32.17 ± 1.34	26.18 ± 0.26	18.03 ± 0.94
ICC 14340	20.26 ± 9.05	20.21 ± 0.96	23.31 ± 0.81	21.76 ± 1.19	24.15 ± 0.85	22.39 ± 1.19	12.03 ± 1.00
ICC 5319	13.06 ± 0.64	13.40 ± 0.94	15.31 ± 1.35	13.26 ± 0.61	14.26 ± 1.30	13.29 ± 1.05	05.01 ± 0.38
ICC 2042	24.21 ± 1.25	25.00 ± 1.20	27.11 ± 0.94	27.28 ± 0.84	28.79 ± 1.52	25.38 ± 1.22	20.62 ± 1.49
ICC 16359	13.59 ± 0.87	13.50 ± 0.81	14.66 ± 1.07	14.38 ± 1.19	15.39 ± 1.31	14.24 ± 1.07	05.13 ± 1.19
ICC C37	24.61 ± 1.09	25.08 ± 1.57	27.31 ± 0.90	25.96 ± 0.81	27.84 ± 1.00	25.51 ± 1.41	20.08 ± 1.18
ICC V2	18.17 ± 0.96	19.00 ± 1.34	20.84 ± 1.36	20.15 ± 1.11	21.61 ± 0.90	19.06 ± 1.69	14.31 ± 0.84

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 35A. Analysis of variance for the data presented in Table 35

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	12	2209.730	184.144	144.0122	1.05294
TREATMENT	6	774.581	129.097	100.9617	-
ERROR	72	92.064	1.279	-	-
TOTAL	90	3076.375			

4.11.2. Reducing sugar

The reducing sugar contents in control seedlings remained stable but exhibited significant changes under heat stress. A pronounced decrease in reducing sugar content was observed in susceptible genotypes directly exposed to lethal temperature treatment. Pre-treatments on the other hand either maintained the steady sugar level or led to a marginal or remarkable increase in reducing sugar contents in all genotypes (Table 36, Fig.15).

4.12. Changes in phenolic profile following pre-treatments and exposure to lethal temperature treatment

HPLC analysis of pre-treated and control seedlings was performed to assess the degree of thermo-protection offered by phenolics in each pre-treatment. By comparing the retention time and co-injection of the standards, five phenolic acids, viz; ferulic, chlorogenic, salicylic, cinnamic and gallic acid were identified in pre-treated as well as non-treated seedlings of various genotypes (Table 37). Among the five phenolics, gallic acid was found to be present in all treatments including controls while chlorogenic acid was found to be completely absent in untreated samples.

Rapid accumulation of ferulic and chlorogenic acids were recorded in tolerant genotypes subjected to SA and PGPR pre-treatments. Seedlings subjected to PGPR treatments also revealed higher rate of accumulation of phenolics and better growth rate. SA pre-treated seedlings showed a significant difference in the phenolic profile while ABA pre-treatment led to a considerable reduction in number of peaks (Figs. 16 and17). Consistent presence and increased synthesis of ferulic acid due to

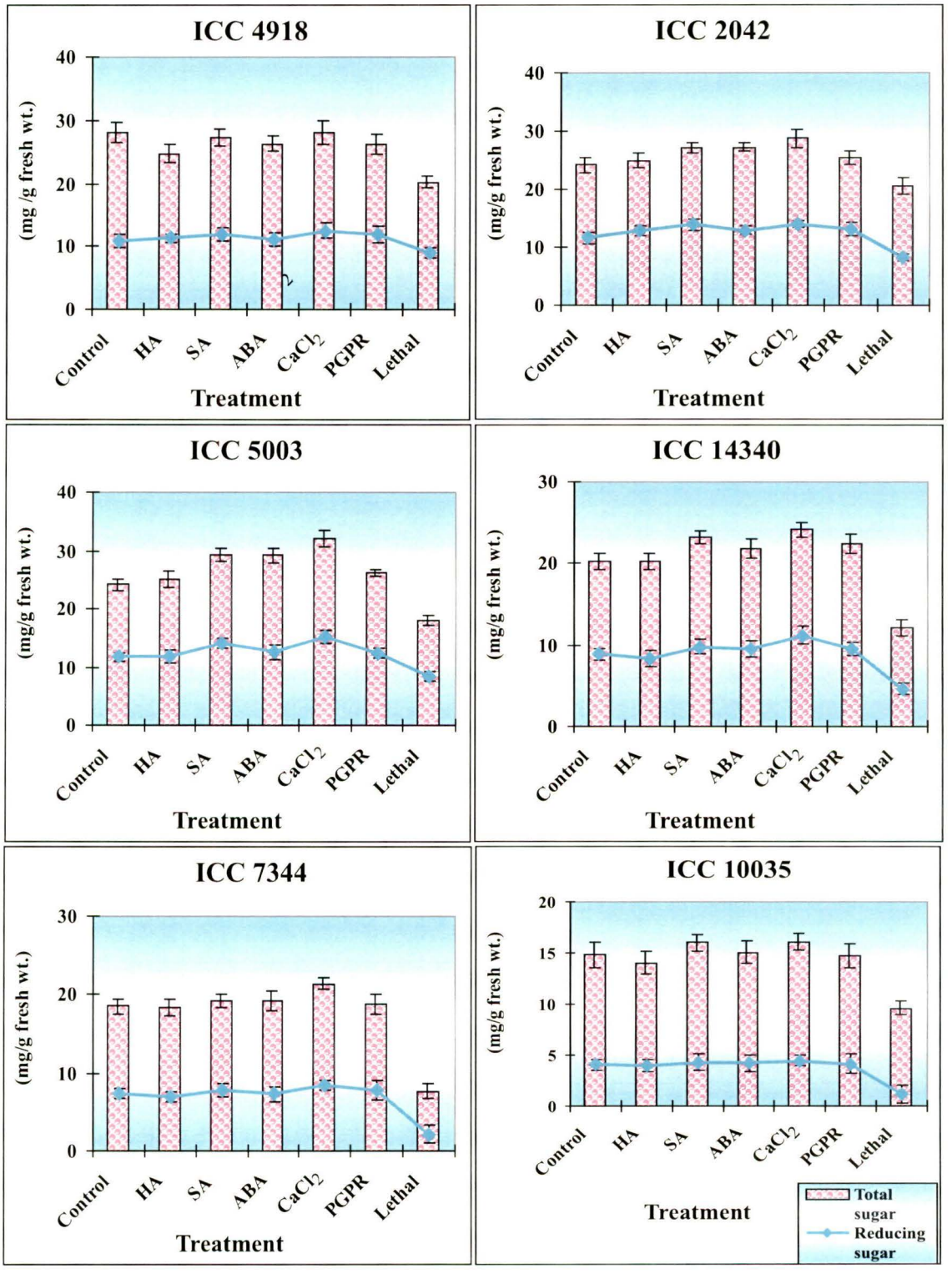


Fig. 15: Changes in total and reducing sugar contents of pre-treated seedlings following lethal temperature treatment

pre-treatments in tolerant genotypes suggest the possible role of ferulic acid and other phenolics in thermo-protection.

Table 36. Changes in reducing sugar content of seedlings following lethal temperature treatment

