Influence of drought and salinity on metabolic processes in different wheat varieties

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DECLARATION

I declare that the thesis entitled "**Influence of drought and salinity on metabolic processes in different wheat varieties**" has been prepared by me under the guidance of Professor U. Chakraborty, Plant Biochemistry Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree of fellowship previously.

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This is to certify that Ms Bhumika Pradhan is submitting her thesis entitled "Influence of drought and salinity on metabolic processes in different wheat varieties" on the basis of her work carried out under my supervision at the Department of Botany, University of North Bengal, for the award of Ph.D degree from the University of North Bengal.

She bears a good moral character and I wish her all the best in life.

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ABSTRACT

Out of several varieties tested, nine different wheat (*Triticum aestivum* L.) varieties – Mohan Wonder (MW), Kedar (KD), Gayetri (GY), Gandhari (GN), Kaweri (KW), PBW 343, UP 2752, Sonalika (SO), Local variety (LV) were selected for our study. Out of these MW, KD, GY, GN, KW were commercially relevant lines and PBW 343, UP 2752 and SO were research varieties and LV was obtained from a local market. The viability of the seeds was tested under laboratory conditions in petri plates and also in earthen pots which was 100% for all the varieties selected. The seeds were allowed to germinate in the petri plates for one week and then transferred to earthen pots under proper condition of water, light and soil conditions.

Drought stress was induced by withholding water and sampling was done on the 3rd, 6th and 9th day. For the control set watering was done regularly and sampling was done on the 0day of withholding water. In a separate treatment salt stress was imposed on one month old plant by transferring the plants to a medium of water containing 50, 100 and 200mM of salt separately and sampling was done on the 0day, 1st day and 3rd day of salt stress in that medium. In the control set (0mM) no such treatment of salt was given. Various morphological, physiological and biochemical parameters were taken to test the response of these varieties to drought and salt stress and their metabolism during the stress condition.

Plants exhibited varying levels of morphological changes however, none of the nine varieties showed sever wilting symptoms morphologically even after 7th day of drought but during the 9th day LV, MW, GY and SO showed sever wilting and yellowing symptoms in the leaf. In KD, KW, GN, UP 2752 and PBW 343 comparatively the wilting and yellowing symptom in the leaf was lesser. Plants under salt stress did not show wilting symptoms on the first day of salt stress for all concentration however, on the 3rd day wilting and yellowing of leaf was significantly higher in case of LV, MW, GY and SO. Physiological parameters such as tolerance index, RWC, CMS and lipid

peroxidation showed a significant difference among the varieties as well as between the duration and severity of both type of stress.

A significantly low tolerance index was observed in case of the leaf of LV, MW, GY and SO which in case of KD, GN, KW, UP 2752 and PBW 343 was higher during the 9^{th} day of drought and also during the salt concentration of 200mM on the 3^{rd} day. The tolerance index of all the nine varieties was higher during the salt stress than during drought. CMS in the leaf of GN, KD, KW, UP 2752 and PBW 343 was higher during drought and salt stress and in case of LV, MW, GY and SO however, with respect to their controls it was lower and decreased with the increase in the duration and concentration of salt respectively. CMS was lowest when the plants were subjected to 9days of drought and salt stress of 200mM for 3days. Lipid peroxidation in the leaf tissue increased in the stressed plants in case of all the varieties with the onset of drought and salt treatments. The increase in the lipid peroxidation with the increase in the duration of drought and during salt stress of different concentration was comparatively lower in case of KD, KW, UP 2752 and PBW 343 than LV, GY, MW and SO where higher lipid peroxidation in the leaf tissue was observed with increase in the duration of withholding water and also with increasing concentration of salt. The highest MDA content, a measure of lipid peroxidation in the leaf was observed when the nine varieties were subjected to higher degree or concentration of stress. Decrease in RWC, CMS and lower tolerance index during drought and salinity stress in our study could be correlated with higher degree of lipid peroxidation and therefore it served as an important physiological parameter to test the tolerance level among the tested nine varieties of wheat.

Following drought stress and salinity stress the accumulation of compatible solutes or osmolytes in the cells of the stressed plant was seen which was significantly higher in the plants under stress conditions than the control set. Content of both leaf and root proline increased in the wheat varieties under both types of stress. In general the accumulation of free proline was higher in the leaves than their accumulation in the roots in all nine tested varieties. The increase in the accumulation of proline following drought and salt stress in the leaf and root tissues of GN, KD, KW, UP 2752 and PBW 343 was

significantly higher than the accumulation of proline in the leaf and root tissues of LV, GY, MW and SO in our study. In the latter, the increase in the proline content in both leaf and root was comparatively lower than the former varieties and in case of LV, lowest accumulation of proline was observed and the highest value for proline was obtained in case of GN.

Determination of profile of free amino acids by HPLC of GN and LV revealed that the amount of free proline along with other important free amino acid increased with the increase in the duration of drought. The accumulation of free amino acids was higher in case of GN than LV. Aspartic acid, glutamic acid, serine, threonine, proline, hydroxyl proline, tyrosine, valine, methionine, leucine, cysteine, isoleucine, DL–phenyl alanine and lysine were found to be most significant during drought in both GN and LV with the peak height and area being comparatively greater in case of GN than LV where it was smaller and some peaks were absent during prolonged period of drought. Along with these, other amino acids were identified such as DL alanine, arginine and alanine.

Other compatible solutes, i.e., soluble sugars, reducing sugar and starch accumulation following drought and salt treatments showed significant differences in between the treatments and between the varieties. The accumulation of total soluble sugars in both leaf and root of all the nine varieties during both drought and salt stress showed significant increase with the increase in the severity of stress treatment in case of GN, KW, KD, UP 2752 and PBW 343 which in case of LV, MW, GY and SO decreased after an initial increase. The accumulation of total soluble sugars in both leaf and root was highest in GN than all the tested varieties and the least accumulation was observed in LV with respect to their control set. The content of total soluble carbohydrates in the leaf was found to be higher than the roots in all cases including the control plants. Similarly the accumulation of reducing sugars in both leaf and root of all the tested nine varieties in our study during stress treatments showed significant increase with the increase in the severity of stress treatments. The content of reducing sugar in the leaf and root in all varieties continued to increase during stress treatments with the increase in case of GN, KW, KD, UP 2752 and PBW 343 being much higher that of the

increase in its content LV, MW, GY and SO decreased after an initial increase. Reducing sugars in both leaf and root was highest in GN than all the tested varieties and the least accumulation was observed in LV with respect to their control set and the leaf reducing sugars was much more than that in the roots in both control and stressed plants. Accumulation of total soluble starch increased significantly in the leaf and roots of all the nine varieties with a slight decrease with the increase in the severity of both drought and salt treatments. This decrease was higher in the leaf than the roots in all cases. GN and KD accumulated more starch in their tissues than all the other varieties in both leaf and root and the lowest accumulation was observed in case of LV.

An increase in the accumulation of total phenols was evident in all the nine varieties with the induction of drought stress. In MW, GY, LV, PBW 343 and UP 2752 the accumulation of total phenol decreased during longer duration of drought. In case of GN, KD and KW the content of total phenol was found to be the higher and the highest value obtained in the leaf of GN during drought stress. However, the accumulation of total phenol in the tested nine varieties during salt stress increased in GN, KD, KW and UP 2752 only and in other varieties total phenol content was higher only at lower concentration of salt treatment. GN leaf showed the highest content of total phenols and in LV lowest content of phenol accumulation was observed. Ortho phenol accumulation increased in all the nine varieties with the induction of drought and salt stress in general. KD, GN and KW leaf showed the highest ortho phenol accumulation during drought and the lowest accumulation of total phenol content was observed in LV and SO. During salt stress the content of ortho phenol increased in all cases except LV. GN leaf showed the highest content of ortho phenols and in LV lowest content of ortho phenol accumulation was observed in our study.

Analysis of total phenol revealed that during both drought and salt stress, the accumulation of ferulic acid was most significant in GN and LV and the change in the accumulation of ferulic acid was found to be significant with increase in the days of drought and the concentration of salt. Along with ferulic acid other phenols which were identified were salicylic acid, chlorogenic acid, caffeic acid which was also found to play a significant change during stress.

Accumulation of these phenols in case of GN was significantly higher than that of LV during drought and salt stress. Phenolic acids from the total phenols were extracted and the types of phenolic acids present during the stress treatment in wheat were studied. Ferulic acid was identified as the most important phenolic compound present in GN and LV during stress and the change in the accumulation of ferulic acid as observed in the HPLC data was found to be significant. Along with ferulic acid, vanillic acid, cinnamic acid, chlorogenic acid and salicylic acid were the other phenolic acid identified in HPLC which were also very significant during drought and salt treatments and the accumulation of these phenolic acids was higher in GN than in LV.

The accumulation of total soluble proteins in the leaf and roots in the case of all the tested wheat varieties showed a general decrease with the increase in the days of withholding water and the increase in the concentration of salt. In case of LV, GY, MW, SO and PBW 343 the decrease in the accumulation of protein in the leaf was higher than the decrease in the other varieties while in roots there was a slight increase in the protein content in MW, GY, GN, LV and SO. During salt stress, the content of soluble protein in roots slightly increased however, it decreased with higher concentration of salt and increase in the days of salt stress. At least a total of about 34 new bands were observed in SDS PAGE of soluble proteins during drought and salinity. 6 and 12 new bands respectively were observed during drought and salinity. Seedlings of wheat subjected to drought and salt stress showed an expression of new protein bands in the SDS PAGE analysis of protein in the leaf in some cases and suppression of certain existing bands at different duration of withholding water and at increasing concentration of salt. More number of new bands was observed in case of GN, KD, KW, UP 2752 and PBW 343 during the SDS PAGE analysis of leaf proteins during both drought and salinity stress which in case of other four varieties was comparatively lesser in number.

The total soluble protein profile in the leaf of two varieties of wheat (GN and LV) during drought stress was determined in Fast protein liquid chromatography (FPLC) where it was observed that the number, height and the area of peak showed a significant difference during the increasing days of water stress with respect to control plant.

Photosynthetic pigments in the leaf of the plants under stress showed a significant change with respect to the control plants. Chlorophyll content as well as the ratio of chlorophyll a/b altered during drought and salt treatments. Chlorophyll content decreased in almost all varieties with the induction of drought and salt treatments. However in case of varieties such as GN and KD, an initial increase was observed. The lowest chlorophyll content was observed in case of LV with prolonged periods of drought and increase in the concentration and days of salt stress when compared the their respective control. The decrease in the content of chlorophyll was higher in LV, MW, GY and SO than the decrease in case of GN, KD, KW, UP 2752 and PBW 343. Significantly, the data obtained in our study for the ratio of chlorophyll a and chlorophyll b suggested that the trend obtained in the ratio of these two pigments could better explain response of plants to drought and salt stress with increasing days of water stress and increasing concentration of salt stress. The ratio of chlorophyll a/b increased initially in all varieties and decline with increase in the severity of stress. However, in LV, GY, SO and MW, the decrease in the ratio of chlorophyll a/b was greater than the decrease in case of other five varieties in our study. The decrease in the ratio was attributed to the fact that the content of chlorophyll a decreased significantly in the varieties during stress.

Results indicate that both drought and salt stress induced oxidative damage in wheat varieties could be overcome by enhanced activities of antioxidative enzymes. The activity of all the antioxidative enzymes seemed to be correlated with the each other during the stress response of the plant. APOX activity increased significantly in all nine varieties however decreased with prolonged water stress in all cases. Similarly during salt stress the activity enhanced with increase in the concentration of salt however decreased on increasing days of salt stress in all the tested varieties. Increase in the activity of APOX was significantly much more pronounced in case of MW, GY, LV and SO. Thus, the enhancement in the activity of APOX suggested a possible underlying mechanism during drought and salt stress in case of these varieties.

With prolonged period of drought stress and with increase in the concentration of salt the activity of GR increased significantly in all varieties but in MW,

GY, LV and SO it decreased with increase in the severity of stress however, in case of KD, GN, KW, UP 2752 and PBW 343 the activity increased significantly.

It was noted that the activity of POX enhanced greatly with increase in the period of water stress in case of GN, KD, KW, UP 2752 and PBW 343 whereas in LV, GY, MW and SO, the activity of POX declined. Activity of POX showed a continuous enhancement with increase in the concentration of salt in all varieties; however in case of MW, LV, SO and GY the activity of peroxidase with prolonged stress showed a significant decline in the later period of stress and higher concentration of salt. In case of peroxidase isozyme analysis in NATIVE PAGE, new bands of peroxidase isozyme were recorded in the stressed leaves with respect to control in case of almost all the varieties with highest number of new peroxizymes recorded in case of varieties like GN KW, KD followed by PBW 343 and UP 2752 and the same trend was observed during salt stress. The activity of peroxidase and the occurrence of bands in NATIVE – PAGE for peroxidase isozyme were correlated.

A general decrease was seen in the activity of CAT during both drought and salt stress in case of MW, GY, LV and SO whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. The activity of CAT following salinity stress showed a continued decline in case of MW, GY, LV and SO with the increase in the concentration and duration of salt stress. However in case of KW, KD, GN, UP 2752 and PBW 343 there was an initial enhancement in the activity of CAT followed by decline in its activity with increasing concentration of salt. In the catalase isozyme analysis in NATIVE PAGE during drought and salinity stress, significant differences was noticed among the varieties as well as during the different days of drought and different concentration of salt. The occurrence of catalase isozyme was more expressed in case of NATIVE PAGE of leaf in GN, KD, KW during drought and in KD and KW in case of salinity stress. In case of MW, GY isozyme for catalase was expressed during the higher concentration of salt and prolonged period of water stress.

SOD activities decreased at all periods of drought and concentration of salt stress in case of MW, GY and whereas in KD, GN, KW, UP 2752, PBW 343 and SO activities of these enzymes increased initially before showing a continued decline. With prolonged stress the activity during both drought and salt stress in case of MW, GY, LV and SO showed a continued decrease whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. SOD is the first enzyme which is expressed in the antioxidant mechanism and it increased initially in the more tolerant varieties and was also involved in contribution to the initial accumulation of H_2O_2. Although H_2O_2 accumulation increased during water and salt stress, after a period of prolonged drought and with the increase in the concentration of salt in the medium there was a decrease in H_2O_2 levels in varieties like KW, GN, KD, PBW 343 and UP 2752 indicating greater antioxidant activity whereas the accumulation of H_2O_2 continued to increase in LV, SO, GY and MW with the increase in the duration of withholding water from the plants and increase in the concentration of salt and days of salt stress. Highest H_2O_2 accumulation was observed in case of LV and lowest was observed in case of GN with increase in the severity of stress. In the microscopic study the leaf of SO, LV, GY and MW showed more darkly stained DAB sites in the tissues than in the leaves from the other five varieties with respect to their control during both the drought and salt stress. DAB polymerization site was largely localized at the tip of the leaf, region surrounding the middle lamella and also the stomata of the leaf in the varieties under stress when compared to the leaf of the control set. The transverse section of the leaf at the stained site showed that the DAB binding sites were localized mostly in the peripheral region of the cell.

Non enzymatic antioxidants such as carotenoids, ascorbate and α–tocopherol showed an enhanced accumulation after the induction of drought and salt stress. Accumulation of carotenoids in the leaves, during drought showed an initial enhancement in all the varieties followed by a decrease after 3 days in varieties MW, GY, LV and SO and after 6 days in KD, KW, GN, UP 2752 and PBW 343. Carotenoid content showed an increase in its accumulation with the increase in the concentration and duration of salt stress in all the varieties but with prolonged days of salt stress and increasing concentration of salt the

accumulation declined at in case of MW, GY, LV and SO and at higher concentration of salt in case of KD, GN, KW, UP 2752 and PBW 343. The accumulation of ascorbate in the leaf was enhanced in all nine varieties even after 9 days of drought stress and increased at all periods of salt stress and enhanced with increasing concentration of salt in all the varieties. The highest accumulation of ascorbate was noted in case of GN, KW, KD, UP 2752 and PBW 343. Accumulation of α–tocopherol in the leaves of the drought stressed plant in general increased but decreased after 3rd day in case of GY, after 6th day in KW, LV, PBW 343 and SO and after 9th day in MW, GY and KD however, in case of UP 2752 and GN it increased during the 9th day of water stress. α–tocopherol content in the salt stressed leaf increased with the increase in the days and concentration of salt stress initially but declined at higher concentration and duration of salt stress in case of MW, GY, LV and SO and in the other five varieties it declined at higher salt concentration.

Total antioxidant activity increased in case of GN, KD, UP 2752 and PBW 343 even after nine days of drought with a slight initial decline in MW during drought and in KW, MW, GY, LV and SO it decline at prolonged periods of drought. The total antioxidant activity declined at higher concentration of salt in LV, MW, SO and GY however it continued to increase in the other five varieties.

Ionic imbalance with respect to the content of Na^+ and K^+ content was observed during drought and salt stress. Na^+ content in case of both water and salt stress increased significantly with the onset of stress treatments. Following water and salt stress treatments the content of Na^+ in case of roots was much higher than that of leaf in all varieties. In MW, GY, LV and SO the increase in the Na^+ content was more than the increase in the other varieties following stress and the content of Na^+ in the roots were higher. The increase in the content of Na^+ was much more in the roots than the leaf and four varieties i.e. MW, GY, LV and SO showed the highest content of Na^+ in both the leaf and roots with increasing concentration and duration of salt stress. K^+ content was higher in the leaf than the roots during the stress. K^+ content in both leaf and root increased significantly during the initial phase of water stress but later with prolonged stress, the content of K^+ in both leaf and root declined; the

decline in case of MW, GY, LV and SO was much more than compared to the other five varieties where the decrease in the content of K^+ was lesser. The decline in K^+ content of the leaf and root during salinity stress in case of MW, GY, LV and SO was much more pronounced than the varieties GN, KD, KW, UP 2752 and PBW 343.

The effect of ABA, SA and proline pre–treatments on amelioration of drought in GN and LV showed a better response of the plant to drought treatments in both the varieties after ABA, SA and proline pre–treatment especially in the case of LV which was considered as the most susceptible variety or in other words the least tolerant variety in our study. The effect of proline pre–treatment in GN and LV showed better results than the pre–treatment with ABA followed by pre–treatment with SA. Pre–treatments of seedlings of GN and LV with solutions of ABA, SA and proline forwarded by drought stress for 3, 6 and 9 days revealed that all three chemicals could provide protection against oxidative stress due to water stress in these varieties with respect to enhanced enzyme activities like CAT, POX, APOX, SOD and GR, decreased accumulation of phenols and of non–enzymatic antioxidants like carotenoids and ascorbate suggesting enhanced antioxidative mechanisms in the two varieties during drought stress. Pre–treatments by these chemicals could also enhance the physiological parameters such as RWC, CMS and was able to decrease the peroxidation of membranes in the leaf and lesser accumulation of H_2O_2 was observed in both GN and LV which was responsible for maintaining a better morphological and physiological property of the plants under drought. Both GN and LV seedlings when pre–treated with solutions of ABA, SA and proline before the induction of drought showed lesser accumulation of soluble proteins and the content of compatible solutes like proline, total soluble carbohydrates, reducing sugars showed a decrease in the seedlings of both the varieties in both leaf and roots.

PREFACE

The ability of plants to survive under severely stressed condition is due to various metabolic adaptations, which, if successful trigger cascades of signaling pathways leading to tolerance of the plant. Drought and salinity are two of the most important abiotic stresses of the present age. Feeding of ever growing population with lesser and lesser arable land will have to depend on increasing plant productivity. For this, selection of plants with ability to tolerate stress conditions, on the basis of knowledge of the mechanisms involved in tolerance is essential. The present study has been undertaken on wheat to understand details of their stress responses.

In this context, I am immensely indebted to my mentor and guide Professor Usha Chakraborty for her encouragement, expert guidance and constant support in every sphere of my research whose valuable inputs and wise counselling have helped me to sail through this endeavour and to whom I shall always remain grateful.

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*(**Bhumika Pradhan**)*

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CHAPTER 1
INTRODUCTION

The two major environmental factors that reduce plant productivity are drought and salinity, and these stresses cause similar reactions in plants due to water stress; moreover, these environmental concerns affect plants more than is commonly thought Serrano (1999). For example, disease and insect loss typically decrease crop yields by less than ten percent, but severe environmental problems can be responsible for up to sixty–five percent reduction in yield. In India, water deficit stress limits crop production in about 67% of the net sown area, while 7mha of agriculture land suffers from soil salinity. An estimated 30 to 60% reduction in potential crop yields is often attributed to abiotic stresses of which water stress is predominant. Productivity of crop plants is strongly influenced by environmental cues like drought, high temperature and salinity.

Diverse environmental stresses like salinity, drought and temperature are major limiting factors in plant productivity and abiotic stress; particularly salinity and drought are major stresses that cause crop losses worldwide (Bartels and Sunkar, 2005; Vinocur and Altman, 2005). Sustaining productivity under water limited conditions and saving irrigation water are the two most important aspects that need to be addressed immediately in agriculture is to feed the burgeoning population of the country. Rain fed agro–ecosystem has a distinct place in Indian Agriculture, occupying 67% of the cultivated area, contributing 44% of the food grains and supporting 40% of the human and 65% of the livestock population (Venkareswarlu, 2005). Environmental degradation, mainly soil pollution and its erosion, desertification, declining biodiversity and overuse of agrochemicals causes biotic and abiotic stress in plants (Shao and Chu, 2005).

In India, water deficit stress limits crop production in about 67% of the net sown area, while 7mha of agriculture land suffers from soil salinity.

Nagaraja *et al* (2010) reports the impact of drought on agriculture and the challenges being faced by the farmers of South India. The percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world (Isendahl and Schmidt, 2006). One third of the world's agricultural land is damaged, and approximately 5% of 1.5 Bha of cultivated land is affected by salt (Tabatabaei, 2006). Drought limits plant growth and field crops production more than any other environmental stresses (Zhu, 2000; Tas and Tas, 2007). Water deficit and salt stresses are global issues to ensure survival of agricultural crops and sustainable food production (Jaleel *et al.* 2007, 2008). According to Chaves *et al.,* (2009) plants are often subjected to periods of soil and atmospheric water deficits during their life cycle as well as, in many areas of the globe, to high soil salinity and the understanding of how plants respond to drought, salt and co–occurring stresses can play a major role in stabilizing crop performance under drought and saline conditions and in the protection of natural vegetation.

Among those stresses that limit plant growth and development, drought is a major factor and is closely related to salt stress (Song *et al.*, 2008). Water stress is the most influential factor affecting crop yield particularly in irrigated agriculture in arid and semi arid regions, it is necessary to get maximum yield in agriculture by using available water in order to get maximum profit form per unit area because existing agricultural land and irrigation water are rapidly diminishing due to rapid industrialization and urban development (Khalil *et al.*, 2010). According to them, optimizing irrigation management due to water scarcity together with appropriate crops for cultivation is highly in demand; the cost of irrigation pumping and inadequate irrigation scheme capacity as well as limited water sources is among the reasons that force many countries to reduce irrigation applications. Global climatic change and growing pressure on natural resources due to population explosion may further exacerbate the incidence of abiotic stresses. Investigating the physiological and biochemical changes that occur during water stress may help in understanding the effect of water stress. This would further help in identifying the genotypes/accessions tolerant to water stress. Rahdari and Hoseini (2012) in their review have identified drought stress as an

important problem especially in countries where crop agriculture is essentially rain–fed and is a dangerous problem for humanity and for nature that global warming causes that is a major a biotic constraint limiting crop production worldwide.

Salt stress on the other hand is also one of the major threats to crop productivity worldwide including India, since about 20–27% of world irrigated land is affected by salinity stress (Ghassami *et al.*, 1995). It is estimated that increased salinization of aerable land will have devastating global effects, resulting in 30% land loss within next 25 years, and up to 50% by the year 2050 (Wang *et al.*, 2003).There are global constraints on fresh water supplies, and this has led to a surge of interest in reusing water (Shannon and Grieve, 1999). According to Munns *et al.* (2006) increased salt tolerance of crops is needed to sustain food production in many regions in the world and suggests that in irrigated agriculture, improved salt tolerance of crops can lessen the leaching requirement, and so lessen the costs of an irrigation scheme, both in the need to import fresh water and to dispose of saline water (Pitman and Lauchli, 2002). Similarly, for the purpose of crop production, yield improvement, developing of drought tolerant varieties is the best option (Siddique *et al.*, 2000). The problem of soil salinity is further increasing because of the use of poor quality water for irrigation and poor drainage. Therefore, it poses serious problem to food security in developing countries like India due to high rate of population growth and stagnation or declining of crop productivity in high productivity areas (Abdin *et al.*, 2000). Soil salinization is one of the major factors of soil degradation. Salinity inhibition of plant growth is the result of osmotic and ionic effects and the different plant species have developed different mechanisms to cope with these effects (Munns, 2002).The knowledge acquired regarding the growth and survival of plants under natural conditions could be used as a tool for screening of plant species for afforestation of saline lands. In warm and dry areas salt concentration increases in the upper soil layer due to high water losses which exceed precipitation (Ebert *et al.*, 2002). In arid and semiarid regions of the world, limited rainfall, high evap–transpiration, high temperature and inadequate water management each contribute to increases in soil salinity

(Meloni *et al.*, 2003). Therefore, plant response to salinity is one of the most widely researched subjects in plant physiology. There is a need to breed salt–tolerant genotypes to sustain crop–productivity and achieve future food–security (Chinnusamy *et al.*, 2005). Munns *et al.* (2006) has reviewed that the salt–tolerant crops have a much lower leaching requirement than salt–sensitive crops so in dry–land agriculture, improved salt tolerance can increase yield on saline soils and in the areas where the rainfall is low and the salt remains in the subsoil, increased salt tolerance will allow plants to extract more water. According to him study of salt tolerance may have its greatest impact on crops growing on soils with natural salinity as, when all the other agronomic constraints have been overcome (e.g. disease resistance and nutrient deficiency), subsoil salinity remains a major limitation to agriculture in all semi–arid regions.

The percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world (Isendahl and Schmidt, 2006). According to Singh *et al.* (2009) 6.73 million ha of land is affected by salinity and alkalinity problem in India, 25 % of ground water used for irrigation is either saline or brackish 11.7 million ha is likely to be affected by salinity and alkalinity in India by 2025, At global level, 810 million ha is affected by sodicity (434 million ha) and salinity (376 million ha). About 20% of the cultivated land worldwide is adversely affected by high salt concentration, which inhibits plant growth and yield. Therefore physiological and biochemical approaches have a great importance in order to understand the complex responses of plants to water deficiency and develop rapidly new varieties (Maralian *et al.*, 2010). According to Shannon and Grieve (1999) among several strategies devised to overcome the problem of salinity stress, the selection of crop species or cultivars with salinity tolerance traits has been considered an economical and efficient strategy and hence suggests that the challenges for using salty water profitably will depend on greater knowledge of salt tolerance. Bayuelo–Jimenez *et al.*, Misra and Dwivedi (2004) have tried to identify the physiological and biochemical differences between salt tolerant and sensitive plants in a n effort to develop rapid screening methods for salt tolerance and suggests that salt tolerance ability depends on genetic and

biochemical characteristics of the species and sufficient genetic variability in relation to salinity exist in many agricultural crops.

Wheat is one of the most important cultivated cereals of the world but almost 32% of wheat cultivars face up to various types of drought stress during the growth season in developing countries (Morris *et al.*, 1991). Water stress during grain development causes large yield losses in cereals including wheat. This reduction is mainly accounted for by a reduction in starch accumulation, since in general over 65% of cereal dry weight is accounted for by starch (Duffus, 1992). Wheat is grown both as spring and winter crop. Winter crop is more extensively grown than spring. Much of the injury on plants under abiotic stress is linked to oxidative damage at the cellular level (Smirnoff 1993). Wheat is a major staple food crop for more than one third of the world population and is the main staple food of Asia (Shirazi *et al.*, 2001). It originated in South Western Asia and has been a major agricultural commodity since pre historic times. The wheat crop is mainly cultivated under rain fed conditions where precipitation is less than 900 mm annually. Global water crisis seriously influences crop productivity, particularly in most of the Asian countries where irrigated agriculture accounts for 90% of total diverted fresh water (Huaqi *et al.*, 2002). Wheat is essential nourishment for more than 1/3 of the world population and crop yield will be considerably influenced in the perspective of global climate change and limitation of water resources in the environment (Chaves and Oliveira, 2004). The lack of adequate moisture leading to water stress is common occurrence in rain fed areas, brought about by infrequent rains and poor irrigation (Wang *et al.*, 2005).

Various approaches have been taken to study the effect of drought and salinity stress on wheat varieties. The main aim of their study has been to study the effect on the plant system and development of new improved crop and to select the more tolerant variety or cultivar of wheat. In different parts of India, productivity is affected by drought stress conditions and the selection of drought–resistant varieties becomes essential. Selection of wheat genotypes/cultivars with better adaptation to water stress should increase the productivity in rainfed areas (Rajaram, 2001). Food insecurity that has increased in recent times owing to competing claims for land, water, labor,

energy, and capital, has created more pressure to improve production per unit of land (Godfray *et al.* 2011; Varshney *et al.* 2011). Jan *et al.* (2013) states that world's population is increasing at an alarming rate and around 70% of the total population lives in the rural areas of developing countries, where poverty, food insecurity and nutritional deficiency are major problems encountered in day–to–day life and low productivity in agriculture is considered to be a major cause of poverty, food insecurity, and poor nutrition, especially in countries where agriculture is the driving force for broad economic growth and poverty alleviation. They further conclude that the ability of plants to tolerate stress conditions is crucial for agricultural production worldwide as they negatively influences the survival, biomass production, accumulation, and grain yield of most crops. Under such circumstances, increasing the yield of crop plants in normal soils and in less productive lands including salinized soils appears as absolute requirement for feeding the world (Jan *et al.*, 2013).

Abiotic stressors induce some changes in plants occurring at different levels, from molecular to organismal ones, and affecting plant productivity Maevskaya and Nikolaeva (2013). Drought and salinity are two of the most important natural stressors. Contemporary models of climate change predict that, in the next decade, the drought frequency, intensity, and duration will increase. Similarly various data shows that the abiotic stressor salinity, challenges the plants in varied ways and the need to combat the deleterious effects and select the plants suitable to grow in saline soils will be one of the most challenging task in the immediate future. Yamaguchi and Blumwald (2005) states that agricultural productivity is severely affected by soil salinity because salt levels that are harmful to plant growth affect large terrestrial areas of the world. It is quite obvious that the study of the mechanisms of plant adaptation and tolerance to drought, as well as the assessment of the ability of plants to recover from water deficit is an important task of modern research Chaves *et al.* (2009).

The impact of water and salinity stress in the days following germination, that challenges seedling survival, is almost certainly one of the major limitations for the establishment of species in many habitats. It was evident from the work of various researchers' that the most affected stage is the seedling stage of the plant during any kind of stress, abiotic stress in

particular, in which water stress and salt stress are of significant importance. Further work in seedling stage, i.e., fifteen to one month old plant is expected to give significant results.

As suggested by Mandhania *et al.*, 2010, the selection of variety among the existing crop which shows stress tolerance and better yield is one of the most economical and efficient strategy to overcome the need to grow crops which are more tolerant to various stresses in the test as well as in the field condition. With physiological parameters such as cell membrane stability, differences in the tolerance for compound stresses such as salinity and water deficiency can be detected. Various workers also have suggested cell membrane stability as a good parameter to test the susceptibility of plant to water stress and salinity stress as well as temperature and other abiotic stresses. Investigating the physiological and biochemical changes that occur during water stress may help in understanding the effect of water stress. This would further help in identifying the genotypes/accessions tolerant to water stress. Physiological mechanisms that underlie traits for salt and drought tolerance by the plant system could be used to identify new genetic sources of salt and drought tolerance.

Drought and salt stress are emerging as the most challenging abiotic stresses which require a detailed study from various aspects. Considering the importance of osmotic stress i.e., drought and salt stress, it is worthwhile to study how different varieties of wheat plants, which is one of the most important cereal crop after maize and rice, respond to these stresses, their mechanisms of action and also any biochemical markers for tolerance may be identified in the different varieties of wheat under treatment. The study and the identification of plants parameters associated to drought and salinity stress in the current scenario, which can be used as a quick and reliable index to differentiate between tolerant and susceptible trait will enable screening of the various available varieties. It will be a beneficial tool or index for the plant breeders as well as the farmers to select the most tolerant variety or cultivar for their purpose. With the increase in the ever growing population, the increased demand of food, decrease in the irrigation water to the crop, increase in the salt content of the soil, reduced rainfall, urbanization leading to less land usage for agriculture, the search for a better crop with more

tolerant traits to stresses, higher yield, lesser water use efficiency is obligatory.

The present investigation was undertaken to identify the tolerant genotypes that may sustain during drought as well as salinity stress as a strategy adopted by various scientists to overcome salinity and drought stress. The present study investigates the effect of drought stress and salt stress on morphological, physiological and biochemical aspects of nine wheat varieties which differ in their relative tolerance to both the stresses and also amelioration study was done by the use of some chemicals prior to the stress treatment. The results obtained will lead to a better understanding of plants' responses under stress conditions and can be of value in programs conducted to breed water and salt tolerant crop varieties and these attributes can also be introduced in species of interest through genetic engineering and molecular breeding programmes.

Objectives of the study:–

1. To screen wheat genotypes for their relative tolerance to various degree of osmotic stress.
2. To study the biochemical parameters associated with water and salinity stresses in terms of changes in the cellular constituents of leaf and roots including protein, carbohydrates, phenol, praline, and chlorophyll.
3. Determination of activities of antioxidative enzymes like catalase, peroxidase, ascorbate peroxidase, glutathione reductase and superoxide dismutase and accumulation of small antioxidants like ascorbate, carotenoids and tocopherol.
4. To determine total antioxidant activities following stress.
5. Analysis of isozymes and proteins to develop markers of tolerance.
6. To determine whether osmotic stress can be ameliorated by compounds such as ABA and the mechanisms thereof.
7. Determination of membrane damage following osmotic stress.
8. Determination of Na & K content in tissues and soil following application of salts.

CHAPTER 2
LITERATURE REVIEW

Plants encounter many unfavourable environmental conditions while growing in their natural environment that interrupt their normal growth and productivity among which drought, high/low temperature and saline soils are the most common type of abiotic stresses that plant encounter (Jan *et al.*, 2013). Wu *et al.* (2007) suggested that agricultural sustainable development is the most important part of global sustainable development since unlike animals, higher plants, are sessile and cannot escape from the surroundings and therefore adapt themselves to the changing environments by a series of molecular responses aimed to cope with these challenges. The physiological basis for these molecular responses is the integration of many transduced events into a comprehensive network of signalling pathways (Wu *et al.*, 2007).

Researchers like Hirayama and Shinozaki (2010) have studied the plant abiotic stress responses in the post–genome era: past, present and future and others like Hubbard *et al* (2010) have tried to understand the early signal transduction mechanisms in terms of newly discovered components and newly emerging questions with regards to genes and their development which may be very useful in terms of understanding at molecular level. According to Kamal *et al.* (2010) water is the most wide–ranging difficulty among abiotic stresses for production of wheat in the world environment and they suggests that the main strategy is to obtain plants with higher performance under water stress conditions by identifying and modifying the molecular mechanisms that take place when the water availability becomes limiting. According to Patade *et al.* (2011) salinity and drought are important environmental factors that limit crop productivity mainly due to alterations in water relations, ionic, and metabolic perturbations; generation of reactive oxygen species (ROS); and tissue damage where plant growth arrest is the first symptom observed in plants exposed to

salt stress and can be considered as a mechanism to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief (Bartels and Sunkar 2005). Patade *et al.* (2011) suggests that shoot growth is more affected than root growth, and continuation of root growth under stress is an adaptive mechanism that facilitates water uptake from deeper soil layers and the emergence of new leaves is slower and the older leaves show early senescence. The need to study the effect of water stress at various stages of growth is one of the important criteria for the assessment of water stress study on the plant. Munns (2002) have suggested that the early responses to water and salt stress have been considered mostly identical. Chaves *et al.* (2009) have reported that drought and salinity share a physiological water deficit that attains, more or less intensely, all plant organs; however, under prolonged salt stress plants respond in addition to dehydration to hyper–ionic and hyper–osmotic stress. Larcher (2003) has reported that environmental stress can disrupt cellular structures and impair key physiological functions (Larcher, 2003). Similarly, Krasensky and Jonak (2012) suggests that abiotic stresses like drought and salinity stress impose an osmotic stress that can lead to turgor loss which may result in the disorganization of membranes, denaturation and loss of activity of proteins and production of excess levels of reactive oxygen species (ROS) leading to oxidative damage. As a consequence, inhibition of photosynthesis metabolic dysfunction takes place and the damage of cellular structures contribute to growth perturbances, reduced fertility, and premature senescence and this leading to reduced performance of the plant in every aspect. Leaf tissue water deficit per se can be triggered not only by low soil water content but also by high vapour pressure deficit of the atmosphere. In the current scenario, apart from the need to study the effect of salt stress and drought stress in the plant system, it should be challenged to a varied combination of stress to select a more tolerant variety and also amelioration of the plant system with various combination of chemicals need to be studied.

The combined study on the assessment of two or more than two kind of abiotic or biotic stresses on the same plant system may have some of the answers to the mechanism of the plant which results in varied degree of

tolerance level of the plant to the given stress. Plant growth and productivity is adversely affected by nature's wrath in the form of various biotic and abiotic stress factors (Jaleel *et al.*, 2009). Jaleel *et al.* (2009) defined the stress to have various meanings, the physiological definition and appropriate term as responses in different situations. According to them the flexibility of normal metabolism allows the response initiation to the environmental changes, which fluctuate regularly and are predictable over daily and seasonal cycles; thus every deviation of a factor from its optimum does not necessarily result in stress. They considered defines stress as being a constraint or highly unpredictable fluctuations imposed on regular metabolic patterns cause injury, disease or aberrant physiology.

Efforts to improve crop performance under environmental stresses have not been that fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood (Yamaguchi and Blumwald, 2005). Furthermore, the biological solutions have been more difficult to develop because a pre–requisite for the development of salt tolerant crops is the identification of key genetic determinants of stress tolerance. In their study Yamaguchi and Blumwald (2005) suggests that the existence of salt–tolerant plants (halophytes) and differences in salt tolerance between genotypes within salt–sensitive plant species (glycophytes) indicates that there is a genetic basis to salt response and currently two basic genetic approaches are being used to improve stress tolerance that includes: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTLs – regions of a genome that are associated with the variation of a quantitative trait of interest) (Foolad, 2004; Flowers, 2004; Lindsay, 2004) and subsequent marker–assisted selection, and (ii) generation of transgenic plants to introduce novel genes or to alter expression levels of the existing genes to affect the degree of salt stress tolerance.

Recent researchers such as Jan *et al.* (2013) have a more molecular and genomic approach to study abiotic stress in plants and suggest that molecular and genomic analyses have facilitated gene discovery and enabled genetic engineering using several functional or regulatory genes that are

known to be involved in stress response and preliminary tolerance, to activate specific or broad pathways related to abiotic stress tolerance in plants. According to their views, through the use of transgenic technology, goals such as production of plants with desired traits that were unattainable with traditional selection programs are achieved and have reviewed the recent advancement in understanding the role of various stress responsive genes and their critical importance for explaining the control mechanism of abiotic stress tolerance and engineering stress tolerant crops based on the expression of specific stress related genes. Similar views are expressed by Bajaj and Mohanty (2005) in their study where they suggests that knowing the fact that developing crops better adapted to abiotic stresses is important for crop production, transgenic technology employing genetic engineering have opened up new opportunities to improve tolerance to abiotic stresses by incorporating genes involved in stress protection from any source into agriculturally important plants. According to Wu *et al.* (2007) the molecular information from higher plant cells, tissues, and organs should be efficiently popularized to levels of individuals, community, and ecosystem, which can play a greater role, and which is also one of the greatest challenges for plant systems biology during the 21st century.

In a study by Munns *et al.* (2006) it was reported that various approaches have been taken to improve the salt tolerance of wheat by introducing genes for salt tolerance into adapted cultivars, including screens of large international collections, detailed field trials of selected cultivars, conventional breeding methods, and unconventional crosses with wheat relatives where the main aim has been to exploit variation in salt tolerance within wheat and its progenitors or close relatives to produce new wheat with more tolerance than modern wheat cultivars. Other researches like Hameed *et al.* (2013) have concluded that there is no report concerning drought induced cell death evident by DNA laddering and parallel changes in antioxidants in wheat and have studied drought stress induced programmed cell death (PCD) where they have analyzed the differences in cell death induction and antioxidant response in drought tolerant and sensitive genotypes of wheat. According to Wu *et al.* (2007) a systematically deeper and comprehensive

understanding of the physiological mechanisms of crops under drought stresses is not enough to manipulate the physiological regulatory mechanism and take full advantage of this potential for productivity and suggests that this study forms the bridge between the molecular machinery of drought and anti–drought agriculture; besides, the performance of the genetic potential of crops is expressed by physiological realization in the field (Shao *et al.*, 2005, 2006, 2007).

2.1. Morphological and Physiological responses of plants to drought and salinity

Drought stress is a very important limiting factor at the initial phase of plant growth and establishment which affects both elongation and expansion growth (Jaleel *et al.*, 2009; Anjum *et al.*, 2003; Bhatt & Srinivasa Rao, 2005; Kusaka *et al.*, 2005; Shao *et al.*, 2008) and also in terms of decrease in stem length (Specht *et al*., 2001; Heuer & Nadler, 1995), reduction in plant height (Wu *et al.*, 2008; Sankar *et al*., 2007 & 08; Manivannan *et al.*, 2007; Zhang *et al.*, 2004; Petropoulos *et al.*, 2008). Water stress greatly suppresses cell expansion and cell growth due to the low turgor pressure. Osmotic regulation can enable the maintenance of cell turgor for survival or to assist plant growth under severe drought conditions in pearl millet (Shao *et al.*, 2008). Salinity stress affects the growth, dry matter accumulation, ionic imbalance, nutrient and phytohormonal status as well as several physiological processes in plants (Kashem *et al.*, 2000; Fukuda *et al.*, 2004). According to the works of Saleh (2013) on leaf relative water content (RWC) during salt stress he also suggests that RWC in leaf is an alternative measure of plant water status, reflecting the metabolic activity in plant tissues (Asfaw 2011, Ghogdi *et al.* 2012, Hossain *et al.* 2006, Win *et al.* 2011). The extent of salt–induced effects on relative water content has been used as one of the vital water relation parameters for assessing degree of salt tolerance in plants (Asfaw 2011, Ghogdi *et al.* 2012, Hossain *et al.* 2006, and Win *et al.* 2011). The osmotic adjustment in both roots and leaves contribute to the maintenance of water uptake and cell turgor, allowing physiological processes, such as stomatal opening, photosynthesis, and cell expansion (Serraj and Sinclair, 2002) and suggests that production of

ramified root system under drought is important to above ground dry mass and the plant species or varieties of a species show great differences in the production of roots. The importance of root systems in acquiring water has long been recognized (Jaleel *et al.*, 2009). In their review Jaleel *et al.* (2009) have given emphasis that a prolific root system can confer the advantage to support accelerated plant growth during the early crop growth stage and extract water from shallow soil layers that is otherwise easily lost by evaporation in legumes (Johansen *et al*., 1992). Similar studies have been made by various authors on the development of root system which increases the water uptake and maintain requisite osmotic pressure through higher proline levels (Djibril *et al.*, 2005), increased root growth due to water stress (Tahir *et al.*, 2002), decrease in dry weight of root under mild and severe water stress (Wullschleger *et al.*, 2005), an increase in root to shoot ratio under drought conditions related to ABA content of roots and shoots (Sharp & LeNoble, 2002; Manivannan *et al.*, 2007; Sacks *et al*., 1997). Under water limited conditions higher plant fresh and dry weights are the desirable characters in a plant. A common adverse effect of water stress on crop plants is the reduction in fresh and dry biomass production (Farooq *et al.*, 2009). Plant productivity under drought stress is strongly related to the processes of dry matter partitioning and temporal biomass distribution (Kage *et al.*, 2004). Similar studies have been made on the diminished biomass due to water stress (Tahir & Mehid, 2001; Mohammadian *et al.*, 2005), reduced biomass was seen in water stressed (Specht *et al.*, 2001; Wu *et al.*, 2008; Webber *et al.*, 2006; Lafitte *et al.*, 2007). According to Jaleel *et al.* (2009) it has been established that drought stress is a very important limiting factor at the initial phase of plant growth and establishment and suggests that in plants, a better understanding of the morpho–anatomical and physiological basis of changes in water stress resistance could be used to select or create new varieties of crops to obtain a better productivity under water stress conditions. Similar views are expressed by Nam *et al.* (2001), Martinez *et al.* (2007) in their study. Understanding plant responses to drought is of great importance and also a fundamental part for making the crops stress tolerant (Reddy *et al*., 2004; Zhao *et al.*, 2008).

Katerji *et al.* (1994) illustrated the salinity effect on water stress and early seedling growth by determining leaf water potential, stomatal conductance, leaf area, and dry matter production. They suggested that the classical explanation of water stress in plants growing in a saline environment is the reduced availability of soil water due to its osmotic potential and the reduction in root growth may provide a supplementary explanation. They also suggested that it is possible that the reduction in root development and a delay in the exploration of soil at greater depth cause less water and fewer nutrients to be available for the plant. The overall effect of salinity on plants is the eventual shrinkage of leaf size, which leads to death of the leaf, and finally the plant. Salinity may also cause reduced ATP and growth regulators in plants (Allen *et al.,* 1994). The salt causes a slower rate or shorter duration of expansion of cells and this compromises the size of the leaves (Volkmar *et al.*, 1998). Volkmar *et al.* (1998), Hasegawa *et al.* (2000), suggested that the cause of growth reduction under salinity stress include ionic imbalances, changes in nutrient and phytohormonal status, physiological processes, biochemical reaction, or a combination of such factors accompanied by a reduction in photosynthesis (Sultana *et al.*, 1999).

Glenn *et al.* (1999) suggests that at higher concentrations, salinity causes both hyper ionic and hyper osmotic stress effects and the consequences of which can be extensive reduction in growth. Several workers showed that salt stress also manifests as an oxidative stress, which contribute to its deleterious effects (Gueta–Dahan *et al.*, 1997; Hernandez *et al.*, 2001; Rios–Gonzalez *et al.*, 2002) on plants directly by oxidation of proteins, lipids and cellular components or indirectly by the formation of secondary reactive compounds in the aqueous media. The toxic effects of salt can occur at relatively low concentrations, depending on the plant species, so the homeostasis of sodium is important for the tolerance of organisms to salt stress. The stress caused by ion concentrations allows the water gradient to decrease, making it more difficult for water and nutrients to move through the root membrane (Volkmar *et al.*, 1998). In turn, the water uptake slows, and as the osmotic effect spreads from the root membrane to the internal membranes, the ion concentration inside the plant alters the solute balances (Volkmar *et al.*,

1998). Once high concentrations of salt have reached the inside of the plant, tissue and organs development is altered. Drought and salinity are connected because in many regions, raising plants requires irrigation. The irrigation water contains calcium, magnesium, and sodium (Serrano *et al.*, 1999); they suggest that as the water evaporates and transpires calcium and magnesium transpire, leaving sodium dominant in the soil. The uptake of ground water by plant roots can increase the salinity of ground water or the soil around the roots due to the exclusion of salt (Niknam and McComb, 2000). These variable conditions make research difficult, and this is compounded by the fact that each species has its own level of salt tolerance.

As suggested by Serrano (1999) the response of plants to salt stress is based on the transcriptional action of many defense proteins, and research has not discovered the basis for them all. According to the study made by him, osmotic stress and ion toxicity are the problems stemming from salt stress, and the resulting decrease in chemical activity causes cells to lose turgor. Cell growth depends on turgor to stretch the cell walls, and lack of turgor implies danger for cell survival. The plant's defense against this salinity attack requires osmotic adjustment, and, to a certain degree, this can be done through synthesis of intracellular solutes (Serrano *et al.*, 1999). In addition to their role in cell water relations, organic solute accumulation may also help towards the maintenance of ionic homeostasis and of the C/N ratio, removal of free radicals, and stabilization of macromolecules and organelles, such as proteins, protein complexes and membranes (Bohnert and Shen, 1999, Bray *et al.*, 2000). Maggio *et al.* (2001) pointed out that at low salt concentrations, yields are mildly affected or not at all but as the concentrations increase, the yields moved towards zero. In fields, the salt levels fluctuate seasonally and spatially, and variation will occur due to the circumstances influencing each particular plant. This variability makes research difficult. Together, it will be a complicated process to match plants with their optimal growing conditions.

The effect of the drought on given plant species depends on variety, intensity and duration of the stress as well as on the developmental stage (Simova–Stoilova, 2008). According to them, it was interesting to observe the antioxidative protection of the same varieties under drought at early seedling

stage in order to evaluate the usefulness of oxidative stress parameters as an additional screening criterion for detecting water stress tolerance or sensitivity in plants. In addition to the toxicity caused by ions, salinity and osmotic stress cause an imbalance of nutrients in plants (Cavagnaro *et al.*, 2006; Song *et al.*, 2006). Simova–Stoilova (2008) studied the oxidative stress and the response of the protective systems to soil drought and subsequent re–watering in an early growth stage of wheat varieties differing in their field drought resistance. Ayala–Astorga and Alcaraz–Meléndez (2010) pointed out that salinity creates the specific problem of ion toxicity, because a high concentration of sodium is bad for the cells.

2.2. Biochemical responses of plants to osmotic stress

Among several methods used to characterise internal plant water status, RWC is an integrative indicator (Parsons and Howe, 1984) and was used successfully to identify drought resistant cultivars (Matin *et al.*, 1989). Sinclair and Ludlow (1985) proposed that leaf relative water content (RWC) was a better indicator of water status than water potential. Singh *et al.*, (1990) observed significant differences in water potential among wheat genotypes under drought stress. It is suggested that decrease of relative water content close stomata and blocking of stomata will reduce photosynthesis rate (Cornic, 2000). Decrease in relative water content in stressed plants was observed in groundnut (Goplakrishna, 2001). Relative water content (RWC) is easier to measure, but not valid when osmotic adjustment occurs (Lafitte, 2002). RWC, although a convenient and widely used method of assessing plant water status, is not useful for salt–treated plants, at least not with the conventional method of detaching leaves and rehydrating on distilled water. This is because in most plants, osmotic adaptation has occurred; i.e. the solute content of cells is higher in saline than non–saline conditions, due largely to the accumulation of Na^+, Cl^-, and also to organic solutes. The increased solute content of the cells in the salt–treated plants causes more water to be taken up than in the control leaves, resulting in an apparent low RWC in the salt treatment as reported by Munns (2006). Leaf water potential is considered to be a reliable parameter for quantifying plant water stress response (Ghobadi *et al.*, 2011). The study by

Sharada and Naik (2011) showed decrease in the RWC under severe drought stress in both the groundnut genotypes.

The degree of lipid peroxidation induced by ROS accumulation is assessed by determining the MDA content in the leaf. Measurement of the concentration of thiobarbituric acid reactive substances (TBARS) such as malondialdehyde (MDA) is routinely used as an index of lipid peroxidation under stress conditions (Lokhande *et al.* 2011). Lipid peroxidation requires active O_2 uptake and involves the production of superoxide radical (O_2^-). It has been demonstrated that salt treatment increases lipid peroxidation or induce oxidative stress in plant tissues (Mittal and Dubey, 1991; Hernandez *et al.*, 1994). This leakiness of membranes is caused by an uncontrolled increase in free radicals, which cause lipid peroxidation (Smirnoff, 1993). The changes in lipid peroxidation were significant but not drastic in *B. maritima*, like in salt–tolerant cotton (Gossett, 1994) and unlike salt–tolerant wild tomato (Shalata and Tal, 1998). However, Bor *et al.* (2003) in their study concluded that the proportional and rational contributions of lipid peroxidation and antioxidative enzyme activities differ in salt tolerant plants. Hence, constitutive and/or induced activity of SOD and other antioxidants such as POX, APOX, CAT and GR is essential. Chen *et al.* (2000) suggested that generation of reactive oxygen species (ROS) lead to lipid peroxidation. Cell membranes are one of the first targets of many plant stresses and it is generally accepted that the maintenance of their integrity and stability under water deficit conditions is a major component of drought tolerance in plants (Bajji *et al.*, 2002). Development of oxidative stress is a result of the imbalance between the formation of reactive oxygen species (ROS) and their detoxification (Mittler 2002). Damage to fatty acid could produce small hydrocarbon fragments including malondialdehyde (MDA) (Alscher *et al.*, 2002), a measure of lipid peroxidaion.

Lipid peroxidation has been found to be associated and correlated to the activity of several antioxidative enzymes such as CAT, APX, SOD, GPX, POX; tolerance to oxidative stress and change in the content of MDA during various kinds of stresses have been studied by many authors. Esfandiari *et al* (2011) have determined that the increase in the concentration of MDA in

higher salt levels due to the low activity of SOD and GR or CAT was not a critical factor for the damage of oxidative stress. Workers like Pandey *et al.* (2010) have also reported increased levels of MDA content in *Avena* during drought stress and thus lipid peroxidation determination of a plant or a cultivar serves as important criteria for the stress studies in plants.

The genetic variation in CMS has been quantified by conductivity meter in different crop plants including wheat (Shanahan *et al.,* 1990). Bajji *et al.* (2002) in their work on the use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat suggested that the extent of the cell membrane damage not only correlated well with the growth responses of wheat seedlings belonging to various cultivars to withholding water but also with the recognised field performances of these cultivars. According to their study, varying the stress conditions influenced both the percent and the kinetics of electrolyte leakage during rehydration and electrolyte leakage exhibited a characteristic pattern reflecting the condition of cellular membranes (repair and hardening). The extent of the cell membrane damage not only correlated well with the growth responses of wheat seedlings belonging to various cultivars to withholding water but also with the recognised field performances of these cultivars Bajji *et al.* (2002). Enhanced water retention and cell membrane stability in tolerant wheat genotypes have also been observed in other studies (Gupta and Gupta, 2005). It has been reported by Helal and Abdel–Aziz (2008) that membranes are subjected to damage rapidly with increasing water stress. It was hypothesized that modulation of the activities of these enzymes at early growth stage may be important in imparting resistance to a plant against environmental stresses therefore, the same authors have studied the relative significance of antioxidative enzymes, MDA, H_2O_2 content, proline, glycine betaine accumulation, photosynthetic activity and membrane permeability at seedling stage in drought–tolerant and susceptible maize genotypes. They suggested that lower cell membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions according to their research findings.

The CMS has been extensively used as selection criterion for different abiotic stresses including drought and high temperature in sorghum (Premachandra *et al.,* 1992), mustard (Hashem *et al.,* 1998), rice (Tripathy *et al.,* 2000), wheat (Blum *et al.,* 2001; Rahman *et al.,* 2006a) and cotton (Ullah *et al.,* 2006; Rahman *et al.,* 2006b).The change in biological membranes stability is a key indicator of cellular damage. Drought and other stresses always results in cellular membrane injures including the increase of membrane permeability (Esfandiari *et al.* 2011, Senadheera *et al.* 2012) and therefore serves as an important parameter for testing the plant for its capacity to avoid or resist the stress related changes.

The limitation of photosynthesis under drought through metabolic impairment is more complex phenomenon than stomatal limitation and mainly it is through reduced photosynthetic pigment contents in sunflower (Reddy *et al.,* 2004). Chaves *et al.* (2009) suggests that the photosynthetic response to drought and salinity stress is highly complex which involves the interplay of limitations taking place at different sites of the cell/leaf and at different time scales in relation to plant development and the intensity, duration and rate of progression of the stress will influence plant responses to water scarcity and salinity, because these factors will dictate whether mitigation processes associated with acclimation will occur or not.

Havaux (1998), Asada (2006), and Kiani *et al.* (2008) suggests that water stress, among other changes, has the ability to reduce the tissue concentrations of chlorophylls and carotenoids, primarily with the production of ROS in the thylakoids (Niyogi, 1999; Reddy *et al.*, 2004); however, reports dealing with the strategies to improve the pigments contents under water stress are entirely scarce. Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers and both the chlorophyll a and b are prone to soil drying (Farooq *et al.*, 2009). Anjum *et al.* (2003) and Farooq *et al.* (2009) have reported that drought stress produced changes in the ratio of chlorophyll ‘a’ and ‘b’ and carotenoids during drought stress. Chlorophyll b content increased in two lines of okra, whereas chlorophyll a remained unaffected resulting in a significant reduction in Chl. a: b ratio in both cultivars under water limiting regimes (Estill *et al.*, 1991; Ashraf *et al.*,

1994). According to Jaleel *et al.* (2009) water deficit is one of the major abiotic stresses, which adversely affects crop growth and yield where the changes are mainly related to altered metabolic functions, one of those is either loss of or reduced synthesis of photosynthetic pigments which results in declined light harvesting and generation of reducing powers, which are a source of energy for dark reactions of photosynthesis and these changes in the amounts of photosynthetic pigments are closely associated to plant biomass yield.

