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

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ABSTRACT

The effect of high temperature stress was studied in three varieties of lentil (*Lens culinaris* Medik.) which were subjected to high temperature ranging from 30^0C to 50^0C. Growth of plants and germination of seeds were found to be retarded at 45^0C and significantly inhibited at 50^0C in which was considered as near lethal temperature. One month old plants of *Lens culinaris* could not tolerate a maximum temperature of 50^0C. Based on tolerance index (TI) and antioxidant activities among the three varieties IPL 81 and IPL 406 were found to be more tolerant than Sehore. Tolerance and susceptibility of varieties were further confirmed by cell membrane stability test. Lipid peroxidation, as determined by accumulation of malonealdehyde, was found to be highest in Sehore. Non-enzymatic antioxidants like ascorbic acid and carotenoid initially increased and then declined in IPL 81 and IPL 406 but abruptly declined in Sehore. Hydrogen peroxide content showed an increase with the increase of temperature in Sehore, but in IPL 81 and IPL 406, H_2O_2 initially declined and then increased. Chlorophyll content also increased initially in 2 of the varieties before declining but in Sehore it decreased at all high temperatures. Phenol contents increased initially but decreased at higher temperatures. Proline content also initially increased and then declined. All results taken together indicate that Sehore is the most temperature susceptible variety, while IPL 81 and IPL 406 are more tolerant.

Keywords: Lentil, High temperature, antioxidants, tolerance

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INTRODUCTION

Among the abiotic stresses such as drought, high and low temperature, salinity etc. the most disturbing climatic change in the earth during the past few decades has been the increase in atmospheric temperature due to global warming. Elevated temperature stress is one of the major factors limiting the growth of plants. Plant can be damaged in different ways either by high day or high night temperature or by either high soil or high air temperature. In nature, however, plants may be exposed to mild stresses before they face severe intensity of stresses and plants may be exposed to multiple environmental stresses either sequentially or simultaneously [1].

Legumes such as lentils (*Lens culinaris* Medik.) are rich source of protein so they occupy an important position in human diet. Lentils are cool season annuals and are more sensitive to hot seasons. Temperature stress is a principal cause for yield reduction in crops [2] and ROS (Reactive oxygen species) generated by these stresses have been shown to injure cell membranes. During the time of temperature stress reactive oxygen species level can increase dramatically which can result in significant damage to cell structure. This leads to oxidative stress. Prolonged accumulation of ROS is very harmful and can cause inactivation of enzymes, lipid peroxidation, protein degradation and damage to DNA [3].Temperature stress leads to an increase in lipid peroxidation and consequent membrane damage due to the dysfunctions of antioxidative enzymes [4-6]. Heat stress induces significant changes in normal physiological processes such as photosynthesis dark respiration, membrane stability and mitochondrial respiration [7]. One mechanism of injury involves the generation and reactions of reactive oxygen species (ROS) [8]. In order to limit oxidative damage under stress condition plants have developed a series of detoxification systems that break down the highly toxic ROS [9].

The present investigation was undertaken to determine the effect of high temperatures on membrane stability, antioxidants, chlorophylls and other biochemical components involved in stress responses of different lentil varieties and to determine the tolerance of these varieties to temperature stress.

MATERIALS AND METHODS

Plant material and induction of cold stress

The seeds of three different varieties of *Lens culinaris* Medik. (IPL 81, IPL 406 and Sehore) were obtained from the Indian Institute of Pulses Research, Kanpur and Oil and Pulses Research centre, Behrampur, West Bengal. Viability was checked in laboratory and seedlings of the different varieties were then raised from this stock of seeds. Seeds were soaked overnight in distilled water after surface sterilization with 0.1% $HgCl_2$ and grown in petridishes. For experimental purposes small seedlings were transferred to pots containing sandy loam soil mixed with farmyard manure. Plants were watered regularly and maintained properly. One month old seedlings were exposed to the different elevated temperatures for 4 hours in plant growth chamber. Immediately after temperature treatment, sampling was done for various analyses.