	Reducing sugar content (mg g^{-1} fresh wt.)						
Genotypes	**Control**	**HA**	**SA**	**ABA**	**$CaCl_2$**	**PGPR**	**Direct lethal**
ICC 4918	10.81 ± 1.03	11.26 ± 0.71	11.95 ± 1.03	11.09 ± 1.10	12.54 ± 1.28	11.86 ± 1.29	08.01 ± 0.85
ICC 4969	08.06 ± 0.63	08.42 ± 1.10	09.51 ± 1.13	08.63 ± 1.22	09.65 ± 1.06	09.12 ± 0.74	05.38 ± 0.48
ICC 7344	07.30 ± 0.46	07.01 ± 0.46	07.76 ± 0.87	07.32 ± 0.91	08.54 ± 0.63	07.82 ± 1.23	02.21 ± 0.21
ICC 1852	09.61 ± 0.88	09.85 ± 1.10	11.44 ± 0.94	10.60 ± 1.06	13.78 ± 1.18	12.34 ± 1.01	07.38 ± 0.80
ICC 10035	04.10 ± 0.51	04.00 ± 0.60	04.30 ± 0.41	04.22 ± 0.28	04.45 ± 0.54	04.15 ± 0.39	01.18 ± 0.09
ICC 6119	05.71 ± 0.57	05.82 ± 0.66	06.87 ± 1.02	05.90 ± 0.96	06.90 ± 0.57	06.38 ± 0.38	02.20 ± 0.32
ICC 5003	11.70 ± 0.65	11.89 ± 1.13	13.99 ± 0.89	12.61 ± 1.27	15.12 ± 1.13	12.45 ± 0.83	0.42 ± 0.81
ICC 14340	08.90 ± 0.73	08.40 ± 0.95	09.81 ± 0.94	09.50 ± 1.00	11.21 ± 1.03	09.50 ± 0.57	04.64 ± 0.46
ICC 5319	04.20 ± 0.40	04.11 ± 0.50	05.31 ± 0.53	04.56 ± 0.68	05.80 ± 0.62	05.43 ± 0.57	01.36 ± 0.19
ICC 2042	11.60 ± 1.00	12.81 ± 0.78	13.95 ± 1.01	12.98 ± 0.63	14.03 ± 0.68	13.12 ± 1.08	08.31 ± 0.63
ICC 16359	04.81 ± 0.38	04.01 ± 0.63	05.21 ± 0.28	04.80 ± 0.47	05.50 ± 0.37	04.89 ± 0.50	01.72 ± 0.10
ICC C37	10.30 ± 1.00	10.45 ± 1.08	11.69 ± 1.02	11.52 ± 0.95	11.81 ± 1.19	10.80 ± 1.05	08.00 ± 0.58
ICC V2	08.98 ± 0.46	09.08 ± 0.74	10.31 ± 1.07	10.40 ± 0.77	10.83 ± 0.81	09.58 ± 0.92	05.02 ± 0.23

Values represent mean ± SE (n = 3). Values are mean of three replicates

Table 36A. Analysis of variance for the data presented in Table 36

SOURCE	D.F.	S.S.	M.S.	F.	C.D.(5%)
BLOCK	12	794.145	66.179	216.4485	0.51488
TREATMENT	6	207.325	34.554	113.0153	-
ERROR	72	22.014	0.306	-	-
TOTAL	90	1023.484			

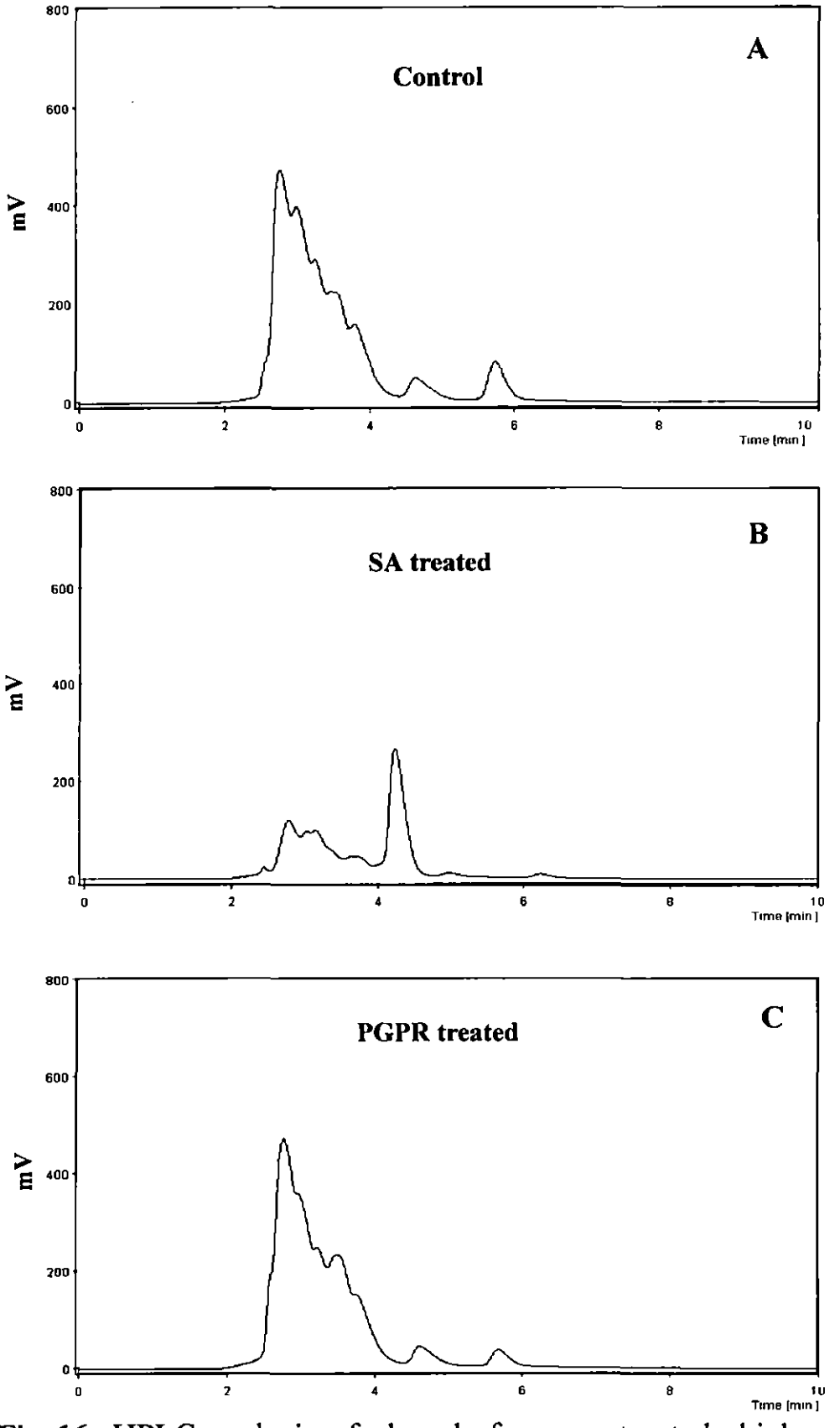


Fig. 16: HPLC analysis of phenols from pre-treated chickpea seedlings (ICC V2) subjected to lethal temperature treatment **A**: Control; **B**: SA and **C**: PGPR