According to Mafakheri *et al.* (2010) a decrease of total chlorophyll content with drought stress implies a lowered capacity for light harvesting and since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments. Oxidative stress under drought is a consequence of the inhibition of photosynthetic activity and the resulting exposure of chloroplasts to excess excitation energy and increased activated oxygen formation via the Mehler reaction (photoreduction of O_2 yielding superoxide radical) along with decrease in photorespiratory H_2O_2 production in peroxisomes (Smirnoff 1993). The reduction in growth of salinized plants may be related to salt–induced disturbance of the plant water balance, and in the extreme to a loss of leaf turgor which can reduce leaf expansion and therefore, photosynthetic leaf area (Erdei and Taleisnik, 1993, Huang and Redmann, 1995).

The physical properties of the photosynthetic apparatus are of crucial importance in desiccation tolerant plants. In general photosynthetic apparatus is very sensitive and liable to drought stress injury. Decline in the chlorophyll content of leaves during abiotic stress was reported by several earlier workers (Ramanjulu *et al*.1998). Earlier works by various authors on salinity stress suggest that salt stress can affect growth, dry matter accumulation and yield and that dry mass of plants is reduced in proportion to the increase in salinity (Sultana *et al* and Pardossi *et al.*, 1999; Asch *et al.*, 2000; Romero–Aranda *et al.*, 2001).

The imbalance caused by salinity affects the nutrients involved in protein synthesis and those involved in photosynthesis, which can lead to inhibition of these processes (Vieira–Santos *et al.*, 2001), as well as to the

degradation of pigments chlorophyll *a* and *b* (Di Martino *et al.,* 2003). Other important sites of ROS production, especially of hydrogen peroxide, are peroxisomes (sources–photorespiration and fatty acid β–oxidation), plasmalemma and cell walls (Mittler, 2002). The mitochondrial respiration is also activated under stress (Mittler, 2002). According to Omami *et al.* (2004) salt may affect growth indirectly by decreasing the rate of photosynthesis and photosynthesis may decrease due to stomatal closure or by a direct effect of salt on the photosynthetic apparatus. Drought stress is a complex syndrome involving not only water deprivation but also nutrient limitation, salinity, and oxidative stresses, close to conserve water as available soil water declines (Luna, 2005). He further suggests that the process of stomatal closure and the enhancement of flux through the photo respiratory pathway increase the oxidative load on the tissues as both processes generate reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2). Nayyar and Gupta (2006) suggested that water stress inhibits the photosynthesis of plants, causes changes in chlorophyll contents and components and damage to the photosynthetic apparatus. Moreover, levels of light that is optimal for photosynthesis in well–watered plants become excessive in plants suffering water deprivation. Greater oxidative load on chloroplasts and mitochondria under drought stress was previously reported (Munné–Bosch and Lalueza, 2007).

Electron transport chains in chloroplasts and mitochondria and excited chlorophyll are the most active intracellular producers of ROS such as superoxide anion radical and singlet oxygen (Simova–Stoilova, 2008). If not quenched, the above mentioned ROS can be converted to the highly toxic hydroxyl radical that can randomly damage cell membranes, proteins, and nucleic acids. Massacci *et al*. (2008), Kiani *et al.* (2008), Tahkokorpi *et al.* (2007) and several others have reported a reduction in chlorophyll content during drought stress. The foliar photosynthetic rate of higher plants is known to decrease as the relative water content and leaf water potential decreases (Lawlor & Cornic, 2002). However, the debate continues as, whether drought mainly limits photosynthesis through stomatal closure or through metabolic impairment (Lawson *et al*., 2003; Anjum *et al.,* 2003). Plant tissues also

contain substantial amounts of carotenoids that serve as non–enzymatic oxygen radical scavengers (Young and Britton, 1990) and reported that carotenoid content in case of the tolerant variety of maize showed an increase when they were subjected to water stress and they found a general decrease in the carotenoid content in leaves of their plant with increasing times of water stress, indicating that such stress resulted in carotenoid degradation as a whole.

Carotenoids have additional roles and partially help the plants to withstand adversaries of drought (Jaleel, 2009). Higher carotenoid content in the tolerant genotype of wheat under drought stress has also been reported by Sairam *et al.* (1997/98). Khalil *et al.* (2010) suggested that drought stress made chloroplast break down and the amount of chlorophyll decrease, therefore formation of chlorophyll a, b and carotenoids decrease. Plant tissues also contain substantial amounts of carotenoids that serve as non–enzymatic oxygen radical scavengers (Young and Britton, 1990). According to Jaleel *et al.* (2009) of the two photosynthetic pigments classes, carotenoids show multifarious roles in drought tolerance including light harvesting and protection from oxidative damage caused by drought and they conclude that increased contents specifically of carotenoids are important for stress tolerance. Tocopherols, another antioxidant present in the chloroplants plays an important role under stress conditions in their study on tobacco (Tanaka *et al,* (1999). Similar reports on the antioxidant property of tocopherol in the plants during stress are given by DellaPenna & Pogson (2006).

2.3. Antioxidative enzymes and antioxidants involved in drought and salinity

Drought stress not only affects cell water potential, induces closure of stomata and a decrease in photosynthesis, nitrate assimilation and various anabolic enzyme reactions (Sairam, 1994; Zlatev *et al.*, 2006) but also induces the generation of active oxygen species, such as superoxide radical, hydrogen peroxide and hydroxyl radical, causing lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Pompelli *et al*. 2010). These reactive oxygen species (ROS) include superoxide anion (O_2^{-}.), hydroxyl radical ($HO^{\cdot}$),

hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Asada 1999), which mediate the degradation of membrane components, the oxidation of protein sulfydryl groups, the formation of gel–phase domains and the loss of membrane function (Quartacci *et al.*, 1995; Sgherri *et al.*, 1996; Navari–Izzo *et al.*, 1999).

Green and Fluhr (1995) in their study identified H_2O_2 as a second messenger in abiotic as well as biotic stress. Environmental stresses are known to induce H_2O_2 and other toxic oxygen species production in cellular compartments and result in acceleration of leaf senescence through lipid peroxidation and other oxidative damage. Bienert (2006) studied the membrane transport of hydrogen peroxide in his work. According to Cheeseman (2007) the relationship between plants and hydrogen peroxide is a challenging one: H_2O_2 has many essential roles in plant metabolism but at the same time, accumulation related to virtually any environmental stress is potentially damaging. In his study he has considered H_2O_2 physiology broadly, both as a stress and as a developmentally and physiologically important metabolite, including its sources and mobility, and the vexing question of tissue level concentrations and considered problems associated with H_2O_2 as a signaling molecule, including mechanisms of H_2O_2 sensing, signaling, and response networks according to the advances in transcript network modelling, and complex systems approaches to understanding the interactions between the transcriptome, proteome and metabolome in responses to H_2O_2.

According to Suzuki *et al.* (1997), Haddad (2004) and Pryor *et al.* (2006) hydrogen peroxide (H_2O_2) which can result from superoxide (O_2^{*-}) is also a very reactive species. Both of these substances are needed as cell regulators but can be toxic as well. Hydrogen peroxide is also generated as a secondary messenger in abscisic acid (ABA)–mediated stomatal closure (Pei *et al.*, 2000). Schroeder *et al.* (2001) suggest that hydrogen peroxide is also considered to fulfil a signalling role in guard cells through the control of stomatal closure. The role of H_2O_2 in stress–induced damage has long been recognized, but it is now also generally accepted that H_2O_2 can be considered an integral component of cell signalling cascades (Mittler, 2002; Vranova *et al.*, 2002) and an indispensable second messenger in biotic and abiotic stress

situations (Pastori and Foyer, 2002). According to Halliwell *et al.* (2000) hydrogen peroxide is the two electron reduction product of O_2. It is potentially reactive oxygen, but not a free radical. Cheeseman (2007) suggests that by comparison with superoxide, $O_2^{\bullet}$ and certainly by comparison with the hydroxyl radical, $^{\bullet}OH$, H_2O_2 is relatively "safe": in the absence of transition metals, it is stable and unreactive, even at concentrations much higher than a biological system would ever generate and suggests that functionally, this imparts on it greater mobility within tissues, and potential utility not only as a substrate in a variety of reactions, but as a molecule for ROS–related signaling.

More recently H_2O_2 has been reported to be intimately involved in a wide range of hormone–dependent developmental signalling processes, as well as in cell wall cleavage and associated cell wall growth (reviewed by Foyer and Noctor, 2003). Sairam *et al* (2002), Fedina *et al* (2003) have reported the importance of hydrogen peroxide accumulation in leaf of salt–stressed plants.Other workers like Kohler *et al.* (2003) also suggest hydrogen peroxide fulfils a signalling role in guard cells through the control of stomatal closure. Luna *et al.* (2005) concluded that CAT regulation serves to limit excessive H_2O_2 accumulation while allowing essential signalling functions to occur. H_2O_2 being a strong oxidant can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity resulting in senescence promotion (Upadhyaya *et al.*, 2007). Hydrogen peroxide (H_2O_2) is a non–radical reactive oxygen species (ROS), produced in a two–electron reduction of molecular oxygen. Several sites have been recognized as H_2O_2 sources, including organelles (mitochondria, peroxisomes and chloroplasts), the apoplastic and the plasma membrane as well as cell–wall associated enzymes (various NADPHoxidases and peroxidases). The overproduction of H_2O_2 has been observed in plants exposed to a number of stress conditions and is considered as one of the factors causing oxidative stress (Snyrychova, 2009). It might be asked why regulation of CAT has this high degree of complexity. The answer must reside in the requirement of a precise control of leaf H_2O_2 levels.

Plant processes are regulated by ROS. Developmental processes, stress responses and biotic interactions regulated by ROS include root growth,

elongation and gravitropism, stress tolerance and systemic acquired acclimation (SAA), tracheary elements development (TE), senescence, hypersensitive response (HR) to pathogens, systemic acquired resistance (SAR) and plant–plant allelopathic interactions. (Gapper and Dolan, 2006; Bais *et al.*, 2003; Alvarez *et al.*, 1998). Much of the ROS generated in photosynthetic plant cells is produced in chloroplasts. Chloroplasts produce singlet oxygen ($^1O_2$) from the excited triplet state chlorophyll (primary source Photosystem II, PSII) and superoxide anion (O^{-2}) in the Mehler reaction (primary source PSI). Mitochondria produce O^{-2} due to electron leakage from the mitochondrial electron transport chain. O^{-2} from both organelles is then rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). H_2O_2, in turn, is detoxified by ascorbate peroxidases (APX) with the ascorbate (AsA) as an electron donor. AsA, oxidized to monodehydroascorbate radical (MDA^-) and eventually to dehydroascorbate (DHA), is then recycled in the Halliwell–Asada pathway (Dat *et al.*, 2000) via a series of enzymatic reactions involving monodehydroascorbate reductase (MDHAR), reduced ferredoxin (Fd), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and non–enzymatic antioxidant glutathione (reduced form GSH, oxidized form GSSG). Peroxisomes and glyoxysomes produce large amounts of H_2O_2 during photorespiration and fatty acid oxidation, respectively. This H_2O_2 is rapidly scavenged by catalases (CAT). The excess H_2O_2 leaking into cytosol from different compartments is metabolized by various peroxidases or may eventually be transported and detoxified into the vacuole.

The detoxification of superoxide radical and hydrogen peroxide is consequently of prime importance in any defense mechanism. ROS brings about inhibition of protein synthesis or causes protein denaturation (Schwanz *et al.,* 1996, Sgherri and Navari–Izzo, 1995) which may be related to a decrease in the number of polysomes (Creelman *et al.,* 1990). Mittler (2002) suggested that due to the short living period of ROS, the damage effects are usually restricted at the sites of their production. In this respect, the antioxidant protection in plant cells is complex and highly compartmentalized, comprising enzymic and non–enzymic components. Maevskaya and Nikolaeva (2013) suggest that the balance between ROS generation and scavenging has a

decisive role for plant survival under stressful conditions. Since oxidative stress is an imbalance between antioxidants and ROS it is as well a toxic mechanism as suggested by Halliwell and Whiteman (2004), Pryor *et al.* (2006) and Halliwell (2007). Oxidative stress causes damage to proteins, lipids, carbohydrates or nucleic acids when those substances are exposed too much to oxidizing gases or when there is not enough protection against oxidants (Pryor *et al.*, 2006). Many researchers like Foyer *et al.* (1997), Sgheeri *et al.* (2000), Srivalli (2013), Polesskaya *et al.* (2007) and Cruz de Carvalho (2008) in their studies have reported a direct correlation between the degree of antioxidant system activity and plant tolerance to drought. However, currently it is shown that the antioxidant response to drought depends on the plant species and cultivar, their physiological state, the degree and duration of stress.

Several workers have done work on the different trends of changes in antioxidative enzyme activities depending on the mode of imposition, duration and severity of the drought stress (Sgherri *et al.*, 2000, Sairam and Srivastava 2001). Due to the short living period of ROS, the damage effects are usually restricted at the sites of their production (Mittler 2002). Other workers like Nayar and Kaushal (2002) also reported that the increased activity of CAT and POX enzymes constitute potential defense mechanism against chilling induced oxidative damage in germinating wheat grains, they further suggested that catalase activity increased under water stress conditions in both tolerant and susceptible genotypes concentrations of catalase and ascorbate peroxidase might have removed the $O2^{\bullet -}$ radicals and its product H_2O_2 induced by water stress which is also suggested by Sairam *et al.* (2000) and Gupta and Gupta (2005). The enzymes superoxide dismutase, a family of enzymes catalysing the dismutation of superoxide anion radical to hydrogen peroxide in organelles and in the cytosol; catalases in peroxisomes which remove the bulk of hydrogen peroxide generated in photorespiration, and peroxidases with broad specificities located in vacuoles, cell walls and the cytosol which use hydrogen peroxide for substrate oxidation play central role in the defense against ROS. The activities of these enzymes and their transcripts are altered when plants are subjected to stress as suggested by Li *et al.* (1998). CAT and APX appear to

play an essential protective role in the scavenging processes when coordinated with SOD activity, they scavenge H_2O_2 generated primarily through SOD action Massacci *et al.* (1995). The activity of SOD is an essential component of these defense mechanisms as it dismutates to produce H_2O_2 and O_2 (Scandalios, 1993); Allen, 1995). Feng *et al.* (2004) reported an increased SOD and CAT activities for a mild water deficit, whereas Guo *et al.* (2006) pointed that severe or prolonged drought stress caused a decline in activities of this enzyme. In this context, Mandhania *et al*. (2006) found that activities of CAT and APX increased with increasing the salt stress in both salt tolerant and salt sensitive wheat cultivars and GR has been suggested to play a pivotal role in the glutathione cycle in the eukaryotic cells. In this respect, the antioxidant protection in plant cells is complex and highly compartmentalized, comprising enzymic and non–enzymic components (Simova–Stoilova *et al.*, 2008). These authors in their study on antioxidative protection in wheat varieties under severe recoverable drought at seedling stage reported that peroxidase (GPX) activity increased, whereas superoxide dismutase (SOD) activity only slightly changed and in recovery CAT activity became significantly higher whereas GPX activity diminished. They were able to reveal three isoforms of SOD, one of catalase and three of GPX. They also determined that SOD activity was little changed as a consequence of drought stress and CAT activity was very low in drought–treated plants and after recovery it was significantly higher than that of control; GPX activity was significantly higher under severe drought and diminished again during recovery and they postulated that it paralleled the changes in electrolyte leakage which was in accordance with the membrane–stabilizing function of this enzyme.

To evaluate the degree of tolerance to NaCl, changes in growth parameters as well as activities of the antioxidant enzymes (SOD, CAT, APX and GR) were monitored by El–Bastawisy (2010). He reported an enhanced degradation of H_2O_2 formed in tolerant wheat either directly from the oxidative stress or as a result of SOD activity; suggesting the faster elimination of ROS in tolerant variety than in the susceptible one. Moreover, he suggested that decreased APX activity in susceptible wheat would result in higher accumulation of H_2O_2 than in the tolerant variety and that such accumulation

could result from a decrease in CAT activity with a consequence shortage in H_2O_2 degradation and/or a decrease in APX activity with inefficiency in H_2O_2 scavenging by AsA. Sharada and Naik (2011) in their study on drought stress in groundnut also determined that the antioxidant enzymes activity increased considerably with the progression of drought stress which enhanced the activities of antioxidative enzymes like SOD, CAT, POX and GR. Researchers like Hameed *et al.* (2013), Kranner *et al.* (2006) also have suggested that plants have evolved both enzymatic and nonenzymatic systems to scavenge the ROS where enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), non–specific (guaiacol) peroxidases (POD), glutathione reductase, *etc*., work in concert with non–enzymatic antioxidants such as glutathione and ascorbate to detoxify ROS and have concluded that the antioxidant defense system may have a crucial role in signaling and execution of plant programmed cell death.

Ascorbic acid is a small, water–soluble anti–oxidant molecule which acts as a primary substrate in the cyclic pathway for enzymatic detoxification of hydrogen peroxide. In addition, it acts directly to neutralize superoxide radicals, singlet oxygen or superoxide and as a secondary anti–oxidant during reductive recycling of the oxidized form of α–tocopherol, another lipophilic anti–oxidant molecule (Noctor and Foyer, 1998). The low molecular antioxidant compounds, such as ascorbate and glutathione, are present in the tissues in millimolar concentrations (Noctor and Foyer 1998).

Ascorbate is the major primary antioxidant that reacts directly with hydroxyl radical, superoxide anion radical and singlet oxygen, as well as secondary antioxidant in the ascorbate–glutathione pathway. Glutathione is the predominant non–protein thiol, redox buffer, and substrate for keeping the ascorbate in reduced form in the ascorbate–glutathione pathway (Noctor and Foyer 1998). In addition ascorbate can act directly as a free radical scavenger, thus, changes in enzymatic and non–enzymatic antioxidative systems may be used to assess effects of drought stress Bowler (1991). Usually enhanced anti–oxidative protection is related to better drought resistance (Sairam and Srivastava 2001). Shalata and Neumann (2001) in their study concluded that there appear to have been no quantitative investigations of the effects of an

additional supply of ascorbic acid on plant resistance to sever salt stress. Dalmia and Savhney (2004) observed an involvement of antioxidant metabolites in ROS detoxification under drought with increased pools of ascorbate and glutathione at the beginning of the water stress and diminution when the stress becomes more severe was observed. Simova–Stoilova (2008) suggested that the ratio between reduced and oxidised ascorbate was more or less conserved, they suggest participation of the low–molecular antioxidative compounds in the defense against ROS under severe drought and rather good functionality of the ascorbate/glutathione cycle, which allowed wheat plants to maintain a low hydrogen peroxide level.

The potential of phenolics to act as antioxidants is mainly due to their properties to act as hydrogen donators, reducing agents and quenchers of singlet O_2 (Rice–Evans *et al.*, 1997). According to Bravo (1998) high accumulation of phenolics at the reproductive stage occurs due to their putative role in reproduction. The synthesis of phenolics is generally affected in response to different biotic/ abiotic stresses including salinity as suggested by Parida *et al.* (2004) and Singh (2004) who in their study determined that tolerant genotypes of chickpea showed a higher level of total phenols, whereas a significant reduction was observed in susceptible genotypes. According to Michalak (2006) phenolics, especially flavonoids and phenylopropanoids are oxidized by peroxidase, and act in $H_2O_2^-$ scavenging, phenolic/Ascorbate/POX system and their antioxidant action resides mainly in their chemical structure. They conclude that there is some evidence of induction of phenolic metabolism in plants as a response to multiple stresses (including heavy metal stress). Although the reduced forms of phenolic compounds act as antioxidants, the oxidized ones (phenoxyl radicals) may exert cytotoxic, pro–oxidant activity when the lifetime of the radicals is prolonged by effectors of spin–stabilization and it was also true for other natural antioxidants like vitamin C, vitamin E and carotenoids (Rietjens *et al.*, 2002). In plants phenolic compounds are structural polymers, which can act as antioxidants, attractants, defence responses and UV screens.

Hydroxycinnamic acids are the most widespread group of phenolic acids with four major phenolic acids in plants: ferulic acid (FA), sinapic acid

(SA), caffeic acid (CA) and p–coumaric acid (PCA). CA is the most abundant PA in fruits with more than 75 % of total phenols and is found in all parts of the fruit (Manach *et al*., 2004). Highest amounts of FA are present in cereals with up to 90 % and more of total phenols (Rondini *et al*., 2004, Manach *et al.*, 2004). According to Engert (2011) the antioxidative ability of PAs is to inhibit lipid oxidation by trapping peroxyl radicals. One way is that the antioxidant like a phenolic compound containing an active hydrogen atom (AOH) spends the hydrogen proton to the radical (R* or ROO*) and the generation of new radicals by chain reaction is stopped in a termination reaction. The antioxidant itself becomes a radical (AO*), which is chemically a lot stabler than the initial radical. The resulting free radical doesn't participate in the chain propagation and diffuses away. It can be reduced by glutathione or ascorbate to its starting substance (Parr and Bolwell, 2000, Koltover, 2010). Another way is to "spend" an electron to the radical (RO*) when the antioxidant itself is a radical (AO*) by radical–radical coupling to form an unreactive addition product (ROOA) (Cupett *et al*., 1997, Parr and Bolwell, 2000).

Konyalioglu (2005) determined that there was a correlation between the amount of total phenol and flavonoid contents and the antioxidant capacity but they were uncertain as to which of the phenols and flavonoids exhibit the greatest antioxidant effect. Phenolic compounds are important at post germination process for lignification of seedlings (Mng'omba *et al.*, 2007) The differential response of plants in phenolic accumulation at different growth stages may bendue to the reason that the accumulation of phenolics depends on plant growth stage (Choi *et al.*, 2006; Barros *et al.*, 2007). Reduced phenolic contents were observed in *Cynara cardunculus* leaves under saline conditions by Falleh *et al.* (2008). Hichem *et al.*, (2009) reported that such variation in concentration of leaf phenolics within a plant under salt stress in relation to leaf age may be due to the reflection of different requirements for counteracting abiotic stresses at different growth stages. Leaf phenolic contents are important protective components of plant cells Ashraf *et al.* (2010).

Tepe *et al.* (2005) suggests that the DPPH test intends to measure the hydrogen atom or electron donor capacity of the extracts to the stable radical DPPH formed in solution. Recent work by Lu *et al.* (2011) in the present

scenario suggest that the DPPH reagent evidently offered a convenient and accurate method for measuring the capacity of the extract to scavenge free radicals in solution, they studied the DPPH radical scavenging activities of the known antioxidative substances (BHT and gallic acid) and the extracts of their test material. The use of DPPH for a radical scavenging measuring method is described by many researchers like Yen and Duh (1994), Yordanov and Christova (1997), Masuda *et al.* (1999), Anderson and Padhye (2004) and Iwashima *et al.* (2005).

2.4. Metabolites rearrangements during stress

Plant metabolites embrace more than 100,000 different substances and they are commonly divided into primary and secondary metabolites (Raven *et al*., 2006). As suggested by Watzl and Leitzmann (2005) primary metabolites (macronutrients) such as carbohydrates, proteins and fats are essential nutrients for the human diet to provide energy for cellular functions. Although plant secondary metabolites or phytochemicals (Greek phyto=plant) build a wide range of chemically different substances and are ubiquitously present in plants they are not essential for humans (Young *et al.*, 2005). Pichersky and Gang (2000) suggest that in contrast to primary metabolites in plants secondary metabolites are present in very small quantities with no specific functions in plants. In plants they are biochemically active substances with the ability of defending plants against pathogens and herbivores, they are growth regulators and pigments to attract insects. Furthermore, they protect against different abiotic factors like heat, UV–light, nutrient and water deficiencies (Raven *et al*., 2006, Treutter, 2005). The most studied phytochemicals are carotenoids and phenolics (Engert and Honermeier, 2010).

Early researchers have suggested that plants overcome the negative effects of water stress also by the accumulation of compatible osmolytes, such as, in particular, sugars and amino acids (Kameli *et al.*, 1995, 1996; Sanchez *et al.*, 2008; Kuznatsov and Shevyakova, 1999) by functioning as both osmotic and osmoprotective agents and the accumulation of such metabolites contributes to the maintenance of cell osmotic balance, prevents the disintegration of cell membranes and enzyme inactivation under conditions of

water deficit. According to Martin *et al.* (1993) drought stress is a decrease of soil water potential so plants reduce their osmotic potential for water absorption by congestion of soluble carbohydrates and proline and in other words osmotic regulation is performed. Therefore osmotic regulation will help to cell development and plant growth in water stress (Pessarkli, 1999). The effect of water stress on *Triticum aestivum* L. was investigated before by Tatar and Gevrek (2008) who concluded that proline accumulation increased after lipid peroxidation content became higher and RWC content of leaves became lower, furthermore, they concluded that proline was mainly involved in protection against oxidative stress than osmotic adjustment during initial steps of water stress. Water deficit induction in plants trigger off a number of biochemical changes and consequently various stress metabolites are accumulated.

Plants during drought stress make changes in some of their physiological and biochemical features (Keyvan, 2010). Patade *et al.* (2011) suggest that plants have evolved complex mechanisms for avoiding the osmotic effects of salt and drought stress, one of them being osmotic adjustment (OA; lowering of osmotic potential, ψP, in plant tissues through accumulation of osmolytes that maintain flow of water into cells). According to them there are two types of osmolytes, organic solutes and inorganic ions which play a key role in osmotic adjustment. Organic solutes known as compatible solutes include sugars, proline, polyols, quarternary ammonium compounds like glycine betaine, and other low molecular weight metabolites serve a function in cells to lower or balance the osmotic potential of intracellular and extracellular ions to tolerate osmotic stresses. Chen and Jiang (2010)that the accumulation of compatible solute accumulation and stress mitigating effects in barley genotypes contrasting in their salt tolerance. Munns and Tester (2008), Lokhande *et al.* (2010) also suggests that the plants exposed to salt stress may use saline ions as an osmoticum; however, synthesis of compatible solute is also required to prevent ion toxicity, while plants exposed to dehydration stress solely rely on synthesis of compatible solutes for maintenance of cell turgor. Sharma and Dubey (2005) have suggested that

some of the osmolytes that accumulate in plant cells in response to stress also play a role in scavenging of free radicals and protecting enzymes.

Rosa *et al* (2009) have reported the complex network and role of soluble sugars, their metabolism, sensing during abiotic stress in the life of plants. Drought stress is a decrease of soil water potential so plants reduce their osmotic potential for water absorption by congestion of soluble carbohydrates and proline and in other words osmotic regulation is performed (Martin *et al.*, 1993). The amount of total soluble sugar/embryonic axes fresh weight increase rapidly answering to the increasing concentrations of NaCl, this result agree with the result of some researchers that indicate that salinity stress induce soluble sugar accumulation (Prado *et al.*, 2000). This is also the case with sorghum seeds; the stress causes a decrease in starch content and an increase in sugar content (Thakur and Sharma, 2005). As explained by Giorgini and Suda (1990), the higher level of soluble sugars detected is probably necessary for the turgor and growth of embryonic axes during emergence. Nevertheless, in cotyledons, sugar levels were not as large as in embryonic axes possibly as explicated by Prado *et al.* (2000); it was due of the weak development followed by a diminished metabolic activity in cotyledons. In addition, Singh (2004) proved that a greater accumulation of sugar lowers the osmotic potential of cells and reduces loss of turgidity in tolerant genotypes. The other possible role of sugar may be as a readily available energy source Dkhil and Dendon (2010). Work of several authors like Todaka *et al.* (2000), Kaplan and Guy (2004), Basu *et al.* (2007), Kempa *et al.* (2008), Madden *et al.* (1985) and Kaplan and Guy (2004) have suggested in details that the main carbohydrate store in most plants and can be rapidly mobilized to provide soluble sugars and the metabolism of carbohydrate is very sensitive to changes in the environment. In addition to diurnal fluctuations in starch levels, salt and drought stress generally leads to a depletion of starch content and to the accumulation of soluble sugars in leaves as suggested by Krasensky and Jonak (2012) sugars that accumulate in response to stress can function as osmolytes to maintain cell turgor and have the ability to protect membranes and proteins from stress damage. The increase in sugar levels accompanied by decrease in starch content in embryos and cotyledons was directly linked to the activity of

α and ß–amylases, which is in agreement with the existing reports of Monerri *et al.*, 1986; Gupta *et al.*, 1993). Kameli and Losel (1995) confirmed that this increase might be considered to play an important role in osmotic adjustment, which is widely regarded as an adaptive response to water deficit conditions.

Krasensky and Jonak (2012) have suggested that the accumulation of amino acids has been observed in many studies on plants exposed to abiotic stress which was also suggested by many early and contemporary authors like Barnett and Naylor (1966), Draper (1972), Handa *et al.* (1983), Rhodes *et al.* (1986), Fougere *et al.* (1991), Kaplan *et al.* (2004), Brosche *et al.* (2005), Zuther *et al.* (2007), Kempa *et al.* (2008), Sanchez *et al.* (2008), Usadel *et al.* (2008), Lugan *et al.* (2010). The reason for the increase in the amino acid might stem from amino acid production and/or from enhanced stress–induced protein breakdown. While the overall accumulation of amino acids upon stress might indicate cell damage in some species (Widodo *et al.*, 2009), increased levels of specific amino acids have a beneficial effect during stress acclimation.

Generally, the plants accumulate some kind of organic and inorganic solutes in the cytosol to raise osmotic pressure and thereby maintain both turgor and the driving gradient for water uptake (Rhodes and Samaras, 1994). Among these solutes, proline is the most widely studied (Delauney and Verma, 1993). The beneficial roles of proline in conferring osmotolerance have been widely reported (Kishor *et al.*, 1995; Bajji *et al.*, 2000). Compatible solutes are overproduced under osmotic stress aiming to facilitate osmotic adjustment (Hasegawa *et al.*, 2000; Shao *et al.*, 2005; Zhu, 2000). It has been shown that proline also have a key role in stabilizing cellular proteins and membranes in presence of high concentrations of osmoticum (Yancey, 1994 and Errabii *et al.*, 2006). Others workers like Shao *et al.* (2006) also determined that plant responses to salt and water stress involve many macromolecules and small compounds, such as proline. Zlatev and Stoyanov (2005) suggested that proline accumulation of plants could be only useful as a possible drought injury sensor instead of its role in stress tolerance mechanism. However, Vendruscolo *et al.* (2007) found that proline is involved in tolerance

mechanisms against oxidative stress and this was the main strategy of plants to avoid detrimental effects of water stress.

Tatar and Gevrek (2008) and Kameli and Losel (1996) showed that wheat dry mater production, relative water content (RWC) decreased and proline content increased under drought stress. Higher proline content in wheat plants after water stress has been reported by Vendruscolo *et al.* (2007) and Patel and Vora (1985). Increasing amount of proline was also established in several stress conditions such as salinity (Poustini *et al*., 2007), cold (Charest and Phan, 1990) and UV (Tian and Lei, 2007) in wheat. Proline continues to be the most studied molecule under abiotic stresses in plants. Positive correlation between magnitude of free proline accumulation and drought tolerance has been suggested as an index for determining drought tolerance potentials between cultivars (Sivaramakrishnan *et al.*, 1998: Ramanjulu and Sudhakar, 2000). Proline and quaternary ammonium compounds, e.g. glycinebetaine, choline, proline, betaine are key osmolytes contributing towards osmotic adjustment (Huang *et al.*, 2000 and Kavikishore *et al.*, 2005). In higher plants the oxygen toxicity is more serious under condition of water–deficit conditions (Helal and Abdel–Aziz, 2008). Recent work by Sharada and Naik (2011) also suggest that one of the immediate responses of plant to drought stress was found to be the accumulation of free proline who in their study found a greater pool of free proline in the drought stressed leaves of groundnut genotypes than control and suggested that proline accumulation under drought conditions by the tolerant and susceptible serves a very reliable and good index for determining drought tolerance potentials between cultivars. Researchers have now concluded and it is now well established that proline is a polyfunctional compound, which has not only protector and osmoregulatory functions but is also an antioxidant involved in the neutralization of certain types of ROS and is believed that proline ability to neutralize ROS is perhaps one of its most important functions (Chen and Dickman, 2005).

According to Saleh (2013) it is well documented that, environmental stresses cause important modification in gene expression and the gene expression is manifested by the appearance of proteins, which are not present before the stimulation. ROS brings about inhibition of protein synthesis or

causes protein denaturation (Schwanz *et al.* 1996, Sgherri and Navari–Izzo 1995) which may be related to a decrease in the number of polysomes (Creelman *et al.* 1990). Generally, drought induces metabolic changes related to protein turnover (alterations in protein synthesis, maintaining the level of some proteins or protein degradation) (Bray, 1997). The contribution of cysteine proteases to total proteolytic activity increases drastically in response to water deficit in wheat (Zagdanska and Wisnievski, 1996). The proteolytic response under drought was found to be different from that of natural senescence by Khanna–Chopra *et al.* (1999). As suggested by Saleh (2013) salinity promotes the synthesis of salt stress–specific proteins, many of these proteins were suggested to protect the cell against the adverse effect of salt stress and the accumulation of these proteins is a common response to salt stress (Kong–ngern *et al.* 2005, Mahmoodzadeh 2009, Meratan *et al.* 2008, Metwali *et al.* 2011, Mohamed 2005). High salt concentrations inhibit enzymes by impeding the balance of forces controlling the protein structure (Serrano *et al.*, 1999). Intracellular proteolysis might have an important role in the reorganization of plant metabolism under stress, however these processes remain poorly understood compared to the role of proteases in germination and senescence (Feller, 2004; Grudkowska and Zagdanska, 2004). Some experimental evidence suggests that drought–sensitive species and varieties have higher proteolytic activity compared to the resistant ones (Hieng *et al.*, 2004); however, data on proteolytic activity relation to drought sensitivity or resistance are still quite limited. According to Yordanova *et al.* (2004) changes in proteins can results from a variety of environmental stresses such as water stress. According to Fazeli *et al.* (2007) reduction of protein content in both of cultivars in leaves and roots suggests that water stress may cause generation of ROS. SDS–PAGE of protein in leaves the appearance of new and colourful protein bands under some of the stress levels suggests that these proteins may be the cause of resistance to drought in sesame however they also determined that SDS–PAGE of root proteins did not show any important differences among the treatments. Simova–Stoilova (2008) has postulated that the data on leaf protein basis reflect the relative proportion of the enzyme in the total protein content. Salekdeh *et al.* (2002) have reported that upon several stress

responses protein, protein–protein interaction and post–translation modification have been also identified (Salekdeh *et al.*, 2002). Qureshi *et al.* (2007) suggests that in the last decade, methodological improvements have allowed comparative proteomic investigations of plants under stress which have allowed us to analyse biochemical pathways and the complex response of plants to environmental stimuli. Similarly in a study made by Kamal *et al.* (2010) they have determined specific proteins induced by each abiotic stress where they have given particular emphasis on the heat shock, drought, cold, salt and others environmental stress by proteomic approaches.

2.5. Ion transporters and osmolytes

The importance of inorganic ions mainly Na^+, K^+, Ca^{2+}, and Cl^- in the contribution for osmotic adjustment has been reported by Chen and Jiang (2010). Serrano *et al.* (1999) suggests that ion transporters selectively transport ions in order to maintain physiologically relevant concentrations whereas Na^+/H^+ antiporter play a vital role in sustaining cellular ion homeostasis, thus allowing plant survival and growth under saline conditions through regulation of cytoplasmic pH, sodium levels, and cell turgor. According to Jan *et al.* (2013) an important strategy for achieving tolerance to abiotic stress is to help plants to maintain and re–establish cellular ion homeostasis during stress conditions. The membrane proteins that are involved in osmotic stress tolerance include water channel proteins and transport proteins and the water channel proteins control cellular water transport in response to drought and salt stress, while as ion transporters play an important role in salt tolerance. In their study Bartels and Sunkar (2005) suggests that there exists three types of mechanisms to prevent excess Na^+ accumulation in the symplast of plant cells which are firstly by restricting the Na^+ permeation and entry into plant cytosol by Na^+ transporters, secondly, the compartmentalization of Na^+ in to vacuole via, Na Na^+/H^+ antiporter and the third by the transport of cytosolic Na^+ back to the external medium or to the apoplast by plasma membrane Na^+/H^+ antiporter. Even though leaves accumulated high sodium concentrations, fruits displayed very low sodium content, demonstrating the potential to maintain fruit yield and quality at high salt levels (Jan *et al.*, 2013).

Shi *et al.* (2003) has suggested that plasma membrane Na^+/H^+ antiporter encoded by the SOS1 gene improves salt tolerance in transgenic *Arabidopsis* and increased salt tolerance was correlated with reduced Na^+ accumulation. Metabolic toxicity of Na^+ is largely a result of its ability to compete with K^+ for binding sites essential for cellular function (Tester and Davenport, 2003) and according to Bhandal and Malik (1988) more than 5 enzymes were activated by K^+ and Na^+ cannot substitute in this role. According to Tester and Davenport (2003), high levels of Na^+ or Na^+: K^+ ratios can disrupt various enzymatic processes in the cytoplasm. Blaha *et al.* (2000) also suggested that protein synthesis requires high concentrations of K^+, owing to the K^+ requirement for the binding of tRNA to ribosomes and probably other aspects disruption of protein synthesis by elevated concentrations of Na^+ appears to be an important cause of damage by Na^+.

According to Ghogdi *et al.* (2012) it is well documented that a greater of salt tolerance in plants is associated with a more efficient system for selective uptake of K^+ over Na^+ (Wenxue *et al.*, 2003) and under salt stress, plants maintain high concentration of K^+ and low concentration of Na^+ in the cytosol. They do this by regulation the expression and activity of K^+ and Na^+ transporters and H^+ pumps that generate the driving force for transport and the regulation of K^+ uptake, prevention of Na^+ influx, promotion of Na^+ efflux from the cell and utilization of Na^+ for osmotic adjustment are the strategies commonly used by plants to maintain desirable K^+/Na^+ ratio in cytosol (Zhu, 2003). They suggest that a high K^+/Na^+ ratio in cytosol is essential for normal cellular functions of the plants. Khan *et al.* (2000) suggests that high salt uptake competes with the uptake of other nutrient ions, especially K^+, leading to K^+ deficiency. Increase in sodium and depletion of potassium contents under salinity stress in case of wheat have been reported earlier (Ashraf and Oleary, 1996), Sairam *et al.*, 2002). Ashraf and Khanum (1997) have reported that the content of sodium is an indicator of salt tolerance in cereals. Poustini and Siosemardeh (2004) have reported that in case of wheat, salt tolerance correlates with sodium exclusion and cultivars, having low ability in this case, can be introduced as sensitive cultivars. Various other reports on the increase in the content of sodium in salt sensitive wheat cultivars have been given by

researchers like Sairam *et al.* (2002), Poustini and Siosemardeh (2004), Ashraf and Oleary (1996) who have reported that salt tolerance could be correlated with lower leaf accumulation of Na^+ who concluded that salt sensitive cultivars lacked the ability of excluding Na^+ and it can be the main reason for their salt sensitivity. Ghogdi *et al.* (2012) studied the effect of salt stress on some physiological traits of wheat (*Triticum aestivum* L.) under green house conditions and concluded that the salinity tolerance in tolerant cultivars as manifested by lower decrease in grain yield is associated with the lower sodium accumulation and higher K^+/Na^+ compared to the sensitive cultivars.

2.6. Tolerance Mechanisms

Tolerance to abiotic stresses is very complex, due to the intricate interactions between stress factors and various molecular, biochemical and physiological phenomena affecting plant growth and development (Razmjoo *et al.*, 2008). When stress avoidance mechanisms are insufficient, stress tolerance mechanisms are required to prevent cellular damage arising from dehydration or ion toxicity (Verslues *et al*. 2006). High yield potential under drought stress is the target of crop breeding. In many cases, high yield potential can contribute to yield in moderate stress environment (Blum, 1996).

Researchers like Gisbert *et al* (2000), Foolad (2004), Flowers (2004), Garcıa–Sanchez *et al* (2006), Metwali *et al* (2011), Godfray *et al* (2011), Gill and Tuteja (2012) have reported in their study the methods to improve crop tolerance, recent advances in genetics, comparative account of different types of abiotic stress, biochemical genetic markers associated with tolerance in different genotypes during abiotic stress. Specifically, there are two mechanisms commonly used by plants to tolerate high salt concentrations. Avoidance is the process of keeping the salt ions away from the parts of the plant where they are harmful. Allen *et al.* (1994) suggested that this can be done through the passive exclusion of ions by a permeable membrane, the active expelling of ions by ion pumps, or by dilution of ions in the tissue of the plant and secondly, tissue tolerance occurs when ions have already accumulated in the tissue of the plant, and they are then compartmentalized into the plant's vacuoles for storage. These two methods prevent the ions from

accumulating and causing damage to the plant. These would be ideal targets for genetic manipulation of plants to become more tolerant of saline conditions. It is reported that high relative water content is a resistant mechanism to drought, and that high relative water content is the result of more osmotic regulation or less elasticity of tissue cell wall (Ritchie *et al.*, 1990). According to Scandalios (1993), particularly SOD and CAT are the most effective antioxidant enzymes in preventing cellular damage. Antioxidative enzymes are the most important components in the scavenging system of ROS. Superoxide dismutase is a major scavenger of O_2^-; it catalyzes the dismutation reaction of superoxide radical anions into O_2 and H_2O_2. Removal of the highly toxic H_2O_2 produced during dismutation is essential for the cell to avoid the inhibition of enzymes such as those controlling the Calvin cycle in the chloroplast (Creissen *et al.*, 1994), and it can be scavenged by catalase and a variety of peroxidases. Because salinity is first perceived in the root, the root sends the signal hormone abscisic acid, which directly or indirectly down regulates the leaf expansion rate (Rausch *et al.*, 1996). Plants have several processes to respond to salt stress. A basic two–phase model describes the overall growth response to salinity as an initial water deficit lasting for a few days or weeks. Then the second phase occurs, where the ion toxicity initiates leaf death (Rausch *et al.*, 1996). All of these broad reactions to salt stress could be target systems to regulate tolerance by the plants: the structural components of the roots, ion transporters, or cell wall and membrane components (Winicov and Bastola, 1997). This overview of plants' response to salt stress broadly categorizes the cellular mechanisms, but there is more detail to the cellular reaction. The early response of plants reacting to salt that has reached their leaves is to exclude it from the cytoplasm (Volkmar *et al.*, 1998). Patade *et al* (2011) have studied the tolerance of sugarcane to salt and drought under iso–osmotic salt and water stress: growth, osmolytes accumulation and antioxidant defense mechanism of the plants. There is a continuous spectrum of plant tolerance to saline conditions ranging from glycophytes that are sensitive to salt, to halophytes which survive in very high concentrations of salt (Volkmar *et al.*, 1998). If salt does enter the plant, there are physiological and metabolic events that can counteract salt at a cellular level (Winicov, 1998).

Widodo *et al* (2009) suggest that under salt stress in barley plants show a metabolic response. Chen *et al* (2007) have also reported mitigating effects in barley genotypes contrasting in their salt tolerance by the accumulation of compatible solutes. Chinnusamy *et al* (2005) in their study suggests the methods to understand and improve salt tolerance in the plants. One means of eliminating the salt that accumulates in plant cells is through storage of the salt ions in vacuoles. This is an important adaptation of plants to salinity. Another method is allowing the salt to build up outside the cells, in the intracellular space. This leads to a gradient of water moving out of the cells to accommodate the change in ion concentration, and eventually too much water leaves the cell and the cell becomes dehydrated (Volkmar *et al.*, 1998). So, this will lead to cell death and the vacuoles comprise most of the cell volume making them good for storage, but the cytoplasm is only one percent of the cell volume making the cytoplasm very sensitive to slight changes in rate of saline transport. The same author further postulates that the rate of salt passing through the membrane must not exceed the rate of salt being collected into the vacuoles, or there will be an imbalance in the cell (Volkmar *et al.*, 1998). As older cells lose their capacity to grow and provide vacuoles, the new growth cannot handle the burden of collecting all the salt ions, this leads to premature death in the cells of leaves, and the plant will quickly succumb to the decreasing ability to compartmentalize the salt (Volkmar *et al.*, 1998). Leaf cell growth is sensitive to salt, because the energy used for compartmentalization takes energy away from cell growth (Volkmar *et al.*, 1998). The root signal tells the shoot to stop growing to conserve energy as well. Growth could be considered a means of regulating the concentration of salt, although high concentrations of salt induce inhibition of growth when the plant needs to continue growth to dilute salt concentrations and find space for vacuoles. Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species. Maevskaya and Nikolaeva (2013) drought tolerance is provided by the multicomponent system of protective mechanisms that cause rearrangements on the physiological, cellular, and molecular levels. Jaleel *et al.* (2009) has concluded in their study that drought stress affects the growth, dry mater and harvestable yield in a

number of plant species, but the tolerance of any species to this menace varies remarkably. According to them a ramified root system has been implicated in the drought tolerance and high biomass production primarily due to its ability to extract more water from soil and its transport to aboveground parts for photosynthesis. Similarly, they further state that in addition to other factors, changes in photosynthetic pigments are of paramount importance to drought tolerance.

Various tolerance mechanisms have been suggested on the basis of biochemical and physiological changes related to drought (Sgheeri *et al.*, 2000). Unfortunately, most crops are not halophytic. Studies in crops suggest that salt tolerance is a multigenic trait (Niknam and McComb, 2000), which makes it more difficult to study and improve. Tolerant species use more than one strategy to tolerate or avoid stress. It is important to keep the levels of ions low in the leaves, particularly in the young ones. This can be done by excluding the ions at the point of uptake and reducing the translocation of ions to the shoot (Niknam and McComb, 2000). The capacity of the plant leaves to accommodate the export of salt from the root is linked to the growth rate, so the ability of the plant to continue to grow would indicate a high level of salt tolerance. In order to judge the tolerance of plants to salinity, the growth or survival of the plant is measured because this is the culmination of many physiological mechanisms occurring within the plant. They further suggested that in low to moderate salinity conditions, salt exclusion is the strategy, hence, the growth and yield are measured as determinants of salt stress. However, he again suggests that under higher salinity conditions, ion toxicity becomes a cause of death, so survival is measured.

Relatively few data describe recovery from drought, especially antioxidative protection (Sgherri *et al.*, 2000, Srivalli *et al.*, 2013). Lots of information states that roots play a crucial role for short–term adaptation to salt tolerance. The concentrated salt surrounds the root membrane, and it is thought that the morphology of the roots affects the amount of salt taken into the plant (Maggio *et al.*, 2001). Some features of the root must be advantageous because they help the root take in water. Salt exclusion from the root is likely to be part of the salt tolerance found in plants. However, when

salt ions make it into the plant, they accumulate in the leaf. As stated above, it is beneficial to the cells of the leaves to compartmentalize the salt ions into the vacuoles. Important mechanisms of tolerance involve Na^+ exclusion from the transpiration stream, sequestration of Na^+ and Cl^- in the vacuoles of root and leaf cells, and other processes that promote fast growth despite the osmotic stress of the salt outside the roots. Plants respond to diverse environmental signals in order to survive stresses such as drought (Pastori and Foyer, 2002). As suggested by Gechev *et al.* (2002) plants cope with stress by activation of the cell antioxidant system. To keep the levels of active oxygen species under control, plants have non–enzymatic and enzymatic antioxidant systems to protect cells from oxidative damage (Mittler, 2002).

According to the study made by Vranova *et al.* (2002); Dalmia and Sawhney (2004) plants also possess several tissue antioxidants for protection against the potentially cytotoxic forms of activated oxygen species, such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, ascorbic acid, α–tocopherol and carotenoids. Strategies to minimize oxidative damage are a universal feature of plant defence responses. Because plants have limited mechanisms of drought stress avoidance, they require flexible means of adapting to changing drought conditions.

Among peroxidases, ascorbate peroxidases and glutathione peroxidase which use ascorbate and glutathione as electron donors, respectively, are well known for their role in H_2O_2 detoxification in plants. Glutathione reductase is responsible for the reduction of oxidized glutathione for the chain reactions of scavenging H_2O_2 by APX and GPX to be completed and continued (Mittler, 2002; Apel and Hirt, 2004). Catalase, which is only present in peroxisomes, dismutates H_2O_2 into water and molecular oxygen, whereas peroxidase decomposes H_2O_2 by oxidation of co–substrates such as phenolic compounds and/or antioxidants. It has been proposed that water–stress conditions, in particular, may trigger an increased formation of the superoxide radical and hydrogen peroxide, which can directly attack membrane lipids and inactive SH–containing enzymes (Imlay, 2003). Bor *et al.* (2003) also suggested that further investigations are necessary to put forward the effect of salt stress by means of ion distribution and osmotic

adjustment, also subcellular compartmentation of antioxidative enzyme activities would be a useful tool for our understanding of salt stress and there are additional studies being conducted and will be conducted in order to explain these phenomenon. To prevent or alleviate injuries from ROS, plants have evolved an antioxidant defence system that includes non–enzymatic compounds like ascorbate, glutathione, tocopherol, carotenoids, flavonoids and enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and polyphenol oxidase (PPO) (Sergi and Alegre, 2003; Agarwal and Pandey, 2004). The utilization of multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants (Sang *et al.*, 2005). Gradual stress imposition gives the possibility for plants to adapt cell processes to the adverse conditions (Simova–Stoilova *et al.*, 2006; Pompelli *et al.*, 2010). Non–enzymatic antioxidants including β–carotenes, ascorbic acid (AA), α–tocopherol (α–toc), reduced glutathione (GSH) and enzymes including: superoxide dismutase (SOD), guaiacol peroxidase (POD), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR) are produced in the plants in response to accumulation of reactive oxygen species in the plants tissues (Xu *et al.*, 2012, 2008).

2.7. Amelioration of drought and salinity

Ali *et al.* (2008) have reported in their extensive study that the role of different compatible solutes in plant tolerance to drought stress is significant because they regulate multitude of metabolic processes including ion transport and have hypothesized that the exogenous application of proline might regulate uptake of mineral nutrients in plants subjected to water deficit conditions. The role of exogenous application of chemicals have been studied by earlier workers during water stress in maize, sorghum and wheat (Agboma *et al*, 1997a) and tobacco (Agboma *et al*, 1997b). Previous work has shown that pre–treating plants in certain endogenous signaling compounds, or pre–exposing plants to mild heat stress, can induce thermotolerance (Gong 1997, Dat *et al*. 1998, Larkindale and Knight 2002). Various workers have reported that the use of certain exogenous chemicals on the plants before stress treatment can

induce tolerance to a much surprising level. In particular, induced thermotolerance has been reported when plants were pre–treated with salicylic acid (SA) (Dat *et al.* 1998, Larkindale and Knight 2002, Aldesuquy *et al*. 2012), abscisic acid (ABA) (Bonham–Smith *et al.* 1998, Bray 1991, Jiang and Huang 2001, Larkindale and Knight 2002), calcium (Gong *et al.* 1997) and ethylene (Larkindale and Knight 2002). Larkindale and Huang (2004) have investigated whether pre–treating plants with specific putative signaling components and heat acclimation would induce tolerance of a cool–season grass, creeping bent grass (*Agrostis stolonifera* var. *palustris*), to subsequent heat stress and whether thermotolerance induction of those pre treatments 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. Mahmood *et al* (2009) in their study on wheat have studied the relation between the application of glycinebetaine as a pre–sowing seed treatment and the growth and regulation of some key physiological attributes in wheat plants grown under water deficit conditions.

Cutler *et al* (2010) have studied abscisic acid as the emergence of a core signalling network. Tuteja (2007) have reviewed the role of ABA in plants under stress. According to his studies the main function of ABA seems to be the regulation of plant water balance and osmotic stress tolerance and suggests that under drought stress, ABA deficient mutants readily wilt and die if the stress persists and under salt stress also ABA deficient mutants show poor growth. Cuevas *et al* (2008) suggest the involvement of putrescine in *Arabidopsis* freezing tolerance and cold acclimation by the regulation of ABA levels in response to low temperature. In addition, ABA is required for freezing tolerance, which also involves the induction of dehydration tolerance genes and the stress induced gene products are also involved in the generation of regulatory molecules like ABA, salicylic acid and ethylene, which can initiate the second round of signalling (Tuteja, 2007). His studies further suggest that small molecules like ABA play important role in this process and

that the application of ABA to plant mimics the effect of a stress condition. Reports suggest that various stress signals and ABA share common elements in the signalling pathway and these common elements cross‑talk with each other, to maintain cellular homeostasis Thomashow (1999) and Shinozaki and Yamaguchi (2000). ABA level is known to induce under stress condition, which is mainly due to the induction of genes for enzymes responsible for ABA biosynthesis. Other workers like Cuin & Shabala (2007) reported that compatible solutes such as glycinebetaine (GB), proline, and trehalose have a mitigating effect on K^+ efflux in *Arabidopsis* under stressed conditions and low concentrations of these organic osmolytes have a role in osmotic adjustment due to the accumulation of inorganic ions, as exogenous application of low concentrations of glycine betaine or proline significantly reduces the extent of the stress induced K^+ efflux from barley roots. Bano and Aziz (2003) have concluded that ABA is involved in salt tolerance of plants and also in the synthesis of solutes for osmotic adjustment.

Different reports suggests that the exogenous application of proline induces abiotic stress tolerance in plants (Claussen, 2005; Ali *et al.*, 2007) although much attention has been paid on the role of proline in stress tolerance as a compatible osmolyte (MacCue & Hanson, 1990; Samras *et al*., 1995), little attention has been given to its role in affecting the uptake and accumulation of inorganic nutrients in plants (Okuma *et al.*, 2000; Khedr *et al*., 2003). Various authors like Ali *et al.* (2007), Ashraf and Foolad (2007), Haque *et al.* (2007) have suggested as well as concluded in their studies that the exogenous application of proline increases its endogenous levels in plant tissues subjected to water stress conditions which contributes to the osmotic adjustment in the plant tissues. Gadallah (1999) have suggested that the ameliorating effects of proline and glycinebetaine on growth, and plant metabolism under salt–stress still remains incompletely understood and have investigated the effects of exogenous application of proline and glycinebetaine on the growth, stability of leaf membranes, leaf relative water content, chlorophyll content and leaf osmotic potential of *Vicia faba* plants grown under salinity along with other biochemical parameters such as soluble and hydrolysable sugars, soluble proteins, total free amino acids, and some mineral

ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-) in shoots. Similar studies have been done by Heuer (2003) in tomato (*Lycopersicum esculentum* L. cv. "F144") where they have investigated the ability of exogenous compatible solutes, such as proline and glycinebetaine, to counteract salt inhibitory effects and have suggested in their studies that neither proline nor glycinebetaine were able to counteract salt stress effects in salt–sensitive fresh market tomato and also conclude that proline is toxic to the plants and may lead to plant death if added in high concentration. However, most of the reports suggest that the application of proline at low concentrations to the plants as pre–treatment or to the seeds before sowing has better results in terms of enhanced or increased levels of plant tolerance to abiotic stresses. Various earlier authors suggested that the exogenous application of proline stimulated the growth of cells in mungbean (Kumar and Sharma, 1989) and plants such as *Pisum sativum* (Fedina *et al.*, 1993), *Zea mays* (Hamed and Wakeel, 1994) and improved the metabolism of plants under stress conditions (Alia, 1991, Rana and Rana, 1996). However, negative effects were also reported by earlier researchers on the exogenous application of proline on the plants followed by stress treatments (Manetas *et al.*, 1986, Wu, 1987, Rodriguez and Heyser, 1988 and Lin and Kao, 2001). However, more contemporary workers suggest that the pre–treatment of plants with lower concentrations of proline were beneficial to the plants and the ameliorating effect of proline was promising to the plants under stress.

Kabiri *et al.* (2012) suggests that osmotic stress associated with drought, and salinity is a serious problem that inhibits the growth of plants, mainly due to disturbance of the balance between production of ROS and antioxidant defense and causing oxidative stress. From his work he suggests that the results obtained in the last few years strongly prove that salicylic acid could be a very promising and protective compound for the reduction of biotic and abiotic stresses in sensitive of crops, because under certain conditions, it has been found to mitigate the damaging effects of various stress factors in plants. In his research, salicylic acid was used in control, and drought stressed plants, and the role of this compound in reduction of oxidative damages in *Nigella* plant was investigated (Kabiri *et al.*, 2012). Sakhabutdinova *et al.* (2003) have studied character of changes in hormonal systems induced by SA

in wheat plants under stress conditions and have concluded that presowing treatment of wheat seeds with SA contributes to the increase in the resistance of plants to stress factors of environment and ABA serves as a mediator in the manifestation of the protective action of SA. According to them SA–treatment induces a sharp accumulation of ABA, which in turn is an inducer of a wide spectra of antistress reactions in plants, which is why it is likely that the effect of SA on the increase of ABA lies at the root of the pre–adaptive action of SA to possible stress situations. The influence of SA on antioxidative enzyme system has been assessed by several works with special emphasis upon abiotic stresses. Janda *et al.* (1999) showed that pre–treatment with SA for 1 d provided protection in maize plants against low–temperature stress and induced increased antioxidant activity. Ananieva *et al.* (2002, 2004) have suggested that salicylic acid mediates tolerance in barley plants to paraquat (Ananieva *et al.* 2002, 2004) and have studied the role of SA in paraquat induced responses by analyzing the capacity of the antioxidative defence system by measuring the activities of several antioxidative enzyme like SOD, APOX, GR, DHAR, CAT and POX. Maintaining a high level of ABA in SA–treated plants under stress contributes to protective reactions aimed to decrease its injurious effect on growth and acceleration of growth resumption (Sakhabutdinova *et al.*, 2003). Fragnière *et al.* (2011) also have suggested the important role SA during biotic and abiotic stress in their study. Chakraborty and Chakraborty (2008) suggested that SA plays an important role in induction of resistance against both abiotic and biotic stresses as part of the signal transduction pathway leading to systemic acquired resistance and the way of signal regulation of plant resistance to unfavourable factors of environment induced by SA is still not clear. They have concluded in their study that SA is a defense mechanism/chemical signal that has roles to play in both biotic and abiotic stresses in plant which during defense triggers a cascade metabolism leading to stress tolerance and have suggested that by continuing to investigate these roles and learning how and why they work might preserve or improve the life of various plants.