Determination of tolerance index (TI) of seedlings

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

$$TI\ (\%)=(FW_t / FW_c\ X100) - 100$$

Determination of cell membrane thermostability

Membrane thermostability was tested by cell membrane stability (CMS) test with the pinnules obtained from seedlings following the method of Martineau et al. [10]. The injury was determined by following the equation

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

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

Determination of lipid peroxidation

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa et al [11]. Leaf tissue was homogenized in 2ml of 0.1% (w/v) tricholoroacetic acid (TCA) and centrifuged. 0.5ml of supernatant was mixed with 2ml of 20% trichloroacetic acid containing 0.5% of (v/v) thiobarbituric acid. The mixture was heated at 95^0C for 30min, quickly cooled and centrifuged at 10000 rpm for 10min. The absorbance of the supernatant was read at 532nm and 600nm. The concentration of MDA was calculated by means of an extinction coefficient of $155mM^{-1}cm^{-1}$ [12].

Extraction and estimation of Non- enzymatic antioxidants

Ascorbate

Ascorbate was extracted and estimated by following the method of Mukherjee and Choudhuri [13]. Leaves were homogenised in a cold mortar and pestle on ice using 10ml of 6% tricholoacetic acid and filtered. To 4ml of the extract, 2ml of 2% dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% thiourea (in 70% ethanol) were added. The mixture was kept in boiling water bath for 15 min and cooled at room temperature. 5ml of 80% (v/v) sulphuric acid was added to the mixture at 0^0C. The absorbance at 530nm was recorded. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler [14]. Extraction was done in methanol and the extract was filtered.

Absorbance of the filtrate was noted at 480nm, 663nm and 645nm in a VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

Carotenoid content = A_{480}-(0.114xA_{663})-0.638(A_{645}) µg g^{-1}fresh weight.

Determination of hydrogen peroxide

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

Extraction and estimation of Chlorophyll

Chlorophyll was extracted according to the method of Harbone [16] in 80% acetone and filtering through Whatman No. 1 filter paper 80% acetone. The filtrate was collected and the total volume was made up to 10ml. The chlorophyll content was estimated by observing the O.D. values at 645nm and 663nm respectively in a UV-VIS spectrophotometer (UV-VIS SPERCTROPHOTOMETER 118 SYSTRONICS) and calculated by using the following formula - TC = (20.2 A_{645} + 8.02 A_{663}) mg /g fresh weight.

Extraction and Estimation of Phenol

Phenol was extracted by following the method of Mahadeven and Sridhar [17] by immersing leaf tissue in boiling absolute alcohol and extracted in 80% alcohol in dark and filtered in dark chamber. The residues are re-extracted with 80% alcohol and then the final volume was made up with 80% alcohol to 10ml. Total phenol was estimated by following the method of Bray and Thorpe [18] by using 50% Folin ciocalteau and 20% Na_2CO_3 and absorbance was measured at 650nm in colorimeter.

Extraction and estimation of Proline

Proline was extracted and estimated following the method of Bates et al [19] where extraction was done in 3% sulfosalicylic acid and filtrate was used as a crude extract. For estimation Ninhyrin was used and absorbance was measured at 520nm.

RESULTS

One month old seedlings of different varieties of lentil were exposed to elevated temperatures ranging from control (20^0C), 30˚C, 35˚C, 40˚C, 45˚C, 50˚C for 4h and responses of the three varieties were determined.

Exposure of seeds of three varieties of lentil to different temperatures for 4 h revealed that nearly 100% germination occurred in control to 35˚C in IPL 406 and IPL 81 but Sehore showed little less percentage of germination. Germination percentage decreased with increase in temperature to about 5 (in Sehore) at 50 ˚C, hence this temperature was considered as near lethal temperature (Table 1). In the other 2 varieties, though germination was about 30% at high temperature, survival percent was much lower. Further, tolerance index was calculated by exposing one month old seedlings to lethal temperature i.e 50˚C and results revealed that Sehore had lower tolerance index values than the other 2, indicating that it was less tolerant to high temperature than IPL 81 and IPL 406.