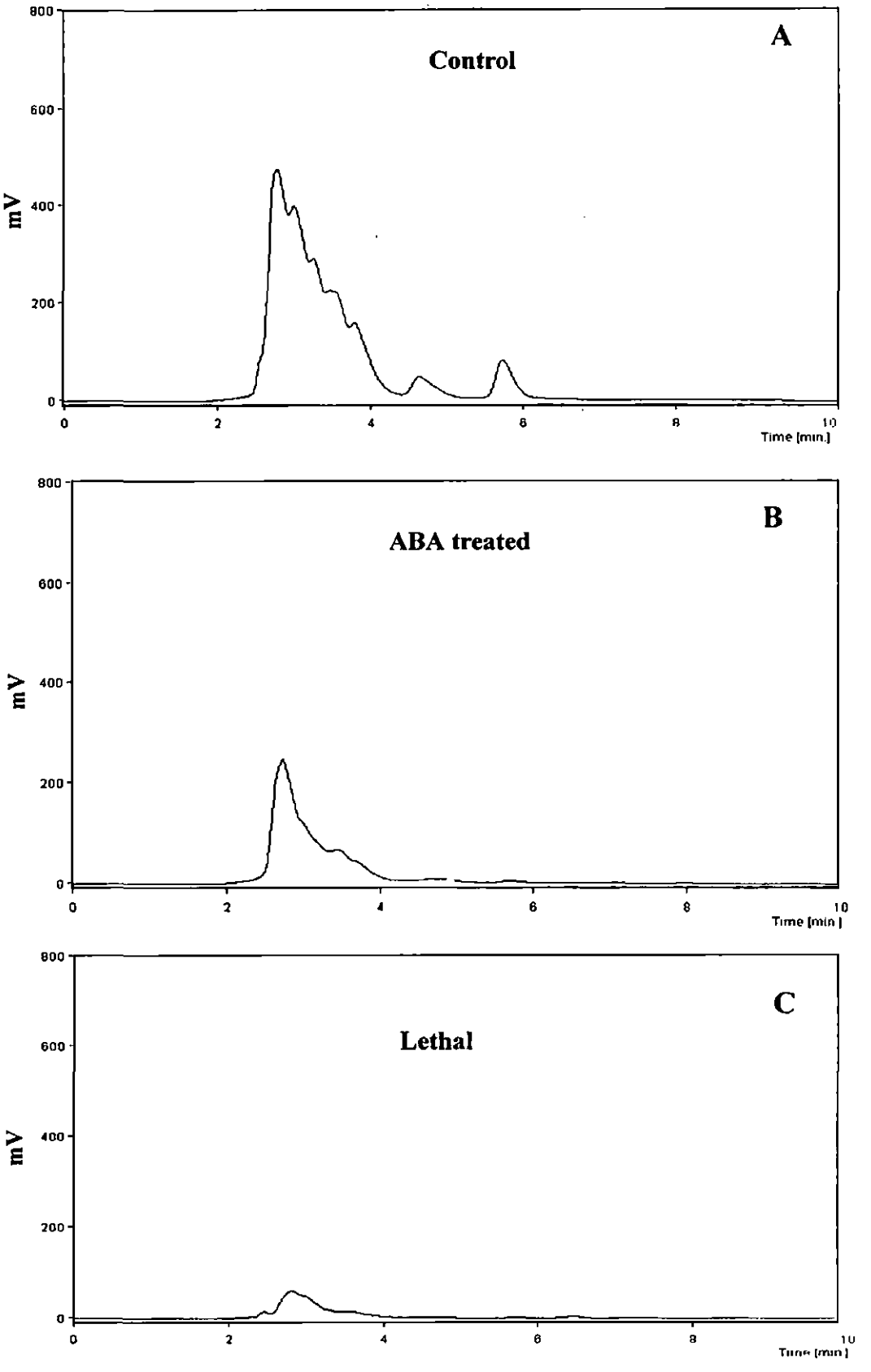


Fig. 17. Peak results of HPLC analysis of phenolic extracts of control and pre-treated seedlings subjected to lethal temperature. **A:** Control; **B**: ABA and **C**: Lethal

Table 37. HPLC analysis of phenols from control and pre-treated seedlings subjected to lethal temperature

Treatment	Peak No.	R. Time	Area (mv.S)	Height (mV)	Area %	Height %
	1	2.450	274.1275	23.738	2.477	4.077
	2	2.790	24069.5844	477.131	88.761	77.911
Control	3	4.640	1224.4737	48.968	4.515	7.996
	4	5.730	1695.0343	82.784	6.251	13.518
	1	2.450	274.1275	23.738	2.477	4.077
	2	2.790	2001.3047	121.127	18.080	20.805
SA	3	3.160	2719.2252	100.564	24.566	17.273
	4	3.650	1001.0582	46.435	9.044	7.976
	5	4.250	4392.5619	268.070	39.684	46.044
	1	2.790	23214.4227	474.067	90.884	84.325
ABA	2	4.620	1145.7434	46.180	4.486	8.214
	3	5.680	1078.3832	39.442	4.222	7.016
	1	2.450	157.5260	14.525	5.997	15.229
	2	2.740	9031.7044	249.652	93.054	93.724
PGPR	3	4.700	375.7401	9.215	3.871	3.460
	4	5.670	245.8212	6.020	2.533	2.260
	1	2.450	157.5260	14.525	5.997	15.229
Lethal	2	2.810	1846.3998	59.001	70.288	61.861
	3	3.590	398.4990	14.285	15.170	14.977

4.13. Analysis of the effect of elevated temperatures and pre-treatments on growth of calli *in vitro*

4.13.1. Elevated temperature treatment

In order to analyze the effect of elevated temperatures on growth of calli, the callus formation was induced from various explants in three differently amended MS medium supplemented with different concentrations and combinations of growth regulators (NAA+BAP, NAA+Kinetin and IAA+BAP). Of the various explants used for induction of callus, shoot tip with a portion of leaf was found to be more responsive than cotyledonory node, hypocotyls and internode.

Callus initiation took place at the cut surface after 7-10 days of incubation and the calli continued to grow till 60 days (Plate XVII). Among the media used,

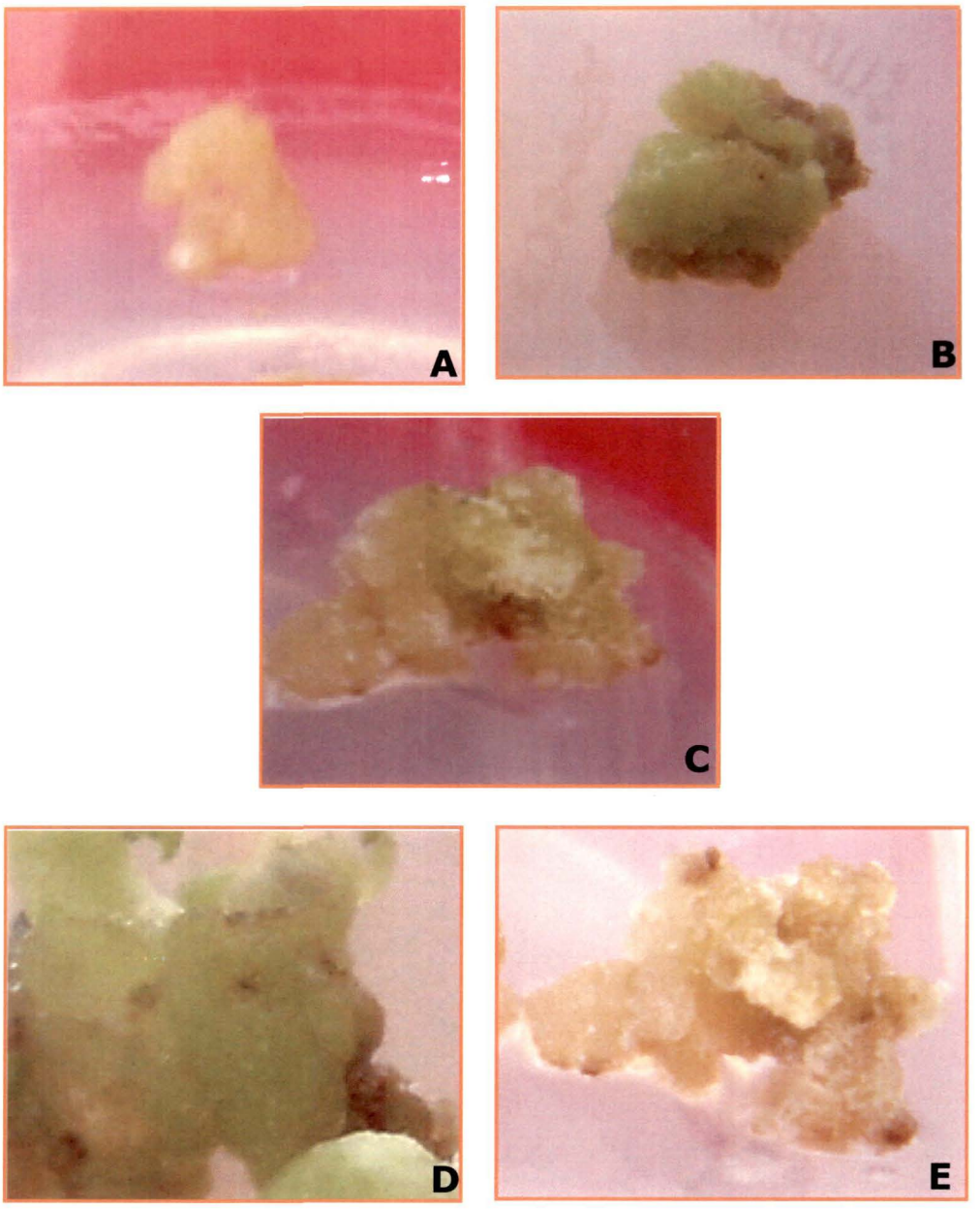


Plate XVII (A-E): Calli of chickpea (*Cicer arietinum* L.) of various ages **A:** 10 d old**; B:** 20 d old**; C:** 30 d old**; D:** 40 d old and **E;** 60 d old.

medium supplemented with NAA (1.0 mg l^{-1}) and BAP (0.5 mg l^{-1}) was found to be the most suitable for callus initiation in both tolerant and susceptible genotypes.

The treatment of callus (20 days old) at elevated temperatures revealed that callus formation and growth was greatly influenced by the temperature. Exposure of calli to elevated temperatures higher than the optimal range of 18-25°C led to rapid browning of calli followed by a drastic decline in growth rate (weight) and death of calli in both tolerant (ICC C37) and susceptible (ICC 7344) genotypes (Plate XVIII). 20 days old callus of tolerant genotype could tolerate a high temperature treatment of 40°C for 2-4 hrs while the calli raised from susceptible genotype could not tolerate elevated temperature treatment of 35°C of equal duration (Table 38).