These mechanisms are the only way that plants can adapt to saline and water scarce conditions themselves, but there have been suggestions of

external manoeuvres to counteract the salinity. According to Ashraf and Foolad (2007) currently, there are no economically viable technological means to facilitate crop production under stress conditions; however, development of crop plants tolerant to environmental stresses is considered a promising approach, which may help satisfy growing food demands of the developing and under–developed countries. Development of crop plants with stress tolerance, however, requires, among others, knowledge of the physiological mechanisms and genetic controls of the contributing traits at different plant developmental stages. Researchers must decide whether to test for the ability to survive under mild salt stress and never know the full potential of the plant to grow. Subjecting plants to stress conditions like salt or drought or concentrations beyond their capability results in the death of the plant and therefore a little knowledge of salt tolerance.

CHAPTER 3
MATERIALS AND METHODS

3.1. Plant material

3.1.1. Source of seeds

Seeds of nine varieties of wheat (*Triticum aestivum* L.) – Mohan Wonder (MW), Kedar (KD), Gayetri (GY), Gandhari (GN), Kaweri (KW), Sonalika (SO), PBW 343, UP 2752, Local variety (LV) were selected for experimental purposes. MW, KD, GY, GN which were commercially relevant lines were obtained from Indo–Japan hybrid seed centre, Siliguri, West Bengal. LV was obtained from a local market in Siliguri, West Bengal. The seeds for SO, PBW 343 and UP 2752 were obtained from UBKV (Uttar Banga Krishi Vishwavidyalaya), Coochbehar, West Bengal.Among many varieties of wheat, seeds of nine wheat varieties (Figure 1) showed the best seed viability under laboratory condition and also in the potted conditions and thus were selected for experimental purposes. The selected seeds of nine wheat varieties before the experiment are shown in Figure 1.

3.1.2. Growth and maintenance of plants

To check the seed viability of the seeds, seed viability test was done in the laboratory and the seedlings were raised in the field in earthen pots. The seeds were stored in air tight bags at –4°C for further use. For planting, these seeds were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3–4 minutes, and then they were washed thrice with sterile distilled water and were then transferred to petri plates under aseptic conditions. The seeds were allowed to germinate in the petri plates for one week and then the seedlings were transferred to earthen pots of 12” height and 8” diameter containing sandy loam soil which was mixed with farmyard manure in the proportion of 2:1 by weight (Figure 2). Plants were maintained in growth chamber at a temperature

of 20–25^{0}C, RH 65–70%, 16 h photoperiod and irradiance of 400 μmol m^{-2} s^{-1}.

Plants were watered regularly twice in a day and weeding was done once a week. Figure 2 shows the control wheat seedlings at various stages of growth in potted conditions in the field before any stress treatment.

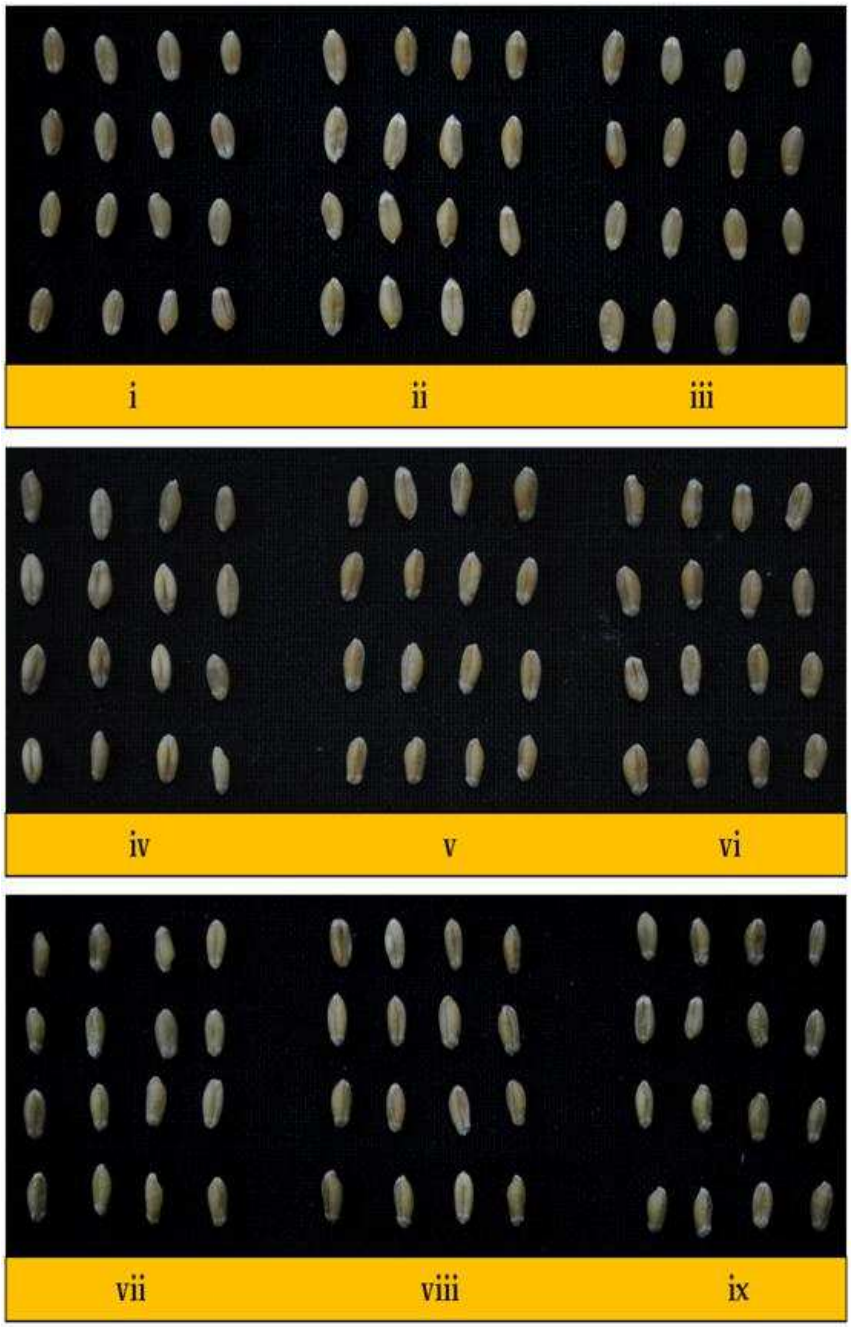

Figure 1. Seeds of nine wheat varieties: i–MW, ii–KW, iii–KD, iv–SO, v–GY, vi–GN, vii–PBW 343, viii–LV, ix–UP 2752

Figure 2. Growth and maintenance of plants in earthen pots shown at different stages of plant growth with no treatment (control plants)

3.2. Treatments

3.2.1. Induction of drought

To impart water stress or drought to the wheat plants, watering of the plant was completely withheld for the test period when the plants were one month old. Sampling of the plant was done on the 3rd, 6th and 9th day to study the response of plants to varied days of drought stress. For the control (0d), one set of plants from each tested varieties were watered regularly and sampling was done on the 0 day of treatment when the plants were one month old.

3.2.2. Salt treatment

To impart salinity stress on the tested nine wheat varieties, one month old plants were treated with sodium chloride (NaCl) solution in water of three

different concentrations, i.e. 50mM, 100mM and 200mM sampling was done on 1st and 3rd day of salt stress in each case. For the control (0mM) set, one set of plants from each variety was kept separately and watered regularly. The sampling of the control set was done on the 0 day of treatment when the plants were one month old.

3.3. Determination of Relative water content (RWC)

A fixed number of leaf discs were floated on double distilled water for 24hrs after taking initial weight (Fresh weight). The leaf discs were taken out and excess water on the surface was removed using blotting paper then the leaf discs were weighed to determine the turgid weight. The leaf discs were dried at 80°C for 24hrs and the weight (Dry weight) of the leaf disc was determined. Relative water content (RWC) of leaves was determined as described by Farooqui *et al*. (2000), calculated by the following formula as RWC (%) = (Fresh weight – Dry weight)/ (Fully turgid weight–Dry weight) X 100.

3.4. Tolerance index determination

Variation in drought and salt tolerance of the seedlings was calculated for the 9th day in case of drought and 200mM concentration for the 3rd day during salinity stress 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 X 100) – 100.

3.5. Determination of cell membrane stability index (CMS)

Membrane thermo stability was tested by cell membrane stability (CMS) test following the method of Premchandra *et al*. (1990) as modified by Sairam (1994). 1g of leaf discs were taken and cleaned in running tap water and then by double distilled water. The leaf discs were then heated in 10mL of de–ionized water at 40°C for 30mins. Electrical conductivity (C1) of the solution was recorded using electrical conductivity meter and then the same samples were place in boiling water bath (100°C) for 10min. Electrical

conductivity (C2) of the sample was recorded. The CMS was calculated as Cell Membrane stability index (CMS) = [1 – (C1/ C2)] × 100.

3.6. Determination of lipid peroxidation

Peroxidation of lipid was measured as accumulation of malondialdehyde (MDA) which was determined by the thiobarbituric acid reaction. 1g of leaf was homogenized in 3 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10, 000 rpm for 10 min. To 0.5 mL of the aliquot of the supernatant, 2 mL of 20% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid were added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mmol^{-1}$ cm^{-1}(Heath and Packer, 1968).

3.7. Extraction and estimation of proline content

3.7.1. Extraction

Free proline was extracted from wheat leaves and roots using 3% sulfosalicylic acid.1g of leaf was homogenized in 5mL of 3% sulfosalicylic acid and filtered through a Whatman No. 1 filter paper following the method of Bates *et al*. (1973). The final volume was made up to 5mL using 3% sulfosalicylic acid and it was used for the estimation of free proline in the sample.

3.7.2. Estimation

For the estimation of free proline in the sample the following test was done. The reaction mixture contained 1mL of filtrate, 3mL of distilled water and 1mL of ninhydrin solution (1g ninhydrin + 10mL acetone + 15mL distilled water) was added. The mixture was kept on boiling water–bath for 30min and cooled under tap water. After cooling it was separated using 5mL of 100% toluene in a separating funnel, the lower coloured layer was taken and the upper layer was discarded. The OD values were measured at 520nm in a colorimeter against a proper blank and quantified from a standard curve of proline.

3.8. HPLC analysis of free amino acids

3.8.1. Extraction

Extraction of free amino acid was done by the method as described by Rozan *et al* (2000) with minor modifications in which a fixed amount of leaf sample (2g) was weighed and homogenised in a pre–chilled mortar and pestle using 4 volumes of ice–cold 70% ethanol. It was stored overnight at 4 °C. The samples were then centrifuged at 20,000rpm for 20min. The pellets were again washed twice with ice–cold 70% ethanol and centrifuged at 20,000 rpm for 20min and the supernatants were pooled. The supernatant was concentrated in a lyophilizer and then finally stored in the deep freezer at –20 °C until use.

3.8.2. Sample preparation and analysis

The sample was prepared by the method given by (Khan *et al*., 1994) with some minor modification and the free amino acid contents of the extracts were analyzed by an HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization). 0.1M ammonium acetate (pH 6.5) was used as Buffer A and 0.1M ammonium acetate (pH 6.5) containing acetonitrile and methanol (44:46:10, v/v) were used as Buffer B.

Sample preparation for HPLC was done by removing 50μL aliquot of the extract and drying it under lyophilizer under ice–cold condition. After drying the extract 20μL of first coupling reagent was added to it which contained 2:2:1 (v/v) of methanol, water, triethylamine (TEA) and mixed. The mixture was then dried under a lyophilizer for 20mins and then reacted with 30μL of PITC reagent which contained 7:1:1:1 (v/v) of methanol, PITC, TEA, water and kept for 30mins at room temperature followed by drying under lyophilizer to remove PITC.

The derivatized samples were then re–dissolved in 500μL of Buffer A (mobile phase for HPLC) and then filtered though Millipore membrane of pore size 0.45μm. A 20μL of the filtered sample was injected into an HPLC system (Shimadzu system, Shimadzu Corp., Kyoto, Japan) was used using a gradient system of buffer A (100–0% after 50min) and buffer B (0–100% after 50min). The separation of compounds was monitored at 254nm.

Standards of several amino acids (1mg/mL) such as aspartic acid, glutamic acid, serine, threonine, DL alanine, arginine, proline, hydroxyl proline, tyrosine, valine, leucine, cysteine, methionine, isoleucine, alanine, DL–phenyl–alanine, arginine, lysine were prepared in the same way for HPLC.

3.9. Quantification of chlorophylls in leaves

3.9.1. Extraction

Extraction of chlorophyll from the leaves was done according to the method of Harborne (1973). 1g of leaf sample was homogenized in 80% acetone and filtered through Whatman No. 1 filter paper in a dark chamber. Addition of 80% acetone from the homogenized sample was done repeatedly from the top of the filter paper till the residue became colourless. The filtrate was collected and the total volume was made up to 10mL using 80% acetone.

3.9.2. Estimation

Estimation of chlorophyll was done by measuring the OD of the filtrate at 663nm and 645nm respectively in a UV–VIS spectrophotometer (UV–VIS Spectrophotometer 118 systronics) against a blank of 80% acetone and calculated using the formula as given by Arnon (1949).

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

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

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

3.10. Extraction and estimation of carbohydrate content

3.10.1. Extraction

3.10.1.1. Total and reducing sugar

Total soluble sugar and reducing sugar was extracted using Harborne (1973) method with minor modifications under normal room temperature and light conditions. 1g of leaf and root tissues were extracted in 10mL of 95% ethanol and the alcoholic fraction was evaporated–off on a boiling water bath at about 70°C in a beaker. The residue was re–extracted with 90% ethanol and the process was repeated and the filtrate was collected and pooled. The

aqueous fraction was re–dissolved in distilled water and made upto 5mL which was then centrifuged at 5000 rpm for 15min. The supernatant was stored in vials at –4°C until use.

3.10.1.2. Starch

The extraction of starch from leaf and root from the sample plant was done by homogenizing 1g of the plant tissue in hot 80% ethanol by the method described by Thimmaiah (1999) with some minor modifications. The homogenized mixture was filtered using Whatman no. 1 filter paper and extraction was done for 3–4 times from the residue by repeated washing using hot 80% ethanol till the washings did not give any colour with Anthrone reagent (0.2% anthrone in conc. H_2SO_4) and the residue was kept. The residue was then dried over a water bath at about 70°C. To the dried residue, 5mL of distilled water, 6.5mL of 52% perchloric acid was added which was then centrifuged for 20minutes at about 5000 rpm. This process was repeated twice and the supernatant was pooled and kept separately. The final volume was made up to 25mL and stored at –4°C for use.

3.10.2. Estimation

3.10.2.1. Total and reducing sugar

Total soluble sugar was estimated by Anthrone's method as given by Plummer (1978). 1mL of test solution was reacted with 4mL of Anthrone's reagent (0.2% anthrone in conc. H_2SO_4) and mixed properly. The reaction mixture was incubated for 10mins in a boiling water bath at about 100° C taking proper precaution followed by cooling it under running tap water. The absorbance was measured at 620nm in a colorimeter and quantified using a standard curve of D–glucose.

Reducing sugar was estimated using Nelson–Somogyi method described in Sadasivam and Manickam (1996) in which 1mL of test solution was reacted with 1mL of alkaline copper tartarate solution (0.4g $CuSO_4$, 2.4g Na_2CO_3, 1.6g Na^+–K^+ tartarate, 18g Na_2SO_4 in 100 ml of distilled water) and then heated in a boiling water bath at 100°C for 20mins taking proper precaution. The reaction mixture was cooled under running tap water and to it

1mL of commercially available Nelson's arsenomolybdate reagent was added and mixed vigorously. 2mL of distilled water was added to make the volume up to 5mL. The colour developed was measured in a colorimeter at 510nm against a blank and was quantified using a standard curve of glucose.

3.10.2.2. Starch

Estimation of starch was done using the method as given by Thimmaiah (1999) in which 1mL of test solution was reacted with 4mL of Anthrone reagent (0.4% in conc. H_2SO_4) and mixed thoroughly. The mixture was then placed in a boiling water bath at 100°C for 8mins taking proper precautions and cooled under running tap water. The absorbance was measured at 630nm in a colorimeter against a proper blank. Starch content was then calculated from the standard curve of starch solution.

3.11. Extraction and estimation of phenols

3.11.1. Extraction

The total phenol content was extracted from the leaf tissues by following the methodology as given by Mahadevan and Sridhar (1982). 1g of leaf tissue was immersed in hot absolute alcohol in the dark at the rate of 5mL of 80% alcohol per gram of tissue for 10mins and then cooled. It was then extracted in dark in 80% alcohol and filtered using Whatman no. 1 filter paper. The residue was re–extracted using hot 80% ethanol and the final volume was made up to 10mL. All the extraction process was done carefully in the dark at normal room temperature. The filtered sample was directly taken as the sample for the estimation of phenols.

3.11.2. Estimation

3.11.2.1. Total phenols

The content of total phenol from the leaf tissue was estimated using the method of Bray and Thorpe (1954). 1mL of extract taken to which 1mL of 50% diluted commercially available Folin–ciocalteu's phenol reagent and 2mL of 20% Na_2CO_3 solution was added which was mixed thoroughly. This reaction mixture was boiled in water bath for 1min and then cooled followed

by dilution with distilled water and the final volume was made up to 25mL. The absorbance was measured at 650nm in colorimeter against a proper blank and quantified using a standard curve of ferulic acid. The estimation of total phenol was done in dark conditions in the laboratory at normal room temperature.

3.11.2.2. Ortho phenols

Estimation of ortho–dihydroxy phenol content was done by Arnow's (1933) method in which the reaction mixture consisted of 1mL of test sample, 2mL of 0.5(N) HCl, 1mL of Arnow's reagent (10g $NaNO_2$, 10g Na_2MoO_4 in 100mL of distilled water) and 2mL of 1(N) NaOH. Volume of the reaction mixture was raised up to 10mL with distilled water after which it was mixed thoroughly and the absorbance was measured in a colorimeter at 515nm against a proper blank and quantified using a standard curve of ferulic acid. The estimation of ortho phenol was done in dark conditions in the laboratory at normal room temperature.

3.12. HPLC analysis of phenols

3.12.1. Sample preparation

3.12.1.1. Total phenol

Phenol extraction and preparation of the sample for HPLC was done by the method described by Pari & Latha (2004) in the dark. Fresh leaf tissues were taken and chopped into small pieces and soaked overnight in absolute methanol at solid material to methanol ratio of 1:3 (w/v) in dark. The suspension was filtered and the filtrate was evaporated using a rotary evaporator. It was re–dissolved in 1mL of HPLC grade methanol and filtered though Millipore membrane (0.45μm) filter. Standards for total phenols (1mg/ mL) such as ferulic acid, salicylic acid, chlorogenic acid and caffeic acid were prepared in the same way for HPLC.

3.12.1.2. Phenolic acid

Separation of phenolic acids was carried out according to Amarowicz and Weidner (2001) in the dark. 300mg extract of the leaf tissue was taken and

then suspended in 10mL of 2mol/L NaOH and kept for 4 h for hydrolysis at room temperature in dark. The suspension was then acidified to pH 2 using 6mol/L HCl. Free phenolic acids and constituents which were liberated from esters were extracted 4times using 15mL of diethyl ether in a separating funnel. Ether from the extraction was removed by evaporation until the extract was dried. The dry residue of free phenolic acids and phenolic acids which was liberated from esters was re–dissolved in 2mL of HPLC grade methanol and filtered through a 0.45μm filter. Standards for total phenols (1mg/mL) concentration such as ferulic acid, salicylic acid, chlorogenic acid, caffeic acid, vanillic acid and cinnamic acid were prepared in the same way for HPLC.

3.12.2. Total phenol analysis

For the analysis of total phenols in HPLC a method followed by Pari *et al* (2007) was used. For the HPLC finger print analysis of phenolic compounds present in extracts a Shimadzu system (Shimadzu Corp., Kyoto, Japan) was used, a flow rate of 1 mL/min, and gradient elution of HPLC grade of acetonitrile–water–acetic acid (5:93:2, v/v/v) [solvent A] and of acetonitrile–water–acetic acid (40:58:2, v/v/v) [solvent B], a 0– 50 min solvent B from 0 to 100%; and injection volume of 20 μL were applied; whereas the separation of compounds was monitored at 280 nm.

3.12.3. Analysis of phenolic acid

Phenolic acids were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan). The mobile phase of HPLC grade of water–acetonitrile–acetic acid (88:10:2; v/v/v) (Amarowicz& Weidner, 2001) was delivered at a rate of 1 mL/min and 20μL of injection volume was applied. The detection was monitored at 320 nm.

3.13. Protein analysis

3.13.1. Extraction of soluble proteins

Soluble proteins were extracted from the leaf and root tissues by the method as given by Chakraborty *et al* (1993) under ice cold conditions. 1g of sample tissue was homogenized using pre–chilled mortar and pestle in liquid

nitrogen with 5 mL of 0.05M sodium phosphate buffer (pH 7.2) to which a pinch of PVPP (polyvinyl polypyrrolidone) was also added. It was then centrifuged at 4°C for 20mins at 10,000 rpm and the volume of the supernatant was made up to 5mL using the chilled extraction buffer and then immediately stored at –4°C until use.

3.13.2. Quantitative estimation of soluble proteins

Quantitative estimation of protein was done by the method of Lowry *et al* (1951). 1mL of the test extract was diluted as required and then reacted with 5mL of freshly prepared alkaline reagent (2% Na_2CO_3 in 100 mL of 0.1N NaOH to which 1mL each of 1% $CuSO_4$ and 2% Na^+– K^+ tartarate was added just before use) and mixed thoroughly after which it was allowed to stand for 15mins at room temperature.

After the incubation period 0.5mL of commercially available Folin–ciocalteu's phenol reagent was added to the reaction mixture and incubated for further 20mins at room temperature. OD value was measured in a spectrophotometer at 690nm against a proper blank and quantified using a standard curve of BSA (Bovine serum albumin).

3.13.3. SDS–PAGE analysis

3.13.3.1. Preparation of stock solutions

For the SDS–PAGE analysis the method as given by Sambrook *et al* (1989) was used with minor modifications.

A stock solution containing 29% acrylamide and 1% N'N'–methylene bis–acrylamide (7.25 g of acrylamide and 0.25g of N'N'–methylene bis–acrylamide in 25mL of warm distilled water) was prepared using warm double distilled water maintaining the pH of the solution below 7. The stock was filtered through Whatman No. 1 filter paper and kept in brown bottle and stored at –4°C for further use and used before one month of preparation.

10 % stock solution of SDS (1g SDS in 10mL of distilled water) was prepared in warm distilled water and stored at room temperature and used before one month of preparation.

Tris buffer for resolving gel was prepared by dissolving 4.542g of tris base in 15mL of distilled water and the pH of the solution was adjusted to 8.8 using concentrated HCl. The final volume of the solution was made up to 25mL with distilled water and stored at –4°C.

Tris buffer for stacking gel was prepared by dissolving 3.029g tris base in 15mL of distilled water and the pH of the solution was adjusted to 6.8 using concentrated HCl. The final volume of the solution was made up to 25mL with distilled water and stored at –4°C.

10% APS (Ammonium per sulphate) solution was prepared just before use by dissolving 0.05g APS in 0.5mL of distilled water.

For the preparation of electrophoresis buffer 3.02g of tris (25mM) and 18.8g of glycine (250mM) was mixed in 1L of distilled water and stored at –4°C. 10 mL of 10% SDS (1g in 10 mL) was added to it just before use.

SDS gel loading buffer contained 50mM Tris–HCl (pH 6.8), 10mM of ß–mercaptoethanol, 2% SDS, 0.1% Bromophenol blue and 10% glycerol. Therefore a 1X solution in 10mL contained 0.5mL of ß–mercaptoethanol, 2mL of 10%SDS, 10mg of bromophenol blue, 1mL of glycerol, 1mL of 50mM Tris–HCl and the volume was made up to 10mL using distilled water and stored at –4°C.

The fixing solution was prepared by mixing glacial acetic acid, methanol and distilled water in the ratio of 10:20:10 and was stored at normal room temperature.

The staining solution was prepared by dissolving 250mg of Coomasie brilliant blue R–250 in 45mL of methanol using magnetic stirrer. 45mL of distilled water along with 10mL of glacial acetic acid was added to it. The prepared mixture of the stain was filtered through Whatman No. 1 filter paper and stored at room temperature until use.

The destaining solution was prepared by mixing methanol, distilled water and glacial acetic acid in the ratio of 45:45:10 and stored at room temperature.

7% glacial acetic acid solution was prepared at the time of use for storing of the gel.

3.13.3.2. Preparation of sample

The soluble proteins were extracted from the leaf tissue by the method as given by Demirevska *et al* (2008). 0.5g of leaf tissue was homogenized in 5mL of ice–cold 100mM Tris–HCl (pH 8) containing 20mM $MgCl_2$, 10mM $NaHCO_3$, 1mM EDTA (di sodium salt), 2mM PMSF (phenylmethanesulfonyl fluoride), 12.5% glycerol (v/v) and 20mM of ß–mercaptoethanol under ice–cold condition. It was then centrifuged at 10,000 rpm at –4°C for 20minutes in a cooling centrifuge. The supernatant was taken and stored at –4°C until use.

For the electrophoresis, the sample preparation was done by mixing the sample protein (35μL) with 1X SDS gel loading dye buffer (15μL). All the samples were floated in boiling water bath at 100°C for 3minutes to denature protein sample. They were then immediately loaded in a pre–determined order into the bottom of the wells using a micropipette.

3.13.3.3. Preparation of gel and electrophoresis

Mini slab gel plate (8cmX 10cm) was prepared for the analysis of protein patterns by SDS–PAGE. For preparation of gel plates, two glass plates were thoroughly cleaned with dehydrated ethanol to remove traces of grease and then dried properly. 1.5mm thick gel plate spacers were placed between the glass plates at three sides and sealed using high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring procedure.

Resolving gel was prepared by mixing the following solution in the same order. 10% resolving gel was made for 1 gel plate by mixing 2.85mL of distilled water, 2.55mL of stock solution of 30% Acrylamide, 1.95mL of stock solution of 1.5M Tris (pH 8.8), 0.075mL of stock 10% SDS solution, 0.075mL of freshly prepared 10% APS and 4μL of TEMED (N,N,N',N'–Tetramethylethylenediamine) was added and mixed gently. It was mixed slowly and immediately after the addition of TEMED it was poured inside the gel plates leaving sufficient space for comb in the stacking gel.

A thin layer of distilled water was over layered on top of the resolving gel after 10mins and then the gel was allowed to stand for 1hr for

polymerisation. After the resolving gel polymerises properly the layer of water was decanted or removed carefully using blotting paper.

5% Stacking gel was prepared by mixing 2.10mL of distilled water, 0.5mL of stock solution of 30% Acrylamide, 0.38mL of stock solution of 1.0M Tris (pH 6.8), 0.03mL of stock solution of 10% SDS, 0.03mL of freshly prepared APS and 3μL of TEMED which was mixed gently. It was then immediately poured on top of the resolving gel and immediately a comb with 1.5mm thickness was inserted to make wells on the stacking gel leaving sufficient space between the stacking gel and the resolving gel. It was then allowed to polymerise for 1hr and over layered with distilled water.

After the polymerisation step the comb and the lower spacer was removed. The wells were cleaned using capillary tube. The gel plate wasthen properly inserted inside the gel electrophoresis system and it was adjusted with electrodes in their respective places.

300mL of Tris–glycine running buffer was added in the gel electrophoresisset up to which 3mL of 10% SDS was added just before useand then it was allowed to pre–run for about 10mins.

35–45μL of protein samples in each lane was loaded on the gel and the electrophoresis was done for about 2–3hrs at 18mA current for a single gel and about 30mA in a dual gel electrophoresis apparatus.

An unstained protein molecular marker was also run in the same way in one lane which was prepared by mixing 10μL of protein molecular marker with 10μL of stock solution of SDS gel loading buffer and 30μL of distilled water. It was then boiled for 1min in a water bath at 100°C for 1min followed by cooling and then 40–50μL of this mixture was loaded in the gel in one lane.

3.13.3.4. Fixing, staining and destaining of the gel

After the separation of protein samples in the gel electrophoresis is completed the gel plate was taken out from the electrophoresis system and then the gel was separated from the glass plate after the stacking layer has been removed carefully. It was then transferred to a gel box of appropriate size and left overnight in30–40mL of fixing solution. The fixing solution was properly decanted from the gel box after fixing the gel for the required time period. 30–

40mL of staining solution was immediately poured on top of the gel and kept for about 4–6hrs on a shaker with a constant speed of 30rpm for proper staining.

After the staining of the gel was done, the staining solution was decanted out from the gel box and destaining solution was added to it and kept on a shaker with 30rpm until destaining was achieved. The destaining solution in the gel was replaced 3–4 times with a fresh destaining solution. After this step the gel was stored in 7% glacial acetic acid solution to stop the reaction and further analysis of the bands were done after it was photographed in an illuminating chamber. Analysis of the bands for their R_m value was done.

3.13.4. FPLC analysis of proteins

3.13.4.1. Sample preparation

Sample preparation for FPLC analysis of proteins was done by the method of Ledesma *et al* (2004) with minor modifications. 2g of sample leaf (stored at –70°C) were ground to a fine powder using liquid nitrogen (N_2) in a pre–chilled mortar and pestle. Protein extraction buffer which contained 60mM of Tris, 2% of sodium dodecyl sulfate (SDS), 2% of ß–mercaptoethanol, 1mM of phenylmethylsulfonylfluoride (PMSF), 5% of sucrose, 1.5% polyvinylpolypyrrolidone (PVPP), 1mM EDTA–Na_2 and the pH adjusted to 8.0 with 1M HCl was added to the sample powder at the rate of 4mL per gram of the tissue and the sample was homogenized in it. The homogenate was centrifuged twice at 20,000 rpm for about 20 min at –4°C. The supernatant was pooled and immediately stored at –70°C until further analysis.

20μL of the sample was taken and diluted 100 times (10^{-2}) using 1.8mL of chilled extraction buffer. The resulting 2mL of the diluted sample was then filtered through a millipore membrane of pore size 0.45μm taking proper precautions. 1mL of the filtered sample was injected into the column for FPLC.

3.13.4.2. Column separation of the protein and analysis

For the FPLC analysis of protein present in the sample AKTApurifier 10 (P–903), with instrument system No. 1275153 (made in Sweden) containing

separation unit with two main modules, i.e., a pump P–900 and a UV–900 monitor. It had an accession volume of 30mL, accession time of 65min, flow rate 0.500mL min^{-1} and pressure 1 MPa.

A flow rate of 0.500mL $min^{-1,}$ elution of Tris–HCl buffer (50mM; pH 7) and 0–50 min solvent run and injection volume of 1mL were applied; whereas the separation was monitored at 280, 254 and 215nm. The separation columns consist of superpose^{TM6} (Amersham Biosciences), 10/300 GL with code no. 17–5172–01. The retention time of the protein was calculated and analysed.

3.14. Extraction of antioxidative enzymes

3.14.1. Catalase

For the extraction of catalase enzyme from the sample, 0.5g of leaves from control and stressed wheat seedlings were homogenized in 5 mL of ice–cold 50 mM sodium phosphate buffer, pH 6.8, containing 1% (w/v) polyvinylpolypyrrolidone (PVPP) using liquid nitrogen in a pre–chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at –4°C. The supernatant was taken out and used directly as crude extract for enzyme assays.

3.14.2. Peroxidase

Extraction of peroxidase enzyme from the leaves from the control and stressed wheat seedlings were done by homogenizing 0.5g of the sample leaf in 5mL of ice–cold 50mM sodium phosphate buffer, pH 6.8, containing 1% (w/v) polyvinylpolypyrrolidone using liquid nitrogen in a pre–chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at –4°C. The supernatant was taken out and used directly as crude extract for enzyme assays.

3.14.3. Ascorbate peroxidase

For the extraction of ascorbate peroxidase enzyme 0.5g of the sample wheat leaf was homogenized in 5mL of ice–cold 50mM of sodium phosphate buffer, pH 7.2 containing 1% (w/v) polyvinylpolypyrrolidone using liquid

nitrogen in a pre–chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at –4°C. The supernatant was used directly as crude extract for enzyme assays.

3.14.4. Glutathione reductase

Extraction of glutathione reductase enzyme was done by homogenizing 0.5g of the sample wheat leaf in 5mL of ice–cold 50mM of potassium phosphate buffer, pH 7.6 containing 1% (w/v) polyvinylpolypyrrolidone using liquid nitrogen in a pre–chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at –4°C. The supernatant was used directly as crude extract for enzyme assays.

3.14.5. Superoxide dismutase

For the extraction of superoxide dismutase enzyme, 0.5g of the sample wheat leaf was homogenized in 5mL of ice–cold 50mM of potassium phosphate buffer, pH 7.6 containing 1% (w/v) polyvinylpolypyrrolidone using liquid nitrogen in a pre–chilled mortar and pestle. The homogenate was then centrifuged at 10,000 rpm for 20 min at –4°C. The supernatant was used directly as crude extract for enzyme assays.

3.15. Assay of enzyme activities

3.15.1. Catalase

Catalase (EC 1.11.1.6) activity was assayed as described by Chance and Machly (1955) by estimating the breakdown of H_2O_2, which was measured at 240 nm. For the assay mixture, 40μL of the enzyme extract was added to 3mL of H_2O_2 buffer (0.1mL of H_2O_2 in 25mL of $NaPO_4$buffer of pH 6.8) and mixed well. The change in absorbance of the sample was immediately measured at 245nm in a UV–VIS spectrophotometer at every 15sec for 3minutes and the assay was done in three replicates. The blank was prepared in the same way where instead of the sample 40 μL of extraction buffer was added in place of enzyme extract. The enzyme activity was expressed as μmol H_2O_2 mg protein^{-1} min^{-1}.

3.15.2. Peroxidase

Peroxidase (EC 1.11.17) activity was assayed spectrophotometrically in 4802 UV VIS spectrophotometer (Cole Parmer, USA) at 460 nm by monitoring the oxidation of o–dianisidine in the presence of H_2O_2 (Chakraborty *et al*. 1993).The reaction mixture consisted of 1.7mL of distilled water, 1mL of 0.2M sodium phosphate buffer (pH 5.4), 0.1mL of H_2O_2, 0.1mL of freshly prepared orthodianisidine (5mg orthodianisidine in 1mL methanol) and 0.1mL of enzyme extract was added in the last after which the change in absorbance was noted down immediately at an interval of 1min for four minutes and the assay was done in three replicates. The blank contained the same assay mixture where 0.1mL of extraction buffer was added in place of enzyme extract. The assay specific activity was expressed as mmol o–dianisidine mg protein^{-1} min^{-1}.

3.15.3. Ascorbate peroxidase

Ascorbate peroxidase (EC 1.11.1.11) activity was assayed as a decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. The reaction mixture consisted of 0.01mL of freshly prepared ascorbic acid (10mg ascorbic acid in 10mL of 0.05M sodium phosphate buffer of pH 7.2), 0.01mL of H_2O_2, 2.97mL of 0.05M of sodium phosphate buffer (pH 7.2) and 0.01mL of enzyme was added just before assay and the change in absorbance of the sample was measured immediately at an interval of one minute for three minutes and the assay was done in a replicates of three. The blank contained the same assay mixture where 0.01mL of extraction buffer was added in place of enzyme extract. Enzyme activity was expressed as mmol ascorbate mg protein^{-1} min^{-1}.

3.15.4. Glutathione reductase

Glutathione reductase (EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000).The reaction mixture consist of 1mL of 0.1M potassium buffer (pH 7.6), 0.2mL of 0.1M EDTA (Ethylenediaminetetraacetic acid), 0.1mL of freshly prepared

6mM glutathione, 0.2mL of 0.1mM NADPH (Nicotinamide adenine dinucleotide phosphate reduced tetrazolium salt) and 0.2mL of enzyme extract. Change in absorbance was determined immediately at an interval of 1minute for 2minutes and the assay was done in a replicate of three. Blank contained all the reaction mixture and 0.2mL of extraction buffer in place of enzyme extract. Enzyme activity was expressed as μmol NADPH oxidized mg protein^{-1} min^{-1}.

3.15.5. Superoxide dismutase

Superoxide dismutase (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium according to the method of Dhindsa *et al*. (1981) with some modification. The assay mixture contained 1.5mL of 0.1M potassium phosphate buffer (pH 7.6), 0.8mL of distilled water, 0.1mL of 1.5M Na_2CO_3, 0.1mL of freshly prepared 3mM EDTA, 0.1mL of 2.25mM NBT (Nitrobluetetrazolium chloride), 0.2mL of 0.2M freshly prepared methionine, 0.1mL of 60μM riboflavin and 0.1mL of enzyme extract was added just before use. The blank was prepared which contained all the assay mixture with 0.1mL of extraction buffer in place of enzyme extract. The absorbance of the samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the nitro blue tetrazolium reduction rate in the controls containing no enzymes.

3.16. Isozyme analysis by polyacrylamide gel electrophoresis (PAGE)

3.16.1. Preparation of stock solution

Stock solution of acrylamide (A) for separating gel was prepared by mixing 7g of acrylamide and 0.185g of bis–acrylamide in 15mL of warm double distilled water and the total volume was made up to 25mL using distilled water. The mixture was stirred using magnet in a magnetic stirrer. The stock was filtered through Whatman No.1 filter paper and stored in air tight brown bottle at –4°C for further use and was used before one month of preparation.

Stock solution of acrylamide (B) for stacking gel was also prepared in the same way by mixing 2.5g of acrylamide with 0.625g of bis–acrylamide in

15mL of warm double distilled water and filtered through Whatman no. 1 filter paper. The volume was made up to 25mL using distilled water and stored in air tight brown bottle at –4°C for further use. It was used before one month of preparation.

36.6 % and 5.98 % of Tris buffer with pH 8.9 (C) and 6.7 (D) respectively was made separately by dissolving 9.15g and 1.495g of tris in 15mL of double distilled water. The desired pH of the buffer was maintained by adding 1(M) HCl to it and later the volume was made up to 25mL using distilled water and stored at –4°C for further usage.

0.15% solution of ammonium persulphate (E) was prepared freshly in 5mL of distilled water just before use.

A stock solution of riboflavin (F) was made by dissolving 4mg of riboflavin in 10mL of distilled water and filtered through Whatman no. 1 filter paper and stored in dark brown bottles at –4°C and used before one week of preparation.

Electrophoresis buffer was prepared on the same day of experiment by dissolving 0.18g of Tris and 0.87g of glycine in 100mL of double distilled water and the final volume was made up to 300mL and stored at –4°C until use.

Gel loading dye was prepared by mixing 0.1g of bromophenol blue and 4g of sucrose in 10mL of distilled water and filtered which was then stored at –4°C.

Preparation of sample material was done by homogenizing 1g of sample plant tissue in 5mL of chilled sodium phosphate buffer (pH 7) in liquid nitrogen in a pre–chilled mortar and pestle according to the method as given by Davis (1964) with some minor modifications. It was then centrifuged at 10, 000 rpm in a cooling centrifuge at –4°C for 20min. The supernatant was directly used as the sample for loading and stored in vials at –4°C in the refrigerator until use.

For the electrophoresis 32μL of the sample was mixed with 15μL of electrophoresis bromophenol blue dye and directly loaded on the lanes of the gel.

3.16.2. Preparation of gel and electrophoresis

Mini slab gel plate (8cm X 10cm) was prepared for the analysis of protein patterns by NATIVE–PAGE. For preparation of gel plates, two glass plates were thoroughly cleaned with dehydrated ethanol to remove traces of grease and then dried properly. 1 mm thick gel plate spacers were placed between the glass plates at three sides and sealed using high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring procedure.

Separating gel was prepared by mixing A, C, E and double distilled water in the ratio of 1:1:4:1 in 7mL for one gel and then mixed slowly. 4–6μL of TEMED was added to it and gently mixed and then immediately poured inside the gel. It was then allowed to stand for 10mins followed by addition of a thin layer of distilled water. It was then allowed to solidify for about 2hr. The thin layer of water was removed using blotting paper.

Stacking gel was prepared by mixing B, D, F and distilled water in the ratio of 2:1:1:4 in 4mL for one gel. 4μL of TEMED was added to it and gently mixed and immediately poured on top of the separating gel. A 1mm thick comb was immediately inserted inside the stacking gel leaving a sufficient space between the resolving and stacking gel. It was then allowed to polymerise for 1hr and over layered with distilled water.

After 1hr the comb and the lower spacer was removed. The wells were cleaned using capillary tube. The gel plate was then properly inserted inside the gel electrophoresis system and it was adjusted with electrodes in their respective places.

300mL of stock chilled Tris–glycine running buffer was added in the gel electrophoresis set up and then it was allowed to pre–run for about 10mins at –4°C. 25–32μL of protein samples made in bromophenol dye was loaded in each lane of the gel and separated for about 2–3hrs at 10mA current for one gel run and for two gel 20mA of current was used in a dual gel electrophoresis apparatus in ice–cold condition at –4°C inside a refrigerator.

After the separation of samples in the gel electrophoresis is completed the gel plate was taken out from the electrophoresis system and then the upper stacking layer was carefully removed with the help of a scalpel. The resolving

gel was carefully taken out and separated from the glass plate and immediately stained for the analysis of isozyme bands.

3.16.3. Staining procedures

3.16.3.1. Peroxidase

The staining of peroxidase isozyme in the resolving gel was achieved by following the method of Reddy and Gasber (1971) in ice–cold condition. 0.52g of Benzidine was mixed in 4.5mL of glacial acetic acid and 20mL of chilled distilled water was added to it and filtered through Whatman no. 1 filter paper. 25mL of 3% H_2O_2 was prepared in chilled distilled water separately. All the solutions were mixed just before use and immediately poured on top of the resolving gel in a gel staining box. Bright blue coloured bands were observed when the bands were stained.

The reaction was arrested by immersing the gel into a large volume of 0.67% NaOH or 7% acetic acid solution for10min. Analysis of the bands for their R_m value was immediately and photographs were taken for further analysis.

3.16.3.2. Catalase

The staining of catalase isozyme in the resolving gel was achieved by following the method of Woodbury *et al* (1971) with minor modification. The resolving gel was soaked in 50mL of chilled 3.3mM H_2O_2 for 20min. The gel was then rinsed in chilled distilled water and washed thrice in it. It was then incubated in freshly prepared 1:1 solution of 1% potassium ferricyanide and 1% ferric chloride for about 20min in the dark at room temperature.

Analysis of the bands for their R_m value was immediately and photographs were taken for further analysis.

3.17. Determination of H_2O_2 accumulation

3.17.1. Quantification

H_2O_2 levels in the leaves were estimated according to Jena and Choudhuri (1981). Leaf tissue (500 mg) was homogenized with 12 mL of 50

mM potassium phosphate buffer (pH 6.5), centrifuged at 6000 rpm for 25 min at –4°C and the supernatant was used for H_2O_2 determination.

3mL of the supernatant was reacted with 1mL of 0.1% $TiSO_4$ (in 20% H_2SO_4) The intensity of the yellow colour was measured at 410 nm in the spectrophotometer and H_2O_2 levels were calculated using extinction coefficient 0.28 μmol^{-1} cm^{-1}.

3.17.2. Microscopic detection

In situ detection of H_2O_2 was carriedout following the method of Thordal–Christensen *et al*. (1997) with minor modifications using diaminobenzidine. Cut leaf discs of 2 cm diameter were vacuum infiltrated with diaminobenzidine (1 mg mL^{-1}, pH 3.8). The leaf discs were then incubated at 30°C in the dark for 24 hr under gentle stirring of 150 rpm; they were then transferred to 90% ethanol at 70°C until the chlorophyll was removed.

H_2O_2 was visualized as reddish–brown colour at the site of diaminobenzidine polymerization. Diaminobenzidine polymerizes instantly and locally atsites of peroxidase activity into a reddish–brown polymer.

3.18. Extraction and quantification of non–enzymatic antioxidants

3.18.1. Carotenoids

Carotenoids were extracted and estimated following the method described by Lichtenthaler (1987). Extraction was carried out in 100% methanol and the extract was filtered. Absorbance of the filtrate was noted at 480 nm in a VIS spectrophotometer and the carotenoid content was calculated using standard formula as follows: $A_{480} – (0.114 \times A_{663}) – 0.638 (A_{645})$.

3.18.2. Ascorbate

Ascorbic acid was extracted and estimated following the method described by Mukherjee and Choudhuri (1983). Leaves were extracted in 6% TCA under ice–cold condition and extraction was done through filter at 0°C. Reaction mixture consisted of 4mL of sample, 2mL of 2% DNPH (in 0.5N HCl) and 1 drop of 10% thiourea (in 70% ethanol). It was kept in boiling water

bath for 15mins and cooled at 0°C followed by addition of 5mL of H_2SO_4.

Absorbance of the sample was measured at 530nm in a VIS spectrophotometer. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

3.18.3. *α*–tocopherol

The extraction and estimation of vit E (*α*–tocopherol) was done by the method as described by Jayaraman (1996) with some minor modifications. Sample leaves were dried in an oven at about 30°C in the dark and grinded in a mixer to a fine powder. 0.5g of the fine powder was then homogenized in 5mL of hexane and shaken vigorously. This extraction using hexane was done twice and then it was filtered using Whatman no.1 filter paper in a dark chamber.

2mL of this hexane layer was taken in a test–tube to which 2mL of absolute ethanol was added and mixed thoroughly. 0.2mL of 2, 2'– dipyridyl solution (0.5% in ethanol) and 0.2mL of ferric chloride solution (0.2% in ethanol) was added one by one to the test tube and shaken well. It was then incubated in dark for 5–10 min. After the development of red colour 4mL of distilled water was added to the above mixture and mixed well.

Two layers are separated out in the test tube containing sample of which the red colour goes to the aqueous layer which was stable up to 30mins. This colour intensity was measured at 520nm in a spectrophotometer against a proper blank and quantified using a standard curve of *α*–tocopherol.

3.19. Estimation of total antioxidant activity

1gram of dried leaf sample powder was extracted in methanol in a dark chamber which was then centrifuged at 4000 rpm for 10mins. The supernatant was taken in a beaker and methanol fraction was evaporated. It was finally dissolved in absolute methanol.

The reaction mixture contained 80μL of sample and 2mL of DPPH (0.0025% in methanol); initial absorbance (Abs t_{0min}) of DPPH was taken in 0min at 515nm in a spectrophotometer and incubated in dark for 30mins. Absorbance (Abs t_{30min}) was taken and the inhibition percentage (%) was

determined from the absorbance of DPPH and the total antioxidant activity was measured by the method described by Blois (1958).

3.20. Estimation of $Na^+$and K^+ content

For the estimation of Na^+ and K^+ content 10g of the tissue samples were taken from the plants from control set and the plants under stress and air dried separately till the samples were completely dried taking proper care of the sample. The samples were than crushed in a grinder and crushed into a fine powder. 1g of dried root and leaf sample powder from each set were taken and dissolved in distilled water which was then used for the estimation of Na^+ and K^+ content. Na^+ and K^+ content were quantified by flame–photometer (Chemi Line, CL – 411) and expressed as mg g^{-1} dry weight.

3.21. Foliar application of proline, ABA and SA

Two sets of plants of GN and LV were grown in the pots and grown for one month. When the plants were one month old, one set of plant was pre–treated separately with foliar spray of 50 mL of 50μM solution of Abscissic acid (ABA), Proline and Salicylic acid (SA) followed by the induction of drought in separate pots for each chemical. Second set of plants was kept separately and drought was induced in them without any pre–treatment with any chemicals for both GN and LV. For each set one control was kept separately where no pre–treatment with chemicals was done and drought was not induced at any period. Sampling was done from both the sets on 3^{rd}, 6^{th} and 9^{th} day of drought and also from the control plants of both the varieties and various biochemical tests were done.

CHAPTER 4
RESULTS

4.1. Screening of tolerant and susceptible varieties of wheat against drought and salinity

4.1.1. Morphological changes in the plant

Plants exhibited varying levels of morphological and biochemical changes as adaptation to stress, none of the nine varieties showed severe wilting symptoms morphologically even after 7 days of water stress but during the 9th day most of the varieties under study showed changes in their morphology due to drought (Figure 3, 4 and 5) and salt stress (Figure 6, 7 and 8). MW, GY, LV and SO in comparison to the other varieties showed severe wilting symptoms and yellowing of leaf on the 9th day of drought stress (Figure 3, 4) and the other five varieties KD, KW, GN, UP 2752 and PBW 343 showed less damage during the 9th day marked as lesser wilting in their leaf and comparatively lesser yellowing of leaf as shown in the picture (Figure 4, 5). The plants under salt stress did not show wilting symptoms in all the three concentration of salt, i.e. 50mM, 100mM and 200mM on the first day, but later during the 3rd day wilting symptoms were visible in all cases. The wilting symptoms were much more pronounced in case of LV, SO, MW and GY than the other five varieties in all the concentration of salt with the highest rate of wilting being more in the salt concentration of 200mM during the 3rd day along with yellowing of leaves (Figure 6, 7 and 8). In the case of KW, KD, GN the rate of wilting, yellowing of leaf and damage due to salt stress was lowest followed by UP 2752 and PBW 343.