Cell membrane stability of three varieties of one month old lentil at the elevated temperature treatments was evaluated by calculating the percentage of relative injury (RI) of the membrane. RI increased with an increase in temperature in all 3varieties, but was much high (86 %) in Sehore compared to IPL 81 and IPL 406 (66%) indicating its susceptibility (Table 2). Besides, degree of lipid peroxidation was determined and it was revealed that high temperatures induced an increase in lipid peroxidation which was higher in Sehore in comparison to the IPL 81 and IPL 406 (Fig.1).

Ascorbic acid content and carotenoids increased initially at 30^0C and 35^0C respectively in IPL 81 and IPL 406 before declining. However in Sehore ascorbic acid contents and carotenoids decreased from 30^0C itself (Table 3 and Fig.2).

Accumulation of H_2O_2 showed an interesting pattern. In IPL 81 and 406, hydrogen peroxide initially declined till 35^0C, after which there was an increase. However, in Sehore, H_2O_2 increased at all high temperatures (Fig.3).

Chlorophyll content decreased with increase in temperature in Sehore, while in the other 2 varieties there was an increase initially before it declined (Fig.4). Quantification of phenolics revealed an initial increase till 40-45^0 after which there was a decline (Fig.5). Proline content of leaves and roots increased initially in all three varieties but at higher temperatures there a decline (Table 4).

DISCUSSION

Plants which are exposed to various environmental stresses throughout the course of their life span have an inbuilt ability to adjust to seasonal and other environmental variables. Such ability to withstand high temperatures results from both prevention of heat damage and repair of heat-sensitive components [20]. The present study was undertaken to determine the influence of high temperatures on three varieties of *Lens culinaris*. Based on germination, seedling survival, tolerance index and cell membrane stability test, one of the varieties-Sehore was identified as most temperature sensitive whereas IPL 81 and IPL 406 were more tolerant. Several previous authors have also confirmed that it is possible to use stress tolerance index and/or cell membrane stability for screening heat tolerant genotypes [21-23].

High temperature generally leads to oxidative stress and induces the production of free radicals. This is turn causes lipid peroxidation of membranes which is one of the first

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Table 1. Germination percentage of lentil seeds at different temperatures

Varieties	% Germination					
	20˚C	30˚C	35˚C	40˚C	45˚C	50˚C
IPL 406	98±3.5	95±2.5	95±4.5	66±2.3	40±1.5	30±1.5
Sehore	90±3.1	80±3.8	60±1.3	50±3.3	25±1.8	05±0.5
IPL 81	97±4.5	91±3.2	80±2.5	77±2.2	50±2.5	35±1.1

Average of 3 replicate sets. ± = S.E.

Table 2. Effect of elevated temperature stress on cell membrane stability of soybean (expressed as % Relative injury)

Varieties	Percent Relative Injury					
	20˚C	30˚C	35˚C	40˚C	45˚C	50˚C
IPL 406	20.4±1.5	32.3±1.4	34.3±1.8	41.2±2.3	55.2±3.2	69.1±2.5
Sehore	36.8±2.8	43.7±1.1	55.7±2.6	61.1±2.7	72.0±2.8	86.2±1.9
IPL 81	34.2±2.9	36.8±2.3	40.6±1.7	48.1±1.9	52.6±2.6	68.0±3.2

Average of 3 replicate sets. ± = S.E.

Table 3 . Ascorbic acid content in soybean seedlings subjected to elevated temperature treatments Average of 3 replicate sets. ± = S.E.