Table 38. Effect of elevated temperatures on growth of calli

Treatments	Day old	Fresh weight of calli (g)	
		ICC C 37	ICC 7344
Control	0	0.035 ± 0.008	0.033 ± 0.007
	20	0.078 ± 0.010	0.064 ± 0.009
	30	0.136 ± 0.101	0.130 ± 0.006
30^0C	0	0.032 ± 0.006	0.031 ± 0.004
	20	0.072 ± 0.011	0.060 ± 0.008
	30	0.139 ± 0.020	0.112 ±0.006
35^0C	0	0.033 ± 0.007	0.030 ± 0.004
	20	0.068 ± 0.006	0.032 ± 0.002
	30	0.126 ± 0.009	0.100 ± 0.004
40^0C	0	0.036 ± 0.002	0.030 ± 0.004
	20	0.062 ± 0.008	0.030 ± 0.005
	30	0.126 ±0.009	0.030 ± 0.003

CD (5%) – 0.03937. Values represent mean ± SE (n = 3). Values are mean of three replicates

4.13.2. Pre-treatments of calli

Effect of heat acclimation (32°C sub-lethal temperature treatment of 2 hrs) and SA pre-treatments (MS medium supplemented with 10^{-5} and 10^{-6} M SA) was tested by exposing the calli obtained from both thermotolerant and susceptible genotypes grown in SA amended and unamended media to lethal temperature. Thermoprotection offered by pre-treatments was further analyzed by recording the

fresh weights of calli regularly after every 15 d interval following exposure to lethal temperature.

Table 39. Effect of heat acclimation and SA on thermopotection of calli

Treatments	Day old	Fresh weight of calli (g)	
		ICC C 37	ICC 7344
Control	0	0.035 ± 0.008	0.033 ± 0.006
	15	0.072 ± 0.013	0.064 ± 0.016
	30	0.136 ± 0.021	0.118 ± 0.032
	60	0.367 ± 0.032	0.329 ± 0.012
HA	0	0.030 ± 0.005	0.032 ± 0.003
	15	0.068 ± 0.014	0.068 ± 0.008
	30	0.140 ± 0.026	0.107 ± 0.018
	60	0.388 ± 0.021	0.352 ± 0.014
SA (10^{-5}M)	0	0.033 ± 0.004	0.033 ± 0.003
	20	0.078 ± 0.010	0.078 ± 0.009
	30	0.162 ± 0.013	0.140 ± 0.19
	60	0.450 ± 0.120	0.386 ± 0.102
Lethal	0	0.032 ± 0.002	0.030 ± 0.003
	20	0.062 ± 0.010	0.032 ± 0.006
	30	0.102 ± 0.013	0.036 ± 0.006
	60	0.136 ± 0.038	0.036 ± 0.002

CD (5%) – 0.08338. Values represent mean ± SE (n = 3). Values are mean of three replicates

Calli of both genotypes exhibited some degree of browning of tissues following lethal temperature treatment. Heat acclimatized calli and calli raised in amended MS media however showed a much higher level of thermotolerance and comparatively much lower reduction in growth rate and browning of tissues (Table 39). Among the pre-treatments, MS medium supplemented with 10^{-5} M SA was found to offer best level of thermoprotection closely followed by 10^{-6} M SA and heat acclimation pre-treatment.

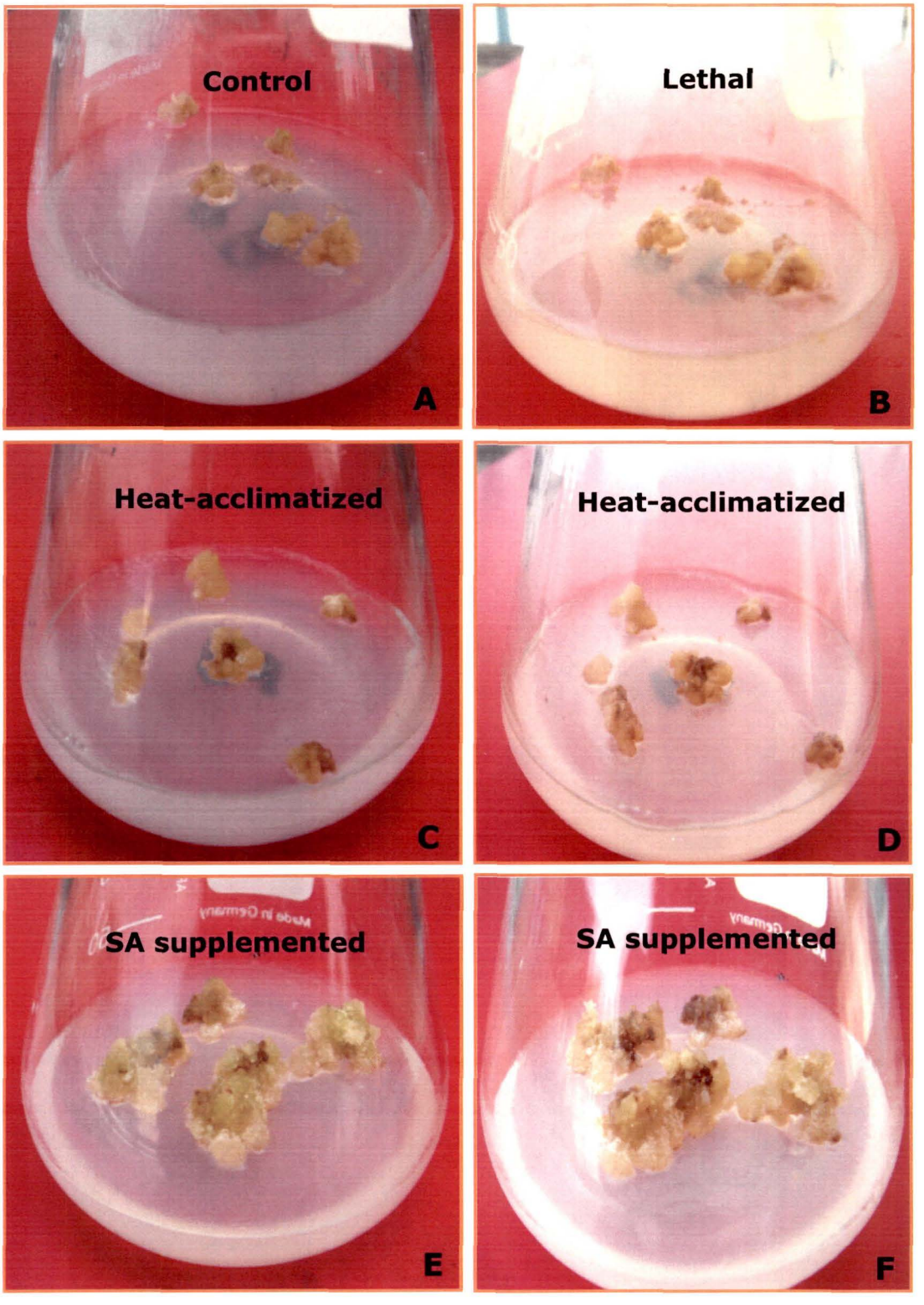


Plate XVIII (A-F) : Calli raised from the shoot tip before and after lethal temperature treatment. **A,C & E:** Before treatment ; **B,D & F:** After treatment

DISCUSSION

Plants are exposed to various environmental stresses throughout the course of their life span and hence have an inbuilt ability to adjust to seasonal and other environmental variables. The ability to withstand and to acclimate to supra-optimal temperatures results from both prevention of heat damage and repair of heat sensitive components (Larkindale *et al.*, 2005). Organisms must also maintain metabolic homeostasis during stress or be able to re-establish homeostasis subsequent to the stress period. Apart from the regular circadian and seasonal perturbations, there may be certain other rapid and unpredicted disturbances in the environment resulting in stressful conditions (Grover *et al.*, 2001). Abiotic stress negatively influences survival, biomass production and grain yield of most crops. Different crop eco-systems are affected by different abiotic stress factors and to a differential extent. The degree of susceptibility of different plant species and of different genotypes within the species is often varied. There is also some level of variation associated with specific developmental stages of the plant.