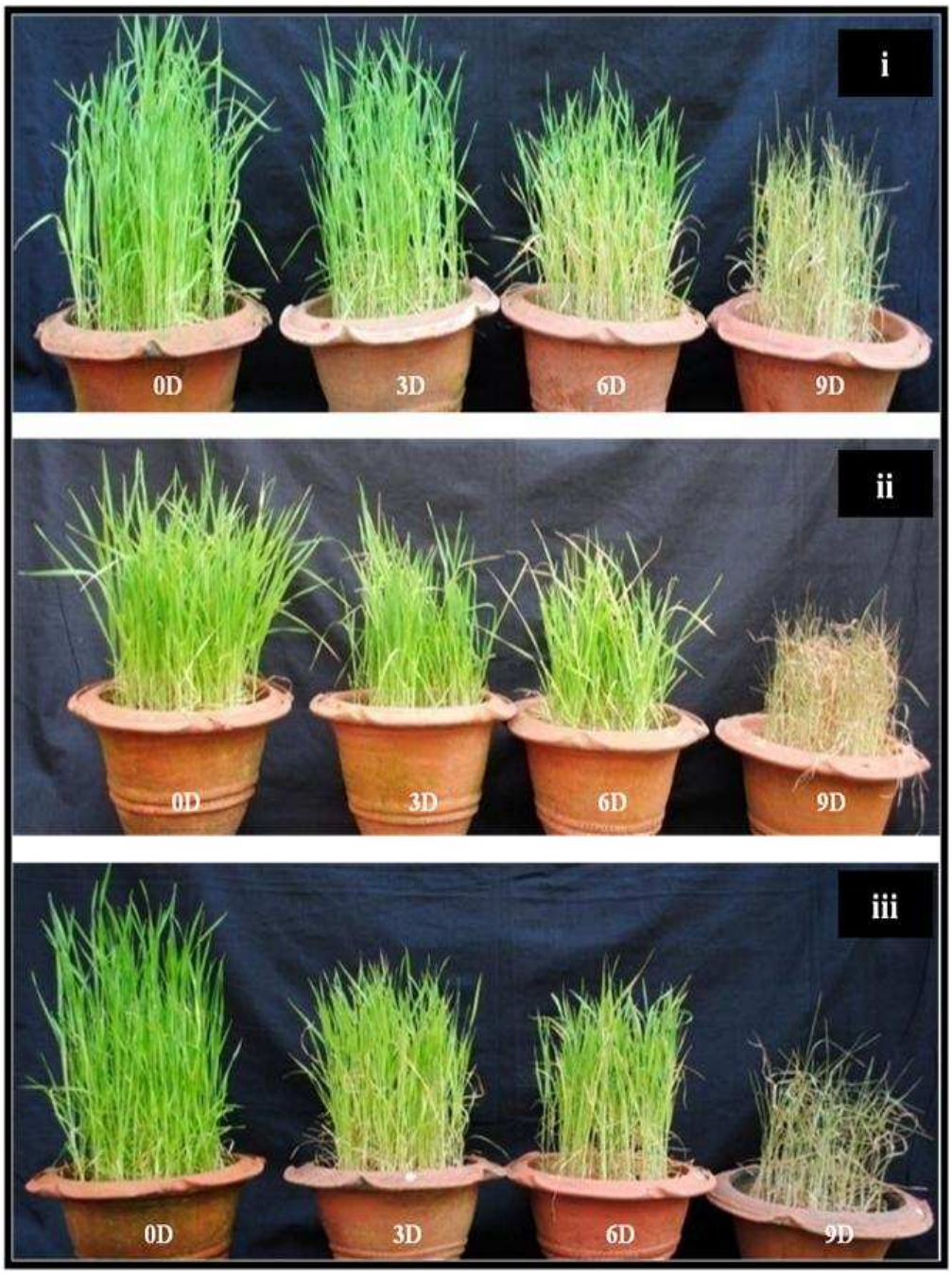

Figure 3. Drought treatment in one month old plant of i–MW, ii–GY and iii–LV for 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

Figure 4. Drought treatment in one month old plant of i–PBW 343, ii–UP 2752 and iii–SO for 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

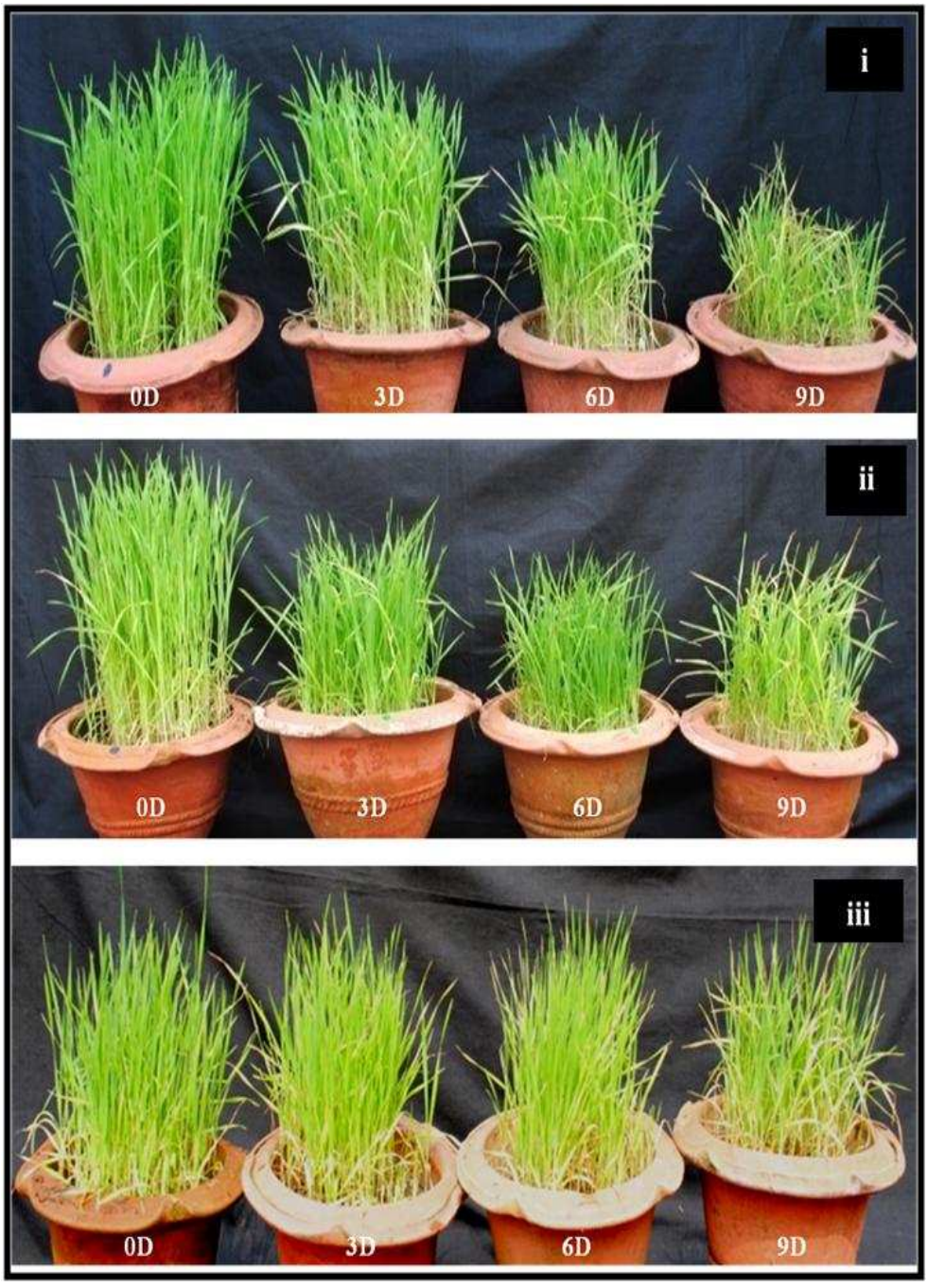

Figure 5. Drought treatment in one month old plants of i–KD, ii–GN and iii–KW for 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

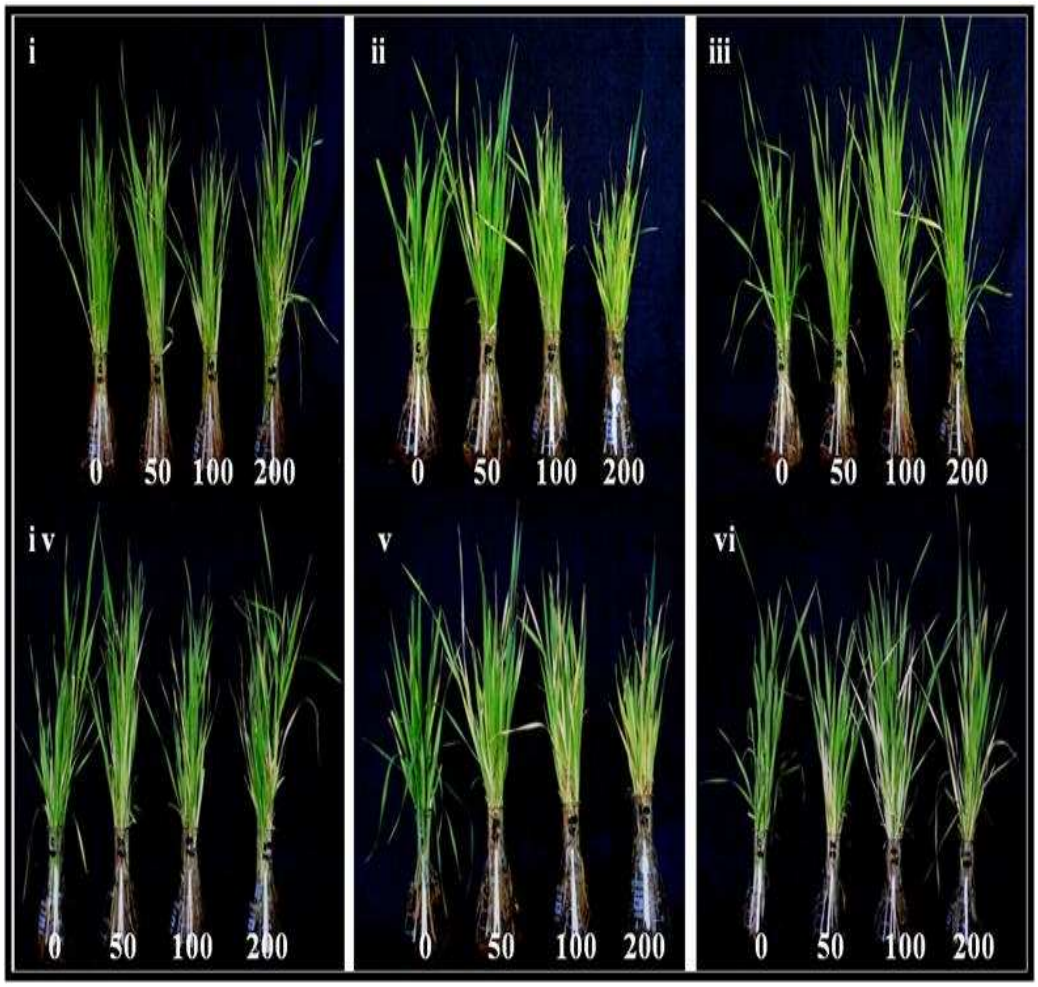

Figure 6. Salt (NaCl) treatment in one month old plant of MW (i–1 day, iv–3 days), GY (ii–1 day, v–3 days) and KD (iii–1 day, vi–3days), 0=0mM, 50=50mM, 100=100mM and 200=200mM respectively

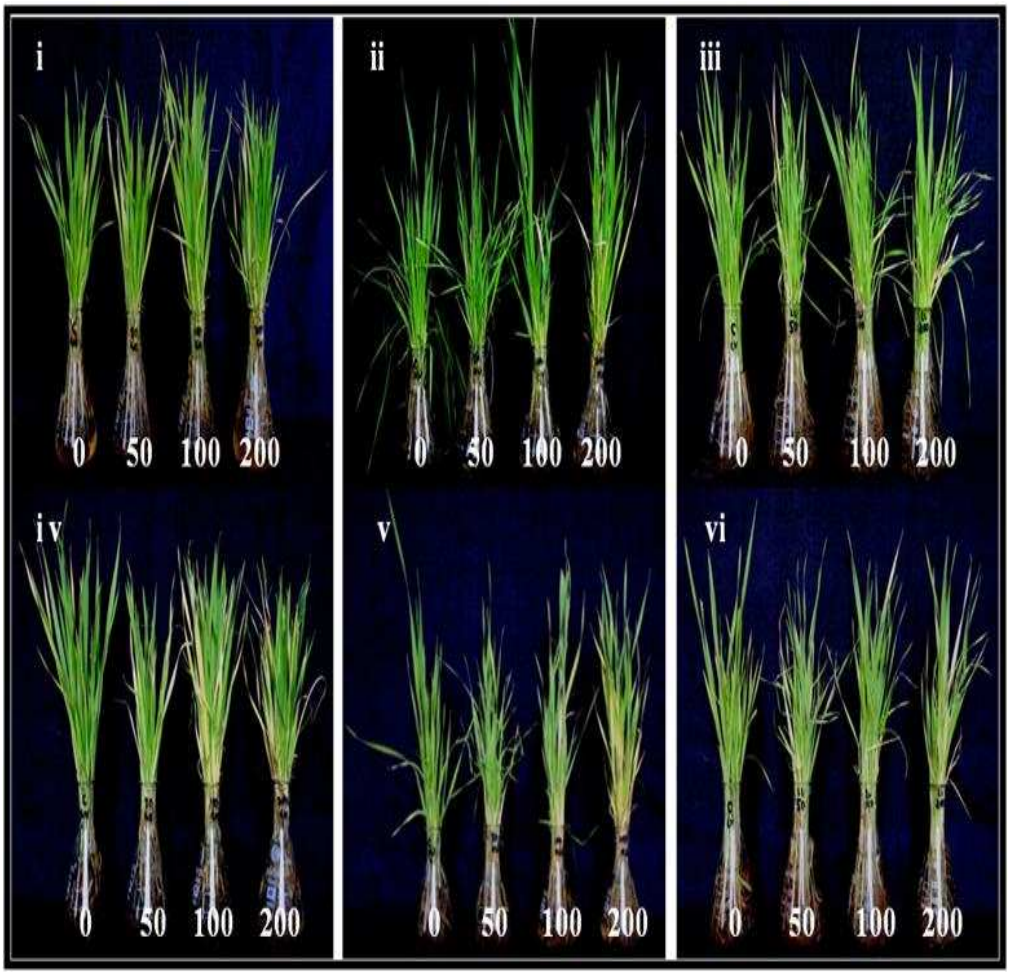

Figure 7. Salt (NaCl) treatment in one month old plant of GN (i–1 day, iv–3 days), KW (ii–1 day, v–3 days) and LV (iii–1 day, vi–3 days), 0=0mM, 50=50mM, 100=100mM and 200=200mM respectively

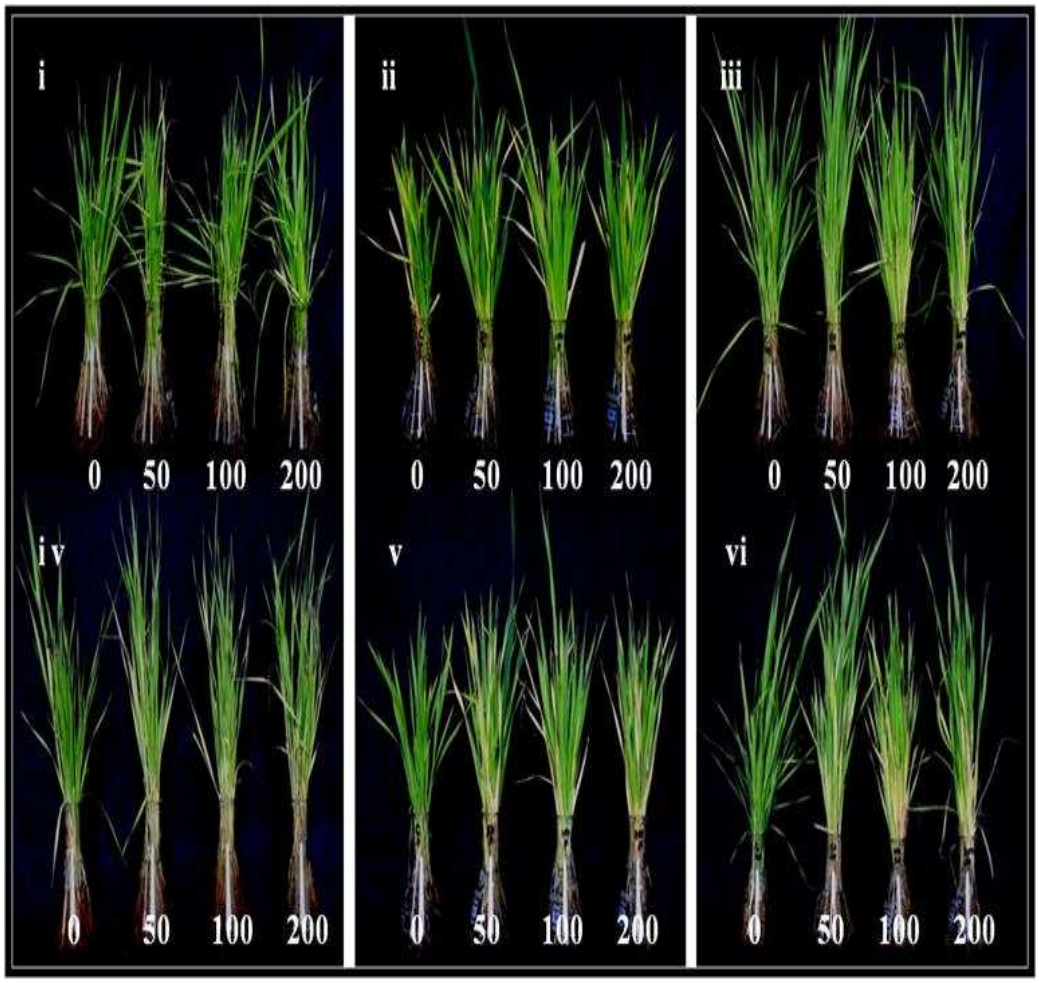

Figure 8. Salt (NaCl) treatment in one month old plant of UP 2752 (i–1 day, iv–3 days), PBW 343 (ii–1 day, v–3 days) and SO (iii–1 day, vi–3 days), 0=0mM, 50=50mM, 100=100mM and 200=200mM respectively

4.1.2. RWC

RWC in the leaf decreased significantly with induction of drought and salinity stress. The increase in the duration of drought stress (Figure 9) and the increase in concentration of salt stress resulted in decrease in RWC content in all varieties of wheat under study. However, the decrease in RWC after 9 days in relation to control (0d) was less in case of KD, GN, PBW 343, UP 2752 and KW (35.7, 36, 40.75, 42.34 and 36.65% respectively) compared with MW, GY, LV and SO (53, 53.4, 52.93 and 52.67%, respectively). RWC in the leaf of all the varieties declined remarkably as the severity of stress in terms of days during drought was increased.

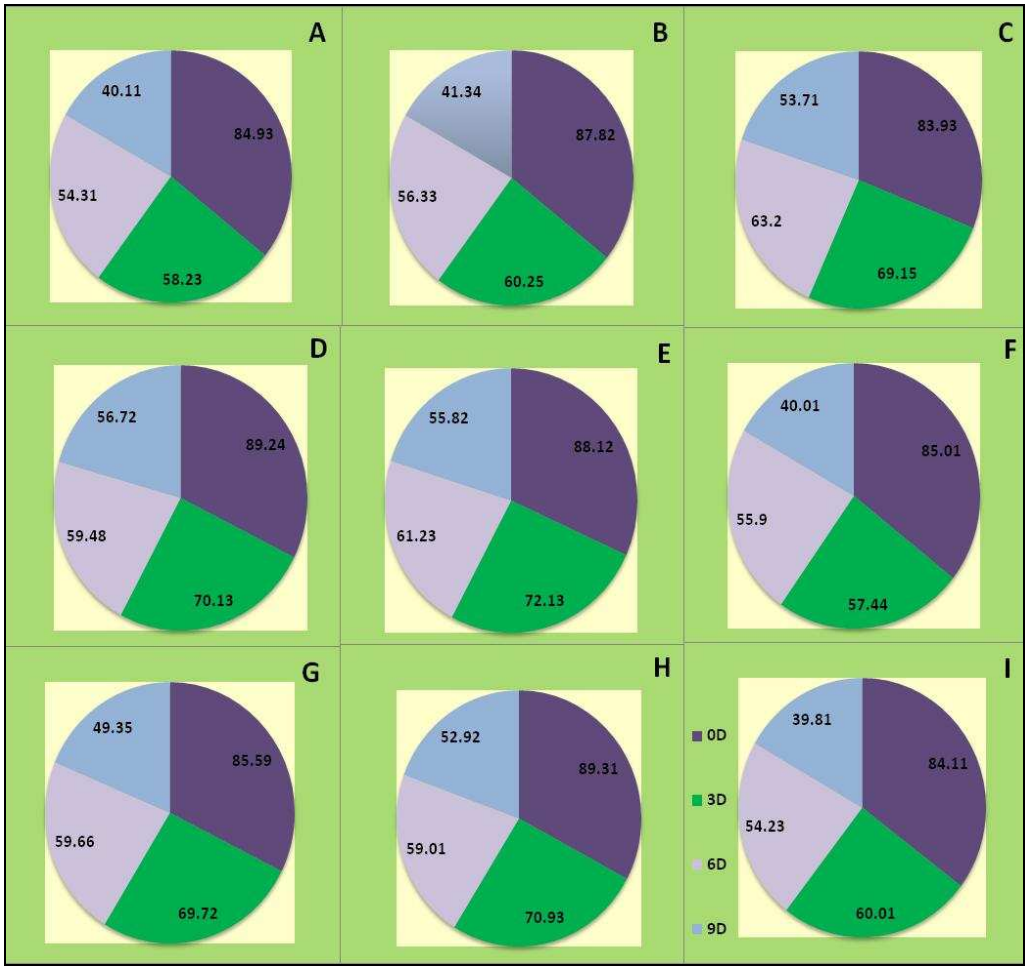

Figure 9. Relative water content of nine varieties of wheat subjected to drought stress treatments: A– MW, B– GY, C– KD, D– GN, E– KW,F– LV,G– UP 2752, H– PBW 343, I– SO. Results are expressed as the mean of three replicates (10 plants each). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

RWC in case of salt stress (Figure 10) also showed similar results. RWC in case of KD, GN, PBW 343, UP 2752 and KW showed a lesser decrease (37.7, 39.38, 46.38, 37.29 and 43.4 % respectively) during 200mM of salt stress for 3rd day with respect to their control set (0d; 0mM of salt) than in case of MW, GY, LV and SO (58.2, 58.87, 61.59, 54.95 % respectively) where increase in the concentration and the duration of salt stress resulted in a greater decline in RWC.

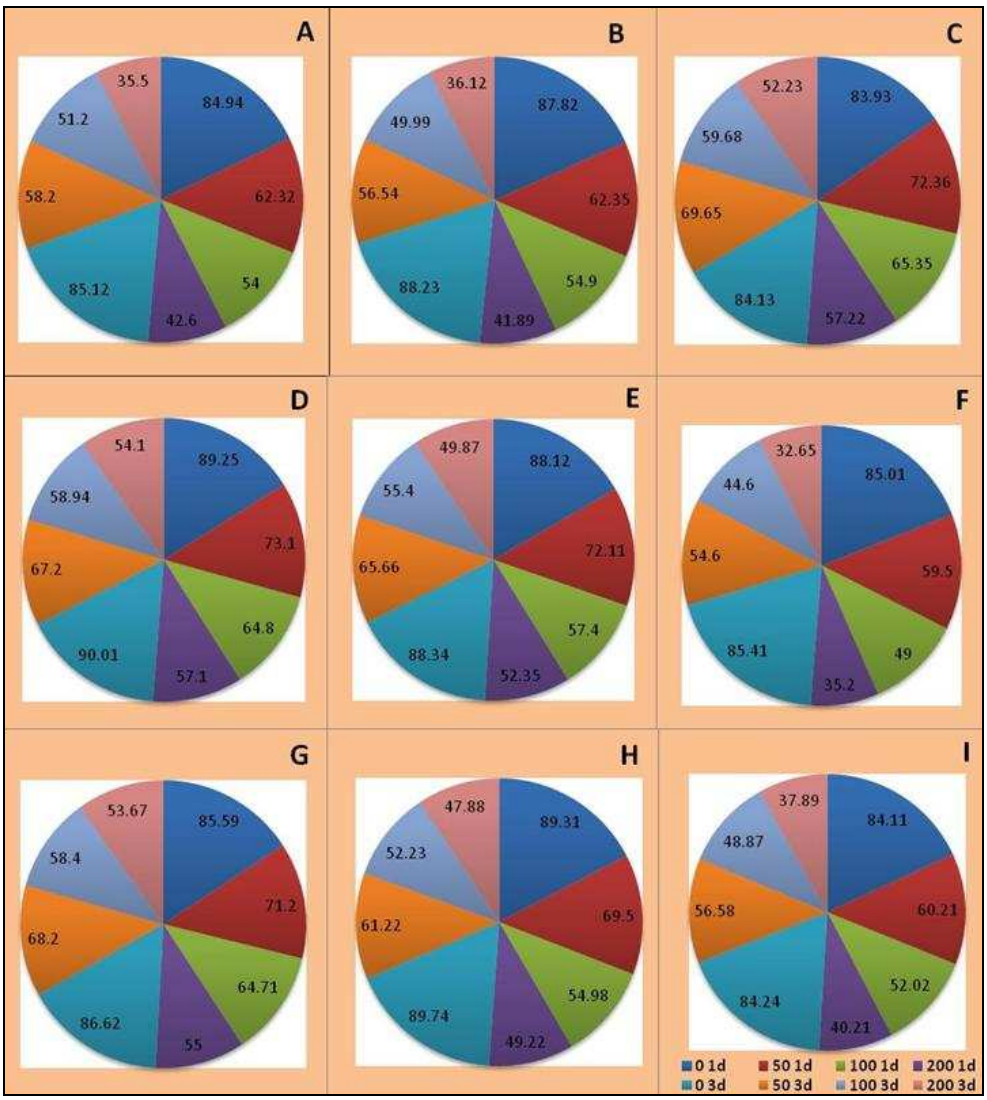

Figure 10. Relative water content of nine varieties of wheat subjected to salt (NaCl) stress treatments: A– MW, B– GY, C– KD, D– GN, E– KW,F– LV,G– UP 2752, H– PBW 343, I– SO. Results are expressed as the mean of three replicates (10 plants each). 0, 50, 100, 200 corresponds to the concentration of salt (NaCl) in mM and 1d, 3d corresponds to the days of salt treatment

Our data showed that the decrease in RWC was significantly lower in case of KD, GN, PBW 343, UP 2752 and KW on the 9th day of water stress and also during the salt concentration of 200mM on the 3rd day of salt stress when compared to their respective controls. However, the decline in RWC was much more in case of MW, GY, LV and SO with the increase in the severity of stress in terms of days and concentration with respect to their controls during drought and salt stress respectively.

4.1.3. Tolerance Index

The value of tolerance index (Table 1) following drought stress for 9 days and salt stress for 3 days at 200mM was significantly lower in case of MW, GY, LV and SO than KD, GN, KW, UP 2752 and PBW 343, the lowest value for tolerance index was recorded in case of LV and the highest value was observed in case of GN which was almost 3times the value of LV. In comparison to drought stress the wheat varieties showed a slightly higher value for tolerance index during salt stress, however, in general, the tolerance index during stress decreased in both water and salt stress in all the wheat varieties. Our data showed that GN showed significantly the highest tolerance index followed by KW, KD, UP 2752 and PBW 343 during both drought and salt stress and the tolerance index was lowest in case of LV followed by MW, GY and SO during drought and salinity stress.

Table 1. Stress tolerance index of nine wheat varieties during drought and salt stress

Varieties/Stress	Tolerance index (TI %) Drought	Salt Stress
Mohan Wonder	– 44.22 ± 4.2	– 32.67 ± 2.1
Gayetri	– 43.14 ± 3.1	– 35.45 ± 1.2
Kedar	– 22.06 ± 0.9	– 12.19 ± 1.2
Gandhari	– 19.01 ± 1.1	– 11.01 ± 1.0
Kaweri	– 21.11 ± 2.3	– 11.99 ± 3.4
Local variety	– 54.10 ± 3.9	– 41.02 ± 2.8
UP 2752	– 31.03 ± 1.3	– 16.32 ± 4.0
PBW 343	– 32.12 ± 2.2	– 18.25 ± 3.7
Sonalika	– 39.31 ± 1.6	– 28.66 ± 1.9

Results are expressed as the mean of three replicates (10 plants each). TI % in case of drought was determined for the 9th day of withholding water and in case of salt stress for the 3rd day at a salt concentration of 200mM

4.1.4. CMS

The cell membrane stability index was more or less same for all varieties during the 0 day of stress treatment in both drought (Figure 11) and salt (Figure 12) treatments. With the induction of stress treatments in the nine different wheat varieties the cell membrane stability in all the varieties showed a significant change. Our results showed that during the 3rd day of withholding water the value of cell membrane stability index was higher in case of KD, PBW 343, UP 2752, GY, and SO than the other four varieties. But during the 6th and 9th day of drought the highest value for cell membrane was recorded in case of KD, GN, KW, UP 2752, PBW 343 followed by the other four varieties. Same trend was observed in case of salinity stress where KD, GN, KW, UP 2752 and PBW 343 showed higher values for cell membrane stability index during the 1st and 3rd day of salt treatment. The CMS index was lower during the third day of salt treatment and lowest at the salt concentration of 200mM in general in all the nine varieties of wheat.

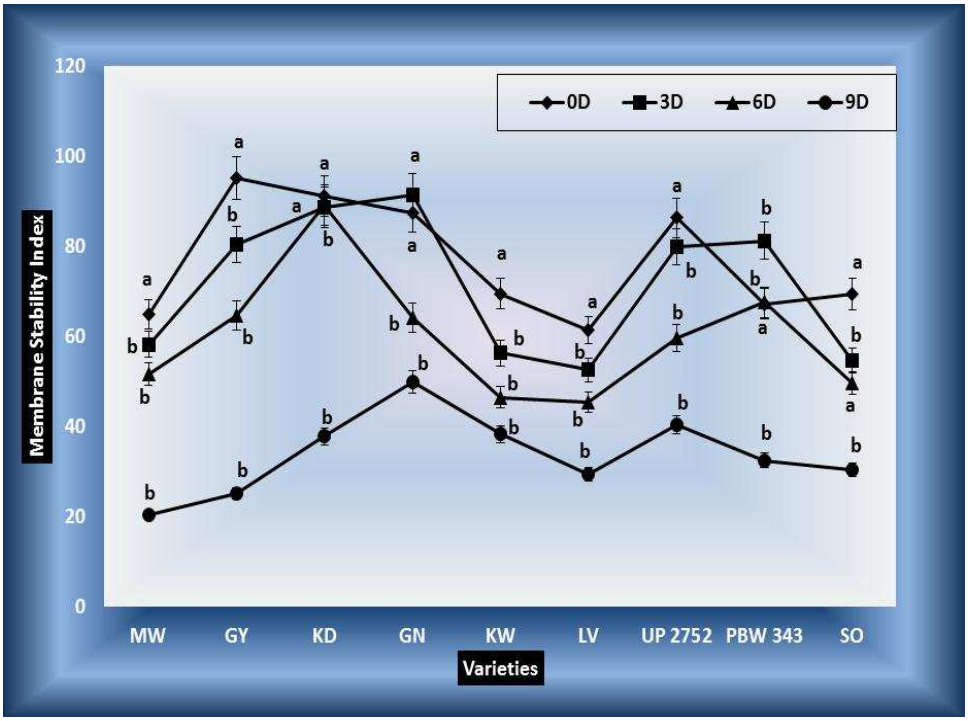

Figure 11. Cell membrane stability in nine wheat varieties subjected to drought stress treatments expressed in terms of cell membrane stability index with K=0.946, Cell constant=1, solution condition=84µS, coefficient–1, 25°C. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$).0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

CMS index in the wheat varieties was higher for salt stress in general than during drought stress. In both the stresses, cell membrane stability index of the cell was greater in case of KD, GN, KW, UP 2752 and PBW 343 with the highest value recorded in GN. LV, GY, SO and MW showed lower value for cell membrane stability index and the lowest value was observed in LV following water and salinity stress treatments .

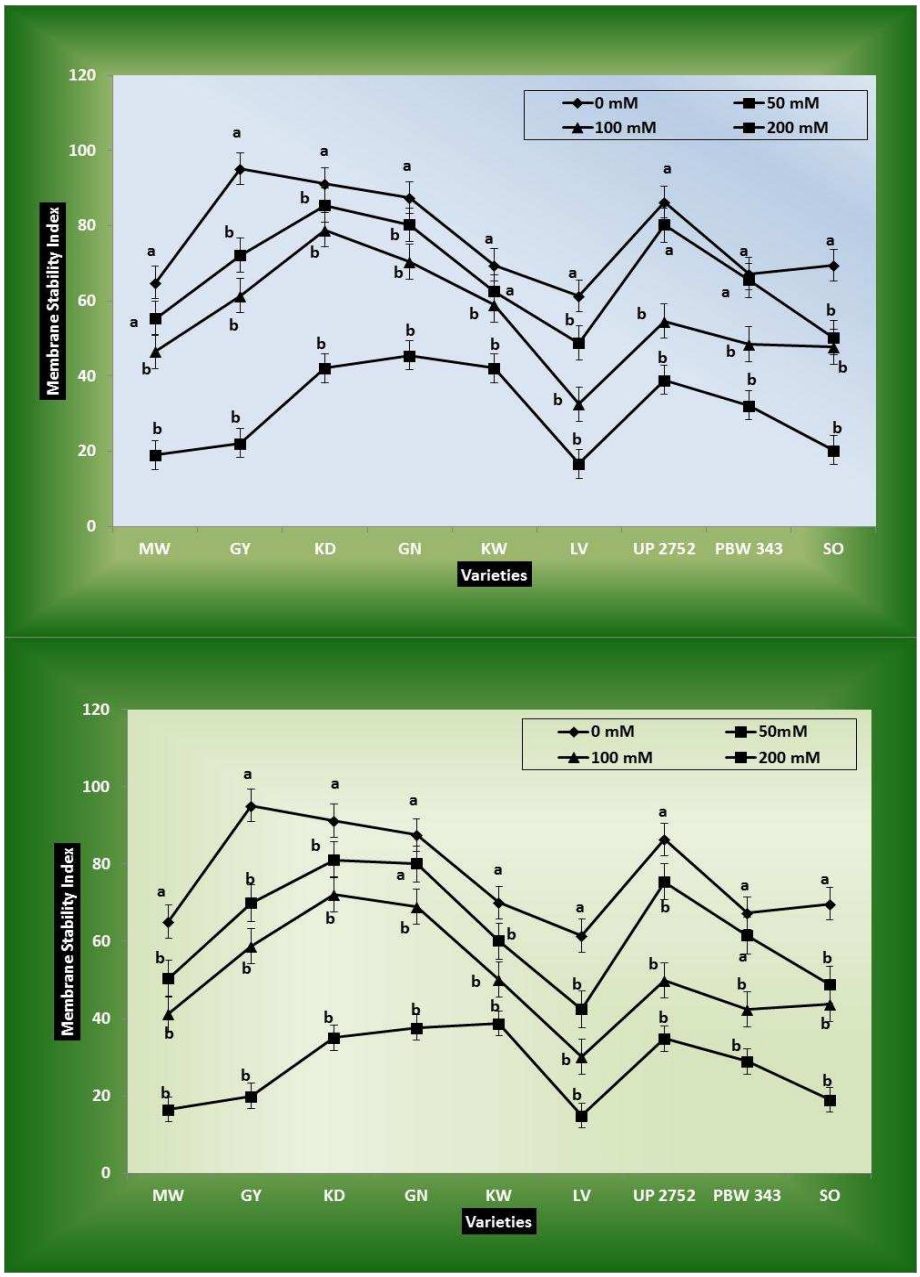

Figure 12. Cell membrane stability in nine wheat varieties subjected to salt (NaCl) stress treatments for 1 day (A) and 3 days (B) expressed in terms of cell membrane stability index with K=0.946, Cell constant=1, solution condition=84µS, coefficient–1, 25°C. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

4.2. Effect of osmotic stress on lipid peroxidation of membranes

4.2.1. Water

In the data presented in our study indicated that although MDA content, a measure of lipid peroxidation, increased in all the nine varieties during water stress in general. After the 9th day of withholding water, MDA content in case of MW, GY, LV and SO was more than three times that of KW, GN, KD, UP 2752 and PBW 343 (Figure 13). MDA content with respect to the control set (0 days of water stress) was more or less similar in all varieties. Our data showed that MDA content in case of LV was highest during the 9th days of water stress and lowest was observed in case of GN and KD which suggest that in LV the peroxidation of lipids in the cell membrane was highest in case of LV during drought which increased with increase in the duration of water stress.

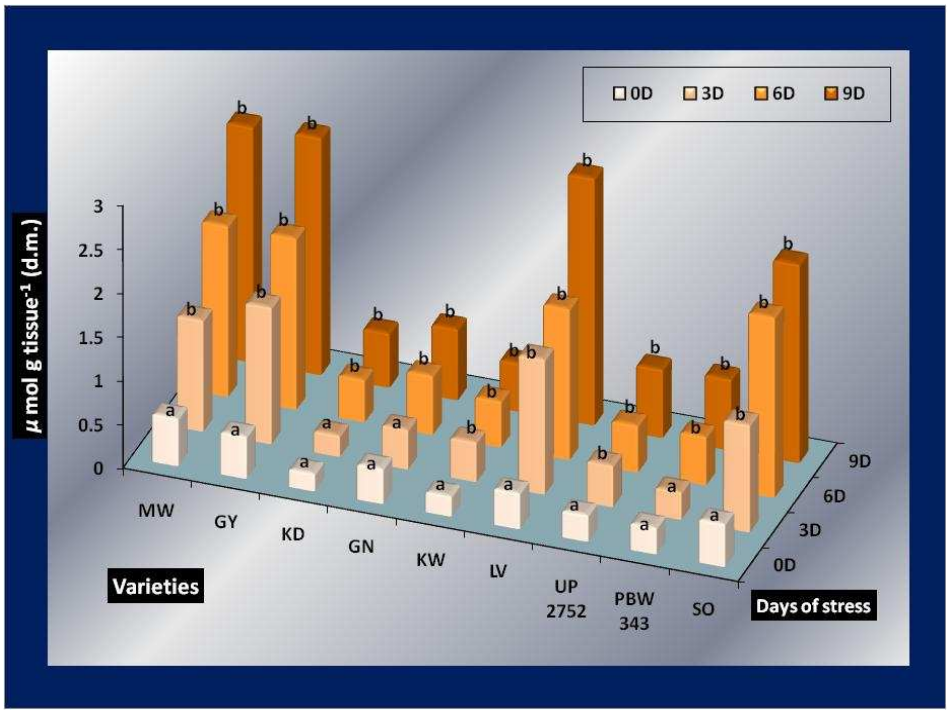

Figure 13. Effect of drought stress on lipid peroxidation (expressed as MDA content) in nine varieties of wheat. Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

4.2.2. Salinity

MDA content following salt stress increased significantly in all the nine varieties with the increase in salt concentration and the duration of salt

stress with the highest value observed during the 3rd day of salt stress of 200mM. As observed in water stress, here too MDA content in case of MW, GY, LV and SO was more than three to four times that of KW, GN, KD, UP 2752 and PBW 343 (Figure 14) during both the 1st and 3rd day of stress.

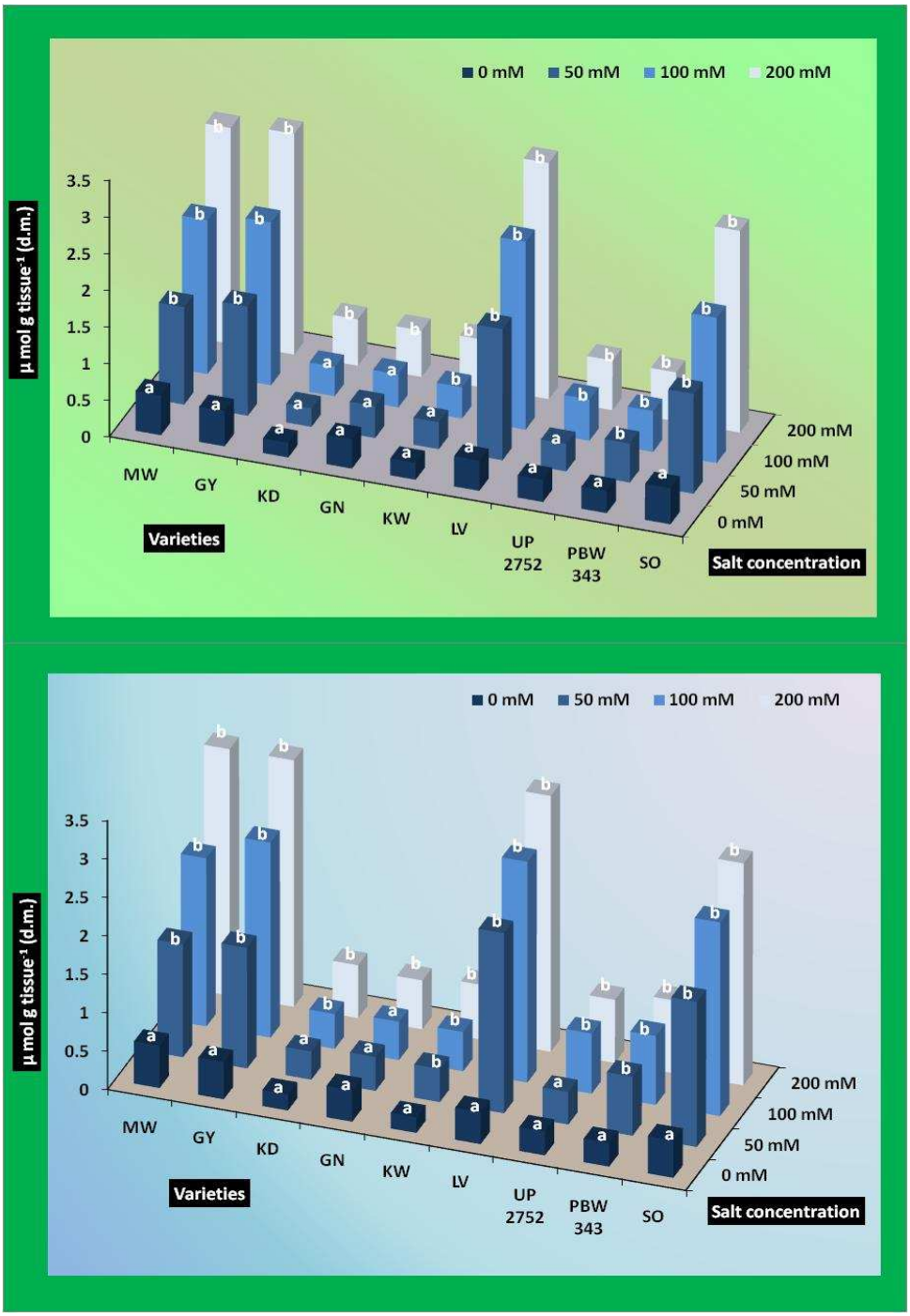

Figure 14. Effect of salt (NaCl) stress on the lipid peroxidation (expressed as MDA content) in the leaf of nine wheat varieties for the 1st day (A) and 3rd day (B). Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

The highest value of MDA content was observed during the 3rd day of 200mM salt concentration in all varieties with the highest value observed in case of LV and the lowest in case GN. It was evident that the content of MDA was much more during drought in wheat varieties than during salt stress.

4.3. Change in proline content during water and salinity stress

Free proline accumulation in leaf and root was enhanced significantly in all nine varieties during prolonged drought stress (Table 2) and salinity stress (Table 3) after an initial non significant decrease in some cases.

Table 2. Content of proline in the leaves of wheat varieties subjected to water stress

Varieties	Part used	Proline Days of Stress			
		0D	3D	6D	9D
MW	Leaf	2.30±0.03[a]	1.90±0.02[a]	3.60±0.04[b]	6.90±0.08[b]
	Root	0.18±0.05[a]	0.29±0.01[b]	0.23±0.03[b]	0.40±0.04[b]
GY	Leaf	2.20±0.06[a]	2.00±0.01[a]	4.80±0.14[b]	5.80±0.01[b]
	Root	0.19±0.04[a]	0.24±0.03[b]	0.28±0.02[b]	0.44±0.04[a]
KD	Leaf	1.90±0.07[a]	1.40±0.08[a]	11.1±0.03[b]	20.3±0.50[b]
	Root	0.11±0.01[a]	0.24±0.02[b]	0.56±0.03[b]	1.08±0.03[b]
GN	Leaf	2.00±0.07[a]	2.60±0.04[a]	6.00±0.07[b]	10.6±0.20[b]
	Root	0.35±0.03[a]	0.53±0.02[b]	0.24±0.04[a]	0.71±0.02[b]
KW	Leaf	2.00±0.02[a]	2.90±0.02[a]	8.10±0.03[b]	10.8±0.02[b]
	Root	0.14±0.01[a]	0.17±0.01[a]	0.23±0.04[b]	0.34±0.03[b]
LV	Leaf	1.70±0.05[a]	1.60±0.06[a]	5.00±0.10[b]	5.10±0.02[b]
	Root	1.46±0.02[a]	0.31±0.01[a]	0.26±0.02[a]	0.40±0.03[b]
UP 2752	Leaf	2.50±0.04[a]	2.30±0.05[a]	9.80±0.01[b]	11.2±0.05[b]
	Root	0.92±0.02[a]	0.53±0.04[a]	0.75±0.01[a]	0.81±0.03[b]
PBW 343	Leaf	1.70±0.06[a]	2.30±0.05[b]	7.50±0.06[b]	11.9±0.10[b]
	Root	1.26±0.02[a]	0.56±0.03[b]	0.29±0.02[a]	0.30±0.02[a]
SO	Leaf	1.50±0.02[a]	2.60±0.05[b]	4.90±0.01[b]	6.10±0.08[b]
	Root	0.38±0.02[a]	0.37±0.02[a]	0.51±0.04[b]	0.58±0.01[b]
CD Value between treatments =		Leaf–2.298351; Root–0.291377			
CD Value between varieties =		Leaf–3.447526; Root–0.437065			

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Proline = mg g^{-1} (d.m.).

After 9 days, in case of water stress proline content of both leaf and root in case of GN, KD, KW, UP 2752 and PBW 343 was 2.5 times higher than that of LV, GY, SO and MW, although in control plants all varieties had more or less similar amounts.

During salinity stress the accumulation of proline in the salt stressed plant increased with the increase in concentration of salt and the duration of salt stress. This study revealed that in GN, KD, KW, UP 2752 and PBW 343 the accumulation of free proline in both leaf and root was almost 3times higher than that of LV, GY, MW and SO in all concentration of salt stress during the 3rd day however, the during the first day of salt stress this trend varied in some varieties. Proline content in wheat varieties in response to salt stress was higher than during the conditions of drought.

Table 2(a). ANOVA of data presented in table 2 for drought in leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	392.4919	3	130.8306	23.4445	2.59E–07	3.008787
Columns	98.645	8	12.33063	2.209615	0.063691	2.355081
Error	133.9306	24	5.58044			
Total	625.0675	35				

Table 2(b). ANOVA of data presented in table 2 for drought in root

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.330636	3	0.110212	1.228808	0.320931	3.008787
Columns	0.990773	8	0.123847	1.380826	0.254524	2.355081
Error	2.152565	24	0.08969			
Total	3.473973	35				

Table 3. Content of proline in the leaves of wheat varieties subjected to salt stress

Varieties	Treatment (mM)	Proline 1D Leaf	Proline 1D Root	Proline 3D Leaf	Proline 3D Root
MW	0	2.30±0.03[a]	0.18±0.05[a]	2.31±0.05[a]	0.17±0.05[a]
	50	2.37±0.02[a]	0.20±0.01[a]	3.14±0.01[b]	0.22±0.01[a]
	100	3.78±0.04[b]	0.19±0.03[a]	3.97±0.03[b]	0.18±0.03[a]
	200	6.67±0.08[b]	0.41±0.04[b]	7.98±0.04[b]	0.42±0.04[b]
GY	0	2.20±0.06 [a]	0.19±0.04[a]	2.19±0.04[a]	0.21±0.04[a]
	50	2.54±0.01[a]	0.21±0.03[a]	3.42±0.03[b]	0.24±0.03[b]
	100	3.89±0.14[b]	0.17±0.02[a]	4.01±0.02[b]	0.19±0.02[a]
	200	6.86±0.01[b]	0.44±0.04[b]	6.97±0.04[a]	0.44±0.04[a]
KD	0	1.90±0.07[a]	0.11±0.01[a]	2.01±0.01[a]	0.12±0.01[a]
	50	8.11±0.08[b]	0.41±0.02[b]	10.40±0.02[b]	0.43±0.02[b]
	100	13.64±0.03[b]	0.45±0.03[b]	15.34±0.03[b]	0.54±0.03[b]
	200	18.75±0.50[b]	0.89±0.03[b]	19.87±0.03[b]	1.01±0.03[b]
GN	C	2.00±0.07[a]	0.35±0.03[a]	1.98±0.03[a]	0.35±0.03[a]
	50	11.10±0.04[b]	0.54±0.02[b]	11.68±0.02[b]	0.61±0.02[b]
	100	13.46±0.07[b]	0.74±0.04[b]	18.14±0.04[a]	0.91±0.04[a]
	200	21.10±0.20 [b]	0.91±0.02[b]	24.1±0.02[b]	1.27±0.02[b]
KW	0	2.00±0.02[a]	0.24±0.01[a]	2.01±0.01[a]	0.24±0.01[a]
	50	6.45±0.02[b]	0.39±0.01[b]	7.23±0.01[b]	0.61±0.01[b]
	100	11.02±0.03[b]	0.40±0.04[b]	13.36±0.04[b]	0.87±0.04[b]
	200	14.12±0.02[b]	0.80±0.03[b]	16.84±0.03[b]	0.98±0.03[b]
LV	0	1.70±0.05[a]	0.21±0.02[a]	1.69±0.02[a]	0.22±0.02[a]
	50	2.01±0.06[a]	0.28±0.01[a]	3.60±0.01[b]	0.38±0.01[b]
	100	3.56±0.10[b]	0.15±0.02[a]	3.74±0.02[b]	0.27±0.02[a]
	200	5.50±0.02[b]	0.35±0.03[b]	4.89±0.03[b]	0.39±0.03[b]
UP 2752	0	2.50±0.04[a]	0.21±0.02[a]	2.48±0.02[a]	0.21±0.02[a]
	50	4.31±0.05[b]	0.41±0.04[b]	4.65±0.04[a]	0.48±0.04[b]
	100	5.12±0.01[b]	0.45±0.01[b]	5.56±0.01[b]	0.50±0.01[b]
	200	7.85±0.05[b]	0.61±0.03[b]	9.78±0.03[b]	0.72±0.03[b]
PBW 343	0	1.70±0.06[a]	0.21±0.02[a]	1.71±0.02[a]	0.22±0.02[a]
	50	3.40±0.05[b]	0.35±0.03[b]	3.97±0.03[b]	0.37±0.03[b]
	100	4.52±0.06[b]	0.44±0.02[b]	4.98±0.02[b]	0.48±0.02[b]
	200	7.78±0.10[b]	0.55±0.02[b]	8.95±0.02[b]	0.70±0.02[b]
SO	0	1.50±0.02[a]	0.23±0.02[a]	1.51±0.02[a]	0.23±0.02[a]
	50	2.94±0.05[b]	0.29±0.02[b]	3.45±0.02[a]	0.31±0.02[b]
	100	5.64±0.01[b]	0.26±0.04[a]	5.97±0.04[b]	0.29±0.04[a]
	200	7.40±0.08[b]	0.39±0.01[b]	6.97±0.01[b]	0.51±0.01[b]
CD Value between treatments =		2.446291	0.099181	2.936868	0.136177
CD Value between varieties =		3.669436	0.148771	4.405301	0.204266

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Proline = mg g^{-1} (d.m.).

Table 3(a). ANOVA of data presented in table 3 for salinity in leaf for 1 day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	367.5001	3	122.5	19.3769	1.33E–06	3.008787
Columns	343.3441	8	42.91801	6.788716	0.000116	2.355081
Error	151.7271	24	6.321962			
Total	862.5713	35				

Table 3(b). ANOVA of data presented in table 3 for salinity in root for 1 day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.675144	3	0.225048	21.65628	5.18E–07	3.008787
Columns	0.551506	8	0.068938	6.633898	0.000137	2.355081
Error	0.249404	24	0.010392			
Total	1.476054	35				

Table 3(c). ANOVA of data presented in table 3 for salinity in leaf for 3 days

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	465.3573	3	155.1191	17.02396	3.86E–06	3.008787
Columns	484.4632	8	60.5579	6.64609	0.000135	2.355081
Error	218.6834	24	9.111808			
Total	1168.504	35				

Table 3(d). ANOVA of data presented in table 3 for salinity in root for 3 days

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.136312	3	0.378771	19.33445	1.36E–06	3.008787
Columns	1.097372	8	0.137172	7.001957	9.15E–05	2.355081
Error	0.470171	24	0.01959			
Total	2.703855	35				

4.4. HPLC profiles of free amino acids following drought stress

Free amino acids detected in HPLC following drought stress for 0, 3, 6 and 9 days in case of wheat (GN and LV) showed different peaks of amino acids which either appeared or disappeared with increasing days of drought stress (Figure 15a–b, 16a–b). There was a marked difference in the number of peaks, peak height and area in both (Table 4–12) the varieties following drought stress and also between the duration of stresses in each variety respectively.

The first peak (peak 1) in each treatment in both the varieties with retention time at 2.5 minutes was not identified; however, the height of the peak decreased with the increase in the duration of water stress in both the varieties Figure (15 and 16). Similarly peak number 19, 20 & 21 with retention time 31, 37 and 41 minutes respectively in all the treatments in both the variety could not be identified and was not taken into consideration for the analysis of any free amino acid (peak 13 and 14).

Peak number 2 with retention time at about 9 minutes was identified as aspartic acid and peak number 3 with retention time at about 10 minutes was identified as glutamic acid and the height as well as the area of both the peaks i.e. peak 2 and 3 decreased with increasing duration of drought treatment in both the varieties. The peak for aspartic acid was absent during the 6^{th} day of drought in case of GN (Figure 15b) and LV (Figure 16b) and the peak for glutamic acid was absent in LV during 6^{th} day of drought (Figure 16b). However both the peaks were present during the 9^{th} day of drought in both the varieties but the height and area of both the peak was very small (15b and 16b). The peak height of glutamic acid was higher than that of aspartic acid in both the varieties and in GN both the amino acid had a greater peak area than LV (Table 4–11).

The height and area of peak number 4 which was identified as serine with retention time at around 14 minutes in each case increased with the increase in the duration of stress in case of GN but the peak height and area decreased in case of LV. Peak height and area of threonine (Peak 5) with retention time of about 15 minutes in both the varieties increased with increase in the duration of stress with the increase in case of GN much greater than that of LV whereas the height and area of the peak number 6 identified as DL alanine showed an increase in case of GN with the increase in the days of drought however the same decreased in case of LV. A small peak with retention time of around 16 minutes was identified as arginine (peak 7) was observed in all the varieties under stress however not much reference could be made due to the small area and height of the peak.

The tallest peak with largest area for amino acid in both the variety was identified as proline (Peak 8) during all the days of stress with retention time of about 17 minutes during the stress treatments**.** The peak height and area of proline following drought stress increased significantly following drought stress in case of GN with the increase in the duration of water stress and the tallest peak was obtained for the 9^{th} day of stress. Similarly out of all the peaks, the height and area of peak number 8 for proline in LV increased significantly in the early days of drought but significantly decreased during the 6^{th} day (Figure 16b) of drought however the height was comparatively lesser than that of the peak height in case of GN (Figure 15a–b & 16a–b). This result was in accordance with the data obtained for the content of free proline in case of GN and LV during the drought stress.

Peak number 9 with retention time around 20 minutes in both the varieties was identified as hydroxy proline which in terms of height and area decreased with increase in the duration of drought in both GN and LV. Tyrosine with retention time around 20.60 minutes (peak 10) in case of both

GN and LV showed an increase in its height and area with increasing duration of drought stress however, the height and area of peak 11 identified as valine with retention time of about 22.50 minutes increased significantly during increasing days of drought stress in case of GN (Figure 15a–b) whereas in case of LV (Figure 16a–b) the increase was insignificant and the peak for valine was absent during the 6^{th} day of drought (Figure 16b).

The peak 12 was identified as methionine with retention time about 22.90 minutes. An unidentified peak (peak 13) with retention time at about 24 minutes was seen along with peak 14 identified as leucine with retention time of about 24.8 minutes and peak 15 was identified as cysteine with retention time at about 26 minutes showed a negligible increase or decrease in both the varieties during the increasing days of water stress and the change in the peak height and area during drought stress was not very significant in drought stress in LV however in case of GN, which was the more tolerant variety among the two, the change in the height and area of these peaks were noteworthy. There was a significant increase in the peak height and area of methionine in case of GN following drought treatments (Figure 15 a–b).

The peaks identified as isoleucine (peak 16) at the retention time of about 26.8 minutes appeared during all days of stress as well as in the respective control sets with a slight increase in the height and area in case of GN however in LV it appeared but the change in the height and area was not significant (Figure 15 and 16). DL–phenyl–alanine (peak 17) at a retention time of around 28.8 minutes and peak number 18 identified as lysine with retention time around 29.6 minutes (Figure 15 and 16) showed a significant increase in its height and area with the increase in the duration of drought in case of GN (Figure 15 a–b) however in LV the change was noticeable only during the 9^{th} day of drought (15b).