Varieties	Ascorbic acid content (mg/g tissue)					
	20˚C	30˚C	35˚C	40˚C	45˚C	50˚C
IPL 406	4.5±0.51	5.2±0.70	3.6±0.09	3.4±0.08	0.7±0.09	0.6±0.05
Sehore	3.4±0.08	3.3±0.31	3.2±0.10	3.0±0.06	0.5±0.02	0.1±0.06
IPL 81	4.9±0.06	5.7±0.22	4.9±0.13	1.8±0.05	0.9±0.04	0.5±0.03

Table 4. Proline content in soybean seedlings under temperature stress

Varieties	Proline content (mg/g tissue)											
	Leaf						Root					
	20˚C	30˚C	35˚C	40˚C	45˚C	50˚C	20˚C	30˚C	35˚C	40˚C	45˚C	50˚C
IPL 406	0.20± 0.05	0.63± 0.04	0.73± 0.07	0.45± 0.04	0.34± 0.03	0.33± 0.04	0.20± 0.01	0.26± 0.01	0.38± 0.02	0.28± 0.02	0.27± 0.02	0.25± 0.01
Sehore	0.10± 0.03	0.35± 0.02	0.25± 0.01	0.25± 0.05	0.25± 0.01	0.19± 0.01	0.12± 0.01	0.33± 0.03	0.32± 0.01	0.23± 0.01	0.13± 0.01	0.06± 0.005
IPL 81	0.16± 0.04	0.16± 0.06	0.23± 0.03	0.29± 0.02	0.58± 0.06	0.46± 0.02	0.16± 0.02	0.13± 0.01	0.11± 0.01	0.19± 0.01	0.26± 0.01	0.23± 0.003

Average of 3 replicate sets. ± = S.E.

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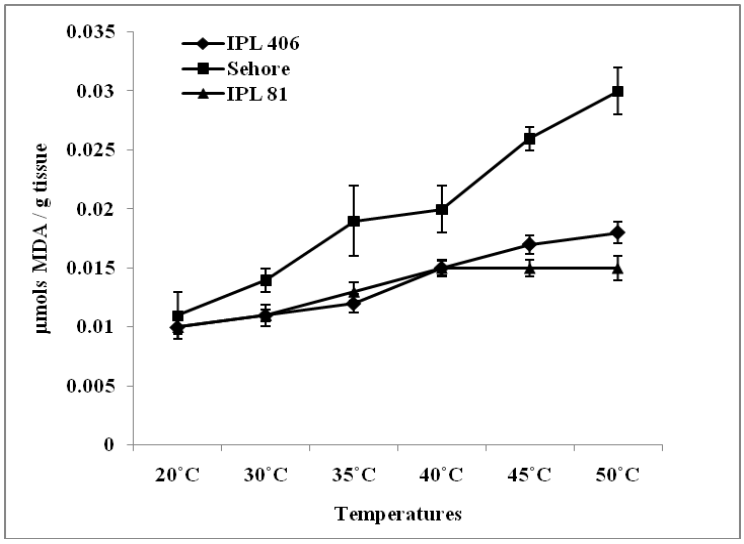

Fig. 1 : Effect of high temperatures on lipid peroxidation of membranes in lentil varieties