Temperature stress is often one of the most important abiotic stresses which the plant is exposed to. Plants can be damaged in different ways by either high day or high night temperature or by either high air or high soil temperatures. In nature, however, plants often experience mild stresses before they face severe intensity of stresses and plants may be exposed to multiple environmental stresses either sequentially or simultaneously (Srivalli *et al.*, 2003). Exposure to sub-lethal abiotic stresses renders plants more tolerant to a subsequent normally lethal dose of the same stress, a phenomenon referred to as acclimation. Acquired thermotolerance can be induced in plants by a short acclimation period at moderately high or sub-lethal temperatures or by treatment with other non-lethal stress prior to heat stress (Kapoor *et al.*,1990; Burke *et al.*,2000; Massie *et al.*, 2003 and Larkindale *et al.*, 2005).

Chickpea (*Cicer arietinum* L.) is one of the important cool season legumes of India and grows best at 18-25°C. With the potentially serious effects of radical global temperature change on agriculture, in the near future it is expected that by the later half of twenty-first century global warming would seriously jeopardize agriculture, forestry and other industries using the natural environment (Iba, 2002). However, although much research is being conducted to evaluate the effects of

global warming on plant growth and productivity, effects to search for specific and practical approaches to improve the adaptability of plants to their temperature environments have only recently begun (Grover *et al.*, 2000, Sharkey, 2000).

In the present study, therefore, attempts have been made to identify genotypes of *Cicer arietinum* L. showing temperature tolerance and to further induce tolerance through various treatments. At the onset, seeds of fifteen genotypes of *Cicer arietinum* were exposed to elevated temperatures upto 55°C and the effect of elevated temperatures on seed germination and seedling growth were evaluated. Germination of seeds was found to be retarded at 50°C and completely inhibited at 55°C which was considered as the lethal temperature for seed germination. Seedlings of most genotypes could not tolerate a maximum temperature of 46°C. Based on tolerance index (TI) of different genotypes, they could be roughly categorized into tolerant (ICC 4918, ICC 1852, ICC 2042, ICC C37, ICC V2 and ICC V10), moderately tolerant (ICC 6119, ICC 14340, ICC 5003 and ICC 4969) and susceptible (ICC 5319, ICC 10035, ICC 16359, ICC 7344 and ICC V1). Tolerance and susceptibility of the different genotypes to temperature stress was further confirmed by cell membrane stability (CMS) test. Similar findings in CMS test to that of tolerance index (TI) confirmed the genotypic variations with respect to temperature tolerance. In a study by Porch (2006), stress tolerance index (STI), stress susceptibility index (SSI) and geometric mean (GM) were used to evaluate the genotypic performance of 14 genotypes of common bean under variable temperature conditions. Their results also indicated that it was possible to identify superior genotypes for heat tolerance based on their indices. In their evaluation of heat tolerance indices, STI and GM although correlated were found to be effective stress indices for the selection of genotypes with good yield potential under stress and low stress conditions. Several previous authors have also confirmed the importance of using thermostability of cell membranes for screening heat tolerant genotypes (Sadalla *et al.*, 1990; Moffet *et al.*,1990 and Agarie *et al.*, 1995). Talwar *et al.* (2002) could group groundnut genotypes into two groups based on the relative injury of cell membranes caused by high temperature.

Chickpea seeds as well as seedlings were treated with known signaling molecules like salicylic acid (SA), calcium chloride ($CaCl_2$), abscisic acid (ABA)

and also to a sub-lethal temperature treatment prior to exposure to lethal temperature in order to induce thermotolerance. It was observed that SA and $CaCl_2$ pre-treatments enhanced the rate of germination while ABA initially inhibited germination to a certain level. Seedlings pre-treated with the chemicals or subjected to heat-acclimation treatment before exposure to lethal temperature showed more tolerance in comparison to the untreated seedlings directly exposed to lethal temperature. High degree of thermotolerance was induced by these treatments in the heat susceptible genotypes – ICC 5319, ICC 10035, ICC 7344 and ICC V1. Similar results have also been reported previously by workers who have shown that pre-treating plants with certain endogenous signaling compounds or pre-exposing plants to mild heat stress can induce thermotolerance (Dat *et al.*,1998a and b; Larkindale and Knight, 2002 and Larkindale and Huang, 2004). SA had been the focus of attention of researchers mainly because of its ability to induce protection against plant pathogens (Raskin, 1992; Katoch *et al.*, 2003). However it has now been shown that SA has an equally important role to play for induction of thermotolerance (Dat *et al.*,1998a and Larkindale and Knight, 2002). Larkindale and Huang (2004) showed that pre-treatment with SA, ABA, ACC, $CaCl_2$, and H_2O_2 improved the tolerance of creeping bentgrass to heat stress. The leaves of treated plants remained greener and shoot density was higher than untreated control plants during heat stress. The observed effects of chemical treatments were similar to the effects of acquired thermotolerance by pre-exposure of plants to mild heat stress. In the present study, also, it was observed that while exposure of seedlings to lethal temperature resulted in wilting and collapse of seedlings, pre-treatment with the chemicals or heat acclimation led to lesser wilting of the seedlings. Besides the other treatments in the present study, seeds or seedlings were also pre-treated separately with a bacterium *Bacillus megaterium* with known plant growth promoting activity. The idea behind the treatment was that since *Bacillus megaterium* increases plant growth and enhances vigour such plants may show more tolerance to temperature. Results of this treatment also showed that it was equally effective as SA or $CaCl_2$ in inducing thermotolerance. Only a few PGPR strains have been studied previously for their capacity to enhance plants' tolerance of environmental stresses. Plants with reduced ethylene level due to application of PGPR showing 1, amino cyclopropane, 1

carboxylic acid (ACC) deaminase activity revealed a substantial tolerance to flooding stress (Grichko and Glick, 2001) and metal contaminants (Nie *et al.*,2002). Hu and Kloepper (2003) reported that PGPR treatments increased tomato seedling survival rate and enhanced the shoot weight even under heat stress conditions.According to them the response of PGPR treated plants subjected to heat stress mimicked the classic heat-shock response.

One of the most important responses of plants to environmental stresses is in their protein metabolism. They respond to environmental stresses either by dis-assembly of pre-formed polysomes resulting in decrease in translation of mRNAs present at the time of induction and their preferential synthesis of stress proteins from newly transcribed stress mRNAs. Since chickpea is cultivated mostly for its proteins it was expected that temperature stress would affect different genotypes to some degree. The response of chickpea seeds and seedlings of various genotypes subjected to high temperature as well as various pre-treatments, with respect to the quantitative changes in protein as well as nature of proteins were analyzed in heat stress condition. Protein content of seeds varied with the genotypes. Protein contents of seeds and seedlings increased following moderate heat treatments but showed a rapid decline at lethal temperature in all genotypes. Protein degradation following prolonged heat treatment was maximum in susceptible genotypes like ICC V1, ICC 7344 and ICC 10035 while it was least in the tolerant genotypes (ICC 4918, ICC 1852 and ICC C-37). Induction of thermotolerance by pre-treatments with chemicals or PGPR treatment was accompanied by an increase in the protein content. New proteins, mostly in the small and intermediate molecular weight range were observed following heat-acclimation, SA, ABA and $CaCl_2$ treatments. The exposure to lethal temperature showed a considerable reduction in the number of protein bands and also revealed genotypic differences in the protein profile. Low molecular weight heat shock proteins have been shown to be exclusively expressed in plants in response to heat stress (Agarwal *et al.*, 2003) Apart from induction by heat stress, there are also reports that show induction of low molecular weight heat shock proteins (HSPs) by other stresses. Almoguera and Jordano (1992) noted expression of Hasp 17.4 transcript in seedlings exposed to ABA and other osmotic shock. Similar results have also been reported by other workers during water and other osmotic stresses (Coca *et*

al., 1996; Pla *et al.*, 1998 and Sun *et al.*, 2001). The positive correlation noted between the synthesis of HSPs and development of thermotolerance in time dependent and temperature dependent manner in several studies as well as in the present study suggest that accumulation of HSPs is an essential component of the protection process. Studies with *Arabidopsis* plants containing an antisense DNA sequence that reduces HSP 70 synthesis showed that the high temperature extreme at which the plants could survive was reduced by 2°C compared with controls although the mutant plants grew normally at optimum temperature (Lee and Schoffel,1996). Presumabably, failure to synthesize the entire range of HSPs that are usually induced in the plants would lead to a much more dramatic loss of thermotolerance. Other studies with both *Arabidopsis* mutants (Hong and Vierling, 2000) and transgenic plants (Queitsch *et al.*,2000) demonstrate that at least HSP 101 is a critical component of both induced and constitutive thermotolerance in plants. The accumulation of 104 kDa protein in rice in response to several abiotic stresses including high temperature was reported by Singla *et al.* (1998). In the present study, however more of low molecular weight proteins were induced during thermotolerance.