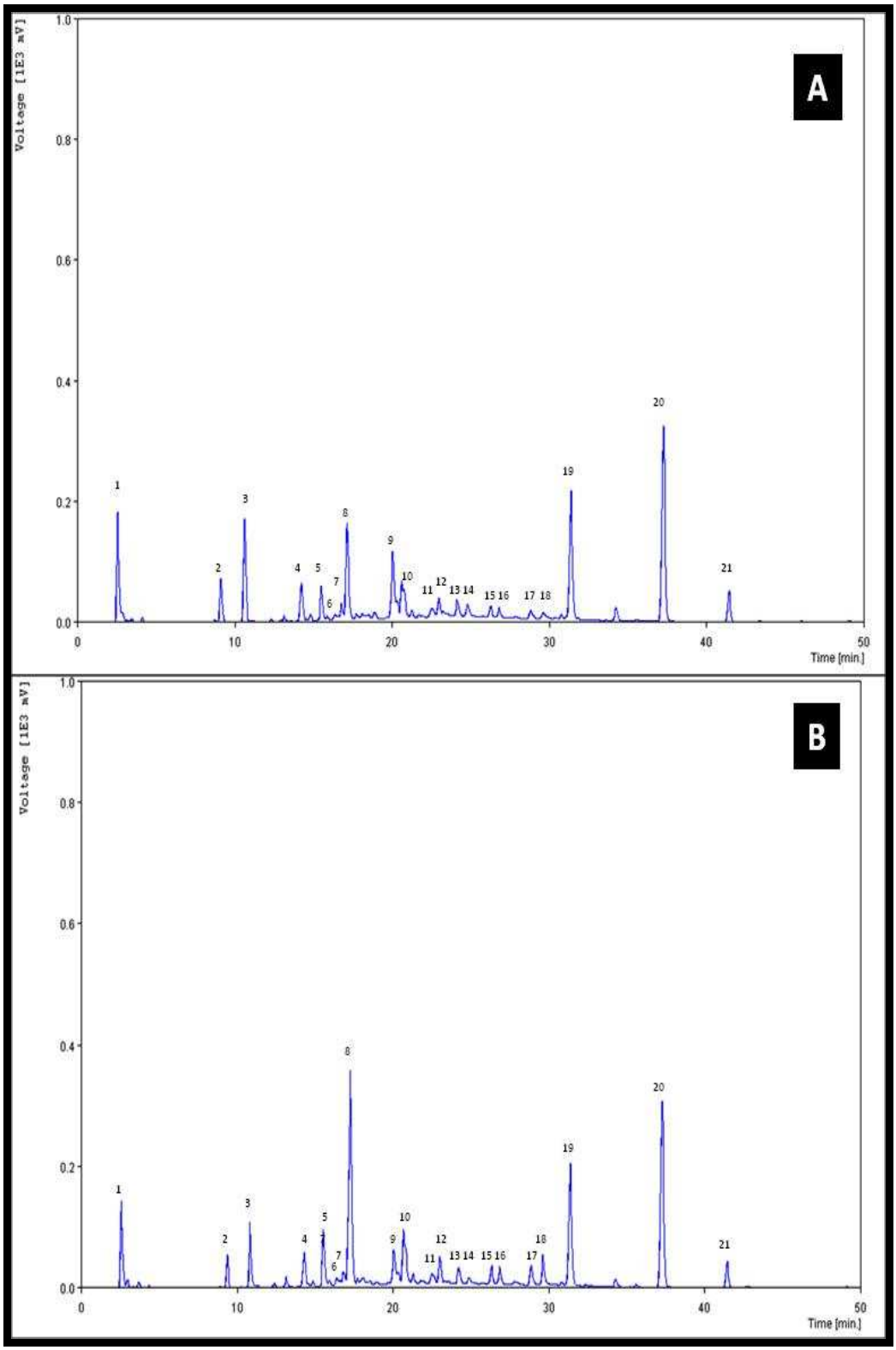

Figure 15 (a). Free amino acid profile detected in HPLC in case of wheat (GN) subjected to A–0D and B–3D of drought stress

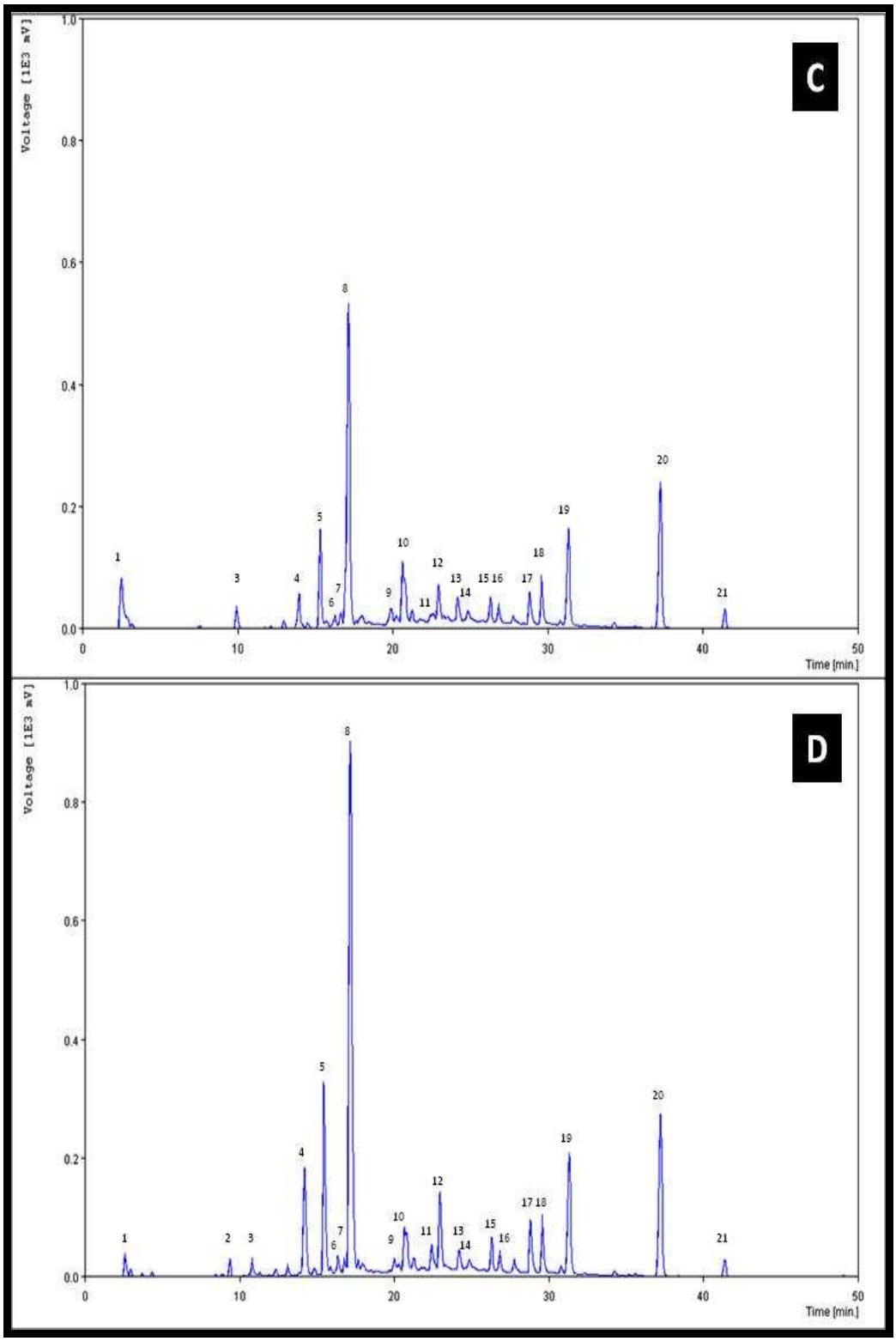

Figure 15 (b). Free amino acid profile detected in HPLC in case of wheat (GN) subjected to C–6D and D–9D of drought stress

Table 4: Peak table of data presented in Figure 15a (A) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.500	2454.7336	189.381
2	9.100	799.0168	76.963
3	10.600	1843.5861	176.215
4	14.220	1023.2114	69.548
5	15.500	887.8070	64.310
6	16.390	288.4517	16.699
7	16.770	569.1539	35.818
8	17.110	2262.0714	168.429
9	20.040	2273.2002	121.021
10	20.620	1431.1736	72.128
11	22.550	673.1000	26.248
12	22.980	598.0892	43.672
13	24.150	960.3388	41.051
14	24.800	1032.9951	32.739
15	26.280	649.7862	30.649
16	26.820	752.4103	28.774
17	28.840	614.5775	21.990
18	29.610	677.9344	19.715
19	31.400	3444.2072	220.399
20	37.290	4805.7144	329.049
21	41.470	770.7729	55.030

Table 5: Peak table of data presented in Figure 15a (B) GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.520	1680.3417	151.115
2	9.330	666.7752	60.997
3	10.800	1373.5412	115.447
4	14.290	1005.5263	63.975
5	15.530	1302.8791	102.427
6	16.400	518.6110	21.937
7	16.810	439.1315	31.598
8	17.250	5269.3238	365.293
9	20.060	1468.5171	68.241
10	20.680	1756.1927	101.653
11	22.490	677.6143	28.395
12	23.010	744.7934	56.736
13	24.230	730.1610	38.193
14	24.870	705.1966	20.995
15	26.330	716.4379	41.837
16	26.860	889.6410	40.350
17	28.880	816.0610	41.231
18	29.640	1096.7126	59.025
19	31.390	3280.4750	209.572
20	37.300	4624.0919	313.067
21	41.480	684.2122	47.109

Table 6: Peak table of data presented in Figure 15b (C) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.440	1828.8683	88.673
3	9.890	641.1135	43.535
4	13.930	1018.0248	64.271
5	15.290	1978.2777	168.970
6	16.250	494.7058	27.164
7	16.620	406.7986	31.123
8	17.110	7727.2651	540.641
9	19.860	821.0983	38.221
10	20.630	2091.0161	114.573
11	21.240	675.1052	35.309
12	22.950	1040.2782	76.375
13	24.200	1184.3491	55.875
14	24.840	1084.5537	33.572
15	26.300	971.1378	55.855
16	26.840	982.2810	44.288
17	28.850	1154.3958	63.233
18	29.610	1278.6776	91.410
19	31.340	2759.7213	168.056
20	37.270	3439.5776	242.879
21	41.450	454.6914	33.894

Table 7: Peak table of data presented in Figure 15b (D) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.530	605.0279	46.156
2	9.330	405.2477	36.051
3	10.780	553.5488	38.124
4	14.190	2542.2227	191.320
5	15.430	4035.9322	335.686
6	16.360	702.2034	41.248
7	16.760	494.5221	37.296
8	17.120	14146.9977	910.730
9	20.010	604.1822	35.952
10	20.660	1809.3209	88.717
11	22.430	1046.3356	59.416
12	22.960	1847.3053	147.476
13	24.220	1040.1130	49.584
14	24.850	1049.2902	32.037
15	26.300	1067.0716	71.316
16	26.840	1031.0702	47.925
17	28.830	1526.1322	99.305
18	29.590	1878.4100	109.042
19	31.330	3312.8786	212.671
20	37.240	4057.6612	277.742
21	41.410	422.0595	30.851

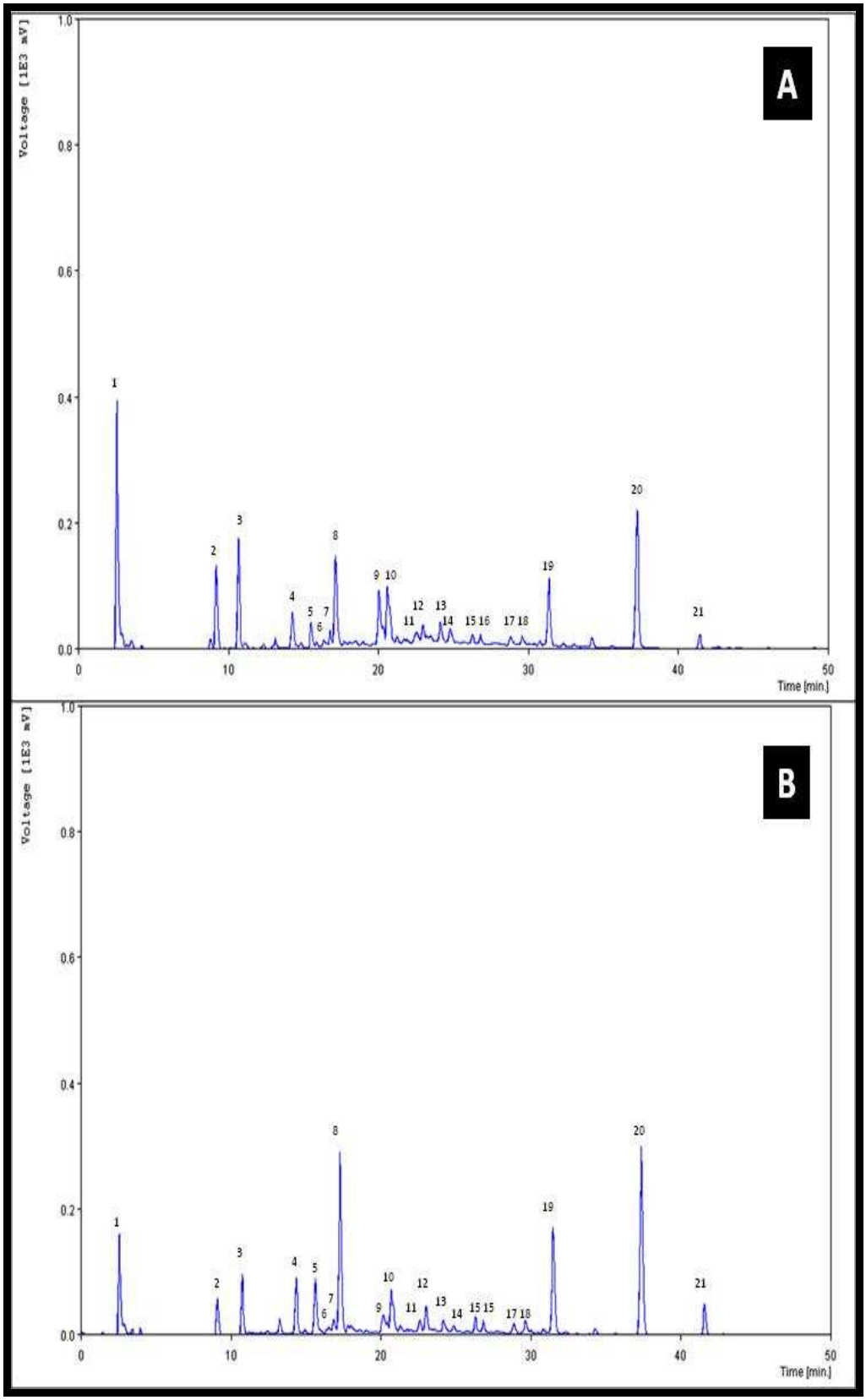

Figure 16 (a). Free amino acid profile detected in HPLC in case of wheat (LV) subjected to A–0D and B–3D of drought stress

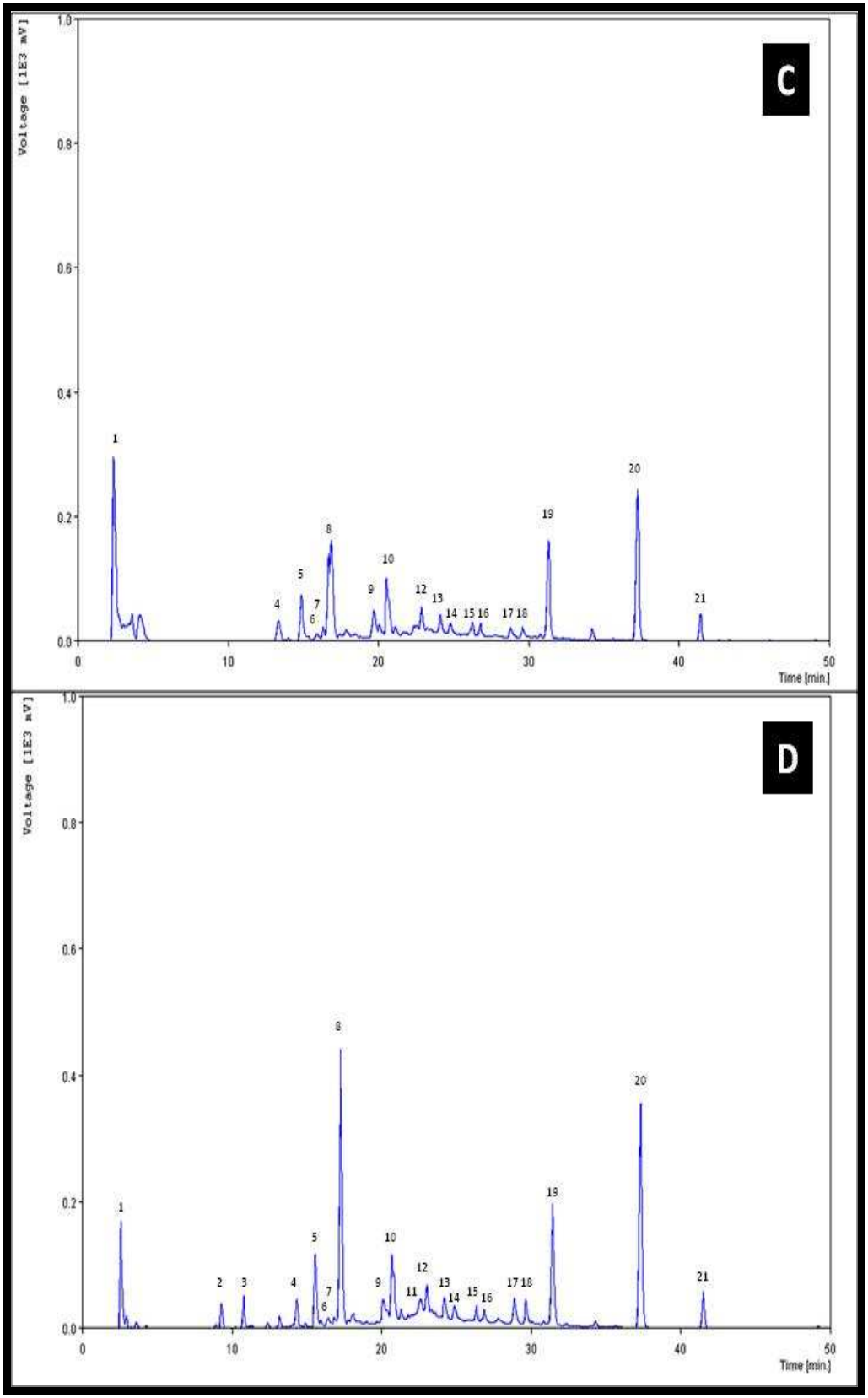

Figure 16 (b). Free amino acid profile detected in HPLC in case of wheat (LV) subjected to C–6D and D–9D of drought stress

Table 8: Peak table of data presented in Figure 16a (A) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.510	4780.6134	401.945
2	9.160	1434.1415	137.644
3	10.640	1887.2006	180.559
4	14.250	994.1142	63.100
5	15.500	588.4011	46.198
6	16.380	398.1190	17.865
7	16.770	432.4622	34.383
8	17.110	2203.1624	153.168
9	20.050	1872.5593	96.820
10	20.600	1839.3910	103.929
11	22.560	786.2054	29.855
12	22.970	615.7713	42.774
13	24.140	986.8426	45.875
14	24.800	1119.8597	35.764
15	26.280	567.2756	26.296
16	26.810	663.0876	25.791
17	28.830	636.0343	22.204
18	29.600	519.5345	23.961
19	31.400	1796.3278	114.494
20	37.290	3368.0055	223.584
21	41.470	358.1553	24.644

Table 9: Peak table of data presented in Figure 16a (B) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.500	2275.1547	167.141
2	9.080	832.3004	64.390
3	10.720	1177.6533	102.893
4	14.340	1303.6096	97.659
5	15.620	1426.6971	95.462
6	16.510	404.3379	17.494
7	16.860	412.7705	29.755
8	17.270	3809.7558	296.460
9	20.170	659.9984	36.805
10	20.690	1329.4643	77.031
11	22.580	533.7188	27.816
12	23.020	865.7804	51.448
13	24.180	593.9813	28.301
14	24.840	674.6569	18.778
15	26.310	568.5359	33.527
16	26.840	534.6730	28.312
17	28.890	509.7613	21.820
18	29.680	546.5808	27.739
19	31.510	2587.9390	174.971
20	37.410	4478.6944	304.667
21	41.630	731.7718	52.131

Table 10: Peak table of data presented in Figure 16b (C) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.340	4573.5095	299.534
4	13.330	799.3064	37.407
5	14.850	1485.5144	78.414
6	15.920	345.7437	14.693
7	16.310	363.4229	26.756
8	16.870	3896.0980	167.019
9	19.710	1549.8314	53.436
10	20.550	2398.8081	104.742
12	22.880	3175.8947	57.897
13	24.130	1155.5793	46.343
14	24.790	958.1485	31.125
15	26.250	723.9730	33.527
16	26.780	748.5402	32.182
17	28.820	653.5972	24.086
18	29.600	918.4992	26.065
19	31.330	2844.6023	165.199
20	37.270	3920.9977	246.418
21	41.460	618.6119	45.811

Table 11: Peak table of data presented in Figure 16b (D) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.520	1978.6435	175.639
2	9.250	520.4148	45.478
3	10.750	655.6983	56.946
4	14.300	780.0219	51.417
5	15.550	1543.6451	123.422
6	16.430	511.2879	22.333
7	16.830	332.8812	22.742
8	17.250	5870.3324	447.977
9	20.120	1374.0169	51.139
10	20.690	2241.2585	121.556
11	22.590	1701.4378	50.767
12	23.020	1103.2839	73.754
13	24.220	1175.2906	53.335
14	24.870	1344.7794	40.541
15	26.340	828.8331	40.547
16	26.870	922.7067	34.208
17	28.900	1104.2693	51.101
18	29.670	839.4179	49.263
19	31.450	3242.5950	201.936
20	37.350	5727.9917	361.974
21	41.550	864.4538	61.225

4.5. Effect of water and salt stress on chlorophyll contents of wheat leaves

Chlorophylls, showed a significant decrease in all varieties following water (Table 12) and salt (Table 13) stress. However, in GN and KD there was an initial non significant increase with the onset of both water and salt stress.

Table 12. Content of total chlorophyll and chlorophyll a/b ratio in wheat varieties subjected to water stress.

Varieties	Treatment (in days)	Total chlorophyll	Chl a/b ratio
MW	0d	0.93 ±0.08 [a]	1.54
	3d	0.56 ±0.01 [b]	1.95
	6d	0.36 ±0.03 [b]	1.20
	9d	0.28 ±0.01 [b]	0.95
GY	0d	1.01 ±0.06 [a]	1.25
	3d	0.56 ±0.08 [b]	1.99
	6d	0.45 ±0.01 [b]	1.33
	9d	0.41 ±0.02 [b]	0.76
KD	0d	0.93 ±0.06 [a]	1.45
	3d	0.98 ±0.07 [a]	1.65
	6d	0.48 ±0.02 [b]	1.45
	9d	0.27 ±0.01 [b]	1.04
GN	0d	1.05 ±0.07 [a]	1.45
	3d	1.21 ±0.04 [a]	1.94
	6d	0.65 ±0.07 [b]	1.86
	9d	0.57 ±0.20 [b]	1.67
KW	0d	0.90 ±0.04 [a]	1.57
	3d	0.77 ±0.02 [b]	1.66
	6d	0.49 ±0.02 [a]	1.54
	9d	0.31 ±0.01 [b]	1.78
LV	0d	1.22 ±0.05 [a]	0.70
	3d	0.41 ±0.04 [a]	1.44
	6d	0.31 ±0.02 [b]	1.55
	9d	0.29 ±0.01 [b]	0.89
UP 2752	0d	2.30 ±0.07 [a]	1.47
	3d	0.75 ±0.06 [b]	1.60
	6d	0.63 ±0.01 [b]	1.59
	9d	0.62 ±0.03 [b]	1.66
PBW 343	0d	1.73 ±0.05 [a]	1.19
	3d	0.86 ±0.03 [b]	1.24
	6d	0.57 ±0.07 [b]	1.97
	9d	0.39 ±0.11 [b]	1.93
SO	0d	0.96 ±0.09 [a]	1.34
	3d	0.62 ±0.05 [a]	1.43
	6d	0.49 ±0.04 [b]	1.38
	9d	0.31 ±0.01 [b]	1.12
CD Value between treatments =		0.306785	0.387911
CD Value between varieties =		0.460180	0.581866

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at $P \leq 0.01$, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Chlorophyll = mg g^{-1} (f.m.).

Interestingly, the chl. a/b ratio showed an initial increase in all varieties before declining during both water and salt stress, and this decrease was greater in case of GY, LV, SO and MW than in the other five varieties. The increase in the ratio of chl. a/b corresponded to the higher value of chl. a content than the content of chl. b and the decrease in the chl. a/b ratio corresponded to the lower content of chl. a than the content of chl. b during the above stresses. The chlorophyll content during salt stress in wheat varieties was higher than during drought stress.

Table 12 (a). ANOVA of data presented in table 12 for total chlorophyll

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	3.007252	3	1.002417	10.08197	0.000174	3.008787
Columns	0.903596	8	0.112949	1.136007	0.375841	2.355081
Error	2.386241	24	0.099427			
Total	6.297089	35				

Table 12 (b). ANOVA of data presented in table 12 for chl. a/b

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.634885	3	0.211628	1.331297	0.287507	3.008787
Columns	0.885396	8	0.110675	0.696224	0.691425	2.355081
Error	3.815139	24	0.158964			
Total	5.335421	35				

Table 13. Content of total chlorophyll and chlorophyll a/b in wheat varieties subjected to salt stress

Varieties	Treatment (mM)	Total chlorophyll		Chl a/b ratio	
		1D	3D	1D	3D
MW	0	0.93 ±0.08 [a]	0.93 ±0.05 [a]	1.54	1.57
	50	0.82 ±0.02 [a]	0.82 ±0.04 [a]	1.96	2.13
	100	0.81 ±0.02 [a]	0.79 ±0.02 [b]	2.68	1.86
	200	1.24 ±0.02 [b]	1.11 ±0.02 [b]	1.47	1.01
GY	0	1.01 ±0.04 [a]	0.99 ±0.05 [a]	1.25	1.29
	50	0.82 ±0.04 [a]	0.60 ±0.04 [b]	2.29	2.21
	100	0.81 ±0.02 [a]	0.80 ±0.04 [b]	1.79	1.75
	200	0.91 ±0.01 [b]	0.87 ±0.01 [a]	1.52	1.62
KD	0	0.93 ±0.04 [a]	0.92 ±0.03 [a]	0.85	0.86
	50	0.97 ±0.04 [a]	0.96 ±0.03 [a]	1.43	1.25
	100	0.70 ±0.03 [b]	0.68 ±0.01 [b]	2.69	2.66
	200	0.52 ±0.02 [b]	0.36 ±0.02 [b]	2.47	2.74
GN	0	1.05 ±0.05 [a]	1.11 ±0.06 [a]	1.07	1.06
	50	1.19 ±0.03 [a]	1.22 ±0.02 [a]	1.04	1.03
	100	0.65 ±0.06 [b]	0.58 ±0.05 [b]	2.66	2.85
	200	0.55 ±0.05 [b]	0.52 ±0.09 [b]	2.90	2.10
KW	0	0.90 ±0.03 [a]	0.91 ±0.03 [a]	1.57	1.49
	50	0.77 ±0.01 [b]	0.72 ±0.01 [b]	1.52	1.64
	100	0.53 ±0.01 [b]	0.54 ±0.01 [b]	2.49	1.89
	200	0.32 ±0.05 [b]	0.31 ±0.08 [b]	2.76	2.84
LV	0	1.22 ±0.04 [a]	1.23 ±0.04 [a]	1.26	1.30
	50	0.82 ±0.03 [b]	0.79 ±0.02 [b]	1.54	1.57
	100	0.75 ±0.04 [b]	0.66 ±0.05 [b]	1.35	1.47
	200	0.50 ±0.02 [b]	0.41 ±0.07 [b]	1.31	1.42
UP 2752	0	2.30 ±0.06 [a]	2.29 ±0.05 [a]	1.47	1.51
	50	0.81 ±0.05 [b]	0.73 ±0.02 [b]	1.61	1.96
	100	0.61 ±0.04 [b]	0.59 ±0.03 [b]	2.45	2.21
	200	0.59 ±0.01 [b]	0.57 ±0.04 [b]	2.89	2.61
PBW 343	0	1.73 ±0.01 [a]	1.72 ±0.05 [a]	1.19	1.22
	50	0.84 ±0.02 [b]	0.79 ±0.04 [b]	1.32	1.41
	100	0.61 ±0.05 [b]	0.57 ±0.05 [b]	1.85	2.03
	200	0.42 ±0.08 [b]	0.39 ±0.08 [b]	2.38	2.22
SO	0	0.96 ±0.08 [a]	0.95 ±0.08 [a]	1.33	1.37
	50	0.72 ±0.05 [a]	0.69 ±0.07 [a]	1.49	1.43
	100	0.51 ±0.04 [b]	0.49 ±0.03 [b]	2.25	1.88
	200	0.52 ±0.03 [b]	0.53 ±0.02 [b]	1.05	1.06
CD Value between treatments =		0.290294	0.292548	0.48253	0.477776
CD Value between varieties =		0.435441	0.438822	0.72379	0.716664

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Chlorophyll = mg g^{-1} (f.m.).

Table 13 (a). ANOVA of data presented in table 13 for total chlorophyll (1 day)

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2.051145	3	0.683715	7.680019	0.000911	3.008787
Columns	0.59768	8	0.07471	0.839201	0.577966	2.355081
Error	2.136604	24	0.089025			
Total	4.785429	35				

Table 13 (b). ANOVA of data presented in table 13 for total chlorophyll content (3 days)

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2.394024	3	0.798008	8.826285	0.000403	3.008787
Columns	0.550703	8	0.068838	0.761375	0.639072	2.355081
Error	2.169904	24	0.090413			
Total	5.114632	35				

Table 13 (c). ANOVA of data presented in table 13 for total chl. a/b ratio for 1 day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	5.275203	3	1.758401	7.148763	0.001356	3.008787
Columns	2.105426	8	0.263178	1.069949	0.415714	2.355081
Error	5.903346	24	0.245973			
Total	13.28398	35				

Table 13 (d). ANOVA of data presented in table 13 for total chl. a/b ratio for 3 days

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	3.28303	3	1.094343	4.538053	0.011746	3.008787
Columns	1.501583	8	0.187698	0.778351	0.625567	2.355081
Error	5.787557	24	0.241148			
Total	10.57217	35				

4.6. Variations in carbohydrate contents of wheat plants subjected to water and salt stress

4.6.1. Total soluble carbohydrates

The content of total soluble carbohydrates in case of drought (Table 14) and salt stress (Table 15) in all the nine varieties for both leaf and root showed a significant initial increase with the increase in the duration and severity of stress which after an initial increase declined in case of MW, GY, LV and SO.

Table 14. Content of total soluble sugar in wheat varieties subjected to water stress

Varieties	Part of plant	Total soluble sugar			
		0D	3D	6D	9D
MW	Leaf	17.00±0.09[a]	53.00±0.06[b]	37.34±0.05[b]	29.66±0.01[b]
	Root	5.34±0.06[a]	10.50±0.02[a]	36.34±0.06[b]	24.66±0.04[b]
GY	Leaf	28.34±0.04[a]	54.00±0.05[b]	33.34±0.10[b]	28.34±0.08[a]
	Root	14.34±0.05[a]	16.06±0.04[a]	36.66±0.03[b]	33.34±0.05[b]
KD	Leaf	17.00±0.09[a]	24.66±0.05[a]	61.34±0.05[b]	84.00±0.10[b]
	Root	15.32±0.02[a]	44.00±0.04[b]	84.68±0.02[b]	117.32±0.02[b]
GN	Leaf	26.00±0.02[a]	43.34±0.04[b]	77.00±0.08[b]	109.00±0.10[b]
	Root	20.00±0.06[a]	69.32±0.03[b]	121.32±0.05[b]	192.68±0.04[b]
KW	Leaf	21.00±0.05[a]	28.30±0.06[b]	65.17±0.02[b]	74.30±0.04[b]
	Root	13.20±0.09[a]	35.94±0.07[b]	79.65±0.06[b]	109.45±0.04[b]
LV	Leaf	11.12±0.05[a]	58.00±0.03[b]	31.12±0.11[b]	27.55±0.10[b]
	Root	5.20±0.03[a]	11.40±0.04[a]	31.10±0.05[a]	27.00±0.08[b]
UP 2752	Leaf	13.71±0.05[a]	58.43±0.04[b]	33.71±0.03[b]	29.52±0.04[b]
	Root	9.00±0.01[a]	13.00±0.03[a]	33.01±0.04[b]	29.00±0.02[b]
PBW 343	Leaf	18.31±0.04[a]	25.00±0.04[b]	48.77±0.10[b]	50.10±0.11[b]
	Root	8.00±0.01[a]	11.70±0.05[a]	31.25±0.04[b]	35.40±0.01[b]
SO	Leaf	18.60±0.04[a]	37.20±0.07[b]	43.00±0.11[b]	44.00±0.09[b]
	Root	6.20±0.03[a]	12.40±0.01[a]	70.00±0.03[b]	58.10±0.10[b]
CD Value between treatments (leaf)/(root)					=17.27776/22.55806
CD Value between varieties (leaf)/(root)					=25.91663/33.8371

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Total soluble sugar = mg g^{-1} (d.m.) and reducing sugar = mg g^{-1} (d.m.).

However, in case of other five varieties the content of total soluble sugar continued to increase even after the 9th day of water stress and after 3rd day of salt stress for all the salt concentration. Increase in the severity of both water and salt stress for 9 days and for 1 & 3 days of different concentration of salt resulted in increase in the accumulation of total soluble sugar in KW, GN, KD, UP 2752 and PBW 343 with the highest values observed during 200mM of salt concentration. The accumulation of total soluble sugar was significantly highest in case of GN in both root and leaf than all the other varieties with the lowest value observed in case of LV. In comparison, the accumulation of total soluble sugar following both water and salt stress in case of leaf and root in MW, GY, LV and SO was not very significant however, in the other five varieties the induction of stress and the increase in the severity of stress resulted in a significant difference in the accumulation of total soluble sugar in the leaf and root. The accumulation of total soluble sugar in case of leaf was much more than the accumulation in case of leaf in KW, GN and KD.

Table 14 (a). of data presented in table 14 for total soluble sugar in leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	6071.643	3	2023.881	6.417627	0.002395	3.008787
Columns	3410.602	8	426.3253	1.351856	0.26681	2.355081
Error	7568.708	24	315.3628			
Total	17050.95	35				

Table 14 (b). ANOVA of data presented in table 14 for total soluble sugar in root

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	20632.9	3	6877.634	12.7938	3.4E–05	3.008787
Columns	26149.62	8	3268.702	6.080452	0.000259	2.355081
Error	12901.81	24	537.5755			
Total	59684.33	35				

Table 15. Content of total soluble sugar in wheat varieties subjected to salt stress

Varieties	Treatment (mM)	Total soluble sugar			
		Leaf		Root	
		1D	3D	1D	3D
MW	0	17.00±0.05[a]	19.01±0.04[a]	5.34±0.05[a]	5.50±0.11[a]
	50	32.10±0.05[b]	34.50±0.03[a]	9.80±0.05[a]	12.30±0.05[a]
	100	49.00±0.06[b]	43.00±0.05[b]	19.20±0.06[b]	15.80±0.06[b]
	200	22.10±0.04[b]	22.00±0.03[b]	17.80±0.02[b]	11.32±0.05[a]
GY	0	28.34±0.05[a]	29.20±0.05[a]	14.34±0.03[a]	14.00±0.06[a]
	50	37.30±0.01[b]	44.70±0.03[a]	14.90±0.05[a]	17.00±0.05[b]
	100	50.02±0.10[b]	39.10±0.02[b]	26.20±0.02[b]	12.40±0.04[a]
	200	26.90±0.07[a]	21.30±0.06[a]	15.50±0.03[a]	9.90±0.08[b]
KD	0	17.00±0.09[a]	16.90±0.01[a]	15.32±0.05[a]	15.02±0.02[a]
	50	41.06±0.06[b]	46.30±0.03[b]	27.40±0.02[b]	31.30±0.05[b]
	100	54.20±0.05[b]	59.00±0.02[b]	49.50±0.01[b]	55.30±0.04[b]
	200	57.32±0.11[b]	62.30±0.04[b]	57.40±0.01[b]	74.20±0.05[b]
GN	0	26.00±0.04[a]	25.90±0.05[a]	20.00±0.06[a]	21.20±0.05[a]
	50	39.80±0.03[b]	40.40±0.06[b]	29.40±0.05[b]	34.00±0.05[b]
	100	55.10±0.07[b]	58.30±0.04[b]	54.00±0.04[b]	61.20±0.04[b]
	200	60.00±0.11[b]	61.10±0.02[b]	59.00±0.02[b]	77.70±0.07[b]
KW	0	21.00±0.04[a]	21.50±0.04[a]	13.20±0.11[a]	12.90±0.01[a]
	50	39.90±0.02[b]	42.10±0.03[b]	32.10±0.05[b]	35.10±0.03[b]
	100	51.20±0.03[b]	55.00±0.05[b]	56.40±0.02[b]	58.10±0.02[b]
	200	58.30±0.05[b]	63.00±0.03[b]	65.10±0.02[b]	62.80±0.05[b]
LV	0	11.12±0.04[a]	12.00±0.02[a]	5.20±0.03[a]	5.50±0.03[a]
	50	33.00±0.02[b]	38.20±0.05[b]	10.10±0.04[b]	14.20±0.06[b]
	100	45.20±0.10[b]	43.10±0.04[b]	14.40±0.03[b]	11.10±0.04[b]
	200	23.60±0.11[b]	20.10±0.05[b]	16.90±0.04[b]	9.70±0.05[b]
UP 2752	0	13.71±0.06[a]	9.00±0.04[a]	9.00±0.04[a]	9.70±0.02[a]
	50	33.60±0.03[b]	13.00±0.03[b]	23.20±0.02[b]	32.4±0.11[b]
	100	50.10±0.04[b]	33.01±0.02[b]	29.90±0.08[b]	26.60±0.03[b]
	200	55.30±0.07[b]	29.00±0.01[b]	31.40±0.04[b]	30.00±0.05[b]
PBW 343	0	18.31±0.02[a]	17.00±0.02[a]	8.00±0.01[a]	7.90±0.04[a]
	50	29.90±0.03[b]	32.60±0.03[b]	11.10±0.01[b]	16.20±0.03[b]
	100	48.20±0.11[b]	39.80±0.05[b]	22.30±0.05[b]	19.90±0.02[b]
	200	30.70±0.09[b]	26.80±0.02[b]	21.80±0.02[b]	15.30±0.06[b]
SO	0	18.60±0.03[a]	17.90±0.05[a]	6.20±0.04[a]	6.70±0.02[a]
	50	34.30±0.05[b]	37.90±0.02[a]	10.12±0.03[a]	13.30±0.04[b]
	100	48.70±0.08[b]	41.50±0.04[b]	20.50±0.02[b]	16.20±0.04[b]
	200	24.10±0.08[b]	21.90±0.08[b]	17.00±0.09[b]	10.30±0.04[b]
CD Value between reatments =		7.876124	9.115584	7.614245	11.5885
CD Value between varieties =		11.81419	13.67338	11.42137	17.38275

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Total soluble sugar = mg g^{-1} (d.m.).

Table 15 (a). ANOVA of data presented in table 15 for total soluble sugar in leaf during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	4541.574	3	1513.858	23.10065	2.95E–07	3.008787
Columns	1234.809	8	154.3511	2.355314	0.049981	2.355081
Error	1572.795	24	65.53313			
Total	7349.178	35				

Table 15 (b). ANOVA of data presented in table 15 for total soluble sugar in leaf during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	3910.333	3	1303.444	14.84867	1.13E–05	3.008787
Columns	1968.43	8	246.0537	2.803012	0.024058	2.355081
Error	2106.765	24	87.78188			
Total	7985.527	35				

Table 15 (c). ANOVA of data presented in table 15 for total soluble sugar in root during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	3306.492	3	1102.164	17.9952	2.46E–06	3.008787
Columns	5036.814	8	629.6017	10.2796	3.99E–06	2.355081
Error	1469.944	24	61.24766			
Total	9813.249	35				

Table 15 (d). ANOVA of data presented in table 15 for total soluble sugar in root during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2753.616	3	917.872	6.469811	0.002298	3.008787
Columns	8147.557	8	1018.445	7.178718	7.56E–05	2.355081
Error	3404.88	24	141.87			
Total	14306.05	35				

4.6.2. Reducing Sugars

The content of reducing carbohydrates in case of both water stress (Table 16) and salt stress (Table 17) in all the nine varieties for both leaf and root showed a general increase with the increase in the severity of the stress i.e., with the increase in the days of withholding water in case of water stress and with the increasing concentration of salt in case of salt stress for one and three days. Following both water and salt stress, the increase in the accumulation of reducing sugar was lower in case of MW, GY, LV and SO than that of KW, GN, KD, UP 2752 and PBW 343 in both leaf and root; the increase in the accumulation of reducing sugar was lower in the roots than the leaf in these varieties.

However, in case of KW, GN, KD, UP 2752 and PBW 343 the accumulation of reducing sugar with the increase in the severity of stress was much higher than the other varieties with the increase in the accumulation of reducing sugar much more pronounced in the roots than that of the leaf in these varieties. It was observed that the accumulation of reducing carbohydrates during salt stress was significantly higher than that of during drought stress in the tested varieties.

Table 16. Content of reducing sugar in wheat varieties subjected to water stress

Varieties	Treatment (in days)	Reducing Sugar	
		Leaf	Root
MW	0d	1.07±0.06 [a]	0.79±0.10 [a]
	3d	1.39±0.08 [a]	0.85±0.08 [a]
	6d	4.09±0.04 [b]	1.70±0.05 [b]
	9d	5.78±0.01 [b]	2.63±0.07 [b]
GY	0d	1.96±0.02 [a]	1.67±0.05 [a]
	3d	1.20±0.04 [b]	0.76±0.08 [b]
	6d	1.81±0.01 [a]	1.37±0.09 [a]
	9d	4.61±0.05 [b]	2.70±0.08 [b]
KD	0d	2.97±0.04 [a]	1.56±0.07 [a]
	3d	9.89±0.03 [b]	8.07±0.05 [b]
	6d	21.86±0.02 [b]	22.95±0.04 [b]
	9d	32.03±0.02 [b]	45.56±0.06 [b]
GN	0d	1.76±0.04 [a]	0.11±0.06 [a]
	3d	10.56±0.08 [b]	7.26±0.07 [b]
	6d	28.70±0.07 [a]	23.71±0.06 [a]
	9d	36.11±0.06 [b]	52.22±0.08 [b]
KW	0d	1.90±0.10 [a]	1.25±0.02 [a]
	3d	7.86±0.08 [b]	6.34±0.02 [b]
	6d	18.95±0.07 [b]	17.36±0.01 [b]
	9d	30.10±0.08 [b]	34.25±0.04 [b]
LV	0d	3.20±0.04 [a]	0.60±0.04 [a]
	3d	1.07±0.05 [b]	0.63±0.02 [a]
	6d	3.06±0.04 [a]	1.22±0.01 [b]
	9d	4.10±0.03 [b]	2.15±0.06 [b]
UP 2752	0d	6.50±0.05 [a]	1.12±0.01 [a]
	3d	1.18±0.06 [b]	0.93±0.10 [b]
	6d	3.09±0.09 [b]	1.40±0.02 [a]
	9d	5.10±0.08 [a]	3.21±0.04 [b]
PBW 343	0d	4.20±0.03 [a]	0.40±0.01 [a]
	3d	0.28±0.04 [b]	0.54±0.02 [a]
	6d	0.19±0.01 [b]	0.39±0.04 [a]
	9d	3.10±0.03 [b]	1.95±0.05 [b]
SO	0d	1.35±0.05 [a]	2.00±0.01 [a]
	3d	2.14±0.06 [a]	1.72±0.03 [b]
	6d	5.79±0.03 [b]	2.89±0.05 [a]
	9d	4.89±0.02 [b]	1.55±0.02 [b]
CD Value between Treatments =		6.434381	9.232227
CD Value between Varieties =		9.651572	13.84834

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Total soluble sugar = mg g^{-1} (d.m.).

Table 16 (a). ANOVA of data presented in table 16 for reducing sugar in leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	736.9763	3	245.6588	5.616721	0.004606	3.008787
Columns	1604.188	8	200.5234	4.584751	0.001717	2.355081
Error	1049.689	24	43.73704			
Total	3390.853	35				

Table 16 (b). ANOVA of data presented in table 16 for reducing sugar in root

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1241.413	3	413.8043	4.595643	0.011154	3.008787
Columns	2374.661	8	296.8326	3.296574	0.01107	2.355081
Error	2161.026	24	90.04275			
Total	5777.1	35				

Table 17. Content of reducing sugar in wheat varieties subjected to salt stress

Varieties	Treatment (mM)	Reducing Sugar			
		Leaf		Root	
		1D	3D	1D	3D
MW	0	1.07±0.01[a]	1.07±0.05[a]	0.79±0.05[a]	0.80±0.11[a]
	50	1.39±0.05[b]	2.11±0.01[a]	0.88±0.05[a]	0.99±0.05[a]
	100	1.99±0.04[b]	2.45±0.05[b]	1.69±0.06[b]	1.73±0.03[b]
	200	3.45±0.02[b]	5.79±0.03[b]	2.01±0.04[b]	2.23±0.03[b]
GY	0	1.96±0.05[a]	2.01±0.05[a]	1.67±0.05[a]	1.60±0.04[a]
	50	2.11±0.06[b]	2.35±0.04[a]	1.71±0.04[b]	0.79±0.01[b]
	100	2.90±0.11[b]	3.34±0.08[b]	1.82±0.05[b]	1.99±0.02[a]
	200	4.12±0.01[b]	4.52±0.07[b]	2.22±0.03[b]	2.69±0.05[b]
KD	0	2.97±0.02[a]	3.10±0.03[a]	1.56±0.03[a]	1.55±0.03[a]
	50	8.57±0.04[b]	11.22±0.04[b]	5.59±0.06[b]	6.11±0.01[b]
	100	18.32±0.03[b]	21.64±0.06[b]	11.20±0.05[b]	15.10±0.05[b]
	200	25.34±0.08[b]	27.56±0.01[b]	19.88±0.03[b]	22.20±0.04[b]
GN	0	1.76±0.01[a]	1.80±0.02[a]	1.11±0.02[a]	1.22±0.02[a]
	50	9.50±0.03[b]	12.48±0.05[b]	6.10±0.05[b]	7.24±0.04[b]
	100	19.98±0.05[b]	23.22±0.06[b]	14.22±0.06[a]	17.98±0.02[b]
	200	26.23±0.04[b]	29.85±0.03[b]	19.98±0.05[b]	28.41±0.05[b]
KW	0	1.90±0.01[a]	2.01±0.01[a]	1.25±0.11[a]	1.21±0.01[a]
	50	8.87±0.03[b]	10.36±0.02[b]	0.75±0.05[b]	6.22±0.01[b]
	100	15.66±0.05[b]	18.50±0.04[b]	1.76±0.03[a]	14.55±0.05[b]
	200	24.66±0.07[b]	26.30±0.05[b]	1.99±0.05[b]	20.11±0.02[b]
LV	C	3.20±0.02[a]	3.96±0.03[a]	1.12±0.03[a]	0.59±0.03[a]
	50	3.99±0.04[b]	4.65±0.04[a]	3.25±0.04[b]	1.02±0.03[a]
	100	3.79±0.10[b]	4.91±0.07[b]	4.45±0.03[a]	1.79±0.02[b]
	200	3.22±0.03[b]	5.10±0.06[b]	16.98±0.07[b]	2.01±0.05[b]
UP 2752	0	6.50±0.06[a]	6.41±0.09[a]	0.80±0.06[a]	1.11±0.02[a]
	50	6.96±0.06[b]	8.88±0.06[a]	0.99±0.05[a]	4.11±0.09[b]
	100	9.11±0.05[b]	10.68±0.03[b]	1.42±0.01[b]	4.87±0.06[b]
	200	12.35±0.02[b]	19.50±0.05[b]	1.99±0.09[a]	20.10±0.03[b]
PBW 343	0	4.20±0.01[a]	4.32±0.02[a]	2.00±0.02[a]	0.79±0.02[a]
	50	5.45±0.02[b]	5.90±0.03[a]	2.41±0.06[b]	1.54±0.05[b]
	100	6.69±0.04[b]	8.01±0.05[b]	2.63±0.03[b]	1.62±0.04[b]
	200	6.98±0.06[b]	10.11±0.06[b]	2.77±0.04[b]	2.54±0.03[b]
SO	0	1.35±0.05[a]	1.40±0.05[a]	1.35±0.05[a]	2.11±0.02[a]
	50	2.80±0.03[b]	4.19±0.04[b]	2.14±0.04[b]	2.61±0.05[b]
	100	3.24±0.08[b]	3.69±0.02[b]	5.79±0.02[b]	2.69±0.02[b]
	200	4.90±0.04[b]	5.87±0.11[b]	4.89±0.07[b]	2.99±0.03[b]
CD Value bet. treatments=		4.458234	4.708482	3.807466	4.811927
CD Value bet. varieties =		6.687351	7.062722	5.711199	7.21789

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Reducing sugar = mg g^{-1} (d.m.).

Table 17 (a). ANOVA of data presented in table 17 for reducing sugar in leaf during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	471.7868	3	157.2623	7.489675	0.001049	3.008787
Columns	827.8046	8	103.4756	4.928063	0.001086	2.355081
Error	503.933	24	20.99721			
Total	1803.525	35				

Table 17 (b). ANOVA of data presented in table 17 for reducing sugar in leaf during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	719.8985	3	239.9662	10.24596	0.000157	3.008787
Columns	1059.795	8	132.4744	5.656325	0.000431	2.355081
Error	562.0938	24	23.42058			
Total	2341.787	35				

Table 17 (c). ANOVA of data presented in table 17 for reducing sugar in root during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	356.3826	3	118.7942	7.756886	0.000861	3.008787
Columns	496.176	8	62.022	4.049841	0.003619	2.355081
Error	367.5523	24	15.31468			
Total	1220.111	35				

Table 17 (d). ANOVA of data presented in table 17 for reducing sugar in root during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	536.9739	3	178.9913	7.317423	0.001194	3.008787
Columns	793.3914	8	99.17393	4.054373	0.003596	2.355081
Error	587.0634	24	24.46098			
Total	1917.429	35				

4.6.3. Starch

The accumulation of starch in the leaf and roots of water stressed (Table 18) and salt stressed (Table 19) wheat varieties showed a general decline after an initial increase in all the cases. The content of starch during water stress increased significantly during the 3rd day of stress followed by a decrease after 6 days of water stress in case of leaf however the decrease in the accumulation of starch in case of root was lower than that of the leaf in all the wheat varieties with the lowest accumulation being observed in case of LV and SO. Similar trend was observed during salt stress for 1 and 3 days for all concentration of salt.

Table 18. Content of starch in wheat varieties subjected to drought stress

Varieties	Part Used	Content of Starch Days of Stress			
		0D	3D	6D	9D
MW	**Leaf**	11.01±0.02[a]	16.23±0.06[b]	11.04±0.03[b]	19.64±0.01[b]
	Root	11.92±0.06[a]	27.26±0.02[b]	10.63±0.03[b]	11.25±0.04[a]
GY	**Leaf**	7.81±0.01[a]	17.36±0.03[b]	9.00±0.02[b]	15.14±0.02[b]
	Root	10.72±0.05[a]	19.88±0.06[b]	11.86±0.02[a]	13.91±0.03[b]
KD	**Leaf**	9.14±0.01[a]	20.05±0.09[b]	7.29±0.08[b]	6.03±0.03[b]
	Root	19.64±0.05[a]	44.73±0.03[b]	28.37±0.02[b]	20.59±0.05[a]
GN	**Leaf**	11.52±0.02[a]	21.14±0.05[b]	9.88±0.06[a]	7.50±0.03[b]
	Root	11.59±0.06[a]	47.56±0.02[b]	24.14±0.02[b]	16.50±0.01[b]
KW	**Leaf**	8.99±0.02[a]	15.58±0.02[b]	7.87±0.06[a]	7.12±0.03[b]
	Root	9.56±0.02[a]	25.23±0.04[b]	10.45±0.03[b]	9.77±0.01[a]
LV	**Leaf**	7.19±0.03[a]	14.66±0.05[b]	8.14±0.11[b]	7.39±0.08[a]
	Root	9.37±0.02[a]	20.79±0.01[b]	11.34±0.07[b]	9.67±0.02[a]
UP 2752	**Leaf**	11.22±0.05[a]	11.01±0.06	8.85±0.04[b]	7.86±0.05[b]
	Root	10.21±0.08[a]	31.12±0.07[b]	24.31±0.05[b]	8.99±0.06[b]
PBW 343	**Leaf**	10.01±0.09[a]	11.31±0.05[a]	9.46±0.06[a]	6.11±0.01[b]
	Root	10.03±0.09[a]	29.40±0.08[b]	21.04±0.05[b]	8.03±0.02[b]
SO	**Leaf**	9.07±0.02[a]	13.16±0.03[b]	7.07±0.04[a]	6.01±0.05[b]
	Root	9.64±0.06[a]	19.75±0.03[b]	10.4±0.05[b]	6.00±0.08[b]
CD Value between treatments=				2.814008	4.338126
CD Value between varieties =				8.442024	6.507189

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Starch = mg g^{-1} (d.m.).

Our result showed that the increase in the days of salt stress resulted in the increase in the accumulation of starch in case of both leaf and root however for the higher concentration of salt the accumulation decreased during the 3rd day. The accumulation of starch decreased with the increasing concentration of salt after a steep rise for the initial concentration, this decrease was more pronounced in case of leaf than that of roots. The highest accumulation of starch was observed for GN and KD following both the stresses and the lowest was observed in LV and SO. All the other varieties showed more or less a similar trend for both kind of stresses respectively. Starch accumulation was higher in the wheat varieties in response to salt stress than drought stress.

Table 18 (a). ANOVA of data presented in table 18 for starch in leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	283.8155	3	94.60518	11.30911	8.1E–05	3.008787
Columns	117.2696	8	14.6587	1.752302	0.137283	2.355081
Error	200.7695	24	8.365395			
Total	601.8546	35				

Table 18 (b). ANOVA of data presented in table 18 for starch in root

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1944.432	3	648.1441	32.60103	1.25E–08	3.008787
Columns	1052.726	8	131.5908	6.618891	0.00014	2.355081
Error	477.1462	24	19.88109			
Total	3474.305	35				

Table 19. Content of starch in wheat varieties subjected to salt stress

Varieties	Treatment (mM)	Leaf		Root	
		1D	3D	1D	3D
MW	0	11.01±0.05[a]	11.15±0.06[a]	11.92±0.06[a]	12.01±0.05[a]
	50	17.20±0.06[b]	19.50±0.02[b]	19.88±0.02[b]	22.10±0.06[a]
	100	20.90±0.03[b]	22.10±0.02[b]	17.32±0.03[b]	15.10±0.02[b]
	200	11.60±0.03[b]	11.01±0.02[a]	12.20±0.02[b]	10.30±0.02[b]
GY	0	7.81±0.05[a]	7.90±0.01[a]	10.71±0.02[a]	11.0±0.03[a]
	50	19.22±0.03[b]	22.11±0.02[b]	20.10±0.02[b]	26.01±0.02[b]
	100	22.54±0.10[b]	24.22±0.05[b]	16.98±0.03[b]	17.88±0.01[a]
	200	15.33±0.05[b]	13.12±0.06[b]	11.65±0.01[b]	11.40±0.06[b]
KD	0	9.16±0.01[a]	9.22±0.01[a]	19.64±0.01[a]	19.99±0.03[a]
	50	21.00±0.02[b]	29.20±0.02[b]	37.88±0.02[b]	42.40±0.02[b]
	100	26.33±0.06[b]	31.10±0.05[b]	26.20±0.03[b]	36.12±0.02[b]
	200	19.99±0.05[b]	18.12±0.08[b]	22.11±0.03[b]	19.50±0.01[b]
GN	0	11.52±0.02[a]	11.25±0.02[a]	11.59±0.01[a]	12.20±0.01[a]
	50	22.64±0.05[b]	27.64±0.06[b]	39.10±0.03[b]	44.00±0.03[b]
	100	28.14±0.04[b]	30.11±0.03[b]	29.60±0.01[b]	38.40±0.03[b]
	200	21.30±0.02[b]	20.10±0.04[b]	24.10±0.01[b]	19.89±0.02[b]
KW	0	8.99±0.02[a]	9.11±0.01[a]	9.56±0.10[a]	10.01±0.02[a]
	50	19.48±0.03[b]	24.65±0.03[b]	15.98±0.04[b]	37.35±0.02[b]
	100	25.47±0.05[b]	29.00±0.06[b]	13.64±0.02[b]	30.10±0.05[b]
	200	19.89±0.02[b]	17.66±0.05[b]	9.70±0.01[a]	17.56±0.06[b]
LV	0	7.19±0.03[a]	7.20±0.02[a]	9.37±0.04[a]	9.54±0.03[a]
	50	9.32±0.04[b]	17.69±0.05[a]	15.98±0.05[b]	17.85±0.04[a]
	100	17.50±0.11[b]	20.76±0.05[b]	13.64±0.02[b]	12.65±0.06[b]
	200	9.36±0.05[b]	8.23±0.06[b]	9.70±0.06[b]	6.35±0.08[b]
UP 2752	0	11.22±0.01[a]	11.00±0.05[a]	10.21±0.03[a]	10.26±0.09[a]
	50	18.23±0.02[b]	20.22±0.05[b]	25.12±0.04[b]	29.96±0.08[b]
	100	25.10±0.04[b]	26.98±0.06[b]	20.11±0.02[b]	22.65±0.06[b]
	200	13.10±0.05[b]	11.98±0.03[b]	15.96±0.08[a]	13.51±0.04[b]
PBW 343	0	10.01±0.06[a]	10.13±0.02[a]	10.03±0.03[a]	10.12±0.05[a]
	50	16.60±0.05[b]	20.65±0.05[b]	21.02±0.05[b]	30.21±0.04[b]
	100	19.88±0.02[b]	23.68±0.04[b]	19.65±0.02[b]	20.32±0.06[b]
	200	12.30±0.04[b]	10.22±0.09[a]	15.20±0.06[b]	14.65±0.02[b]
SO	0	9.07±0.02[a]	9.11±0.10[a]	9.64±0.04[a]	9.71±0.01[a]
	50	18.10±0.05[b]	20.30±0.09[b]	19.16±0.03[b]	25.10±0.06[b]
	100	21.65±0.04[b]	26.00±0.08[b]	16.36±0.01[b]	18.67±0.03[b]
	200	10.09±0.01[b]	9.31±0.10[a]	12.30±0.05[b]	10.20±0.02[b]
CD Value between treatments=		2.089539	2.046043	3.17669	3.78034
CD Value between varieties =		3.13430	3.069064	4.76504	15.5796

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Starch = mg g^{-1} (d.m.).

Table 19 (a). ANOVA of data presented in table 19 for starch in leaf during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	866.9172	3	288.9724	62.64978	1.69E–11	3.008787
Columns	280.7693	8	35.09616	7.608916	4.81E–05	2.355081
Error	110.7001	24	4.612504			
Total	1258.387	35				

Table 19 (b). ANOVA of data presented in table 19 for starch in leaf during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1590.422	3	530.1406	119.8742	1.41E–14	3.008787
Columns	282.7853	8	35.34816	7.992847	3.26E–05	2.355081
Error	106.1394	24	4.422474			
Total	1979.346	35				

Table 19 (c). ANOVA of data presented in table 19 for starch in root during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1035.547	3	345.1824	32.37892	1.33E–08	3.008787
Columns	869.9357	8	108.742	10.20025	4.27E–06	2.355081
Error	255.8572	24	10.66072			
Total	2161.34	35				

Table 19 (d). ANOVA of data presented in table 19 for starch in root during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2098.753	3	699.5843	46.3386	3.88E–10	3.008787
Columns	1223.973	8	152.9966	10.13409	4.52E–06	2.355081
Error	362.3334	24	15.09723			
Total	3685.059	35				

4.7. Effect of stresses on phenolics of wheat varieties

4.7.1. Phenol contents

The content of total phenol increased with increase in the severity of the stress in all varieties. During water stress the total and ortho phenol content (Figure 17) showed a significant increase with the increase in the days of stress however the total phenol content in case of MW and GY showed an initial decrease followed by an increase and in case of LV, PBW 343, UP 2752 the content of total phenol decreased during the 9th day of withholding water. The accumulation of phenol was highest in case of KD, GN and KW during the 9th day of water stress with the other four varieties showing comparatively lower accumulation. Similarly, ortho phenol content in wheat varieties following water stress increased significantly in all the varieties with the highest accumulation being observed in case of KD, GN and KW and the lowest accumulation of total phenol content was observed in LV and SO for all the days of stress. During salt stress the total phenol content (Figure 18) showed a similar trend as during the water stress. However, with the increase in the concentration of salt for the 1st and 3rd day the total phenol content increased only in case of KD, GN, KW and UP 2752 with a little lower accumulation during the salt concentration of 100mM in all the days of stress. The accumulation of total phenol in the other wheat varieties increased for the lower concentration of salt but later decreased during higher salt concentrations. This trend was similar for the salt stress during both the 1st and the 3rd day of salt stress for all concentrations. The accumulation of ortho phenol (Figure 19) increased with the increase in the concentration of salt during the 3rd day in all cases except LV where it decreased for the salt concentration of 200mM and during the 1st day the content of ortho phenol increased in case of GN, KD, KW, UP 2752, PBW 343 and SO with increasing concentration of salt and in all other varieties it decreased during 100 and 200mM of salt concentration. The accumulation of total and ortho phenol was highest in case of GN, KD and it was lowest in case of LV and SO during both the stresses.