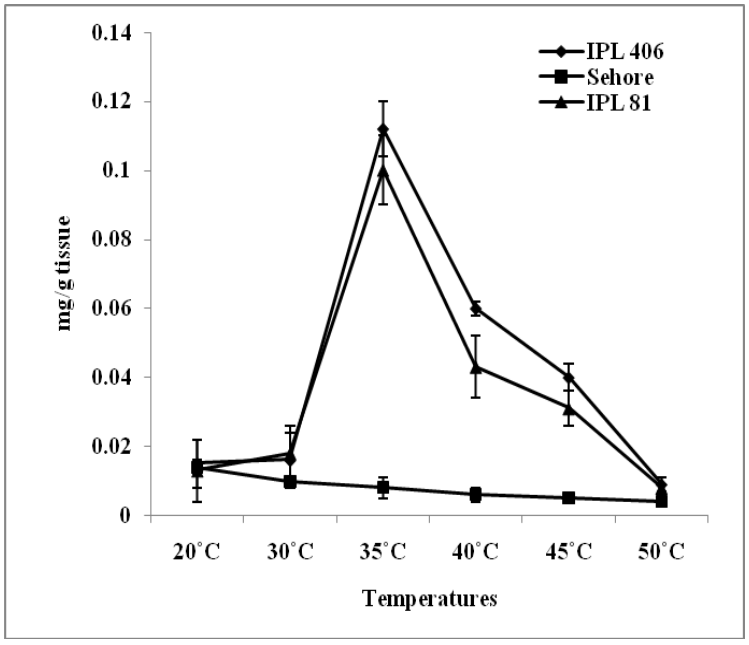

Fig. 2 : Effect of high temperatures on carotenoid contents in lentil varieties

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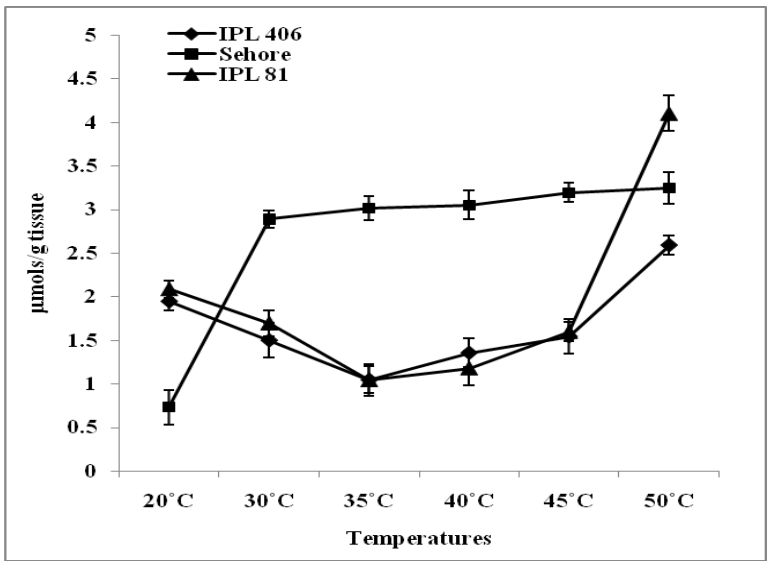

Fig. 3 : Accumulation of H_2O_2 in lentil varieties subjected to elevated temperatures

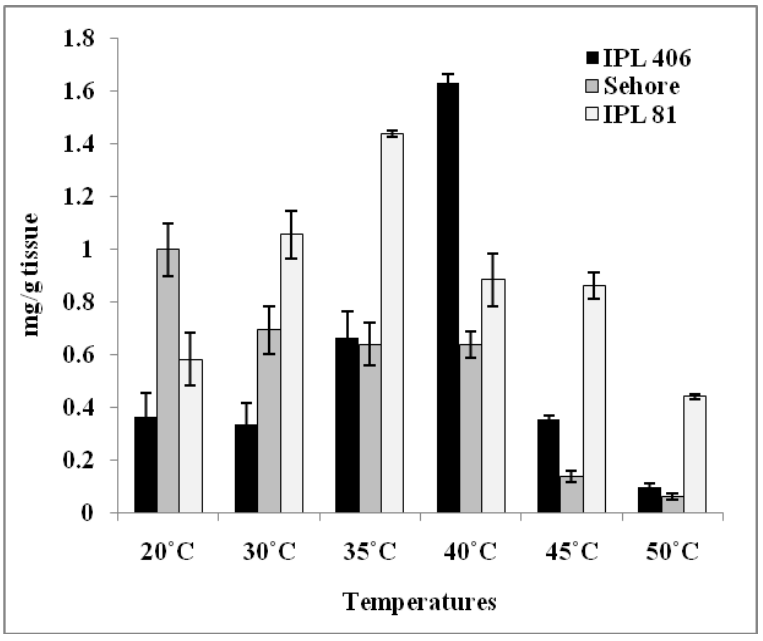

Fig. 4 : Total chlorophyll contents of leaves of lentil plants subjected to elevated temperatures