Heat stress is known to have a complex impact on cell function indicating that many processes are involved in thermotolerance. Some processes may be specific to basal thermotolerance, others may be induced during acquired thermotolerance and many may be involved in both (Larkindale *et al.*, 2005).Though the best characterized aspect of thermotolerance is the production of heat shock proteins, several lines of evidence indicate that HSP synthesis is only one aspect of protection against heat induced damage.High temperatures are known to affect membrane linked processes due to alteration in membrane fluidity and permeability (Alfonso *et al.*,2001; Sangwan *et al.*,2002). Enzyme function is also sensitive to changes in temperature. Heat induced alterations in enzyme activity can lead to imbalance in metabolic pathways or heat can cause complete enzyme inactivation due to protein denaturation (Vierling, 1991; Kampinga *et al.*,1995). Membrane and protein damage lead to the production of active oxygen species (AOS) that cause heat induced oxidative stress (Dat *et al.*, 1998 a, 1998b; Gong *et al.*, 1998a,b Larkindale and Knight, 2002; Suzuki and Mittler, 2006). These different types of

damage translate into reduced photosynthesis, impaired translocation of assimilates and reduced carbon gain leading to altered growth and production (Hall, 2001).

Heat stress induced oxidative damage and scavenging by antioxidative enzymes and non enzymatic antioxidants were evaluated in chickpea genotypes in the present study. Activities of antioxidative enzymes like ascorbate peroxidase (APOX), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidae (POX) were analyzed both during temperature stress and induction of thermotlerance by various treatments. It was observed that APOX activities of seedlings decreased on exposure to high lethal temperature but mild temperature treatment prior to lethal temperature resulted in increased activity in most of the genotypes. More significant increases were obtained by SA and $CaCl_2$ pre-treatments. Maximum activity was seen in ICC 4918 following $CaCl_2$ pre-treatment. APOX is one of the most important antioxidative enzymes of plants that detoxify H_2O_2 using ascorbate for reduction. 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 signaling (Srivalli *et al.*, 2003). APOX gene expression and activity has been reported to be rapidly induced by various stress conditions including chilling (Prasad *et al.*,1994), drought (Mittler and Zilinskas, 1994) and salt stress (Lopez *et al.*, 1996). Larkindale and Huang (2004) reported that in bentgrass APOX activity increased over the first 2 days and 5 days of heating for ACC and $CaCl_2$ respectively but only 12 hrs for H_2O_2 pre-treatment. SA and ABA after pre-treatments had no effect on APOX activity earlier but maintained activity at a significantly higher than in controls after 24 hrs of heating. Jiang and Zhang (2001) also obtained increased activities of APOX in leaves of maize seedlings following ABA treatment. Panchuk *et al.* (2002) reported that heat stress triggers the expression of APX2 gene at the mRNA level and this correlated with the appearance of a new APOX isozyme in *Arabidopsis*. It was shown previously that mRNA levels of pea and *Arabidopsis* APX 1 gene were induced by heat stress and oxidative stress and there was evidence that in *Arabidopsis* heat induction of APX 1 requires an HSE sequence present in the promoter upstream region of APX 1 (Storozhenko *et al.*, 1998).

Peroxidase activity in chickpea seedlings was found to vary among the different genotypes. Genotypes tolerant to temperature stress were found to have higher constitutive activity than the susceptible ones. Exposure to high temperature increased activities in the tolerant genotypes while it decreased in the susceptible ones. All pre-treatments followed by lethal temperature led to an increase in activity in all genotypes. Maximum activity was obtained in SA pre-treatments. Seed treatment with SA was also found to enhance POX activity in seeds and roots of chickpea (Keshamma *et al.*, 2004). In a study with wheat genotypes Gupta and Gupta (2005) reported that exposure to high temperature increased POX activity which was higher in the tolerant genotype C-306. Peroxidases are often the first enzymes to alter their activities under stress. Enhanced activities have been observed in rice seedlings under anoxia (Lee and Lin, 1995) and low temperature stress (Oidaira *et al.*, 2000). Chakraborty *et al.*(2002) also obtained increased POX activity following water stress in tea plants. In mulberry, increase in salinity was found to induce higher activity of POX (Harinasut *et al.*, 2003). In the present study it was observed that pre-treatments not only enhanced activities but also induced two new isozymes of the enzyme. Thus, POX would seem to be generally involved with the plants' response to various types of environmental stresses. Though in the present study, increased POX activity was obtained following induction of thermotolerance, this was not in conformity with the results of Larkindale and Huang (2004) in creeping bent grass. They found that SA and ABA pre-treatments had no effects on POX activity, ACC treatment significantly increased activity while $CaCl_2$, H_2O_2 and heat acclimation reduced activity particularly during later phase of heating.

Catalases are tetrameric heme containing enzymes that catalyze the breakdown of H_2O_2 to H_2O and O_2. Catalase is indispensable for ROS detoxification during stress (Willekens, 1995). This is also due to the fact that there is proliferation of peroxisomes during stress which might help in scavenging of H_2O_2 diffusing form the cytosol (Lopez Hupertez, 2000). However, reports on effects of stresses on CAT activities vary. Increased, decreased or unchanged CAT activities under drought stress have been observed (Smirnoff, 1993; Zhang and Kirkham,1994; Castillo,1996). Jiang and Huang (2001) showed that CAT activities declined under drought, heat and a combination of the two stresses. Results of the present study also

revealed that CAT activity decreased during high temperature stress in all genotypes. On the other hand, CAT activity also decreased during induction of thermotolerance by pre-treatments. Isozyme analysis of catalse also did not reveal any induction of new isozymes. Dat *et al.* (1998b) 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 molecualar 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. CAT activity reached a minimum during the thermoprotection period but the reason for this still remains unknown. It would seem possible that the changes in CAT activity may vary according to intensity of stress, time of assay and induction of new isozymes. The dual role of H_2O_2 as a signaling molecule as well as the toxic metabolite could make it a very variable enzyme. H_2O_2 production being an ongoing process in plants, inhibition of CAT activity-one of the main routes of H_2O_2 degradation could result in H_2O_2 accumulation which would then activate defense related genes by acting as a second messenger (Keshamma *et al.*, 2004). The observed decrease in CAT activity during induction of thermotolerance in the present study may also be due to the above mechanism of accumulation of H_2O_2 during initial stages. Larkindale and Huang (2004) also obtained lower CAT activities in *Agrostis stolonifera* plants treatd with SA, $CaCl_2$, H_2O_2 and HA over the control plants prior to heating and within 48 hrs of heat stress. ABA and ACC pre-treatments maintained higher CAT activity than controls after 48 hrs of heating.Increased activity following ABA treatments have also been reported by Jiang and Zhang (2001) in maize seedlings.

Superoxide dismutases (SOD) are very important reactive oxygen species (ROS) scavenging enzymes and they catalyze the dismutation of O_2^- into H_2O_2. It has been reported that under stress conditions different plants and tissues respond differently with regard to SOD induction suggesting that different mechanisms may be involved in protection against oxidative stress (Blokhina *et al.*, 2003). The reduced activity of SOD leading to accumulation of singlet oxygen has been shown in flooding stress in maize (Yan *et al.*, 1996). While no significant differences in

SOD activity in two cultivars of rice differing in sensitivity to chilling was observed (Saruyama and Tanida, 1995), other studies have reported increase in SOD activity in tolerant cultivars compared to susceptible ones (Hernandez *et al.*, 1993). Similar results were also reported by Pal *et al.* (2004) who reported that though SOD activity increased under salt stress in all three tested genotypes increase in the tolerant genotype was higher. In the present study, while the exposure of chickpea seedlings to lethal temperature decreased SOD activity, induction of thermotolerance by various pre-treatments enhanced activity over control. Maximum enhancement was obtained due to $CaCl_2$ and SA pre-treatments while heat-acclimation did not significantly increase activity. It was also observed that increase was higher in the tolerant cultivars. It would seem that the increased activity of SOD might contribute to the protection of plants from oxidative injury during induction of thermotolerance. SOD was also reported to be enhanced continuously with increase in temperature in two wheat genotypes though the magnitude was comparatively lower in the susceptible genotypes (Gupta and Gupta, 2005). Authors suggested that the comparatively higher increment of SOD activity in tolerant genotypes might have decreased the possible toxic concentration of O_2^- radical more effectively. Mazorra *et al.* (2002) also reported a role of SOD activity in imparting temperature stress tolerance to tomato. Larkindale and Huang (2004) however, 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.