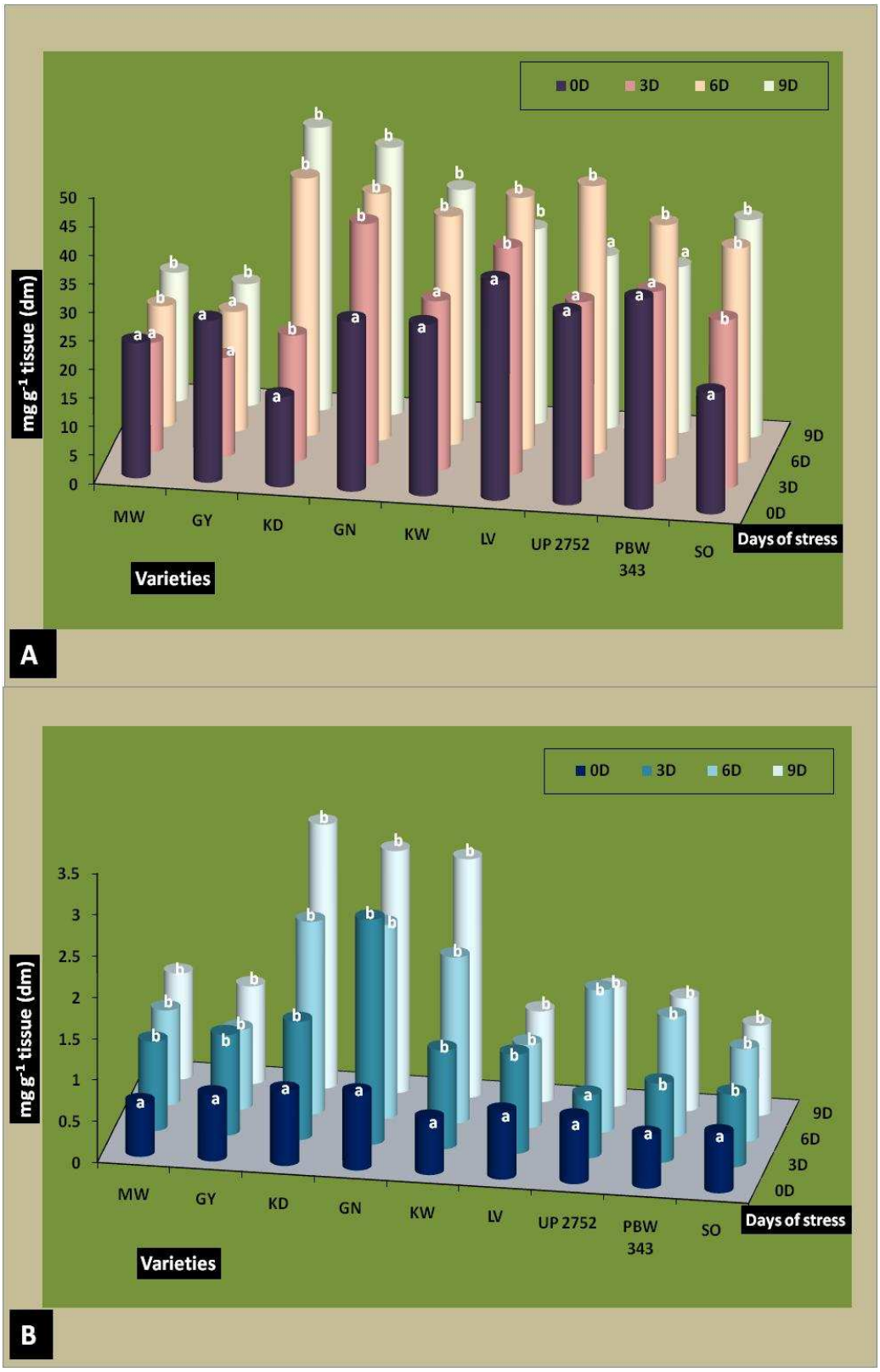

Figure 17. Content of total phenol (A) and ortho phenol (B) in the leaves of nine wheat varieties subjected to drought stress. Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

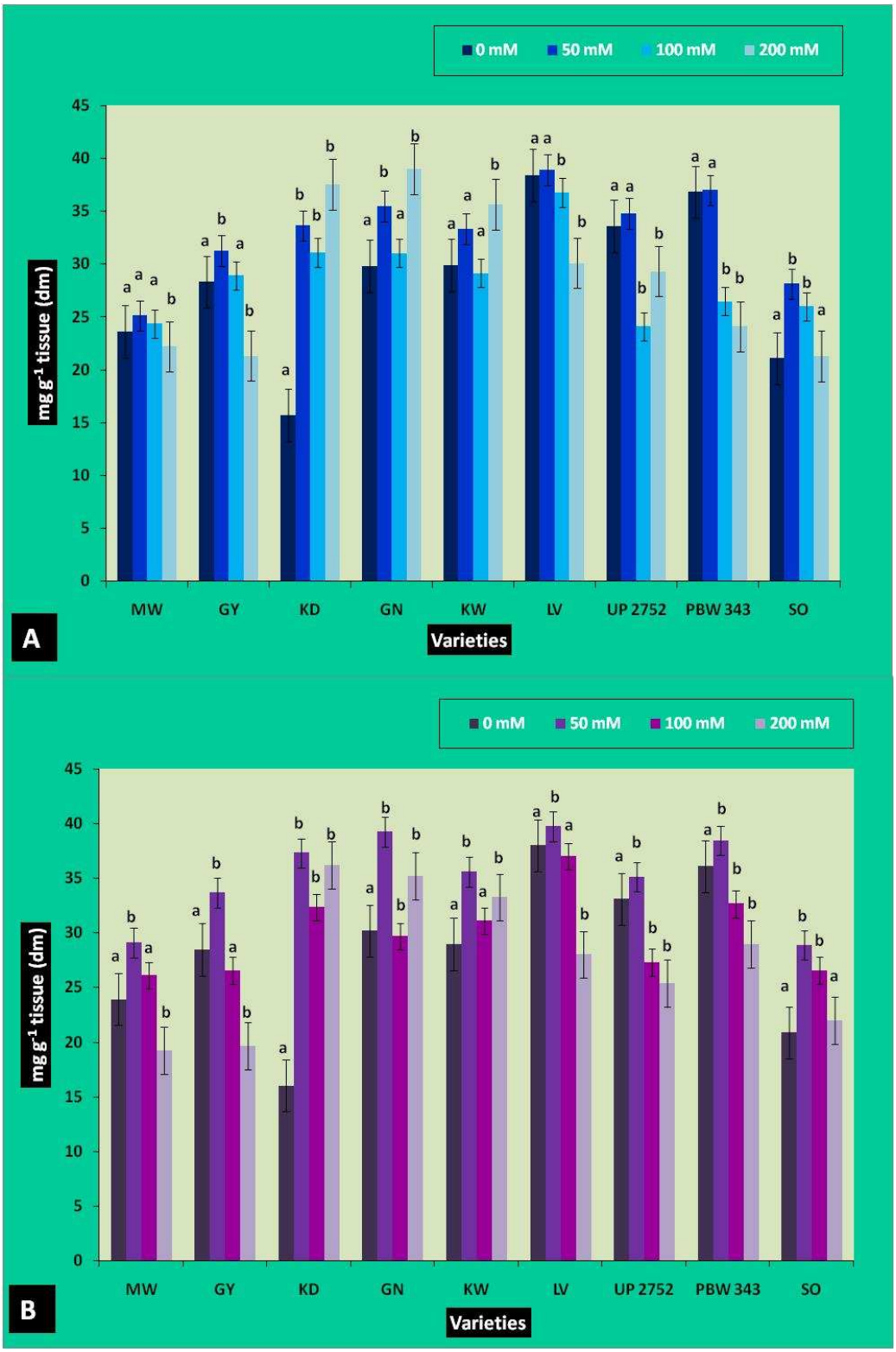

Figure 18. Content of total phenol in the leaves of nine wheat varieties subjected to salt stress for 1 day (A) and 3 days (B). Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

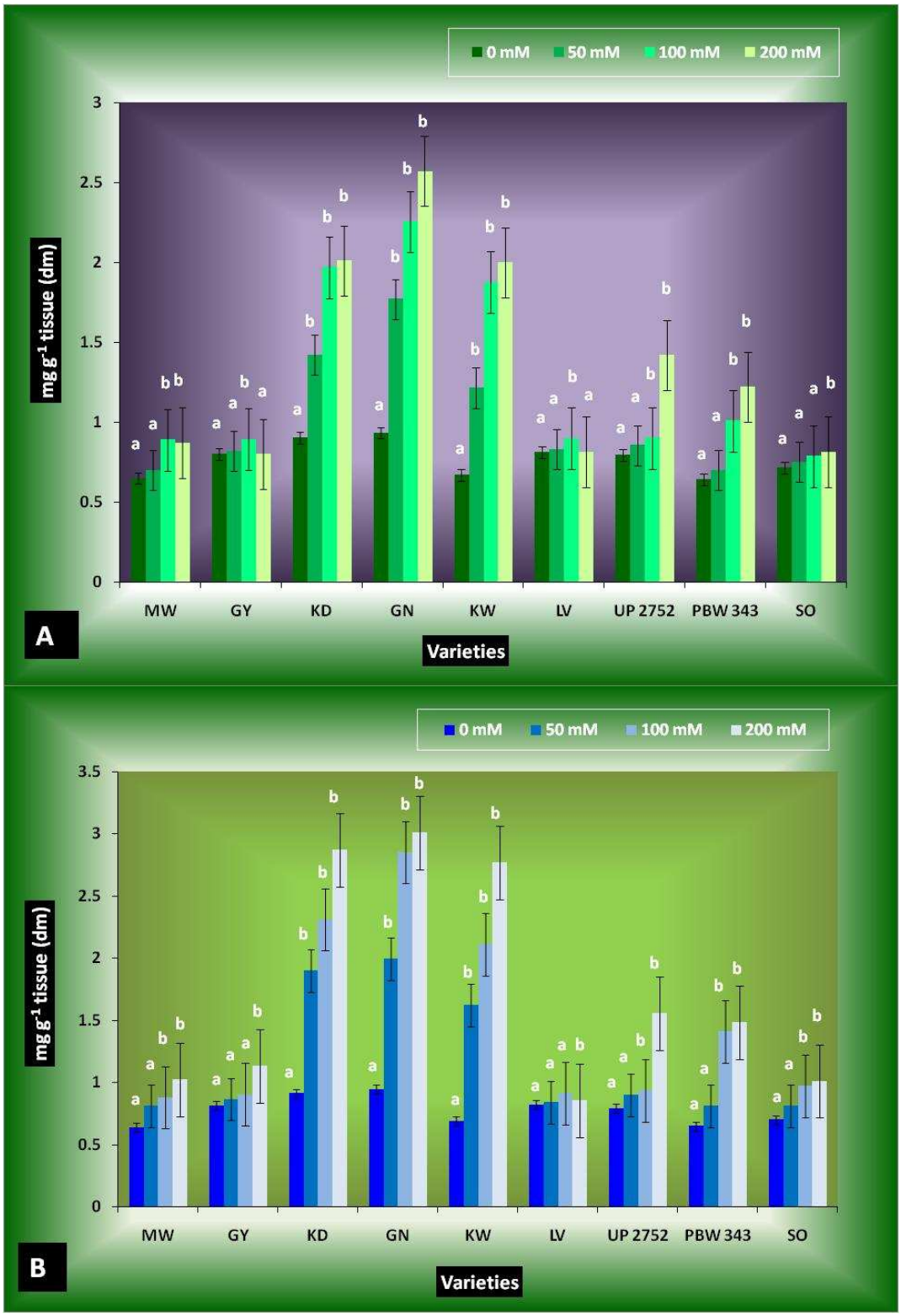

Figure 19. Content of ortho phenol in the leaves of nine wheat varieties subjected to salt stress for 1 day (A) and 3 days (B). Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

4.7.2. HPLC profiles

4.7.2.1. Total phenols

The profile of total phenols in HPLC in the leaf of LV and GN was studied during drought and salinity stress in order to determine and identify the source of the phenols in the leaves. It was evident that the content of total phenols during water and salinity stress enhanced in case of all the wheat varieties in our study with the highest content recorded in case of GN in our study and the least in case of LV following both water and salt stress separately which was proven in the studies made by high performance liquid chromatography in the same varieties under stress. Number, height and area of the peaks obtained in HPLC study in case of GN during increasing days and concentration of water (Figure 21) and salt stress (Figure 23) were much more than that of LV where the number, height and the area of the peaks were comparatively lesser during water (Figure 20) and during salt stress (Figure 22) respectively. With increase in the duration of drought, one peak identified as the ferulic acid was most prominent in both the variety with retention time of about 22.98 (peak 11), 24.82 (peak 7), 23.52 (peak 7) and 23.30 (peak 8) minutes in case of LV during 0, 3, 6 and 9 days respectively (Figure 20a, 20b) and in case of GN the same peak had the retention time of about 23.26 (peak 8), 23.89 (peak 8), 23.89 (peak 10) and 24.69 (peak 10) minutes respectively (Figure 21a, 21b) with increase in the days of withholding water. With the increase in the severity of drought the height and area of this peak increased in case of GN whereas in case of LV (Table 20–27) the peak height and area decreased. The peak for ferulic acid in case of salt stress in GN and LV also showed the same trend (Figure 20–21). The height and area of peak for ferulic acid (peak 7) in LV during salt stress with retention time of about 24 minutes (Table 28–33) decreased with the increase in the concentration of salt stress in LV and during the 3rd day of stress, the height and area of the peak increased in the salt concentration 100 and 200mM (Figure 22a and 22b). The same peak for ferulic acid (peak 7, 6, 5 during 1st day and peak 8, 7, 6 during 3rd day for 50, 100 and 200mM respectively) in case of GN following salt stress was comparatively smaller in height and its area however with the increase in the concentration of salt in the 3rd day an increase was observed (Figure 23a and

23b). Peak number 20, 11, 13 and 15 in LV during drought (Figure 20a, b) and peak number 16, 16, 18 and 19 during salt stress (Figure 22a, b) was identified as salicylic acid with retention time shown in the respective tables (Table 20–27).

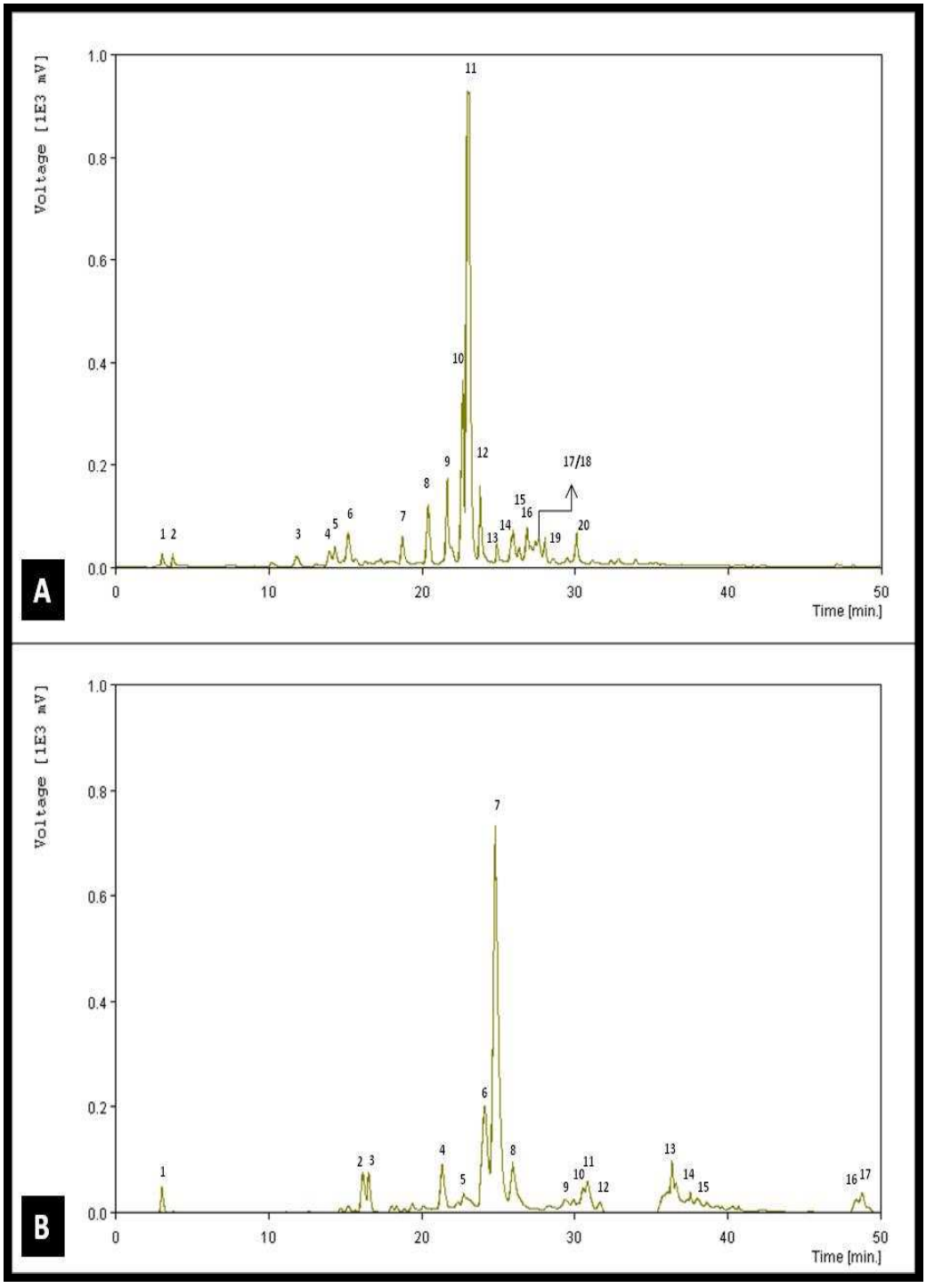

Figure 20(a). HPLC of total phenols in the leaf of one month old plant of LV following drought stress for A–0 day and B–3 days

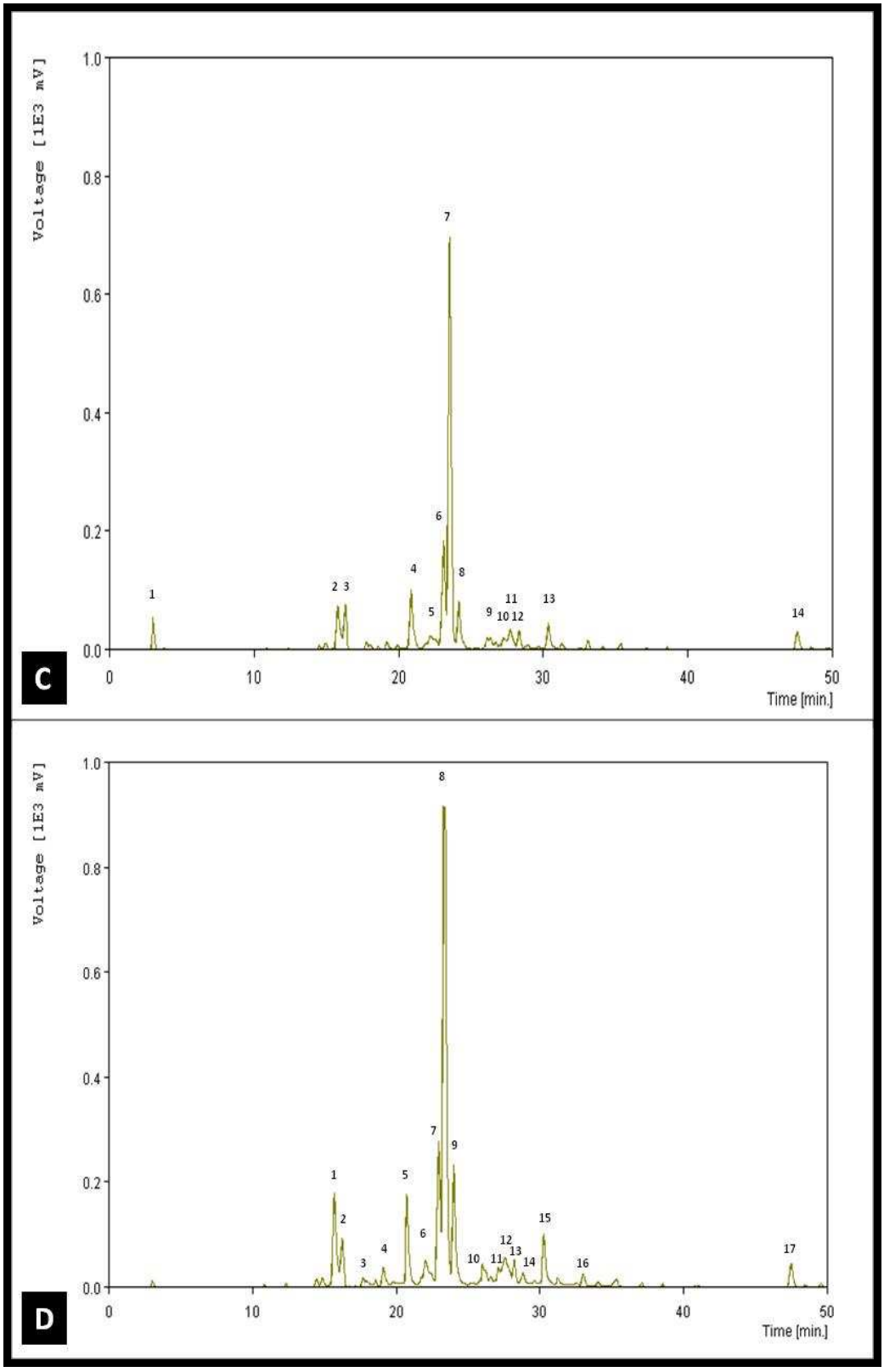

Figure 20(b). HPLC of total phenols in the leaf of one month old plant of LV following drought stress for C–6 days and D–9days

The height and area of this peak increased only during the later phase of drought stress in case of LV; however, in GN the height of this peak increased with the increase in the duration of stress and this peak was prominent. In case of salinity stress, peak number 14, 11, 13 during 1st day (Figure 22a) and peak number 12, 12, 13 (Figure 22b) during the 3rd day respectively in case of LV was identified as salicylic acid which was prominent peak however the change in the height and area was not very significant (Table 28–33). The peak for salicylic in case of GN with peak number 15, 10, 8 during 1st day (Figure 23a) and 16, 14, 13 during 3rd day (Figure 23b) respectively was identified as the peak for salicylic acid which was also a prominent peak and the height and area of this peak increased significantly with the increase in the duration of salt stress however increasing concentration of salt decreased its appearance in the HPLC analysis. During drought in LV (Figure 20a, b) peak number 8, 4, 4 and 5 during drought was identified as chlorogenic acid which was significantly prominent and was well observed with increase in the increase in the duration of water stress. In GN peak number 5, 5, 7 and 6 (Figure 21a, b) was identified as chlorogenic acid which during drought increased significantly in its height and area (Table 20–27) with increasing days of stress. The peaks for chlorogenic acid identified with peak number 4 in case of LV during salt stress (Figure 22a, b) and peak number 4, 3, 2 during 1st day and 5, 4, 3 during 3rd day of salt stress of 50, 100 and 200mM salt concentration in GN (Figure 23a, b) was very prominent during salt stress and increased with increase in the duration and concentration of salt stress. The area and peak heights are shown in the corresponding tables. Caffeic acid was also identified during the HPLC run as peak number 7 in 0 days of drought and peak 4 in the 9th day in case of LV and was absent or less prominent during the 3rd and the 6th day of drought stress (Figure 20a, b) which was prominent in the control plant of LV and reappeared during the 9th day. Peak number 4 in control (0D) and 3rd day, peak number 6 during the 6th day and peak 5 in the 9th day of drought was identified as caffeic acid in case of GN (Fiugre 21a, b). The peak for caffeic acid in case of GN was prominent during the initial days of drought however; it was less prominent during the 9th day. During salt stress, peak number 3 during 1st day of salt stress in LV and peak number 5, 3 and 3 during 3rd day was identified as caffeic acid (Figure

22a, b) and in case of GN, peak number 3, 2 during 1st day of 50 and 100mM of salt concentration was identified as caffeic acid and peak number 4, 3, 2 during the 3rd day of 50, 100 and 200mM of salt stress respectively corresponded to caffeic acid (Figure 23a, b). Similarly numbers of peaks were observed during drought and salt stress which were prominent during both drought and salt stress which were unidentified in our standards for phenols. However the phenolic acid analysis was more specific and a number of phenolic acids were analyzed in our study.

Table 20: Peak table of data presented in Figure 20a (A) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	3.000	303.6590	25.556
2	3.670	373.1670	26.233
3	11.800	439.2776	21.693
4	13.940	471.8729	29.676
5	14.320	752.1698	39.230
6	15.150	1200.2916	67.376
7	18.740	990.1350	59.954
8	20.390	1909.5825	122.463
9	21.640	3145.2429	172.915
10	22.640	5084.8773	364.692
11	22.980	17123.7593	930.018
12	23.820	2514.6365	158.283
13	24.930	860.5621	45.148
14	26.000	1686.4858	71.224
15	26.360	562.9983	38.428
16	26.860	1114.4745	78.135
17	27.430	586.8967	50.205
18	27.630	840.2752	54.662
19	28.040	888.2747	58.040
20	30.110	1410.2560	67.036

Table 21: Peak table of data presented in Figure 20a (B) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.990	709.0265	57.057
2	16.130	2966.9456	83.331
3	16.151	2722.4151	83.335
4	21.340	2465.9560	98.697
5	22.750	1951.3912	43.728
6	24.150	5585.6991	209.837
7	24.820	20360.7756	743.137
8	25.970	3904.7446	102.480
9	29.420	1198.3382	31.869
10	29.930	589.7099	32.687
11	30.870	2864.0153	67.281
12	31.680	664.0000	26.358
13	36.380	4582.4382	104.228
14	37.630	1216.1555	44.312
15	38.100	1639.1381	31.915
16	48.520	610.6400	26.781
17	48.860	1051.6001	40.261

Table 22: Peak table of data presented in Figure 20b (C) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.980	652.1378	60.485
2	15.790	1390.4876	80.199
3	16.290	1319.8140	80.593
4	20.900	1971.8214	105.935
5	22.210	1229.5697	26.741
6	23.140	2864.7277	188.433
7	23.520	9873.5399	703.141
8	24.190	1618.3532	85.834
9	26.390	1130.0073	22.852
10	27.300	432.4815	23.292
11	27.730	887.5220	37.317
12	28.380	558.7761	36.367
13	30.420	964.8697	46.352
14	47.660	631.9537	33.650

Table 23: Peak table of data presented in Figure 20b (D) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.700	3221.6470	185.915
2	16.210	1707.6259	98.255
3	17.720	664.1004	23.741
4	19.110	857.3840	42.915
5	20.770	3299.8170	182.686
6	22.060	2134.2356	55.957
7	22.920	4215.0234	269.22
8	23.300	20983.1665	923.555
9	24.040	4032.0835	240.205
10	26.060	1769.5359	48.372
11	27.160	789.1271	41.258
12	27.610	2056.7328	61.827
13	28.270	927.4030	55.680
14	28.840	928.3590	30.984
15	30.330	2243.5639	106.620
16	33.040	723.2449	29.670
17	47.570	905.4995	48.640

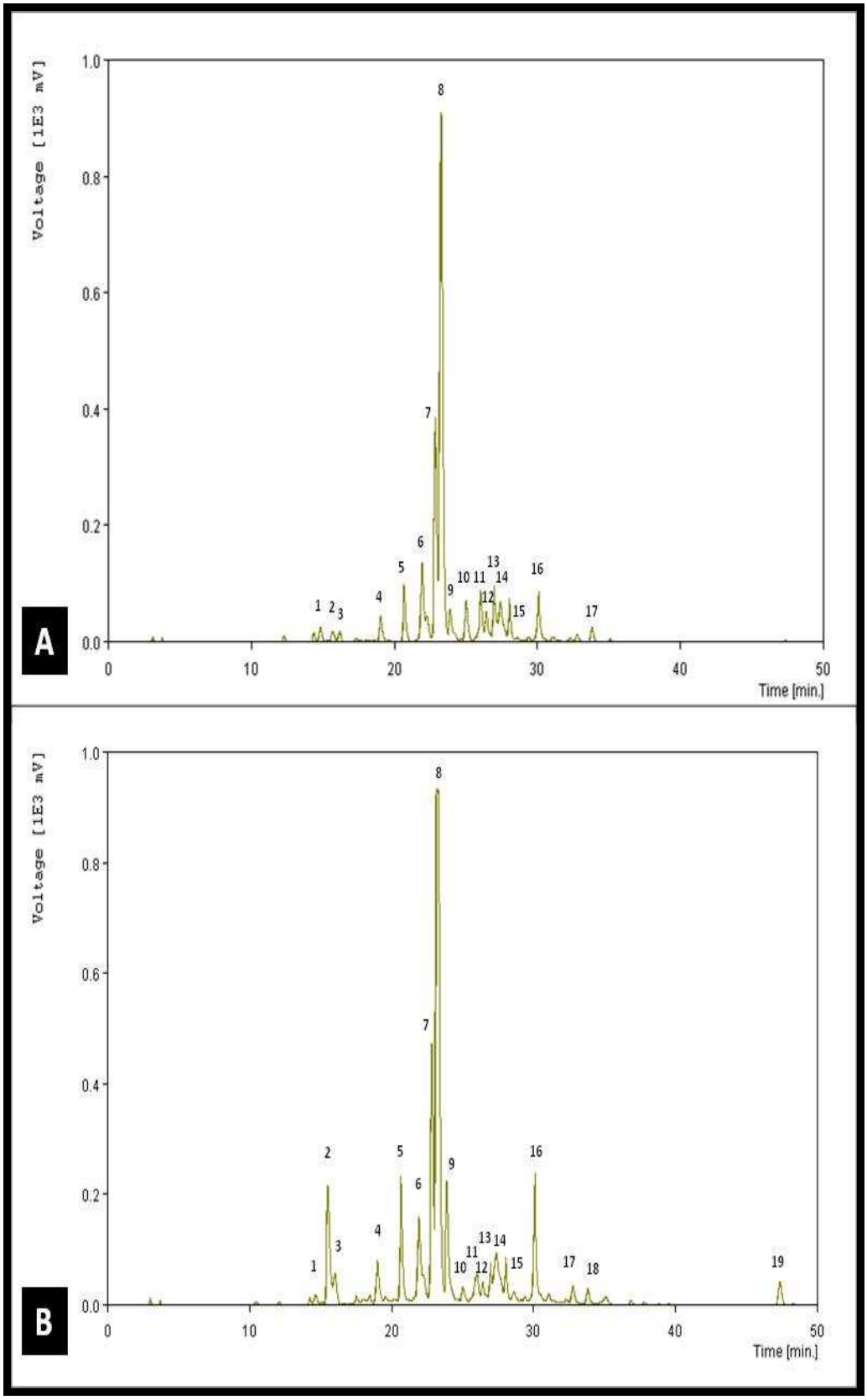

Figure 21a. HPLC of total phenols in the leaf of one month old plant of GN following drought stress for A–0 day and B–3 days

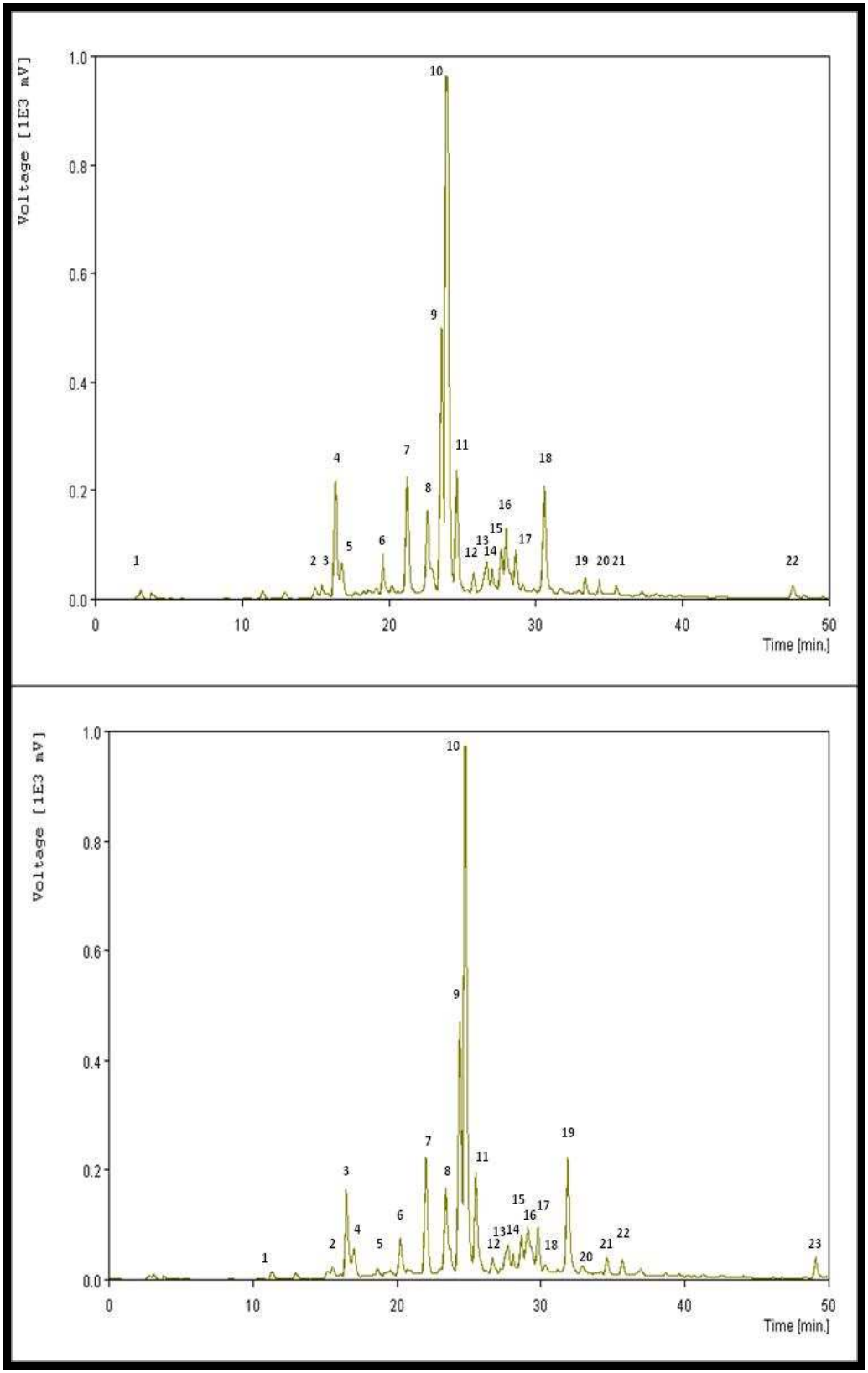

Figure 21b. HPLC of total phenols in the leaf of one month old plant of GN following drought stress for C–6 days and D–9 days

Table 24: Peak table of data presented in Figure 21a (A) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	14.830	523.1013	30.960
2	15.680	454.7880	24.927
3	16.160	409.3989	24.795
4	19.050	783.6187	50.117
5	20.710	1681.5221	104.089
6	21.970	2192.0253	142.672
7	22.910	5308.4744	391.850
8	23.260	13768.7649	919.410
9	23.950	1269.5236	62.628
10	25.070	1157.6486	77.313
11	26.090	1720.9895	93.521
12	26.470	943.3739	57.901
13	27.010	1639.7674	100.846
14	27.460	1570.2496	74.480
15	28.100	1181.1836	79.749
16	30.120	1841.0583	91.803
17	33.870	505.7158	30.479

Table 25: Peak table of data presented in Figure 21a (B) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.420	514.2305	26.363
2	16.320	3557.5919	218.075
3	16.790	1115.7852	67.366
4	19.590	1346.4181	83.882
5	21.240	3703.5444	226.566
6	22.610	4110.0996	164.115
7	23.580	6781.1151	502.080
8	23.890	18537.8569	966.918
9	24.610	3752.5152	238.567
10	25.760	790.5866	47.841
11	26.680	1715.3778	68.534
12	27.070	1103.2856	55.262
13	27.670	1289.9084	93.137
14	27.990	2801.7172	130.672
15	28.670	1352.1600	90.615
16	30.630	3921.5457	209.516
17	33.440	855.7588	39.443
18	34.380	667.2610	34.578
19	35.550	706.4179	22.689
20	47.600	398.1242	22.008

Table 26: Peak table of data presented in Figure 21b (C) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	3.070	345.0835	17.100
2	14.960	358.0790	21.095
3	15.420	514.2305	26.363
4	16.320	3557.5919	218.075
5	16.790	1115.7852	67.366
6	19.590	1346.4181	83.882
7	21.240	3703.5444	226.566
8	22.610	4110.0996	164.115
9	23.580	6781.1151	502.080
10	23.890	18537.8569	966.918
11	24.610	3752.5152	238.567
12	25.760	790.5866	47.841
13	26.680	1715.3778	68.534
14	27.070	1103.2856	55.262
15	27.670	1289.9084	93.137
16	27.990	2801.7172	130.672
17	28.670	1352.1600	90.615
18	30.630	3921.5457	209.516
19	33.440	855.7588	39.443
20	34.380	667.2610	34.578
21	35.550	706.4179	22.689
22	47.600	398.1242	22.008

Table 27: Peak table of data presented in Figure 21b (D) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	11.330	248.9855	13.413
2	15.550	456.3200	21.828
3	16.490	2800.7867	163.385
4	17.010	983.4809	55.051
5	18.680	514.9884	18.954
6	20.250	1324.5289	76.216
7	22.040	3392.1218	223.634
8	23.400	3823.7283	166.478
9	24.380	6559.8938	472.292
10	24.690	19289.0348	976.909
11	25.500	3384.3758	194.446
12	26.660	812.9294	38.461
13	27.730	1431.3328	61.607
14	28.120	708.6736	45.477
15	28.670	1342.7970	80.109
16	29.160	2870.3657	93.935
17	29.830	1506.0134	94.003
18	30.360	647.2980	24.973
19	31.900	4045.5554	221.921
20	32.910	829.6450	23.476
21	34.660	857.3288	37.078
22	35.730	628.8024	33.456
23	49.190	660.9716	38.468

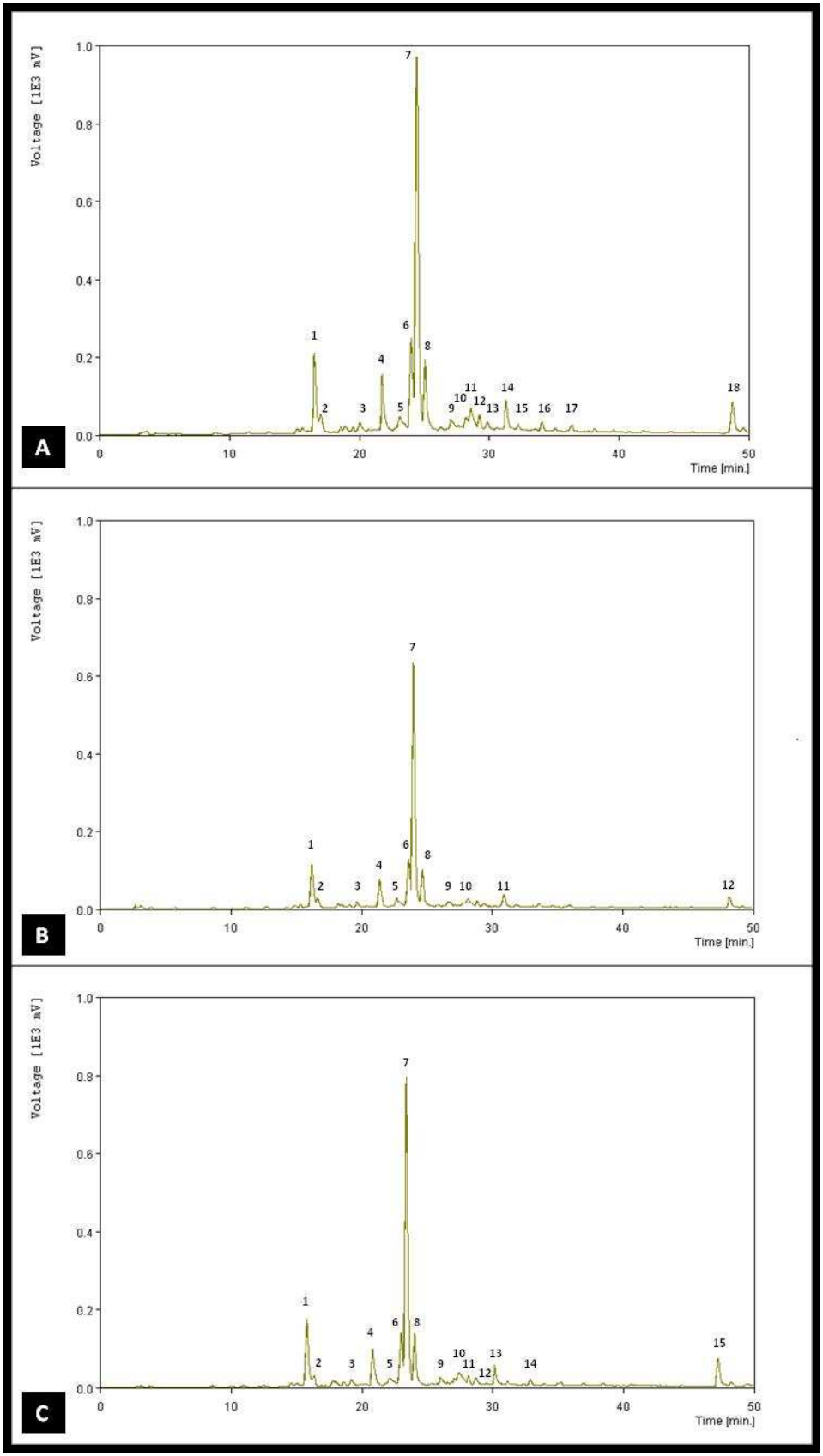

Figure 22 (a). HPLC profile of total phenols in the leaves during salt stress in one month old plant of LV following NaCl treatment; A–50mM, B–100mM C–200mM for the 1st day

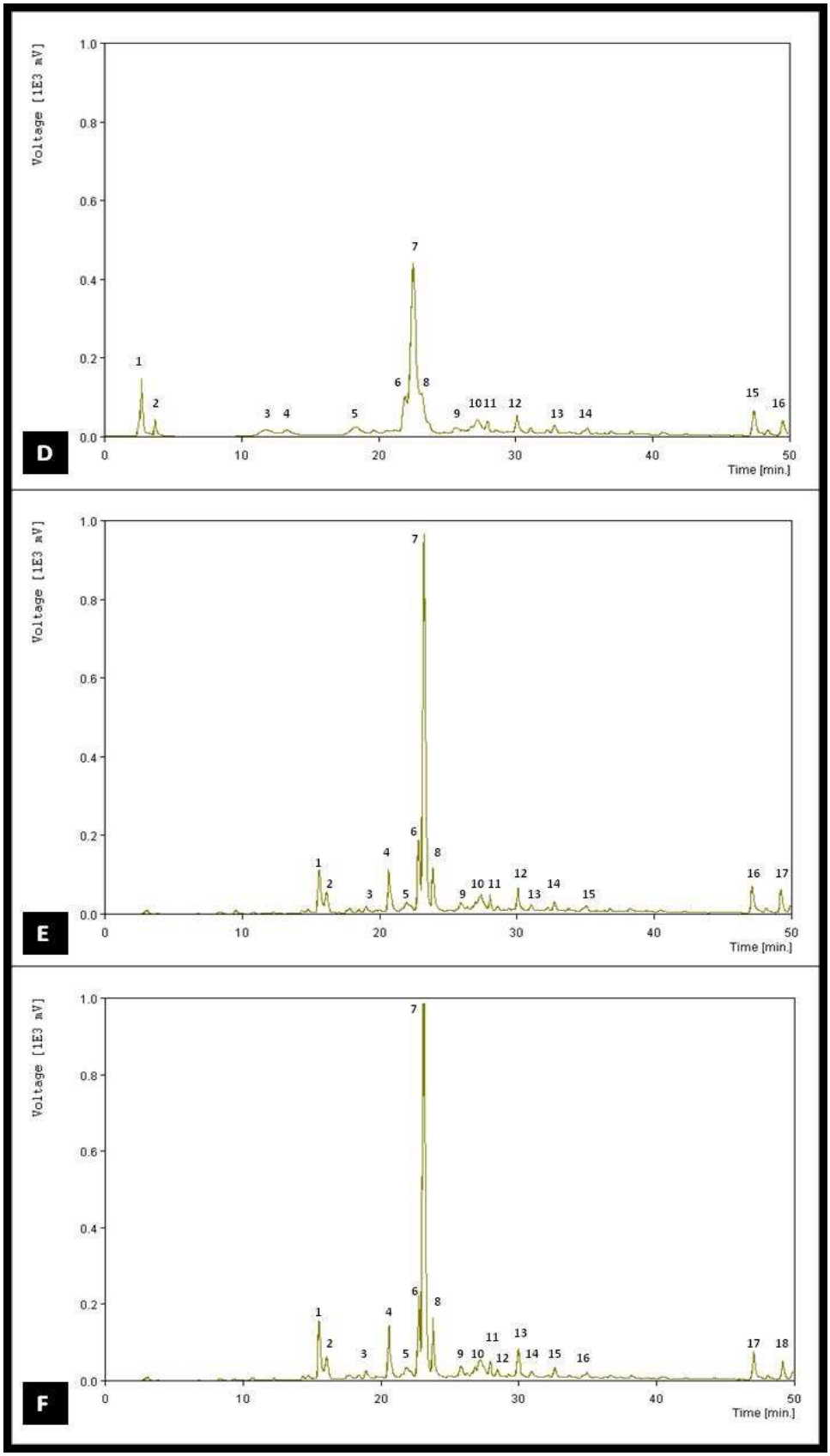

Figure 22 (b). HPLC profile of total phenols in the leaves during salt stress in one month old plant of LV following NaCl treatment; D–50mM, E–100mM F–200mM for the 3rd day

Table 28: Peak table of data presented in Figure 22a (A) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	16.530	4486.2155	207.242
2	18.560	331.7083	20.592
3	20.050	688.8392	31.530
4	21.780	3151.8747	152.564
5	23.120	1632.4992	43.734
6	24.030	3802.4440	246.441
7	24.450	16419.6329	971.204
8	25.090	3451.4475	189.814
9	27.070	1772.5937	37.097
10	28.260	728.3323	42.385
11	28.610	1657.2986	65.420
12	29.300	821.1341	49.977
13	29.870	877.4953	30.097
14	31.350	1870.7228	84.348
15	32.270	753.3488	22.853
16	34.080	692.1439	29.139
17	36.370	754.8201	20.936
18	48.800	1436.8299	78.477

Table 29: Peak table of data presented in Figure 22a (B) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	16.160	1824.6152	113.910
2	16.660	557.0002	26.834
3	19.680	358.2666	17.069
4	21.380	1384.4939	76.665
5	22.670	889.5586	26.019
6	23.620	1951.7154	128.008
7	24.000	8896.2739	634.069
8	24.660	1814.0872	99.955
9	26.640	552.8736	16.320
10	28.190	781.2073	24.259
11	30.910	805.0514	36.399
12	48.250	545.0029	27.835

Table 30: Peak table of data presented in Figure 22a (C) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.780	3196.7621	175.017
2	17.810	406.5090	15.607
3	19.210	359.8530	19.519
4	20.850	1950.3365	96.238
5	22.120	817.6982	21.417
6	23.030	2012.7062	138.022
7	23.390	10990.7868	797.445
8	24.060	2142.9909	136.324
9	26.020	744.3247	23.993
10	27.430	1130.0694	34.277
11	28.190	420.4068	26.344
12	28.730	510.6626	21.483
13	30.160	1045.8800	53.620
14	32.890	351.3350	16.281
15	47.330	1262.7338	70.646

Table 31: Peak table of data presented in Figure 22b (D) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	2.640	2406.1781	145.538
2	3.610	702.6333	41.847
3	11.760	1275.1473	16.385
4	13.290	1008.7914	15.509
5	18.340	1501.6873	23.078
6	21.960	2221.9322	101.268
7	22.500	16332.5465	440.225
8	23.140	2564.2141	115.341
9	25.590	1005.3236	19.687
10	27.260	2020.6758	40.926
11	27.940	778.5287	35.902
12	30.120	1263.7829	51.919
13	32.870	682.4148	26.374
14	35.250	531.1103	18.544
15	47.490	1238.7410	64.495
25	49.600	577.0111	36.209

Table 32: Peak table of data presented in Figure 22b (E) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.620	1674.2253	110.047
2	16.120	892.7828	54.944
3	19.020	280.5231	15.907
4	20.690	1833.6599	108.771
5	21.970	891.6411	23.501
6	22.880	2616.0812	183.960
7	23.250	12917.4106	963.877
8	23.920	1724.4318	111.369
9	26.970	404.5409	23.246
10	27.420	1140.7120	40.816
11	28.070	537.9539	39.838
12	30.110	897.0819	57.227
13	31.050	227.2842	14.299
14	32.780	321.1536	22.543
15	35.090	269.1202	13.523
16	47.230	1206.3558	66.512
17	49.330	902.5013	57.722

Table 33: Peak table of data presented in Figure 22b (F) for LV

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.520	2451.7024	157.844
2	16.050	1080.6384	62.589
3	18.940	537.0669	25.634
4	20.580	2473.3486	143.174
5	21.850	1089.2534	33.603
6	22.760	3168.1879	222.629
7	23.090	15249.0815	987.425
8	23.810	2700.9380	163.587
9	25.780	815.8082	37.218
10	27.300	1706.9559	53.206
11	27.950	812.7629	50.101
12	28.490	531.7824	26.455
13	29.980	1682.1945	82.193
14	30.940	780.8076	23.328
15	32.670	590.5786	31.700
16	47.140	1310.2386	72.220
17	49.240	742.2390	47.131

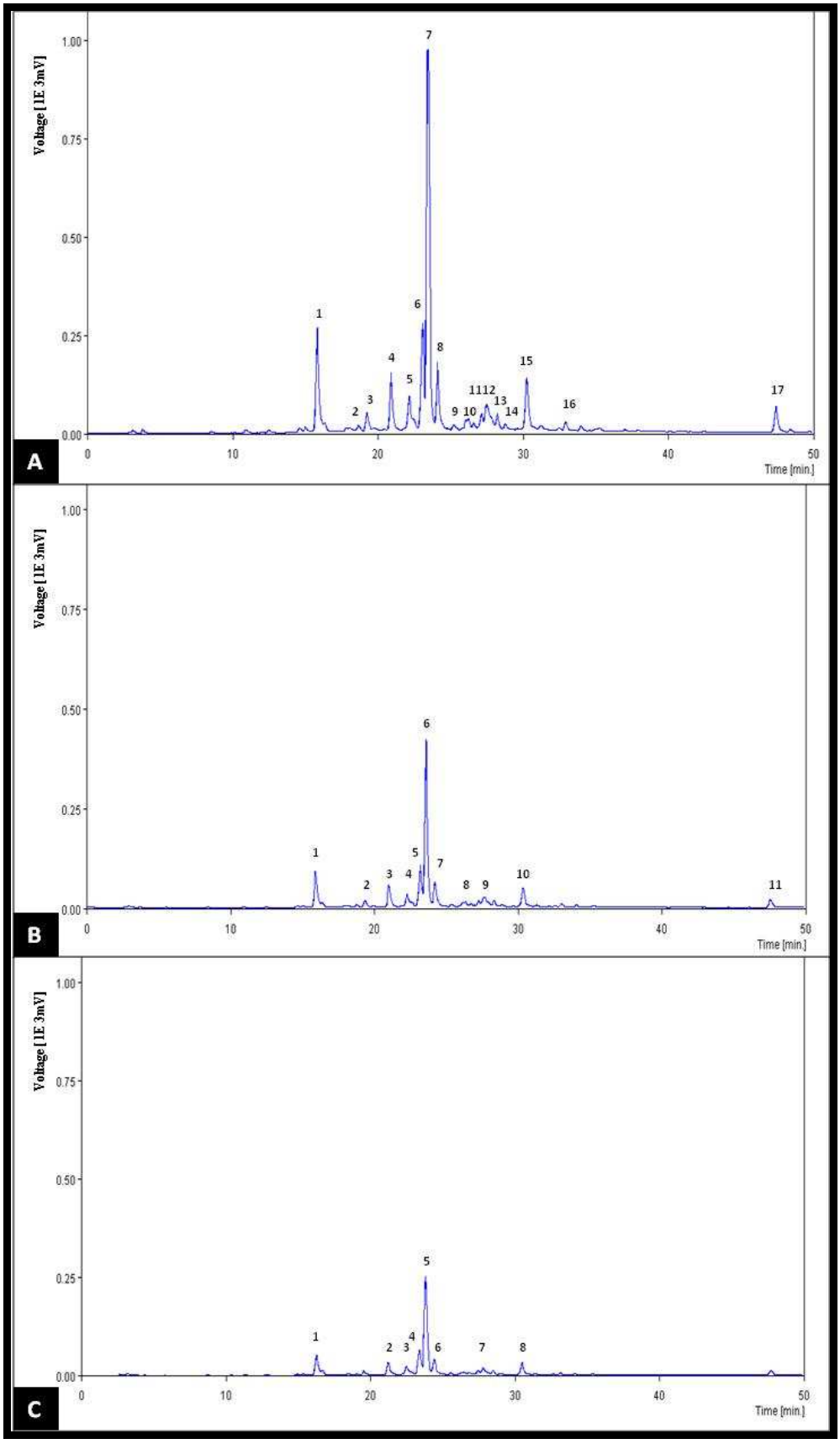

Figure 23 (a). HPLC profile of total phenols in the leaves during salt stress in one month old plant of GN following NaCl treatment; A–50mM, B–100mM C–200mM for the 1st day

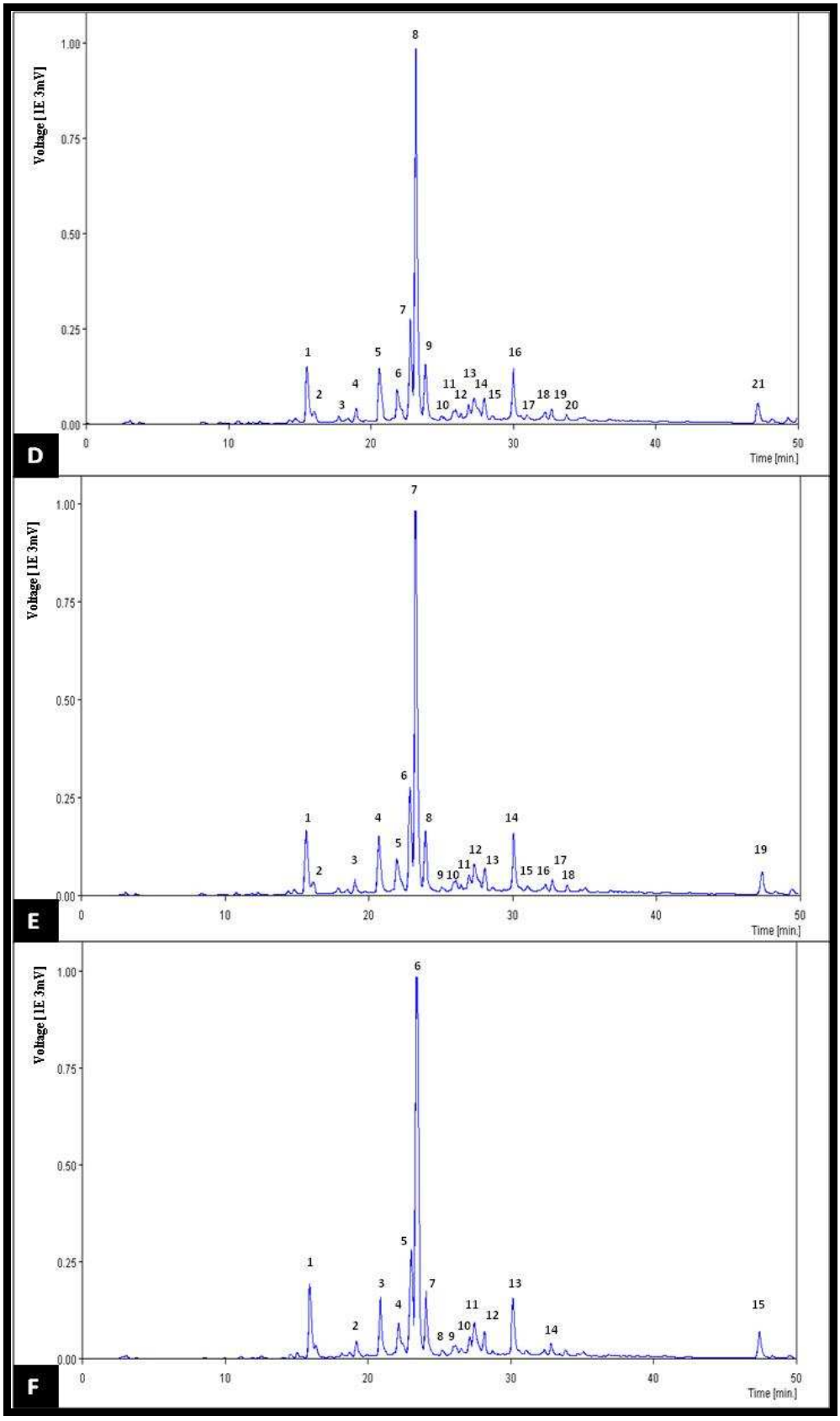

Figure 23 (b). HPLC profile of total phenols in the leaves during salt stress in one month old plant of GN following NaCl treatment; A–50mM, B–100mM C–200mM for the 3rd day

Table 34: Peak table of data presented in Figure 23a (A) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.800	4695.3641	267.643
2	18.670	439.8508	19.395
3	19.240	1177.4229	51.760
4	20.890	2512.8329	153.861
5	22.140	2201.4577	94.184
6	23.070	3952.4180	277.884
7	23.410	15244.6573	975.764
8	24.100	3045.2502	179.685
9	25.220	474.6405	20.348
10	26.230	1387.1074	35.271
11	27.140	816.2642	48.192
12	27.480	2035.0635	72.078
13	28.240	831.2607	49.638
14	28.790	664.3033	21.627
15	30.260	2632.8764	140.129
16	32.950	633.9049	27.333
17	47.460	1177.3621	66.153

Table 35: Peak table of data presented in Figure 23a (B) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.900	1436.0791	91.936
2	19.340	318.7543	18.317
3	20.000	135.5207	4.386
4	21.010	950.3179	56.769
5	22.270	852.1264	34.809
6	23.200	1607.4486	107.946
7	23.590	5858.5861	422.118
8	24.220	1142.6491	64.348
9	27.260	255.3694	17.246
10	27.650	719.4571	26.003
11	28.340	303.1147	18.214
12	30.360	937.0563	49.564
13	47.590	371.3557	19.654

Table 36: Peak table of data presented in Figure 23a (C) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	16.250	846.2721	52.557
2	21.220	647.7336	33.640
3	22.490	568.7914	22.391
4	23.400	986.4447	64.229
5	23.820	3732.8482	251.796
6	24.410	804.8136	42.071
7	27.800	452.1203	19.125
8	30.500	638.7081	32.950

Table 37: Peak table of data presented in Figure 23b (D) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.510	2332.2165	150.829
2	16.020	556.2953	33.043
3	17.730	430.2381	20.568
4	18.950	729.7159	40.306
5	20.610	2631.8371	145.925
6	21.860	2266.2969	90.794
7	22.780	3885.4913	275.085
8	23.150	14279.7832	986.767
9	23.840	2671.0889	156.035
10	24.950	520.5766	20.478
11	25.970	545.9192	36.400
12	26.330	388.7045	26.055
13	26.870	898.6843	49.270
14	27.260	1933.0027	66.781
15	27.980	1076.7879	66.641
16	30.020	2503.0694	143.735
17	30.980	647.9043	22.097
18	32.240	929.0924	28.936
19	32.710	730.8788	36.848
20	33.770	632.6816	22.560
21	47.230	955.9214	52.165

Table 38: Peak table of data presented in Figure 23b (E) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.640	3096.4323	166.658
2	17.870	395.1929	18.104
3	19.040	725.2846	40.127
4	20.700	2616.0018	149.840
5	21.970	2325.9726	92.559
6	22.880	3934.5614	276.618
7	23.240	14951.2329	984.501
8	23.940	2805.9840	164.314
9	25.070	496.1610	20.650
10	26.070	1467.4813	36.380
11	27.000	973.5837	51.657
12	27.370	1971.1566	78.786
13	28.070	1107.0678	68.985
14	30.110	3194.6146	157.341
15	31.050	659.8055	21.874
16	32.320	866.7498	26.933
17	32.790	941.6456	39.420
18	33.830	635.3400	23.405
19	35.090	521.7972	17.660
20	47.400	1009.7380	57.217

Table 39: Peak table of data presented in Figure 23b (F) for GN

Peak No.	Retention time	Area (mV.s)	Height (mV)
1	15.800	4695.3641	267.643
2	19.240	1177.4229	51.760
3	20.890	2512.8329	153.861
4	22.140	2201.4577	94.184
5	23.070	3952.4180	277.884
6	23.410	15244.6573	975.764
7	24.100	3045.2502	179.685
8	25.220	474.6405	20.348
9	26.230	1387.1074	35.271
10	27.140	816.2642	48.192
11	27.480	2035.0635	72.078
12	28.240	831.2607	49.638
13	30.260	2632.8764	140.129
14	32.950	633.9049	27.333
15	47.460	1177.3621	66.153

4.7.2.2. Phenolic acids

The extraction and analysis of phenolic acids from the total phenols obtained during stress treatments in the leaves of treated plants showed interesting and significant results. One of the most prominent peaks observed during the analysis of phenolic acids in case of GN and LV was identified as ferulic acid, followed by vanillic acid, cinnamic acid, chlorogenic acid and also salicylic acid. Peak number 1 in each treatment was identified as the vanillic acid with retention time of about 2.99 or 2.95 minutes (Table 40–51). The peak for vanillic acid was two of the highest peak observed in our study of phenolic acid which was very prominent and its height increased with increased in the concentration of salt in case of LV during the 1st day (Figure 24a) and for each concentration of salt it showed enhancement on the 3rd day (Figure 24b). However, during the 3rd day the highest peak in LV was observed in case of lowest concentration of salt, i.e. 50mM but the peak height decreased with increase in the concentration of salt (Figure 24b). In case of GN, however, the peak for vanillic acid decreased slightly with increase in the concentration of salt on the 1st day (Figure 25a). On the 3rd day of salt stress for the corresponding concentration of salt the height of peak decreased how ever there was a significant increase in its height on the 3rd day of salt concentration of 200mM (Figure 25b). It was significant observation that in GN, during the 3rd day of salt stress, the tallest peak for vanillic acid was observed during the 3rd day.