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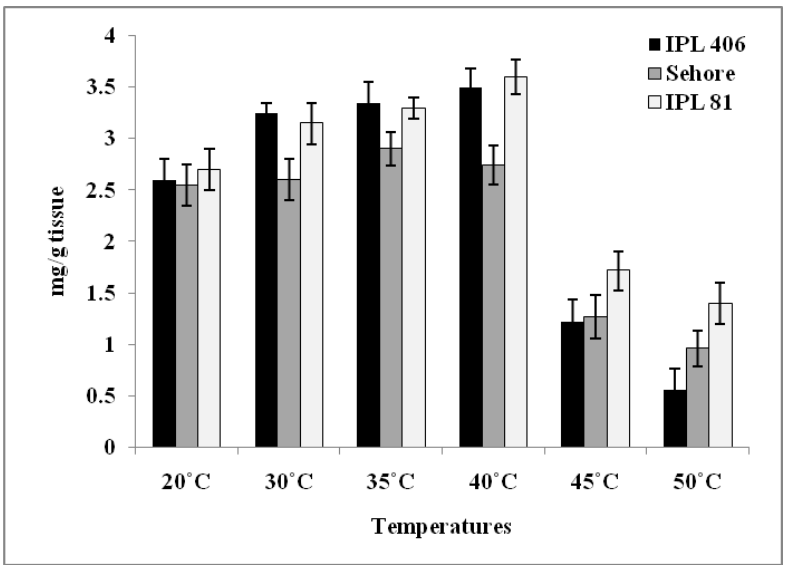

Fig. 5 : Total phenols in leaves of lentil plants subjected to elevated temperatures

effects of high temperature damage in cells. In the present study increase in lipid peroxidation was obtained at all high temperatureswhich was more marked in Sehore. Larkindale and Knight [24] reported that treatment of *Arabidopsis* at 40^0C caused a significant increase in lipid peroxidation. Similar results were also reported by other workers [9, 25]. Plants protect cell and subcellar systems from the cytotoxic effects of the active oxygen radicals using antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, catalase and metabolites like glutathione, ascorbic acid, a-tocopherol and carotenoids [26]. In lentil varieties subjected to high temperatures ranging from 30-50^0C, it was observed that in the 2 tolerant varieties there was an initial increased accumulation of ascorbic acid and carotenoids which declined with further increase. The increase in carotenoids at 35^0 was highly significant. However, in Sehore, the 2 antioxidants decreased with temperature increase. This is conformity with the results of previous workers [27]. In the present study, experiments revealed that in the tolerant varieties, H_2O_2 accumulation initially decreased though it increased at further high temperatures. On the other hand, in the susceptible variety, H_2O_2 accumulation was enhanced at all high temperatures. It is quite clear that in the tolerant varieties, H_2O_2, which is an ROS, does not accumulate initially but in the susceptible variety its accumulation is enhanced.

Decrease in chlorophyll content due to high temperature has been observed in rye [28] and wheat [29]. Bhullar and Jenner [30] reported accelerated chlorophyll degradation under high temperature stress. Significant reduction in chlorophyll content under late and very late planting was observed in all genotypes and at all stages of plant growth in wheat by Almeselmani et al.[31]. However, tolerant genotypes HDR 77 and HD 2815 maintained comparatively higher chlorophyll content and showed less reduction compared to other genotypes under increasing temperature of late and very late plantings. Premature loss of chlorophyll due to heat sensitivity in wheat crop has been reported earlier [32]. In the present study also elevated temperatures led to a decrease in chlorophyll content in the susceptible varieties though in the 2 tolerant varieties there was an increase. Other

components like phenol and proline which help plants in withstanding stress increased initially before declining.

In conclusion, it can be stated that among the three tested varieties, Sehore is more susceptible to high temperature stress than IPL 81 and IPL 406. This has been confirmed by tolerance index, cell membrane stability test, greater lipid peroxidation, and lesser accumulation of antioxidants in Sehore.

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