Glutathione reductase (GR) activity was found to be enhanced in pre-treated seedlings while a decrease in activity was observed during high temperature exposure. However, the decrease in activity of susceptible genotypes was greater. SA, ABA and $CaCl_2$ pre-treatments were most effective in enhancing activities. Jiang and Zhang (2001) reported increased activities of GR in maize seedlings following ABA treatment. Jiang and Huang (2001) obtained decreased activity of GR under heat stress, which would have resulted in H_2O_2 accumulation. Since GR catalyzes the NADPH dependent reaction of the disulphide bond of GS-SG and is responsible for maintaining the reduced pool of glutathione, increased GR activity would facilitate improved stress tolerance (Tyystjärvi *et al.*, 1999). Increase in GR

activity has been reported during water stress in various plants (Pastori and Trippi, 1993; Jiang and Huang 2001) and has also been correlated with increased resistance to paraquat exposure (Broadbent *et al.*, 1995). Increase in the activity has been linked with the increase in synthesis of proteins (Edwards *et al.*, 1994). Over expression of GR in chloroplast has been reported to confer increased antioxidant protection to cold induced photo inhibition (Foyer *et al.*, 1995).

Besides antioxidative enzymes a few non-enzymatic antioxidants are also known to be involved in scavenging of ROS of which ascorbate and carotenoids are most well known. Ascorbate is one of the most extensively studied antioxidants and has been detected in majority of plant cell type organelles and apoplast. Carotenoids also function as antioxidants in several cases. The present study indicated that both ascorbate and carotenoids were reduced following exposure to high lethal temperature, which was greater in the susceptible genotypes. However, all pre-treatments increased both of the antioxidants to a certain degree. ABA did not induce any significant changes in the content of antioxidants in the leaves of maize seedlings within first 12 hrs of treatment (Jiang and Zhang, 2001). Reduction in ascorbate content in response to drought was reported in sorghum (Zhang and Kirkham, 1996) and wheat leaves (Bartoli *et al.*,1999). Agarwal and Pandey (2003) on the other hand obtained increased ascorbate content in *Cassia* seedlings subjected to water stress which were able to adapt themselves to the stress. Mahan *et al.* (2006) also showed that oxidative damage resulting from temperature extremes in cotton seedlings (*Gossypium hirsutum* L.) cultivar Fibermax 958 could be by mitigated by minor alterations in the antioxidant metabolism.

The results of the present study and those of other studies point to the role of ROS scavenging antioxidant mechanism in inducing tolerance to stresses including temperature stress. It is interesting to note that while ROS have the potential to cause oxidative damage to cells during environmental stress, they may also play a key role in plants as signal transduction molecules involved in mediating responses to pathogen infection, environmental stresses, programmed cell death and developmental stimuli (Mittler *et al.*,2004; Torres and Dangl, 2005; Suzuki and Mittler,2006). The rapid increase in ROS production referred to as 'oxidative' burst was shown to be essential for many of these processes and genetic studies have

shown that respiratory burst oxidases homologue (Rboh) genes encoding NADPH oxidases are the main producers of signal transduction associated ROS in cells during these processes. Thus the two somewhat opposing faces of ROS i.e., the damaging toxic molecule on one hand and beneficial signal transduction molecule on the other might explain the somewhat confusing results obtained in some cases in relation to the detoxifying antioxidant systems.

Oxidative stress which induces the production of free radicals can result in lipid peroxidation causing membrane damage. Larkindale and Knight (2002) found that treatment of *Arabidopsis* at 40°C caused a significant increase in lipid peroxidation after 2 days which increased even further after 3 days. In contrast, seedlings treated in the same way but subjected to a prior treatment at 30°C for 1hr showed no significant increase in lipid peroxidation over three days. Results of the present study indicated that lethal temperature led to an increase in lipid peroxidation as measured by malondialdehyde (MDA) content in all genotypes. This effect was partially overcome by the pre-treatments 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 bentgrass where lipid peroxidation increased during heat stress but was lowered by pre-treatments with chemicals and sub-lethal temperature. Drought and heat stress have also been reported to increase MDA content in tall Fescue and Kentucky blue grass similar to what has been found in other species (Rensburg and Kruber 1994; Behl *et al.* 1996; Gong *et al.*, 1997; Liu and Huang, 2000; Jiang and Zhang, 2001).The increase in MDA content according to Jiang and Huang (2004) may be related to reduction in SOD, CAT, APOX and GR activities.

Disruption of membrane stability was found to be a major result of imposition of temperature stress in chickpea seedlings. Relative injury to the membranes was much lesser in the tolerant genotypes than in the susceptible ones. Similarly, induction of thermotolerance by pre-treatments also decreased the injury to membranes. Higher thermostability 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 are most sensitive to high temperature

stress (Yang *et al.*, 2005). Inhibition of photosynthesis by high temperature stress is of common occurence for plants in tropical and sub-tropical regions and the temperate zones where the plants are exposed periodically to high temperatures (Larcher, 1995). Hence the effect of temperature stress on chlorophyll contents and Hill activity were also determined in the present study, as these would indicate disruptions in photosynthesis. Both chlorophyll contents and Hill activity decreased due to lethal temperature treatment. Pre-treatments reversed the effects to a certain degree and among all treatments SA and $CaCl_2$ were most effective. Since Hill activity was affected it would seem that PS II was inhibited. Previous studies also report that photosystem II is inhibited by severe heat stress with temperatures higher than 45°C (Havaux, 1993, 1996; Sharkey, 2000). 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.

Studies were also conducted on the effect of temperature stress on other important biochemical components i.e. proline, carbohydrates and phenols. Proline showed an increase in accumulation in all treatments including lethal temperature treatment. Proline being a stress metabolite is known to increase under various stresses (Kramarova *et al.*, 1999; Chakraborty *et al.*, 2002; Agarwal and Pandey, 2003). Total sugar contents of seedlings were found to decrease during the lethal temperature treatment, which was most significant in the susceptible genotypes. Pre-treatments could increase the contents to a certain degree. Similar trend was also observed in case of reducing sugars. Xu and Huang (2001) suggested that roots play more important role than shoots in the mediation of carbohydrate responses to high air temperatures or high soil temperatures. High soil temperature alone or combined with high air temperature causes imbalance between photosynthesis and respiration and decrease in carbohydrate availability, which could contribute to the decline in shoot and root growth under high temperature conditions. Analyses of phenols which are known in some cases to offer protection to various stresses were also carried out. Few of the phenolic acids commonly present were detected by HPLC analysis of

which Ferulic and Chlorogenic acids were recorded in tolerant genotypes subjected to SA and PGPR treatments. Sarma *et al.*, (2002) reported that PGPR could elicit alterations in phenolic profiles of chickpea subjected to biotic stress. Chakraborty *et al.* (2001) obtained increased levels of phenols in tea leaves subjected to temperature stress upto 45°C, and a decline thereafter.

Finally, induction of thermotolerance obtained in seedlings was confirmed in callus cultures. It was observed that temperatures of 36-40°C were lethal to callus formation but a prior acclimatization at 32°C for 2 hrs made the calli more tolerant to the lethal temperature. Similarly, supplementation of media with SA(10^{-5} and 10^{-6}M) also conferred tolerance for growth of calli. Lopez-Delgado (1998) also reported that low concentration of acetyl SA in culture medium improved tolerance of a 5 week high temperature (35°C) treatment.

In conclusion, it might be stated that exposure of chickpea seedlings to a lethal temperature of 46°C resulted in an overall change in several metabolic pathways. Temperature stress was observed to cause an oxidative stress that was however overcome by pre-treatment with SA, ABA, $CaCl_2$ as well as HA which could induce thermotolerance to the seedlings by exposure to a sub-lethal temperature. Among all treatments, SA and $CaCl_2$ were the most effective. This is not surprising since both SA and Ca^{2+} play important roles as secondary messengers. These might act in some signaling pathways limiting heat induced oxidative damage. It is clear that many protective pathways contribute to survival of plants at higher temperatures and probably the relative importance of the different pathways change throughout plant development. Depending on the stress applied and age of the plant it appears that different aspects of heat induced damage impact plant survival and different types of heat induced damage prevent or repair damage through different cellular systems. Probably signaling pathway may contribute to basal and acquired thermotolerance (Larkindale *et al.*,2005). Chen *et al.* (2006) revealed an important role of galactolipids in thermotolerance and suggested that the digalactosyldiacylglycerol (DGDG) level and/or the ratio of DGDG to monogalactosyldiacylglycerol (MGDG) may play an important role in basal as well as acquired thermotolerance in *Arabidopsis*. Signalling pathways involving SA and AOS are critical for events during both basal and acquired thermotolerance (Liu *et*

al.,2006). Overall results of the present study also indicate the possibility of selecting a few biochemical markers for temperature tolerance in chickpea. High constitutive POX activity, high membrane stability and high tolerance index was evident in all tolerant genotypes which were also enhanced during induced thermotolerance. This could therefore, be used as biochemical index for screening thermotolerant genotypes.