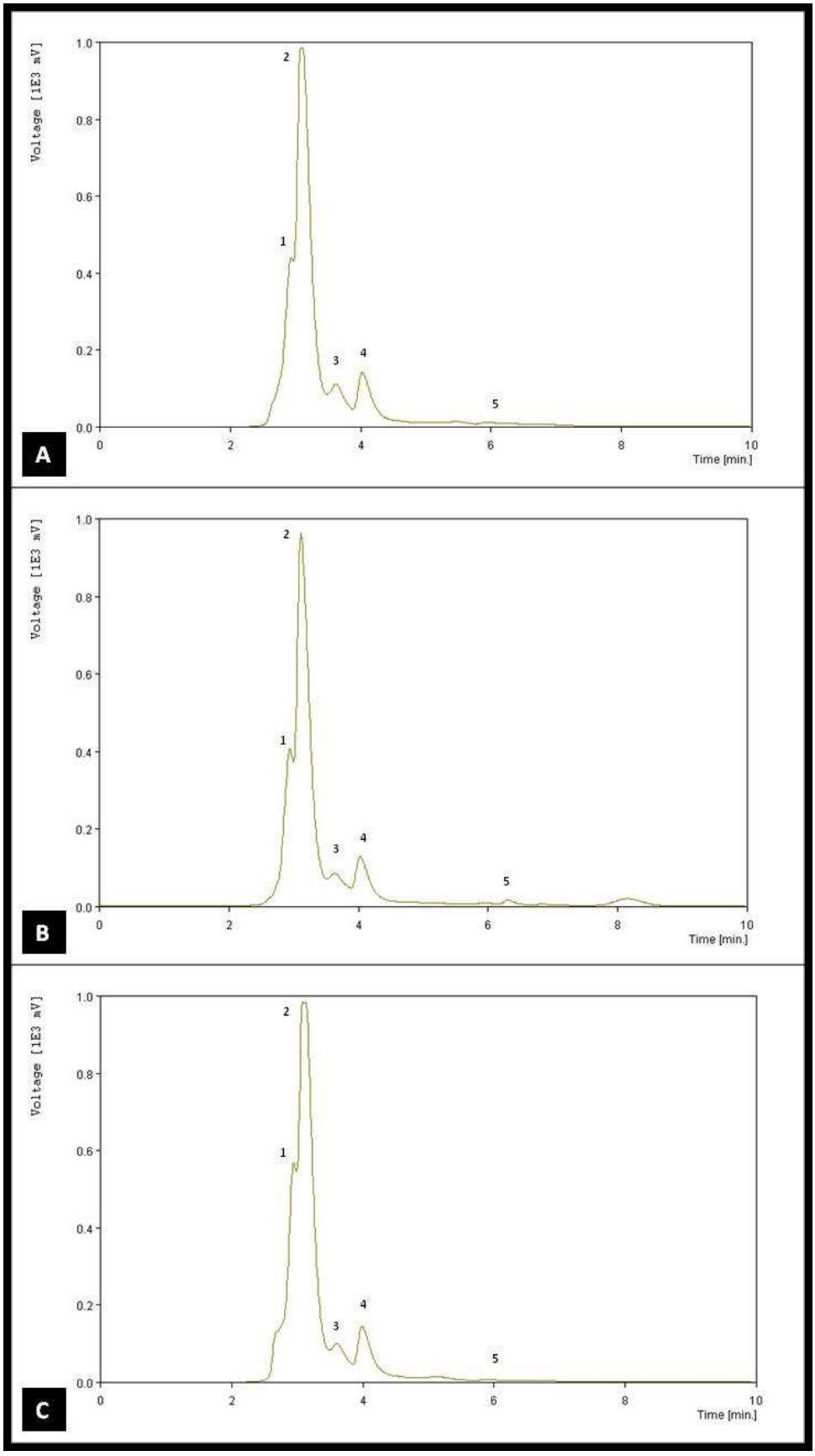

Figure 24(a). HPLC profile of phenolic acid in the leaves of wheat (LV) following salt stress in one month old plant; A–50mM, B–100mM, C–200mM for 1st day

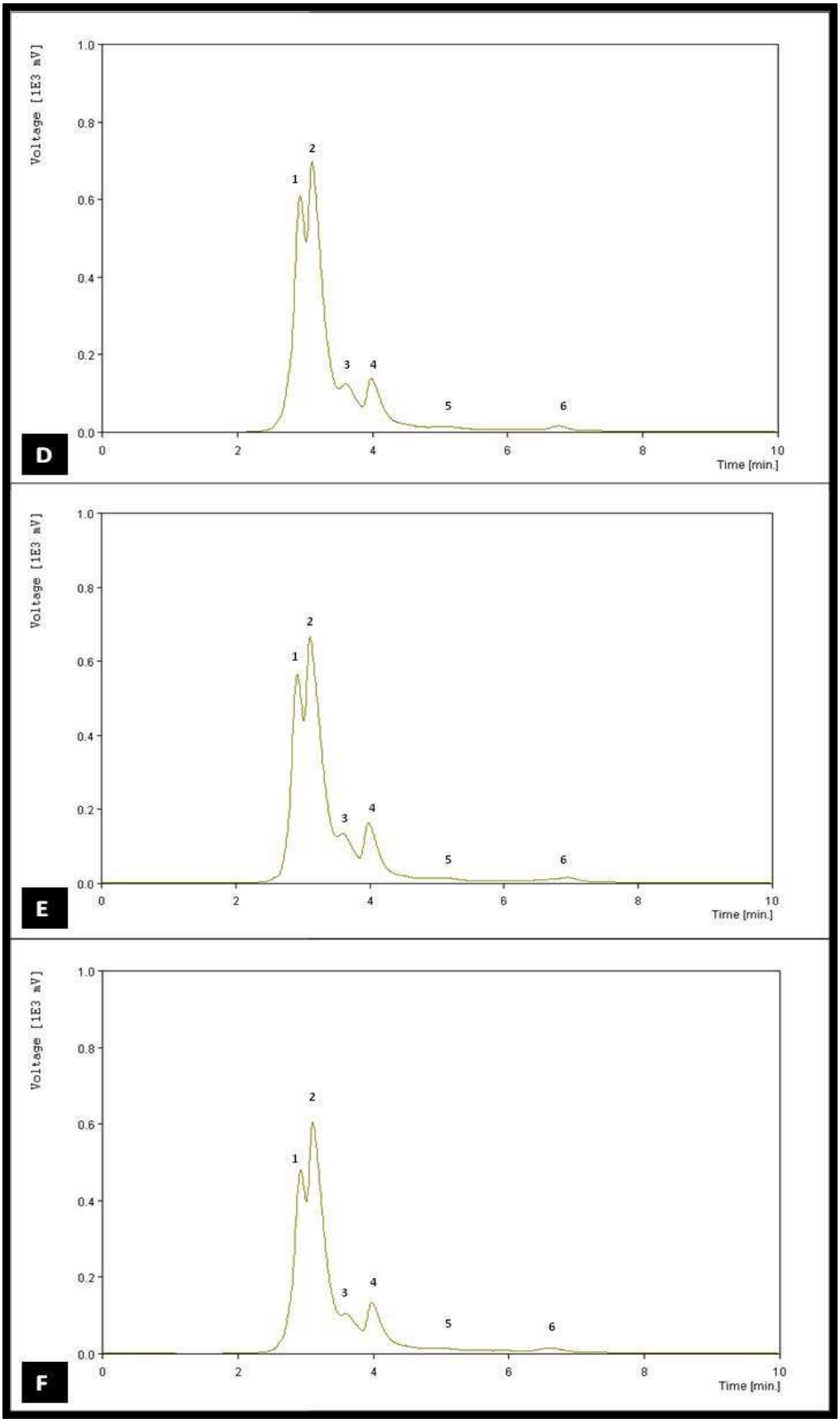

Figure 24(b). HPLC profile of phenolic acid in the leaves of wheat (LV) following salt stress in one month old plant; D–50mM, E–100mM, F–200mM for 3rd days

Vanillic acid was the tallest peak observed in GN during the 3rd day among all the peaks obtained in HPLC. The tallest peak (peak 2) obtained in case of GN and LV was identified as ferulic acid (Figure 24 and 25). The peak of ferulic acid in case of LV with retention time of about 3minutes was the most prominent peak and the height of this peak remain unchanged during the 1st day of salt stress in all the concentrations but on the 3rd day of salt stress the height of this peak decreased significantly in all the concentration. During the 3rd day the height of this peak was highest in the salt concentration of 50mM but it reduced in case of 200mM of salt concentration. (Figure 24a, b). In case of GN, the height of ferulic acid decreased with increasing concentration of salt during the 1st day which decreased further on the 3rd day of salt stress, however, interestingly the height of this peak during the 3rd day increased with increasing concentration of salt and was significantly highest at the salt concentration of 200mM (Figure 25a, b). The peak number 3 in GN and LV in all treatments was identified as the peak for chlorogenic acid with a retention time of about 3.75 minutes which remained unchanged or showed a little change in its height during different concentration of salt in the 1st and 3rd day of salt stress (Figure 24 and 25).

Peak number 4 was identified as cinnamic acid with retention of about 4 minutes and it appeared in all the concentration of salt and also during all the days in our study. The height of this peak in both GN and LV showed a little change during salt stress however in GN there was an enhancement during the 3rd day of salt stress (Fig 25b). Peak number 5 and 6 was identified as the peak for salicylic acid in all the trea4tments in both GN and LV. Peak number 6 was absent in case of LV during the 1st day of salt stress and appeared during the 3rd day (Figure 24a) and in GN it was absent on the 1st day of 50mM concentration of salt but present in all other concentration and days of stress (Figure 25a). Peak number 5 showed a change during all the concentration with increase in its height with increasing concentration of salt in both 1st and 3rd day. Out of all the peaks the peak for ferulic acid, vanillic acid, chlorogenic acid, cinnamic acid and salicylic acid was found to be the most prominent during salt stress in HPLC analysis with significant change in their height.

Table 40: Peak table of data presented in Figure 24a (A) for LV

Peak No.	Retention time	Height (mV)
1	2.950	425.122
2	3.080	988.725
3	3.750	110.11
4	4.050	140.11
5	5.460	13.733
6	5.940	11.647

Table 41: Peak table of data presented in Figure 24a (B) for LV

Peak No.	Retention time	Height (mV)
1	2.990	409.25
2	3.110	964.910
3	3.756	98.99
4	4.060	138.9
5	5.930	8.531
6	6.310	15.813

Table 42: Peak table of data presented in Figure 24a (C) for LV

Peak No.	Retention time	Height (mV)
1	2.950	580.22
2	3.080	988.118
3	3.750	108.29
4	4.050	122.21
5	5.090	14.151
6	5.890	7.630

Table 43: Peak table of data presented in Figure 24b (D) for LV

Peak No.	Retention time	Height (mV)
1	2.990	600.00
2	3.100	699.955
3	3.755	110.99
4	4.061	115.89
5	5.080	13.405
6	6.760	14.751

Table 44: Peak table of data presented in Figure 24b (E) for LV

Peak No.	Retention time	Height (mV)
1	2.99	565.55
2	3.100	667.524
3	3.756	140.59
4	4.060	161.25
5	5.070	13.805
6	5.850	5.925

Table 45: Peak table of data presented in Figure 24b (F) for LV

Peak No.	Retention time	Height (mV)
1	2.99	361.000
2	3.100	606.685
3	3.756	100.101
4	4.061	120.15
5	4.990	13.389
6	6.600	13.510

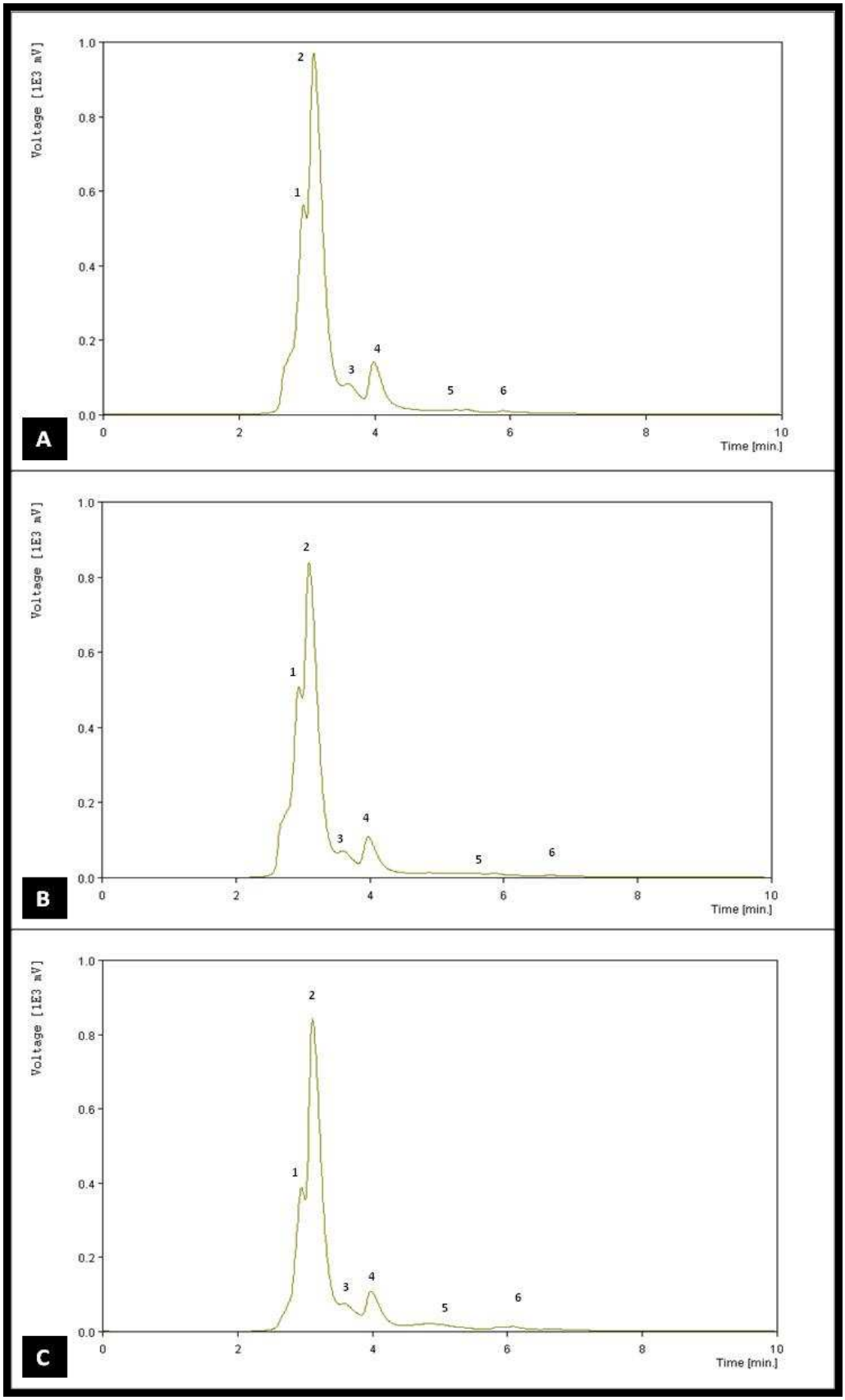

Figure 25(a). HPLC profile of phenolic acid in the leaves of wheat (GN) following salt stress in one month old plant; A–50mM, B–100mM, C–200mM for 1st day

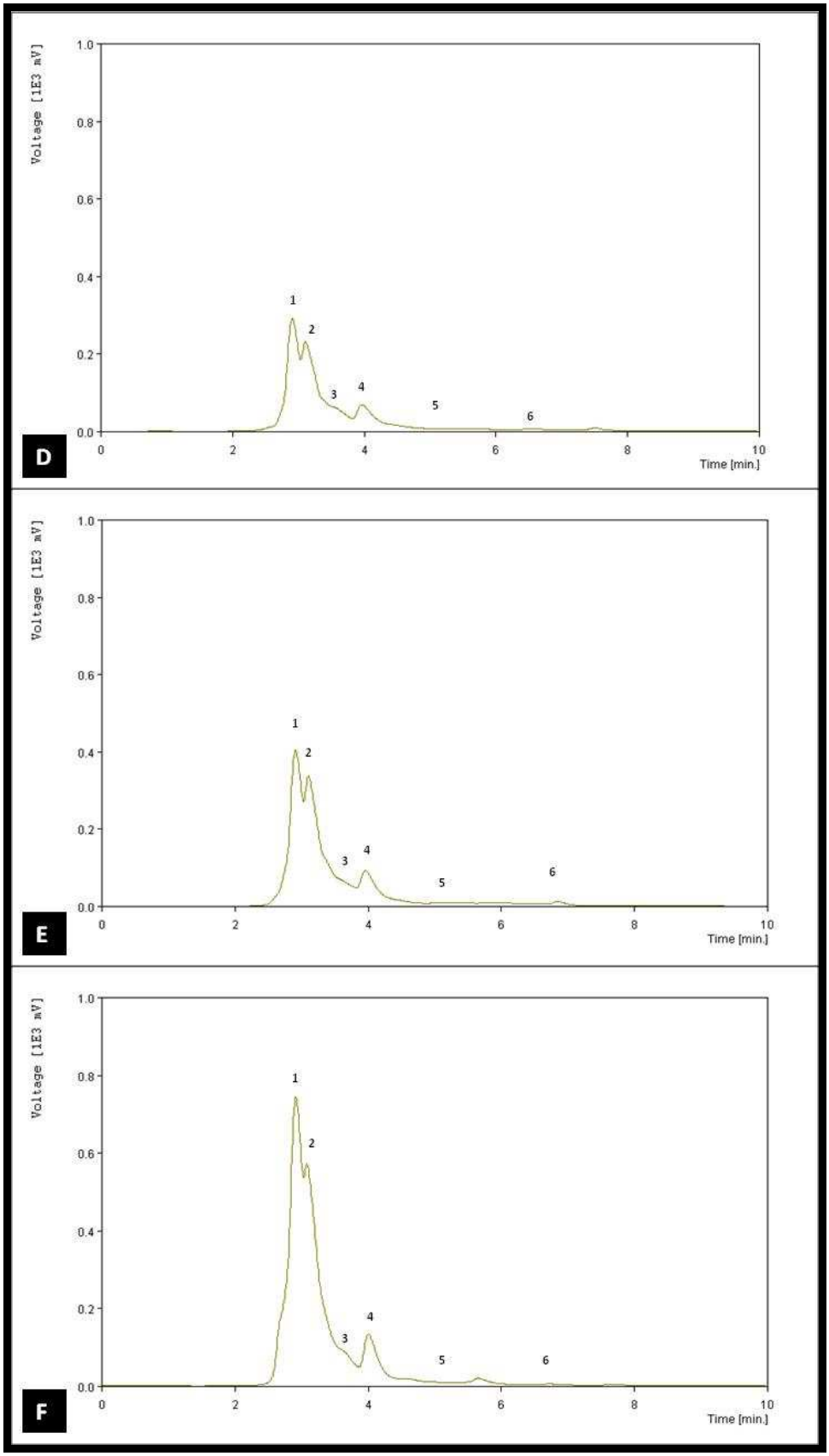

Figure 25(b). HPLC profile of phenolic acid in the leaves of wheat (GN) following salt stress in one month old plant; D–50mM, E–100mM, F–200mM for 3rd day

Table 46: Peak table of data presented in Figure 25a (A) for GN

Peak No.	Retention time	Height (mV)
1	2.990	531.11
2	3.100	974.320
3	3.756	80.12
4	4.060	115.55
5	5.360	12.931
6	5.890	9.792

Table 47: Peak table of data presented in Figure 25a (B) for GN

Peak No.	Retention time	Height (mV)
1	2.950	509.95
2	3.090	841.904
3	3.754	69.15
4	4.061	105.55
5	5.470	11.598
6	6.690	5.713

Table 48: Peak table of data presented in Figure 25a (C) for GN

Peak No.	Retention time	Height (mV)
1	2.990	391.12
2	3.110	846.247
3	3.756	79.98
4	4.065	105.59
5	4.850	20.363
6	6.080	12.881

Table 49: Peak table of data presented in Figure 25b (D) for GN

Peak No.	Retention time	Height (mV)
1	2.900	291.544
2	3.070	225.55
3	3.749	64.55
4	4.060	65.91
5	5.560	5.613
6	6.520	8.769

Table 50: Peak table of data presented in Figure 25b (E) for GN

Peak No.	Retention time	Height (mV)
1	2.910	405.937
2	3.060	341.00
3	3.710	81.10
4	4.010	105.15
5	5.370	9.033
6	5.870	9.687

Table 51: Peak table of data presented in Figure 25b (F) for GN

Peak No.	Retention time	Height (mV)
1	2.910	746.526
2	3.060	565.51
3	3.720	104.25
4	4.020	115.54
5	5.650	20.016
6	6.730	5.090

4.8. Studies on proteins of wheat plants following drought and salinity

4.8.1. Protein contents

The content of soluble proteins in case of wheat varieties decreased following water stress (Figure 26) and the decrease continued with the increase in the days of drought. The decrease in the soluble protein content in case of GN, KW, UP 2752 and KD was more or less similar and did not show much significant difference during the 6^{th} and 9^{th} day. However, the protein content changed significantly in case of MW, GY, LV, PBW 343 an SO with the increase in the days of withholding water from the plant. The content of soluble protein in the roots in case of MW, GY, GN, LV and SO increased during water stress during the 6^{th} and the 9^{th} day while in case of all the other varieties it showed a general decrease. The accumulation of soluble proteins in case of leaf was higher than the root. Accumulation of soluble protein in the leaves during salt stress for 1^{st} and 3^{rd} (Figure 27) day showed a general decrease with the increase in the severity of salt stress whereas the content of soluble protein in the roots (Figure 28) for 1^{st} and 3^{rd} day showed a difference. In case of KD, GN, KW, UP 2752, PBW 343 and SO the accumulation of soluble protein in the roots decreased with the increase in the concentration of salt whereas in case of MW, GY and LV the content of soluble proteins increased significantly when the salt concentration was higher in the 1^{st} day but the accumulation again showed a decline on the 3^{rd} day of salt stress.

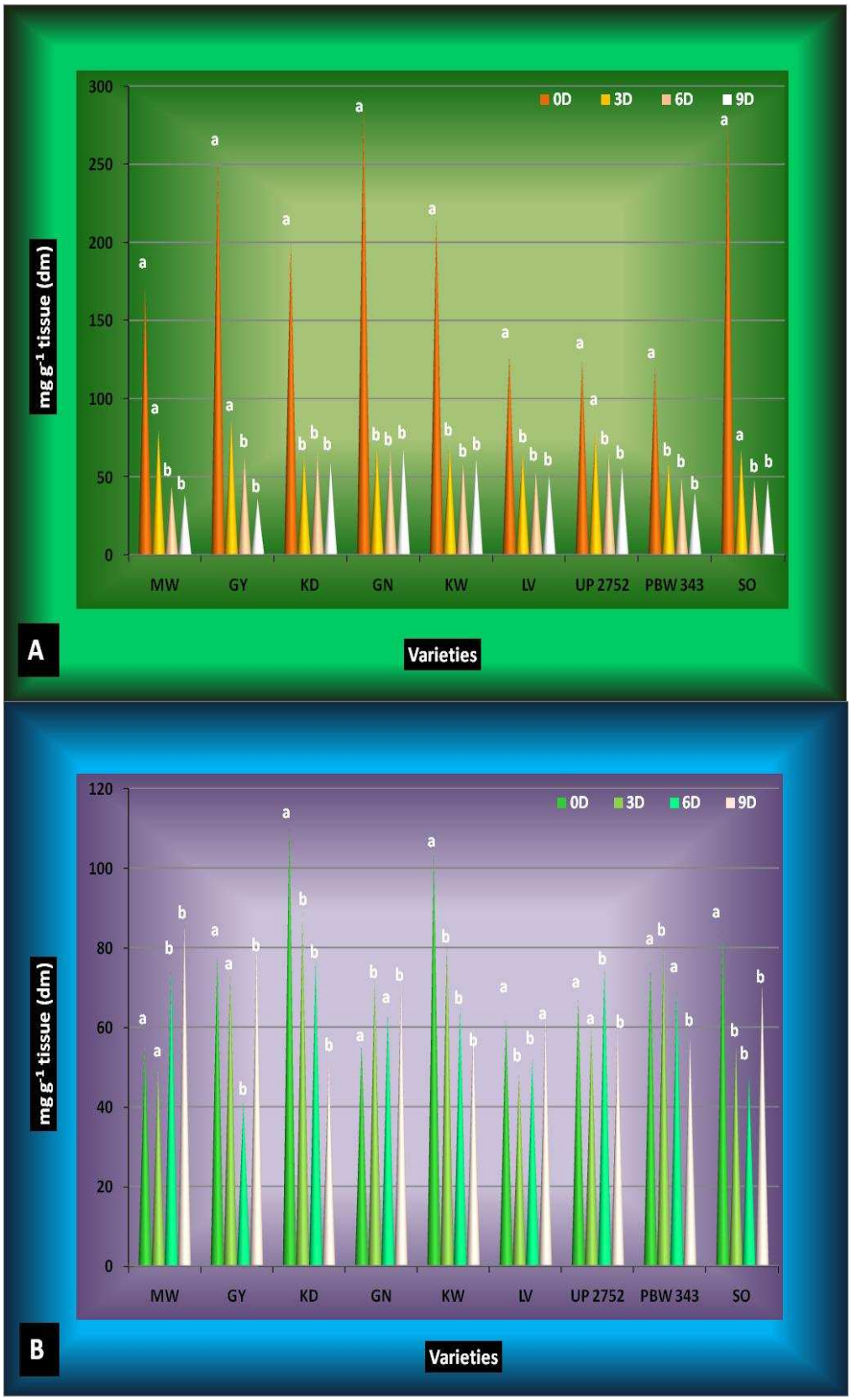

Figure 26. Content of total soluble protein in leaf (A) and root (B) of nine wheat varieties subjected to drought stress. Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

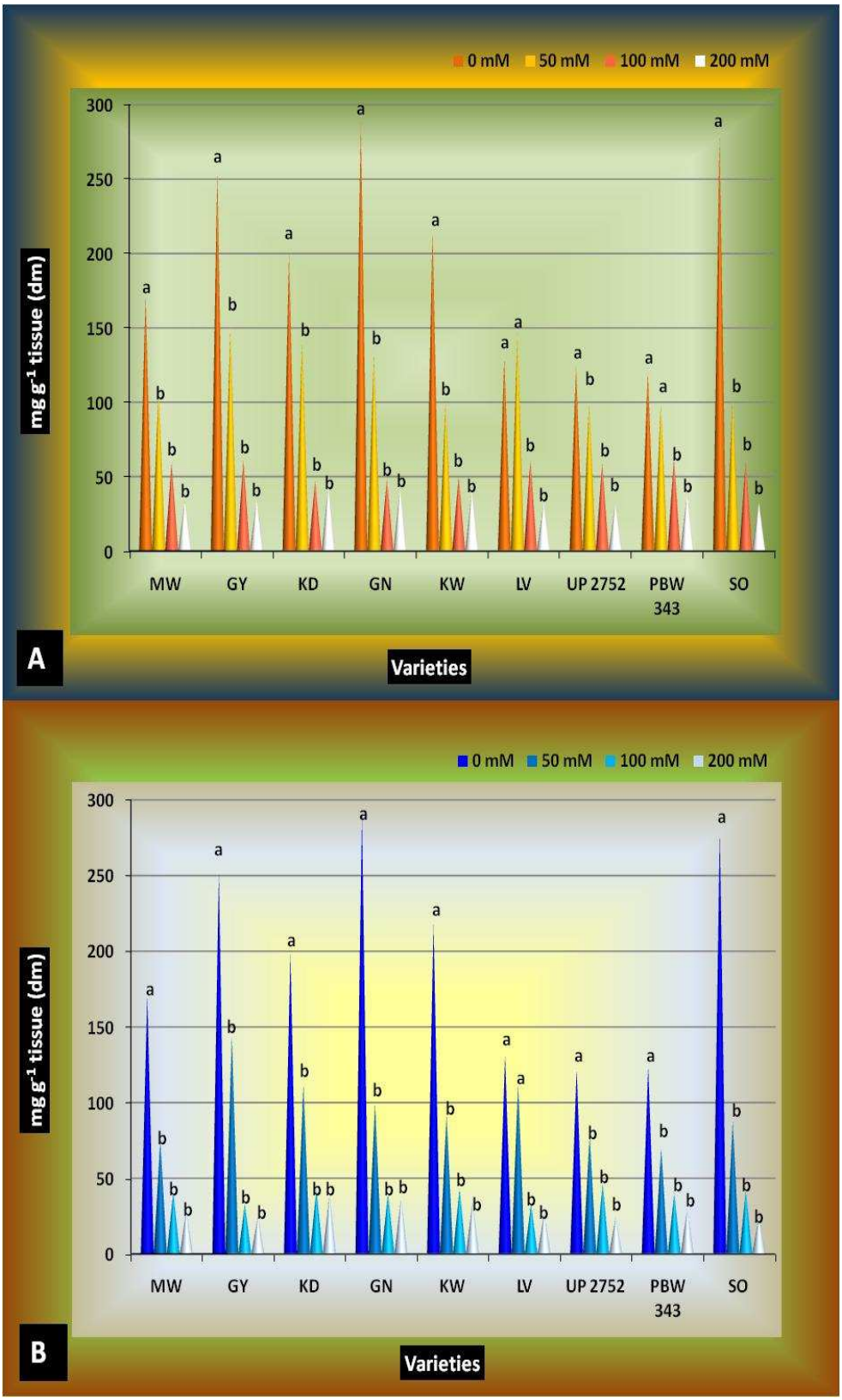

Figure 27. Content of total soluble protein in the leaves of nine wheat varieties subjected to salt stress for 1 day (A) and 3 days (B). Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

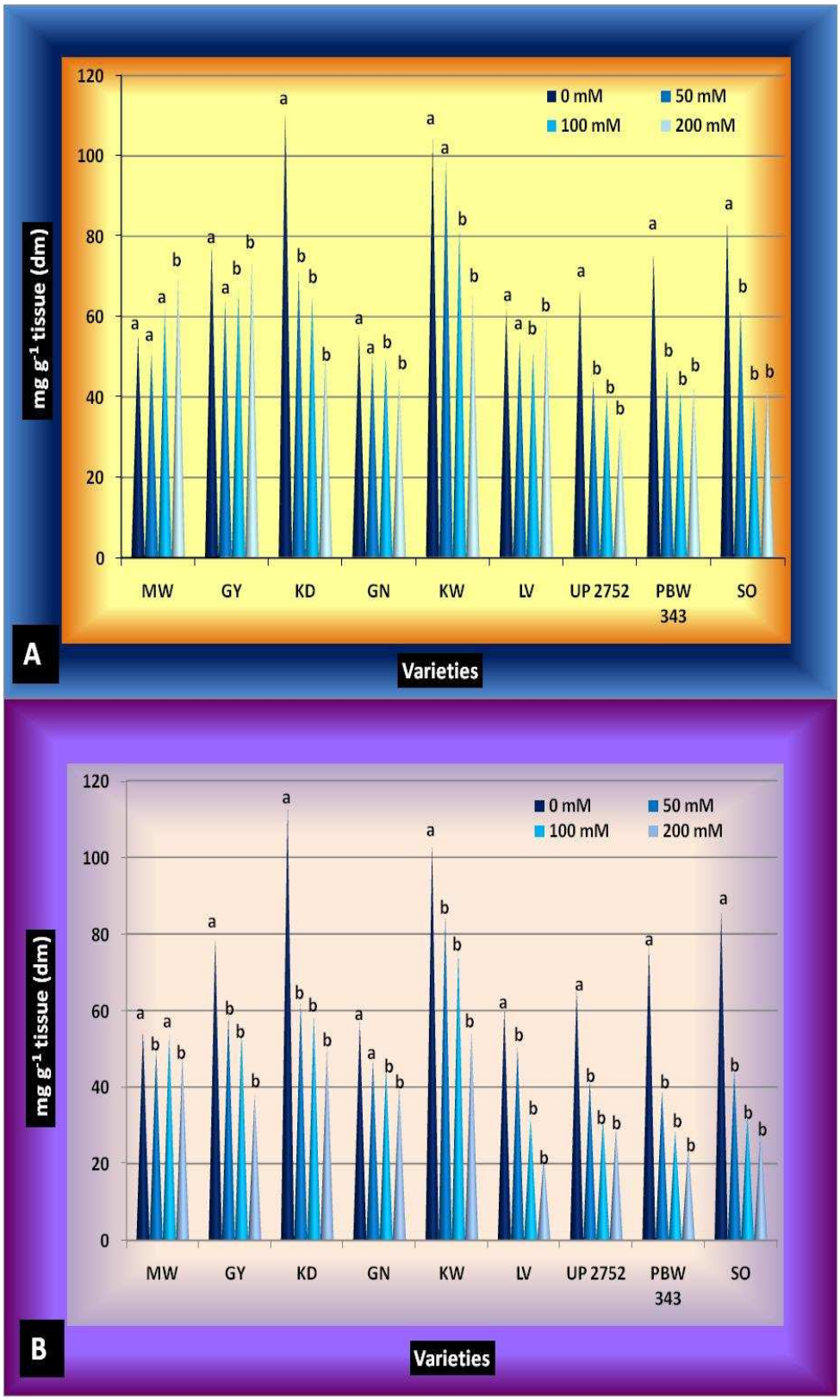

Figure 28. Content of total soluble protein in the roots of nine wheat varieties subjected to salt stress for 1 day (A) and 3 days (B). Dm, dry matter; Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

4.8.2. Protein profile determined by SDS PAGE

Analysis of protein profiles by SDS PAGE in the seeds and leaves of wheat varieties was done for both drought and salinity stress with respect to the control plants of each variety. The accumulation of protein in the seeds was also studied without any treatment (Figure 29) in seven varieties. Seedlings of wheat subjected to drought and salt stress showed an expression of new protein bands in some cases and suppression of certain existing bands at different duration of withholding water and at increasing concentration of salt for 1 and 3rd day. At least a total of about 34 new bands were observed in SDS PAGE of proteins during drought (Figure 29–34) and salinity (Figure 35–42), out of which 8 new bands with molecular mass (approx. in KDa) 7.3, 7.9, 10.1, 14.4, 16.1, 16.2, 17.5, 18.9, 19.2, 49.8, 69.9, 89.9, 95.0, 97.8, 99.0, 105.2 occurred and were common in both the stresses. Out of the 34 new bands 6 bands with molecular mass (in KDa) 16.8, 17.0, 28.8, 40.2, 49.9 and 96 were observed only in case of drought (Table 52–57) and 12 new bands were observed only in case of salinity stress (Table 58–65) with molecular masses (in KDa) of 8.6, 12.3, 15.9, 17.8, 19.6, 22.1, 25.2, 27.6, 28.8, 32.2, 34.5 and 43.6. More number of new bands was observed in case of GN, KD, KW, UP 2752 and PBW 343 during the SDS PAGE analysis of leaf proteins during both drought and salinity stress which in case of other four varieties was comparatively lesser in number.

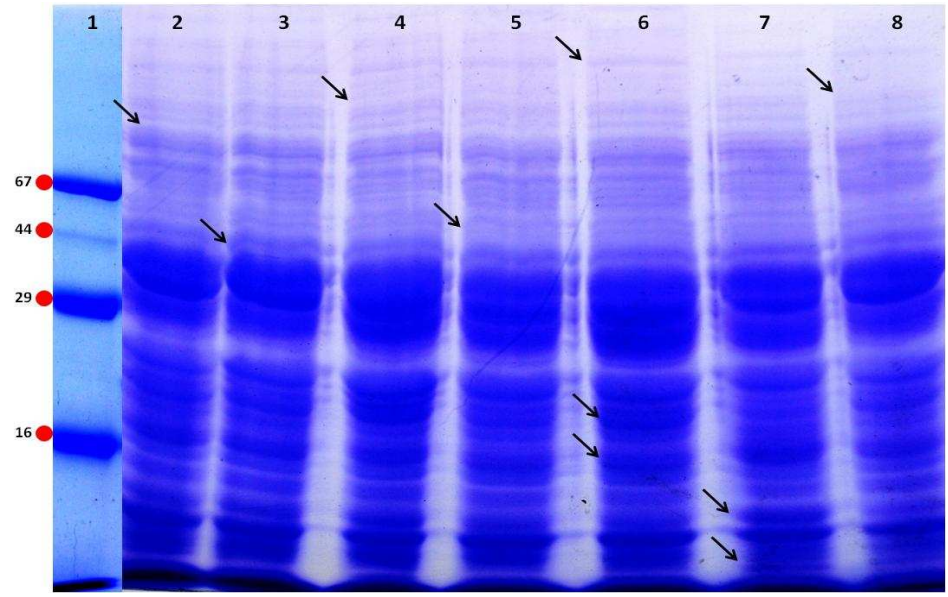

Figure 29. SDS PAGE of seeds. 1–Marker, 2–GN, 3–GY, 4–KD, 5–KW, 6–MW, 7–SO, 8–PBW 343

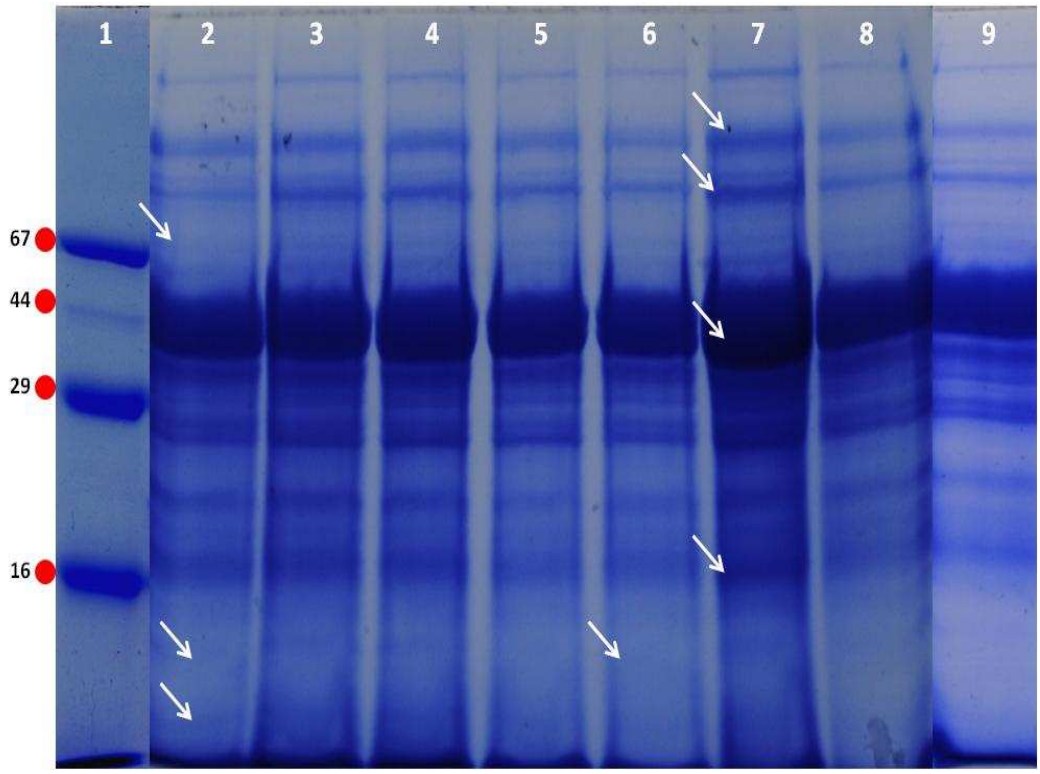

Figure 30. SDS PAGE in the leaves of LV and GN for drought. 1–Marker, 2 & 6–9d; 3 & 7–6d; 4 & 8–3d; 5 & 9–0d of LV & GN respectively

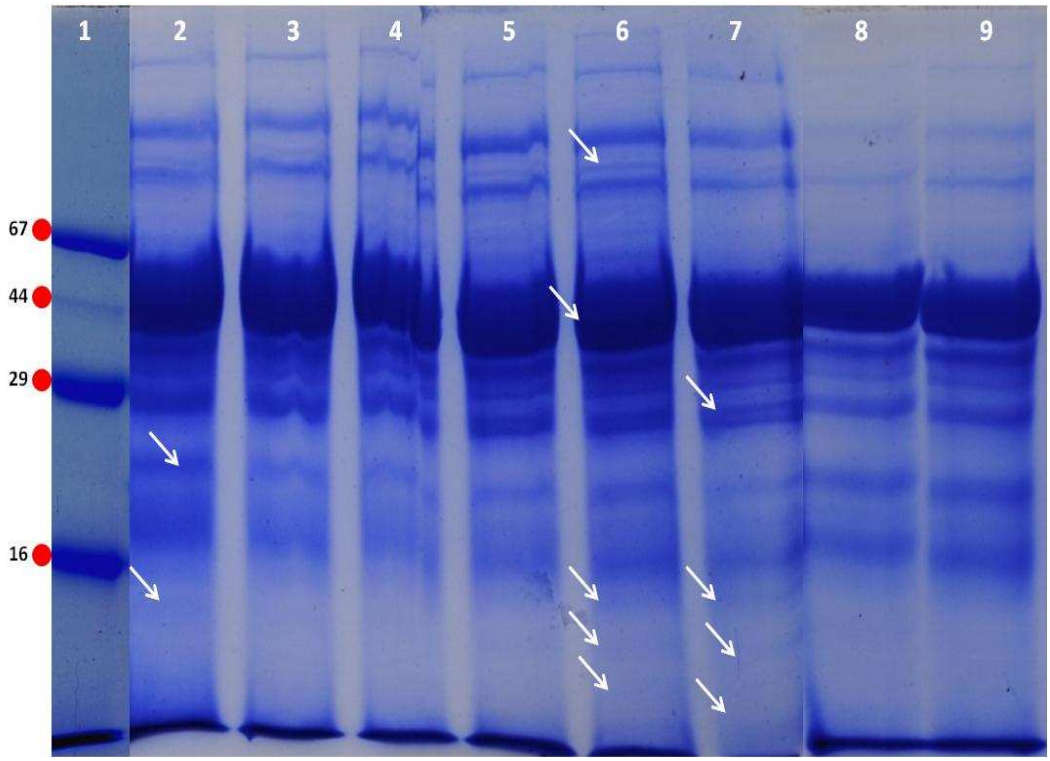

Figure 31. SDS PAGE in the leaves of GY and PBW 343 for drought. 1–Marker, 2 & 6–9d; 3 & 7–6d; 4 & 8–3d; 5 & 9–0d of LY & PBW 343 respectively

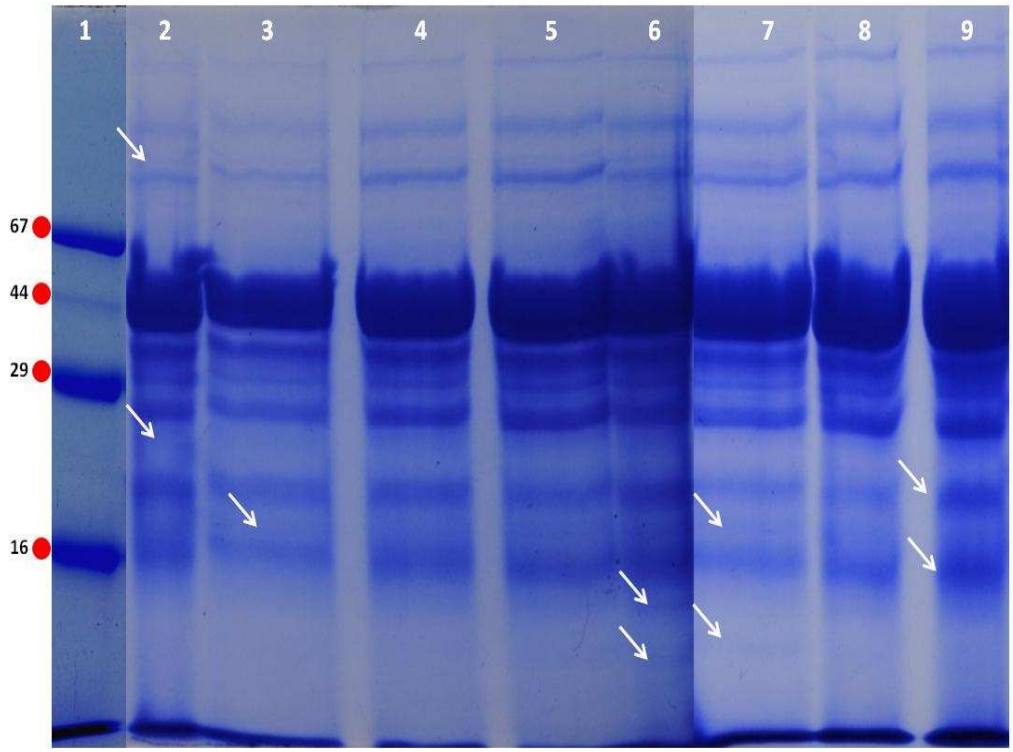

Figure 32. SDS PAGE in the leaves of KD and SO for drought. 1–Marker, 2 & 6–9d; 3 & 7–6d; 4 & 8–3d; 5 & 9–0d of KD & SO respectively

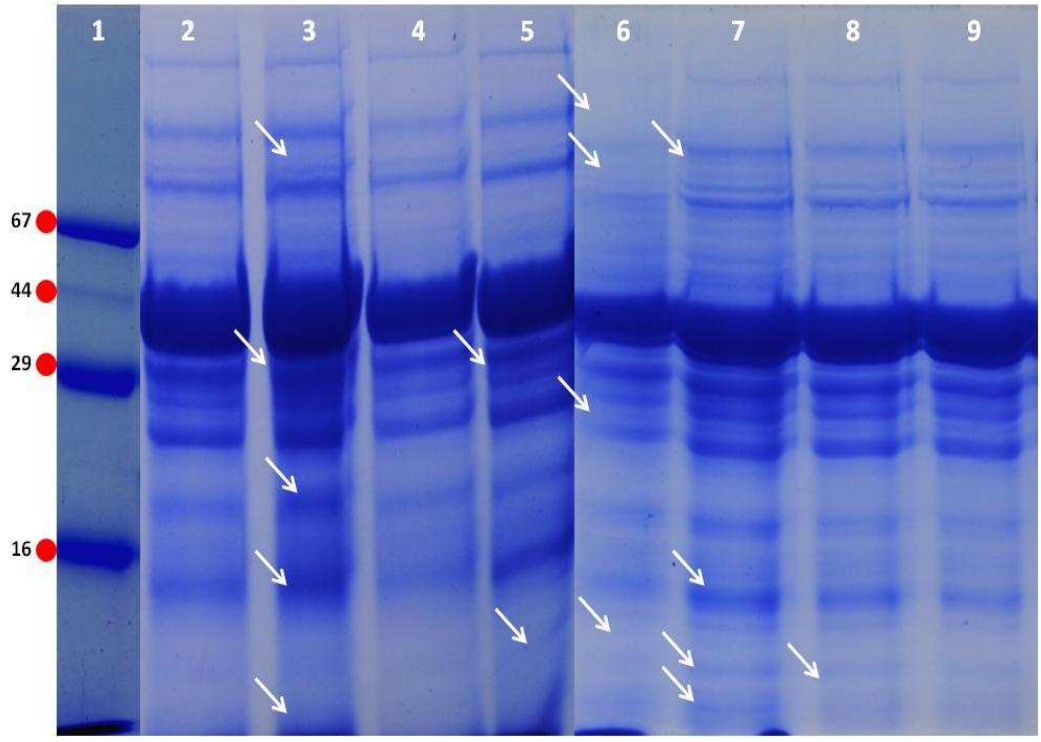

Figure 33. SDS PAGE in the leaves of KW and MW for drought 1–Marker, 2 & 6–9d; 3 & 7–6d; 4 & 8–3d; 5 & 9–0d of KW & MW respectively

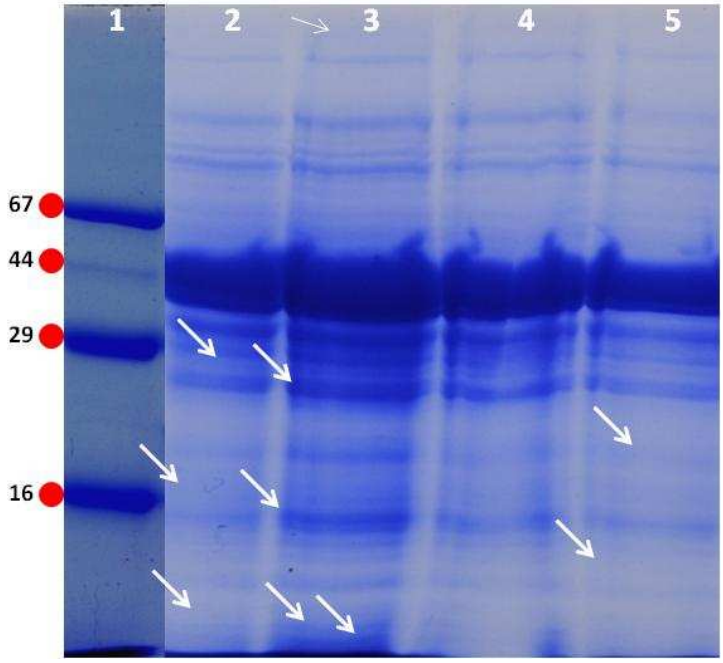

Figure 34. SDS PAGE in the leaves of UP 2752 drought. 1–Marker, 2–9d; 3–6d; 4–3d; 5–0d of UP 2752

Table 52. Analysis of protein in SDS PAGE in Fig 29

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
GN	2	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.0, 15.9, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 95.5, 97.1, 98.1, 116.4, 117.2, 121.3
GY	3	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.0, 15.9, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 49.9*****, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 95.5, 97.1, 98.1, 116.4, 117.2, 121.3
KD	4	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.0, 15.9, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 49.9*****, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 96.0*****, 97.1, 98.1, 116.4, 117.2, 121.3
KW	5	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.0, 15.9, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 49.9*****, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 95.5, 97.1, 98.1, 116.4, 117.2, 121.3
MW	6	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.0, 15.9, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 49.9*****, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 95.5, 97.8*****, 98.1, 116.4, 117.2, 121.3
SO	7	40	2.1, 4.2, 6.1, 7.9*****, 8.8, 10.1, 12.2, 14.4, 15.9, 16.8*****, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 49.9*****, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 94.1, 95.5, 97.8*****, 98.1, 116.4, 117.2, 121.3
PBW 343	8	40	2.1, 4.2, 6.1, 7.3, 8.8, 10.1, 12.2, 14.4, 15.9, 16.8*****, 17.1, 18.2, 19.1, 20.5, 21.1, 24.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 55.2, 58.4, 67.5, 69.9, 70.2, 73.6, 74.1, 78.1, 81.8, 85.4, 90.1, 95.0*****, 95.5, 97.8*****, 98.1, 116.4, 117.2, 121.3

***New bands**

Table 53. Analysis of protein in SDS PAGE in Fig 30

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
LV 0D	2	24	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
LV 3D	3	25	2.1, 7.3*, 7.9**, 10.1**, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
LV 6D	4	25	2.1, 7.3*, 7.9**, 10.1**, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
LV 9D	5	21	2.1, 10.1**, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
GN 0D	6	24	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
GN 3D	7	27	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 17.0*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 99.0*, 116.4, 117.2
GN 6D	8	27	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 17.0*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 99*, 116.4, 117.2
GN 9D	9	25	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 17.0*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2

***New bands**

Table 54. Analysis of protein in SDS PAGE in Fig 31

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
GY 0D	2	22	2.1, 14.4, 16.1, 16.8, 18.2, 20.5, 25.2, 28.8, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
GY 3D	3	20	2.1, 14.4, 16.8, 18.2, 20.5, 25.2, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4, 117.2
GY 6D	4	19	2.1, 14.4, 16.8, 18.2, 20.5, 25.2, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0, 95.5, 98.1, 116.4, 117.2
GY 9D	5	20	2.1, 14.4, 16.8, 18.2, 20.5, 25.2, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4, 117.2
PBW 343 0D	6	24	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
PBW 343 3D	7	23	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 98.1, 116.4, 117.2
PBW 343 6D	8	20	2.1, 14.4*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 85.4, 95.0**, 95.5, 98.1, 116.4, 117.2
PBW 343 9D	9	21	2.1, 14.4*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 98.1, 116.4, 117.2

***New bands**

Table 55. Analysis of protein in SDS PAGE in Fig 32

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
KD 0D	2	21	2.1, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
KD 3D	3	22	2.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 116.4, 117.2
KD 6D	4	24	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 116.4, 117.2
KD 9D	5	24	2.1, 7.3*, 10.1*, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 116.4, 117.2
SO 0D	6	22	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
SO 3D	7	20	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
SO 6D	8	20	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
SO 9D	9	22	2.1, 14.4, 16.1*, 16.8, 18.9*, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2