SUMMARY

1. A brief review of literature pertaining to the line of investigation has been presented which mainly deals with biochemical responses of plants to elevated temperature stress and metabolic changes associated with induction of thermotolerance.

2. Materials and methods used in this investigation and experimental procedure followed have been discussed in detail.

3. Different genotypes of chickpea (*Cicer arietinum* L.) were screened for thermotolerance by performing cell membrane stability (CMS) test and testing their tolerance index (TI). The test revealed a distinct genotypic variation for heat tolerance amongst the fifteen genotypes tested.

4. Seed germination percentage of different genotypes was tested following elevated temperature treatments ranging from 35-55°C for 2 hrs duration. Germination percentage was also recorded in case of seeds imbibed in 100 µM/L of SA, 50 µM/L of ABA, 100 mM $CaCl_2$ solution overnight and seeds bacterized with Plant Growth Promoting Rhizobacteria (PGPR)-*Bacillus megaterium* following exposure to lethal temperature of 55°C for the same duration.

5. Some of the important biochemical parameters such as changes in enzymes related to defense and antioxidative stress response like peroxidase (POX), ascorbate peroxidase (APOX), superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR), variations in non-enzymatic antioxidants like ascorbate and carotenoids, changes in stress amino acid proline, sugar content, chlorophyll pigment content, Hill activity and quantitative and qualitative alteration of proteins were tested in seedlings following lethal temperature treatment of 46°C for 2 hrs duration.

6. Peroxidation of cell membranes due to heat stress injury and changes in phenolic profile of seedlings subjected to lethal temperature were also analyzed separately by spectrophotometric and High Performance Liquid Chromatography (HPLC) analysis respectively.

7. Desi types of chickpea with dark coloured seed coats were found to be comparatively more tolerant to to heat stress than the kabuli types. However, kabuli types were found to contain more protein per mg fresh weight.

8. Germination of seeds following elevated temperature treatments revealed decrease in germination percentage with increase in temperature. At a lethal temperature of 55°C only 10% germination occurred in thermotolerant genotypes while no germination occurred in susceptible genotypes.

9. Salicylic acid (SA) and $CaCl_2$ pre-treatments and bacterization of seeds with *Bacillus megaterium* enhanced the rate of germination in most genotypes. Abscisic acid (ABA) on the other hand exhibited inhibitory effects on germination in early stages which declined with the onset of germination.

10. Quantification of protein contents of different plant parts revealed maximum protein content in seeds followed by leaves, stem and roots.

11. Protein contents of both seeds and seedlings increased gradually following moderate heat stress but showed a rapid decline at lethal temperature in all genotypes.

12. SDS-PAGE analysis of seed proteins of various genotypes subjected to pre-treatments followed by lethal temperature revealed the appearance of new proteins having molecular masses of 18, 25, 26 and 82 kDa (HA); 13.3,18, 20, 25, 26, 26.5, 44, 82, 84 and 96 kDa (SA) and 18, 25, 26, 27.5, 82 and 96 kDa in ABA pre-treatments. Pre-treated seedlings exposed to lethal temperature also revealed the expression of some new proteins. Low molecular masses proteins of 15.6 and 17.3 k Da (approx.) and other proteins having molecular masses of 21.2, 22.3, 25.1, 39.8, 42.1, 44.6, 55, 66 and 70.7 k Da. (approx.) were observed in SA pre-treated seedlings challenged with lethal temperature. ABA and $CaCl_2$ pre-treatmnents also led to the expression of new proteins of molecular masses 10.6, 21.1, 22.3, 29.4, 39.8, 45.3 and 55 kDa and 11.2, 22.3, 33.5, 35.4, 39.8, 44.6, 45.3, 55 and 66 kDa respectively. Exposure to lethal temperature directly without any pre-treatments led to loss of some protein bands.

13. Changes in activities of antioxidative enzymes like POX, APOX, SOD and GR in pre-treated seedlings revealed that enzymatic activities gradually increase with increase in temperature and reach peak activity after which the activity declines sharply recording the lowest activity at lethal temperature. Quantum of increase in enzymatic activity was however highest in thermotolerant and lowest in heat susceptible genotypes. CAT activity, in contrast, showed a remarkable decline in its activity in pre-treated seedlings following exposure to lethal temperature.

14. POX isozyme analysis by native PAGE revealed the presence of five isozyme bands having R_m values of 0.166, 0.300, 0.336, 0.500 and 0.566 in control and pre-treated seedlings subjected to elevated temperatures (35-45°C) for 2 hrs SA, $CaCl_2$ pre-treatments and seed bacterization led to the induction of new isozymes of R_m values 0.083 and 0.316. Intense isozyme bands having R_m 0.166 and 0.300 were recorded only in thermotolerant genotypes following lethal temperature treatment.

15. CAT isozyme analysis, in contrast, revealed no induction of new isoforms in pre-treated seedlings challenged with a lethal temperature. Both control and pre-treated seedlings revealed the presence of only a single isozyme band of R_m value 0.466.

16. Cell Membrane Stability (CMS) test revealed 45% (C_a) and 83% (C_a) relative injury respectively in thermotolerant and susceptible genotypes subjected to lethal temperature treatment. Pre-treatments of seedlings however led to a remarkable reduction in membrane injury.

17. Pre-treatment of seedlings before exposure to lethal temperature treatment also led to a considerable reduction in peroxidation of cell membranes in all genotypes.

18. High accumulation of free proline and significant increase in sugar contents were recorded in pre-treated seedlings in comparison to untreated control samples.

19. Analysis of photosynthetic pigments (total chlorophyll, chlorophyll a and b) and Hill activity showed a high degree of thermosensitivity of chlorophyll molecules and a rapid decline in Hill activity following lethal temperature treatment. Pre-treatment of seedlings however showed a reasonable reduction in the impact of heat stress.

20. Biochemical analysis of non-enzymatic antioxidants like asorbate and carotenoid contents also revealed similar increasing trend in pre-treated seedlings followed by a drastic decline at lethal temperature.

21. HPLC analysis of pre-treated and control seedlings showed the presence of five phenolic acids viz. ferulic, chlorogenic, salicylic, cinnamic and gallic acids. Among the five phenolics, gallic acid was found to be present in all treatments including controls while chlorogenic acid was found to be completely absent in untreated samples.

22. Calli raised from various explants (shoot tip with a portion of leaf, cotyledonory node, hypocotyls and internode) in three differently amended MS medium supplemented with different concentrations and combinations of growth regulators (NAA+BAP, NAA+Kinetin and IAA+BAP) when subjected to elevated temperatures (30-40°C) showed rapid browning and decline in growth rate in both thermotolerant and susceptible genotypes. Calli raised in MS media supplemented with 10^{-5} and 10^{-6} M of SA and heat acclimatization (32°C- 2 hrs) however showed a much higher level of thermotolerance and comparatively much lower reduction in growth rate and browning of tissues.

23. Foliar spray treatments and seed bacterization with *Bacillus megaterium* offered a certain degree of thermoprotection. Thermoprotection induced were found to be concentration dependent up to a certain level. Very high concentration of foliar spray led to scorching of leaves and induction of oxidative stress.

24. Thermopotection provided by various pre-treatments may be due to coordinated action of antioxidative enzymes like POX, APOX, CAT, SOD and GR and non- enymatic antioxidants like ascorbate and carotenoids.

25. The high antioxidative enzymatic activity, low relative injury and peroxidation of membranes, lower sensitivity of pigment molecules and increased accumulation of non-enzymatic antioxidants like ascorbate and carotenoids could be directly linked with enhanced tolerance to heat induced oxidative damage. This could therefore, be used as biochemical markers for screening thermotolerant genotypes of chickpea.

26. Results of the investigation have been properly analyzed and their implications have been thoroughly discussed.

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