***New bands**

Table 56. Analysis of protein in SDS PAGE in Fig 33

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
KW 0D	2	21	2.1, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
KW 3D	3	24	2.1, 7.3*, 15.9, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0*****, 95.5, 97.8, 98.1, 116.4, 117.2
KW 6D	4	24	2.1, 7.3*, 15.9, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0*****, 95.5, 97.8, 98.1, 116.4, 117.2
KW 9D	5	25	2.1, 7.3*, 15.9, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 40.2*, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0*****, 95.5, 97.8, 98.1, 116.4, 117.2
MW 0D	6	22	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
MW 3D	7	24	2.1, 14.4, 16.1*, 16.8, 17.5*, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 105.2*, 116.4, 117.2
MW 6D	8	22	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
MW 9D	9	20	2.1, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2

***New bands**

Table 57. Analysis of protein in SDS PAGE in Fig 34

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
UP 2752 0D	2	22	2.1, 7.3, 10.1, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
UP 2752 3D	3	24	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
UP 2752 6D	4	22	2.1, 7.3*, 10.1*, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
UP 2752 9D	5	19	2.1, 16.2*, 15.9, 16.8, 18.2, 20.5, 29.3, 34.5, 43.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2

***New bands**

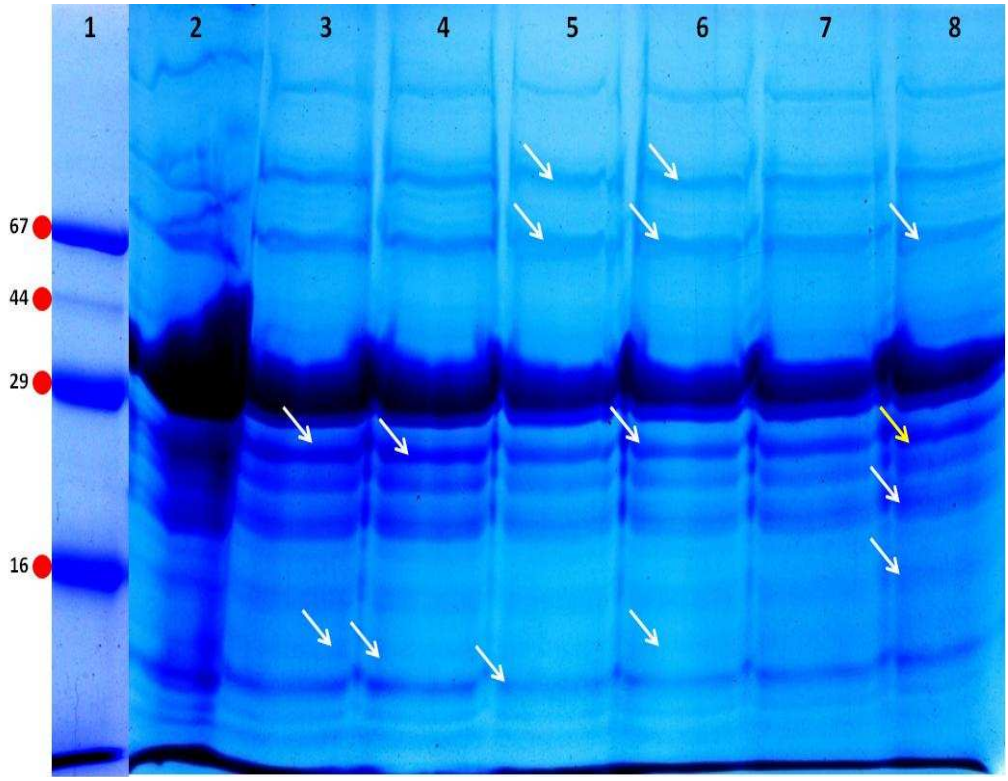

Figure 35. SDS PAGE in the leaves of GN following salt stress. GN: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

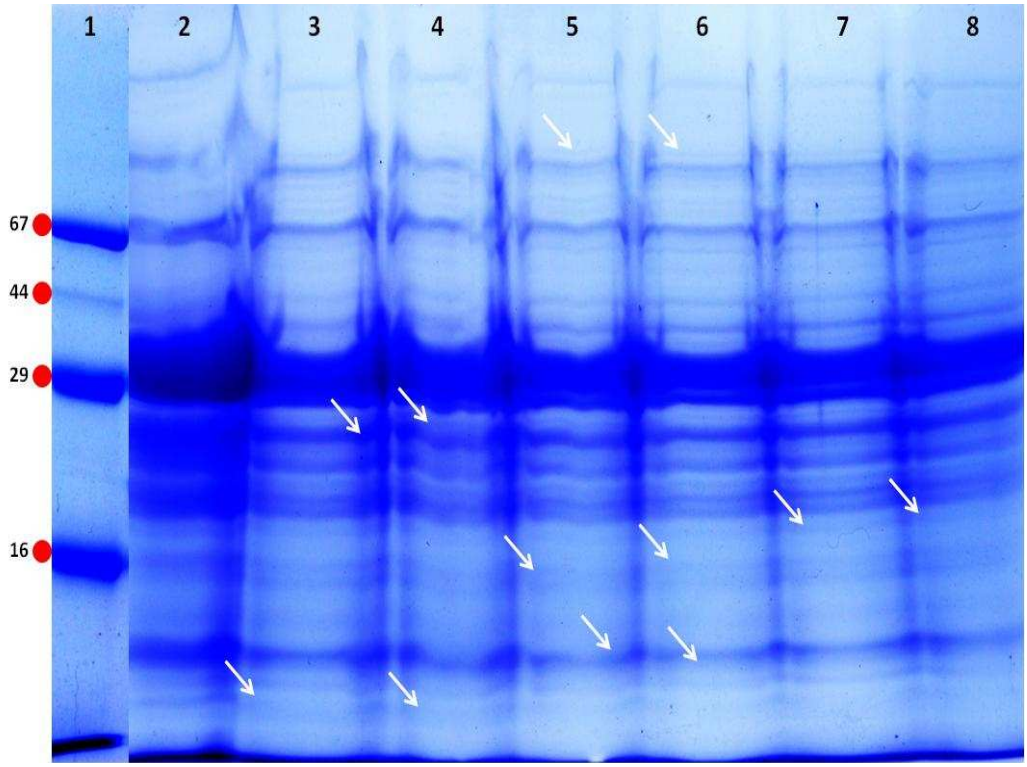

Figure 36. SDS PAGE in the leaves of KD following salt stress. KD: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

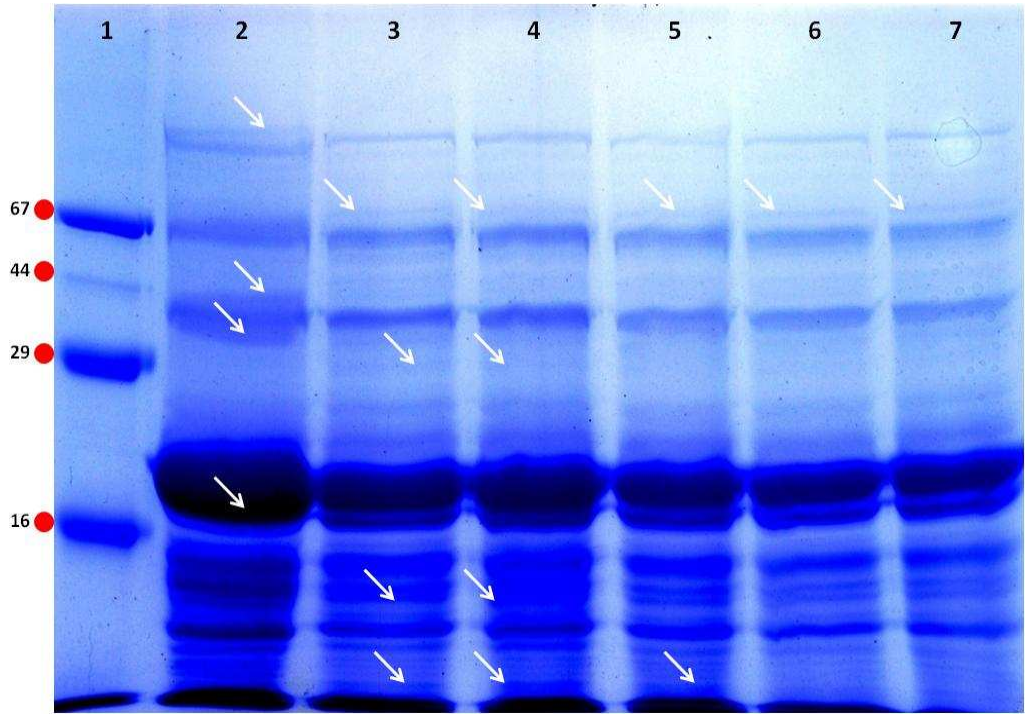

Figure 37. SDS PAGE in the leaves of KW following salt stress. KW: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

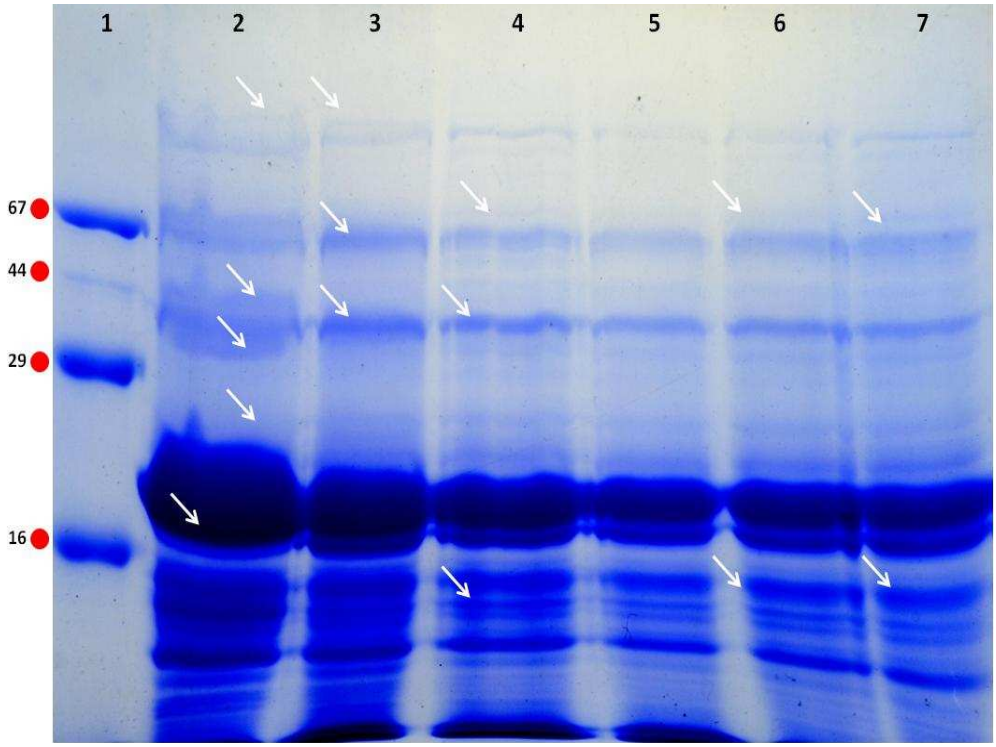

Figure 38. SDS PAGE in the leaves of PBW 343 following salt stress. PBW 343: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

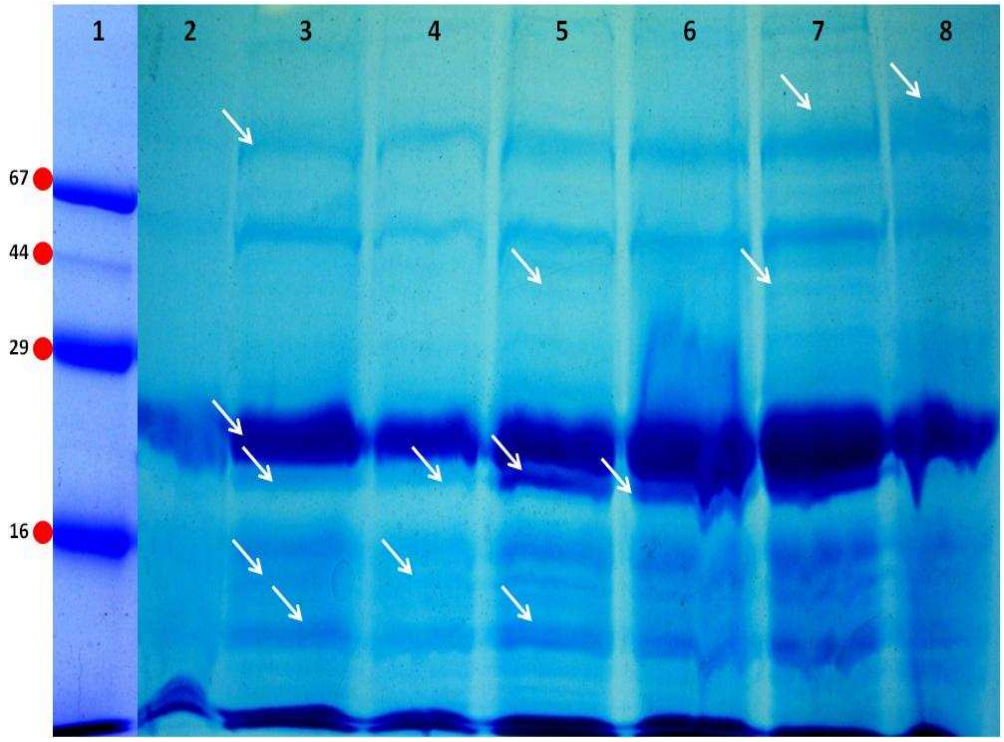

Figure 39. SDS PAGE in the leaves of LV following salt stress. LV: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

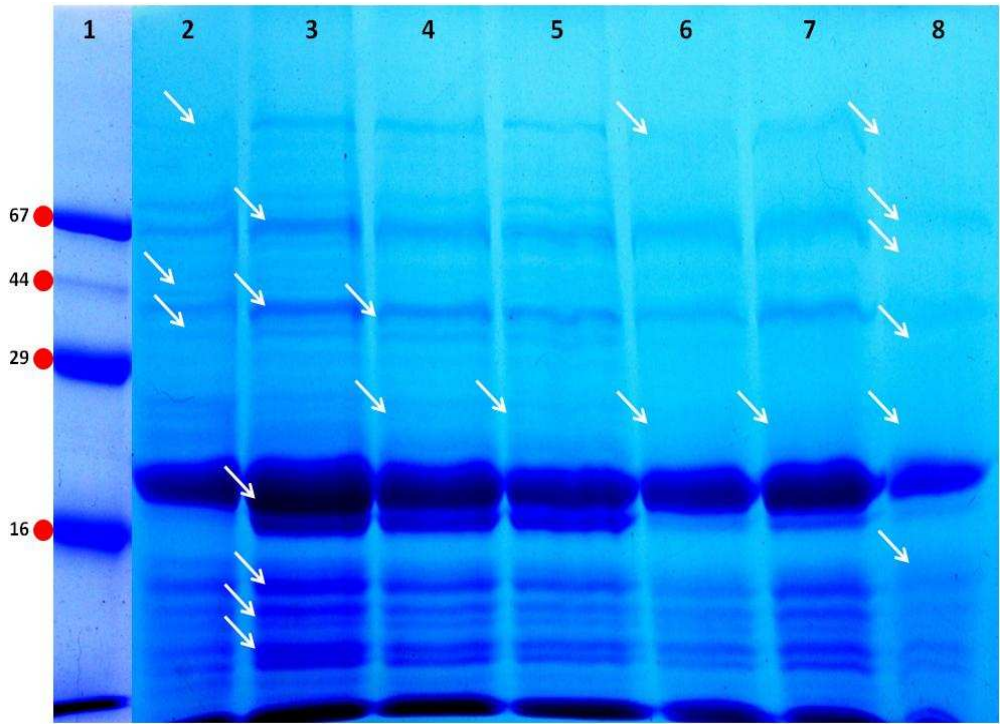

Figure 40. SDS PAGE in the leaves of UP 2752 following salt stress. UP 2752: 1–Marker, 2–0mM, 3 & 4–50mM (1d &3d), 5 & 6–100mM (1d & 3d), 7 & 8–200mM (1d & 3d) respectively

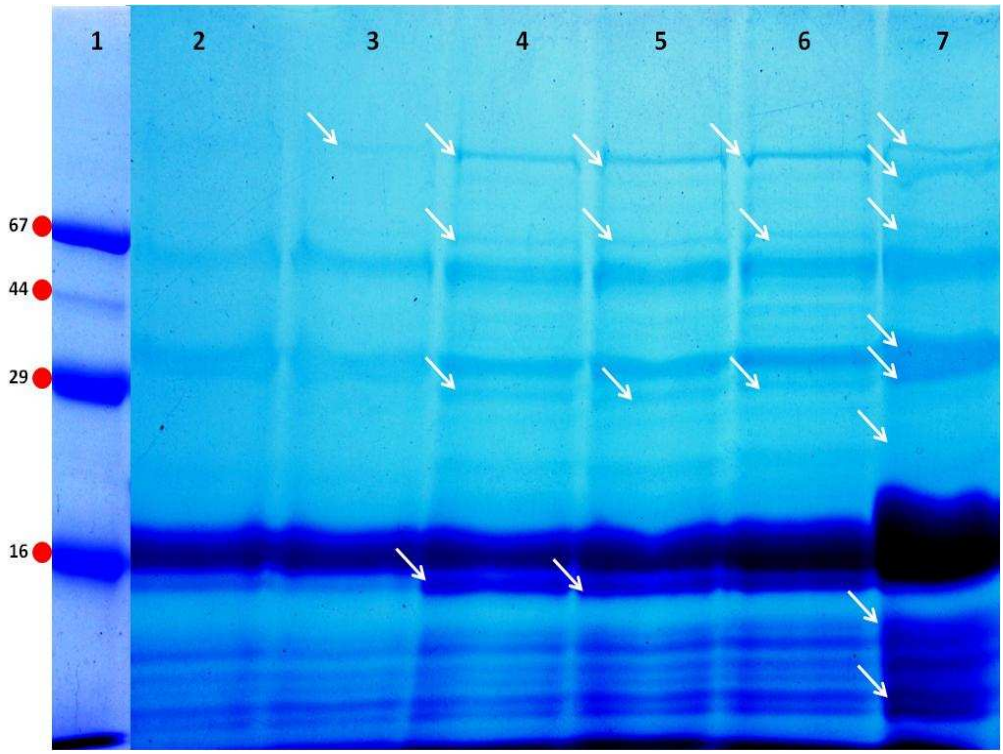

Figure 41. SDS PAGE in the leaves of SO following salt stress. 1–Marker, 2 & 3–50mM (1d &3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

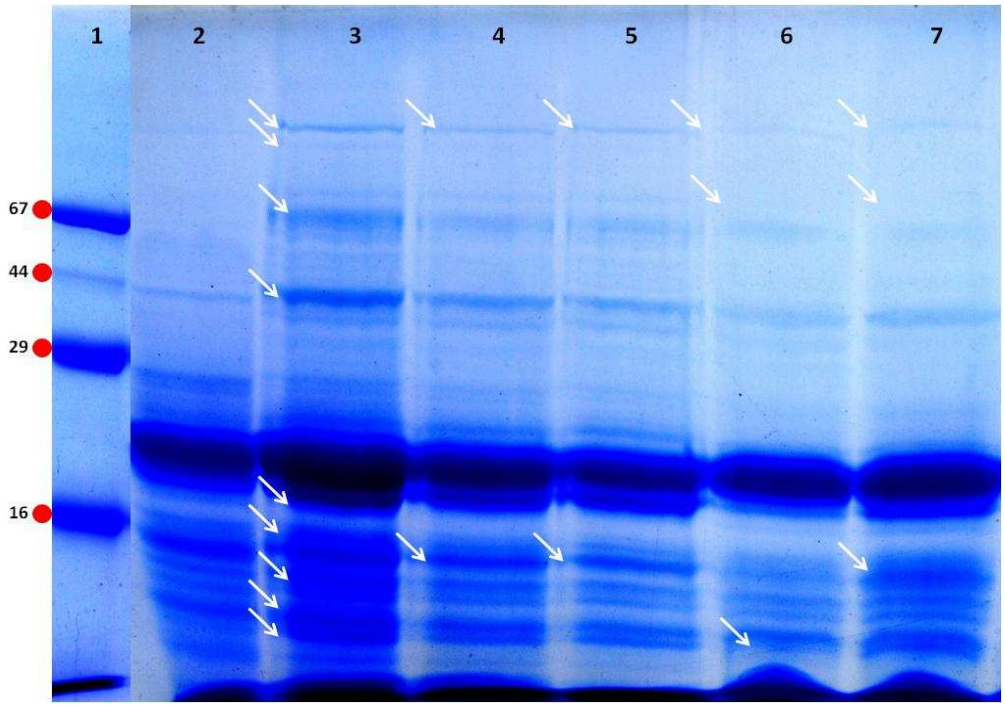

Figure 42. SDS PAGE in the leaves of MW following salt stress. MW: 1–Marker, 2 & 3–50mM (1d &3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

Table 58. Analysis of protein in SDS PAGE in Fig 35

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
GN 0mM	2	24	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
GN 50mM; 1D	3	27	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0, 18.2, 20.5, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8, 98.1, 99.0*, 116.4, 117.2
GN 100mM; 1D	4	28	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0, 18.2, 20.5, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 99.0*, 116.4, 117.2
GN 200mM; 1D	5	28	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0, 18.2, 20.5, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 99.0*, 116.4, 117.2
GN 50mM; 3D	6	28	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0, 18.2, 20.5, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8, 98.1, 99.0*, 116.4, 117.2
GN 100mM; 3D	7	28	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0, 18.2, 20.5, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 99.0*, 116.4, 117.2
GN 200mM; 3D	8	29	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.0,17.5*, 18.2, 20.5, 22.1*, 25.2, 27.6*****, 28.5, 29.3, 34.5, 43.6, 46.6, 49.8*, 58.4, 69.9*, 78.1, 85.4, 95.0*****, 95.5, 97.8*****, 98.1, 99.0*, 116.4, 117.2

***New bands**

Table 59. Analysis of protein in SDS PAGE in Fig 36

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
KD 0mM	2	20	2.1, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 117.2
KD 50mM; 1D	3	23	2.1, 7.3*, 10.1*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2
KD 100mM; 1D	4	24	2.1, 7.3*, 10.1*, 12.3*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2
KD 200mM; 1D	5	24	2.1, 7.3*, 10.1*, 12.3*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2
KD 50mM; 3D	6	21	2.1, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2
KD 100mM; 3D	7	24	2.1, 7.3*, 10.1*, 12.3*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2
KD 200mM; 3D	8	24	2.1, 7.3*, 10.1*, 12.3*, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8, 98.1, 116.4, 117.2

***New bands**

Table 60. Analysis of protein in SDS PAGE in Fig 37

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
KW 50mM; 1D	2	18	2.1, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.0*, 78.1, 85.4, 95.0,.1, 116.4, 117.2
KW 100mM; 1D	3	25	2.1, 7.3*, 7.9*, 8.6*, 15.9, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0**, 95.5, 97.8, 98.1, 117.2
KW 200mM; 1D	4	24	2.1, 7.3*, 7.9*, 8.6*, 15.9, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0**, 95.5, 97.8, 117.2
KW 50mM; 3D	5	16	2.1, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.0*, 78.1, 85.4, 95.0,.1, 116.4, 117.2
KW 100mM; 3D	6	24	2.1, 7.3*, 7.9*, 8.6*, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0**, 95.5, 97.8, 98.1, 117.2
KW 200mM; 3D	7	24	2.1, 7.3*, 7.9*, 8.6*, 16.8, 18.2, 19.2*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 89.9*, 95.0**, 95.5, 97.8, 98.1, 117.2

***New bands**

Table 61. Analysis of protein in SDS PAGE in Fig 38

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
PBW 343 0mM	2	22	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 98.1, 116.4, 117.2
PBW 343 50mM; 1D	3	21	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 58.4, 69.9, 78.1, 85.4, 95.0, 98.1, 116.4, 117.2
PBW 343 100mM; 1D	4	23	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.8*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 85.4, 95.0, 95.5, 98.1, 116.4, 117.2
PBW 343 200mM; 1D	5	21	2.1, 14.4, 15.9, 16.8, 17.8*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 98.1, 116.4
PBW 343 50mM; 3D	6	19	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 58.4, 78.1, 85.4, 95.0, 98.1, 117.2
PBW 343 100mM; 3D	7	23	2.1, 7.3, 10.1, 14.4, 15.9, 16.8, 17.8*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 85.4, 95.0, 95.5, 98.1, 116.4, 117.2
PBW 343 200mM; 3D	8	24	2.1, 7.3, 10.1, 14.4, 15.9, 16.2*, 16.8, 17.8*, 18.2, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 85.4, 95.0, 95.5, 98.1, 116.4, 117.2

***New bands**

Table 62. Analysis of protein in SDS PAGE in Fig 39

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
LV 0mM	2	15	2.1, 7.3, 10.1, 16.8, 18.2, 20.5, 28.5, 29.3, 43.6, 46.6, 69.9, 95.0, 95.5, 97.8, 116.4
LV 50mM; 1D	3	21	2.1, 7.3, 7.9*****, 10.1, 14.4,* 15.9*, 16.8, 18.2, 20.5, 28.5, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4
LV 100mM; 1D	4	23	2.1, 7.3, 7.9*, 10.1, 14.4*, 15.9*, 16.8, 18.2, 20.5, 25.2*, 28.5, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
LV 200mM; 1D	5	23	2.1, 7.3, 7.9*, 10.1, 14.4*, 15.9*, 16.8, 18.2, 20.5, 25.2*, 28.5, 29.3, 34.5*, 43.6*, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2
LV 50mM; 3D	6	21	2.1, 7.3, 7.9*****, 10.1, 14.4,* 15.9*, 16.8, 18.2, 20.5, 28.5, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4
LV 100mM; 3D	7	19	2.1, 7.3, 7.9*, 10.1, 15.9*, 16.8, 18.2, 20.5, 25.2*, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 97.8, 98.1, 116.4, 117.2
LV 200mM; 3D	8	18	2.1, 7.3, 14.4*, 15.9*, 16.8, 18.2, 20.5, 25.2*, 29.3, 34.5*, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4, 117.2

***New bands**

Table 63. Analysis of protein in SDS PAGE in Fig 40

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
UP 2752 0mM	2	22	2.1, 7.3, 10.1, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8, 98.1, 116.4
UP 2752 50mM; 1D	3	24	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 19.6*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 78.1, 85.4, 97.8**, 98.1, 116.4, 117.2
UP 2752 100mM; 1D	4	22	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
UP 2752 200mM; 1D	5	19	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
UP 2752 50mM; 3D	6	19	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 18.2, 19.6*, 20.5, 25.2, 28.5, 29.3, 34.5, 43.6, 78.1, 85.4, 97.8**, 98.1, 116.4
UP 2752 100mM; 3D	7	21	2.1, 7.3*, 10.1*, 14.4*, 15.9, 16.8, 20.5, 28.5, 29.3, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4, 117.2
UP 2752 200mM; 3D	8	21	2.1, 10.1*, 14.4*, 15.9, 16.8, 18.2, 20.5, 28.5, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9*, 78.1, 85.4, 95.0**, 95.5, 97.8**, 98.1, 116.4

***New bands**

Table 64. Analysis of protein in SDS PAGE in Fig 41

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
SO 50mM; 1D	2	21	2.1, 7.3, 10.1, 14.4 15.9, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 97.8
SO 100mM; 1D	3	22	2.1, 7.3, 10.1, 14.4 15.9, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 97.8, 98.1
SO 200mM; 1D	4	22	2.1, 7.3, 10.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4, 117.2
SO 50mM; 3D	5	24	2.1, 7.3, 10.1, 14.4, 16.1*, 16.8, 18.9*, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4, 117.2
SO 100mM; 3D	6	24	2.1, 7.3, 10.1, 14.4, 16.1*, 16.8, 18.9*, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4, 117.2
SO 200mM; 3D	7	22	2.1, 7.3, 10.1, 14.4, 16.1*, 16.8, 18.9*, 19.8*, 25.2, 28.8*, 29.3, 32.2*, 34.5, 43.6, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8**, 98.1, 116.4

***New bands**

Table 65. Analysis of protein in SDS PAGE in Fig 42

Source of protein	Lane No.	No. Of protein bands	Approx. Molecular masses (KDa)
Marker	1	4	16, 29, 44, 67
MW 50mM; 1D	2	14	2.1, 14.4, 16.1, 16.8, 18.2, 20.5, 25.2, 28.8, 29.3, 34.5, 43.6, 46.6, 58.4, 98.1,
MW 100mM; 1D	3	23	2.1, 14.4, 16.1*, 16.8, 17.5, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 105.2*, 116.4
MW 200mM; 1D	4	19	2.1, 14.4, 16.1*, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 95.5, 97.8*****, 98.1, 116.4, 117.2
MW 50mM; 3D	5	20	2.1, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****, 98.1, 116.4, 117.2
MW 100mM; 3D	6	17	2.1, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****
MW 200mM; 3D	7	19	2.1, 14.4, 16.1*, 16.8, 18.2, 20.5, 25.2, 28.8*, 29.3, 34.5, 43.6, 46.6, 58.4, 69.9, 78.1, 85.4, 95.0, 95.5, 97.8*****

***New bands**

4.8.3. Protein profile determined by FPLC

The total soluble protein profile in the leaf of two varieties of wheat (GN and LV) during drought stress was determined in Fast protein liquid chromatography (FPLC) where it was observed that the number, height and the area of peak showed a significant difference during the increasing days of water stress with respect to control plant. It was seen that the change in height and number of peak in case of GN (Figure 43–46) during 0, 3, 6 and 9 days of withholding water was comparatively lower than LV (Figure 47–50), however the total content of protein as determined by the peak height and area was higher in case of GN than LV which was in accordance with the content of total soluble protein in the leaf of GN and LV during water stress (Figure 26).

A significant difference in the number and height of peak in FPLC analysis was observed in case of LV during the 9^{th} day. The main peak was obtained at a retention time of about 44–48 minutes and was most significant and other important peaks which showed significant changes occurred at about 36 to about 40 minutes in each case (Table 66).

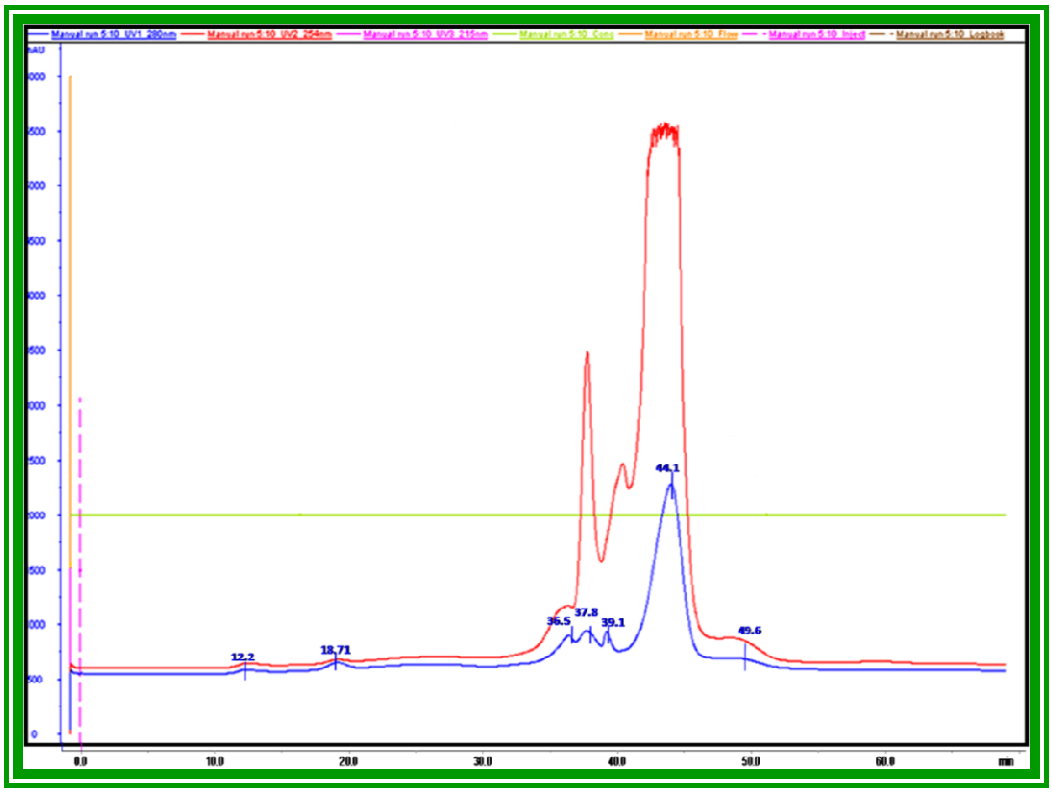

Figure 43. Protein profile in the leaves of wheat (GN) detected in FPLC subjected to drought stress for 0 day (control)

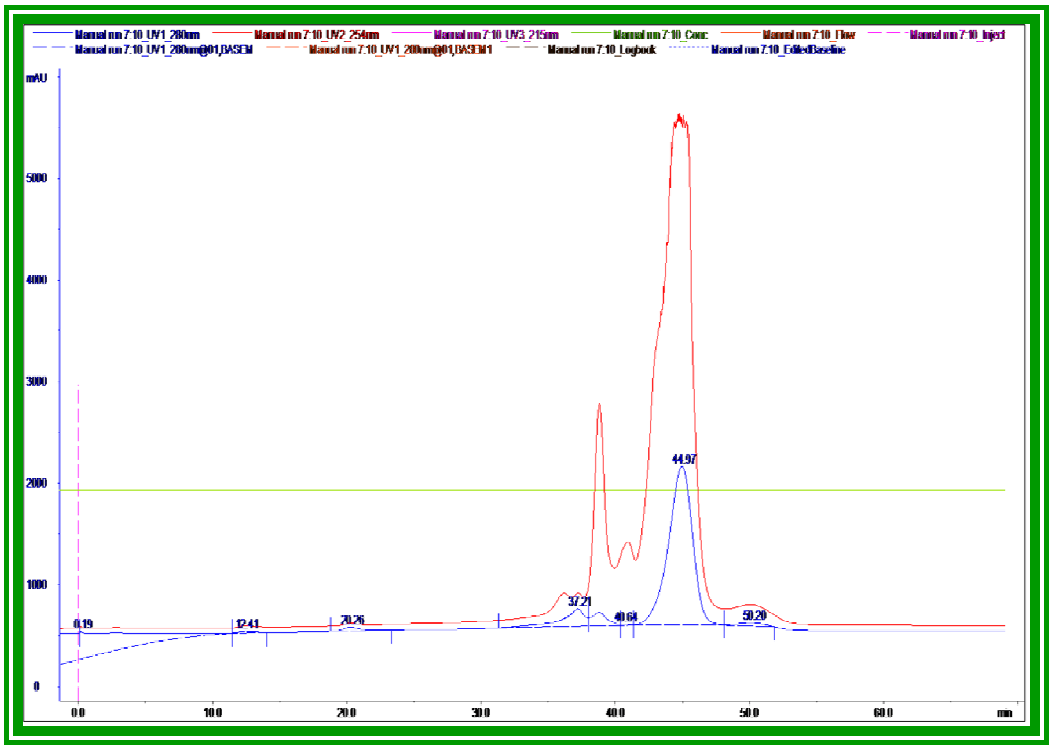

Figure 44. Protein profile in the leaves of wheat (GN) detected in FPLC subjected to drought stress for 3 days

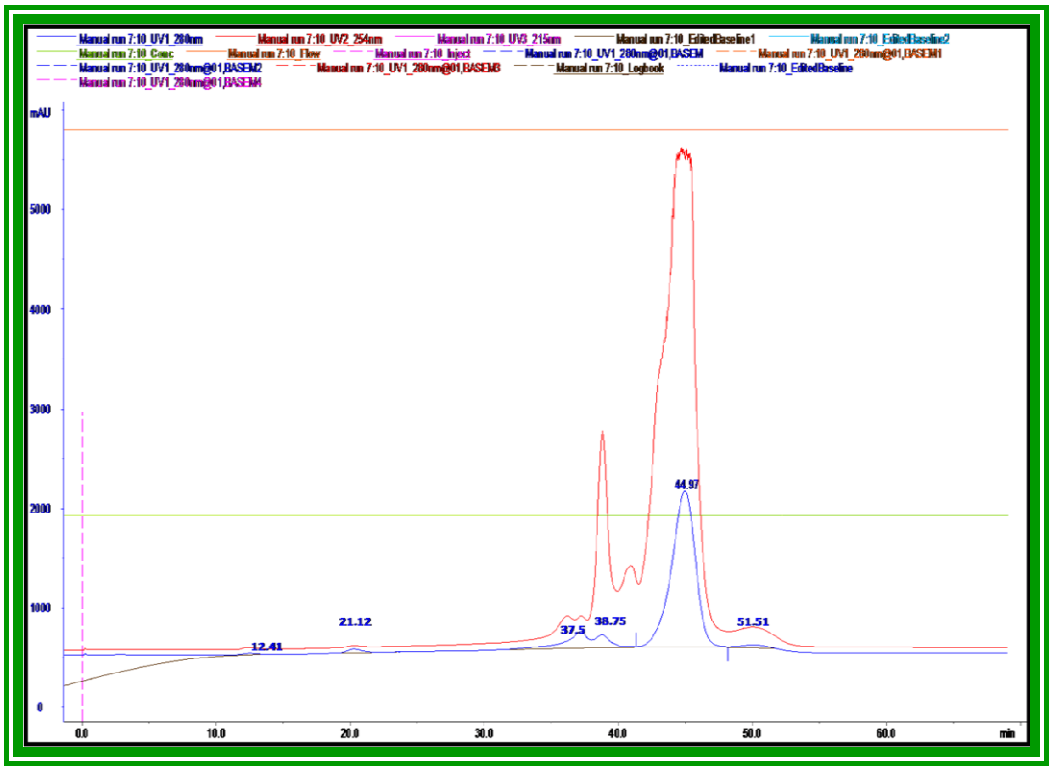

Figure 45. Protein profile in the leaves of wheat (GN) detected in FPLC subjected to drought stress for 6 days

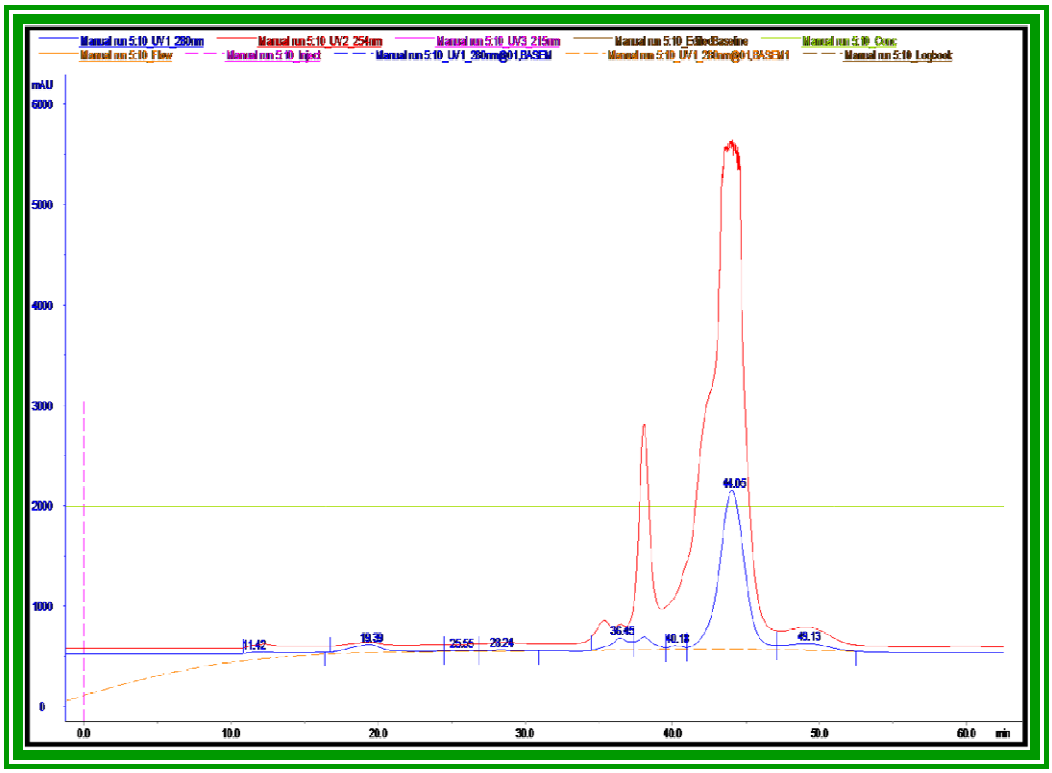

Figure 46. Protein profile in the leaves of wheat (GN) detected in FPLC subjected to drought stress for 9 days

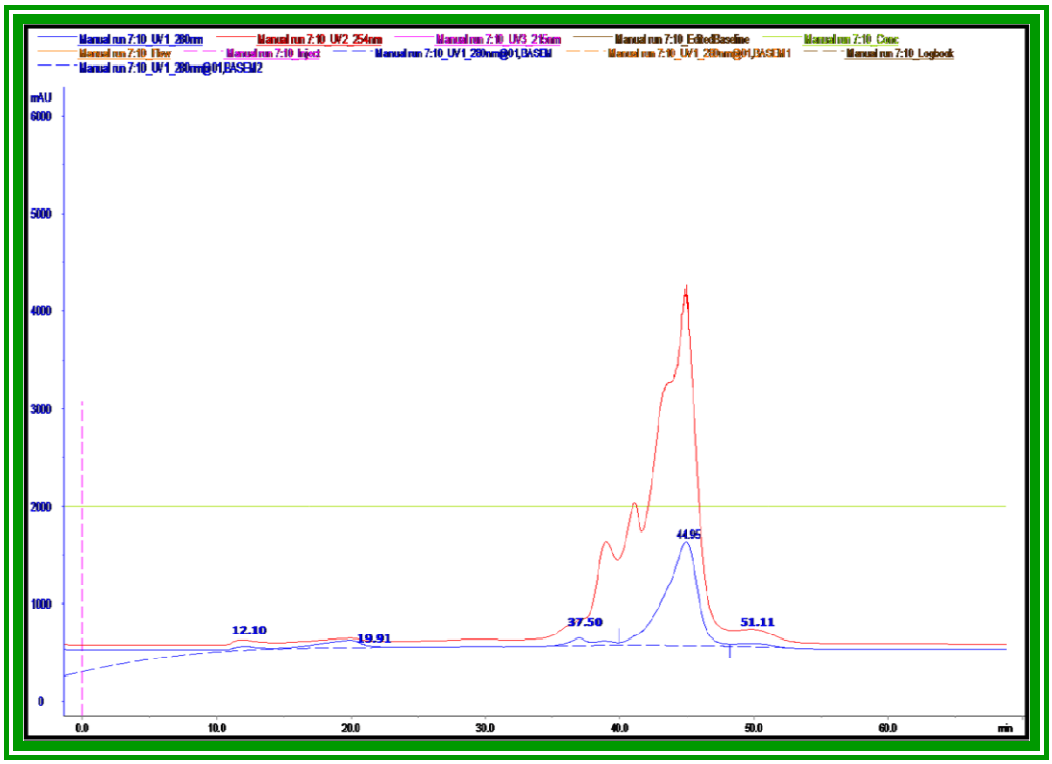

Figure 47. Protein profile in the leaves of wheat (LV) detected in FPLC subjected to drought stress for 0 day (control)

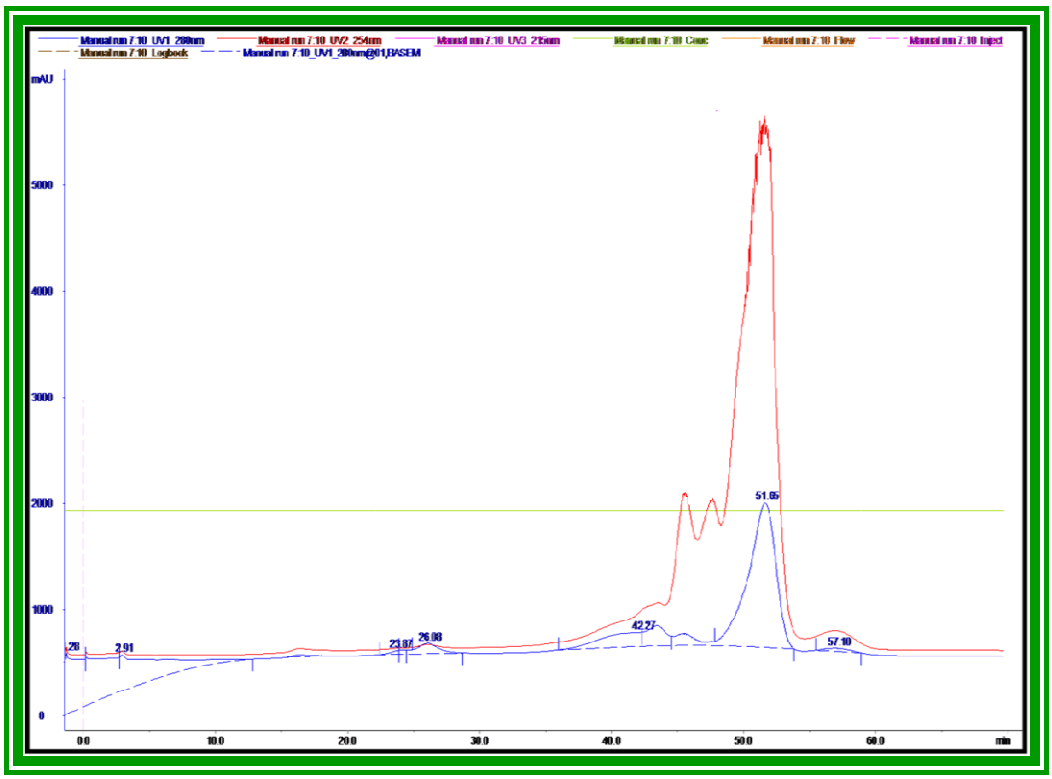

Figure 48. Protein profile in the leaves of wheat (LV) detected in FPLC subjected to drought stress for 3 days

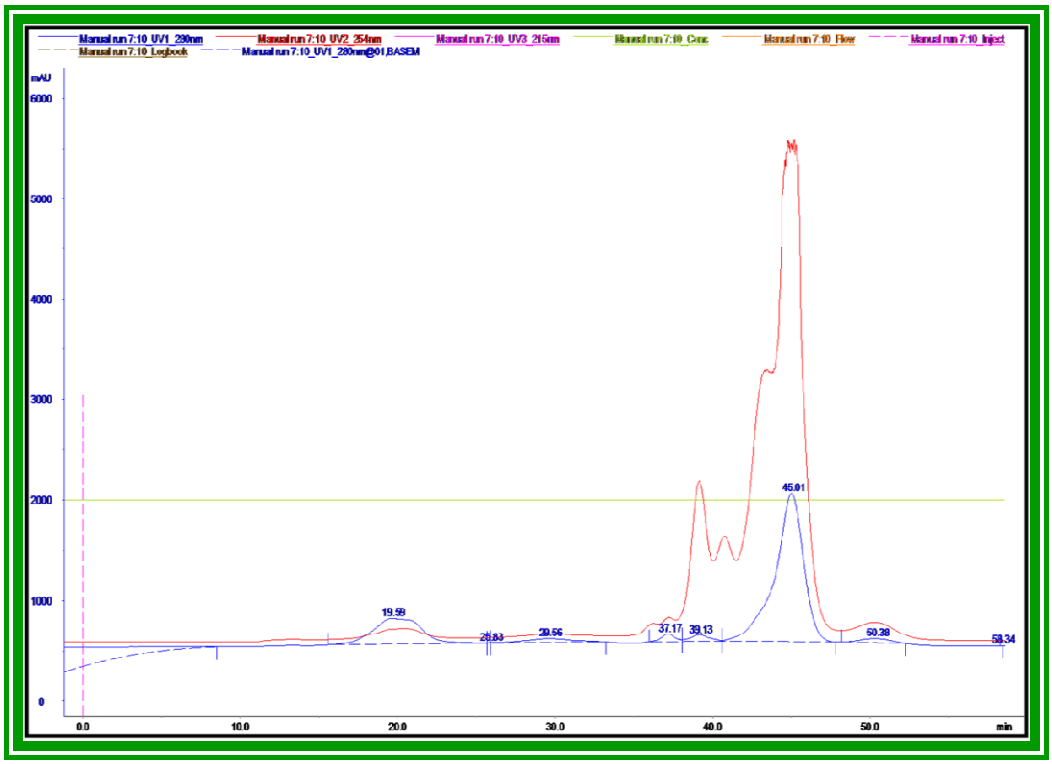

Figure 49. Protein profile in the leaves of wheat (LV) detected in FPLC subjected to drought stress for 6 days

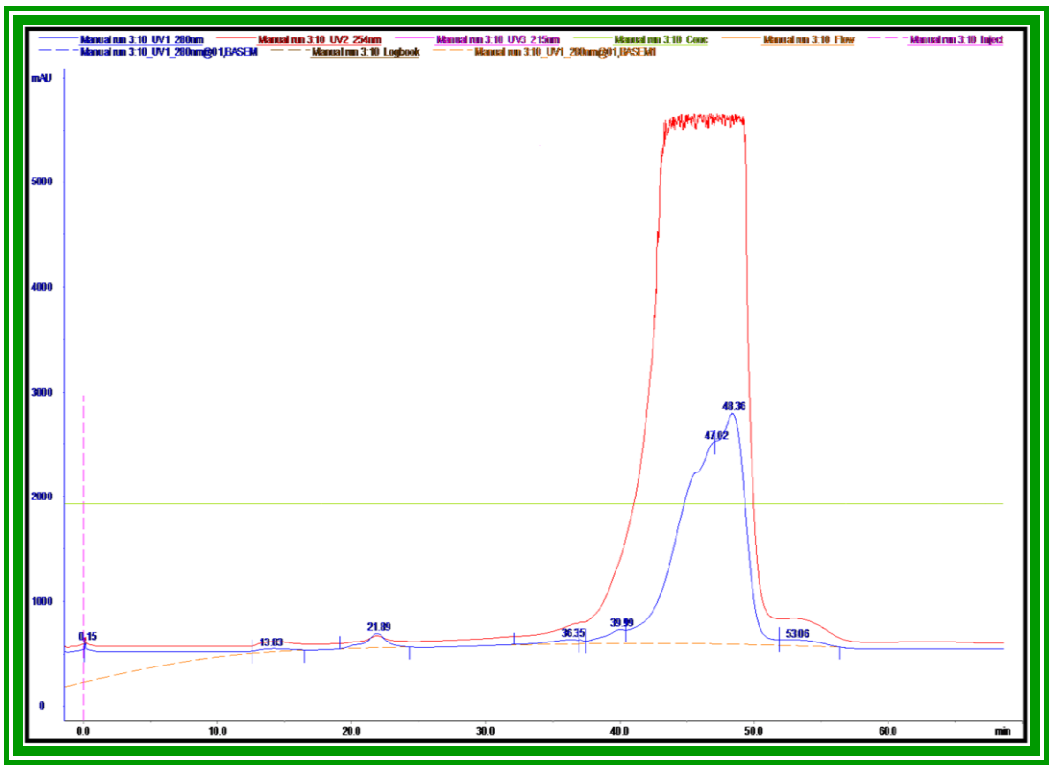

Figure 50. Protein profile in the leaves of wheat (LV) detected in FPLC subjected to drought stress for 9 days

Table 66. Peak table of data presented in Figures 43–50

Source of protein for FPLC	Number of peaks	Retention time
GN 0D	7	12.20, 18.71, 36.5, 37.8, 39.10, 44.10, 49.60
GN 3D	7	0.19, 12.41, 20.26, 37.21, 40.64, 44.97, 50.20
GN 6D	6	12.41, 21.12, 37.50, 38.75, 44.97, 51.51
GN 9D	8	11.42, 19.39, 25.55, 28.24, 36.45, 40.18, 44.05, 49.13
LV 0D	5	12.10, 19.98, 37.50, 44.95, 51.11
LV 3D	6	2.91, 23.87, 26.08, 42.27, 51.65, 57.10
LV 6D	8	19.59, 25.83, 29.56, 37.17, 39.13, 45.01, 50.38, 58.34
LV 9D	8	0.15, 13.83, 21.89, 36.35, 39.99, 47.02, 48.36, 53.06

4.9. Changes in anti–oxidative enzymes of wheat following osmotic stress

Activity of antioxidative enzymes like POX, CAT, APOX, GR and SOD following osmotic stress i.e. water stress and salinity stress showed a difference in their activities during different stages of water stress and salinity stress. The activity of antioxidative enzymes showed a change either by a declined in their activity or by enhancement in their activity during different days of withholding water in case of water stress and different concentration of salt for 0, 1, and three days. The activity of all the antioxidative enzymes seemed to be correlated with the each other during the stress response of the plant.

4.9.1. Enzyme activities during drought

In this study, activities of ascorbate peroxidase (APOX) and glutathione reductase (GR) (Table 67) increased significantly in all nine varieties initially after 3 days of drought stress. With prolonged water stress, the activities of ascorbate peroxidase decreased in all varieties and the activity of glutathione reductase decreased in varieties MW, GY, LV and SO whereas its activity continued to increase in case of KD, GN, KW, UP 2752 and PBW 343. It was noted that the activity of peroxidase (POX) (Table 67) enhanced greatly with increase in the period of water stress in case of GN, KD, KW, UP 2752 and PBW 343 whereas in LV, GY, MW and SO, the activity of POX declined.

Table 67. Activities of antioxidative enzymes in water stressed wheat varieties

Varieties	Days of stress	CAT	POX	APOX	GR
MW	0d	1.67±0.07[a]	0.020±0.006[a]	0.116±0.007[a]	0.14±0.02[a]
	3d	1.43±0.02[b]	0.029±0.013[a]	0.730±0.010[b]	0.40±0.03[b]
	6d	1.19±0.01[b]	0.038±0.015[b]	0.263±0.006[b]	0.33±0.01[b]
	9d	1.09±0.02[b]	0.026±0.009[a]	0.168±0.004[a]	0.23±0.01 [b]
GY	0d	1.77±0.02[a]	0.023±0.005[a]	0.089±0.005[a]	0.18±0.03[a]
	3d	1.46±0.04[b]	0.048±0.007[a]	1.017±0.003[b]	0.22±0.01[a]
	6d	1.41±0.02[b]	0.051±0.006[b]	0.385±0.004[b]	0.15±0.01[a]
	9d	1.27±0.01[b]	0.044±0.008[a]	0.244±0.006[b]	0.11±0.01[a]
KD	0d	1.77±0.03[a]	0.045±0.007[a]	0.189±0.011[a]	0.13±0.01[a]
	3d	2.76±0.06[b]	0.062±0.006[a]	0.633±0.005[b]	0.28±0.02[b]
	6d	1.25±0.03[b]	0.095±0.007[a]	0.067±0.009[a]	0.76±0.04[b]
	9d	1.13±0.02[b]	0.193±0.007[a]	0.044±0.004[b]	1.53±0.08 [b]
GN	0d	1.18±0.01[a]	0.035±0.010[a]	0.065±0.004[a]	0.17±0.01[a]
	3d	2.19±0.03[b]	0.042±0.011[a]	0.166±0.003[a]	0.32±0.02[b]
	6d	1.73±0.02[b]	0.072±0.009[b]	0.107±0.004[a]	0.51±0.03[b]
	9d	1.50±0.04[b]	0.126±0.004[a]	0.068±0.008[a]	0.97±0.04[b]
KW	0d	1.61±0.03[a]	0.055±0.012[a]	0.120±0.009[a]	0.13±0.05[a]
	3d	2.55±0.05[b]	0.071±0.008[a]	0.211±0.011[b]	0.34±0.02[b]
	6d	1.45±0.02[b]	0.145±0.007[b]	0.062±0.005[b]	0.77±0.02[b]
	9d	1.10±0.03[b]	0.199±0.009[b]	0.049±0.006[b]	1.53±0.04[b]
LV	0d	1.34±0.02[a]	0.019±0.011[a]	0.084±0.004[a]	0.11±0.02[a]
	3d	1.24±0.04[a]	0.030±0.007[a]	1.000±0.013[b]	0.22±0.04[b]
	6d	0.66±0.05[b]	0.029±0.009[b]	0.301±0.002[b]	0.19±0.05[b]
	9d	0.50±0.03[b]	0.023±0.014[a]	0.204±0.003[b]	0.10±0.03[a]
UP 2752	0d	1.53±0.03[a]	0.049±0.018[a]	0.098±0.005[a]	0.14±0.04[a]
	3d	2.44±0.06[b]	0.064±0.009[a]	0.605±0.004[b]	0.31±0.02[b]
	6d	1.48±0.05[a]	0.089±0.011[b]	0.090±0.007[a]	0.57±0.04[b]
	9d	0.99±0.04[b]	0.188±0.019[b]	0.050±0.001 [b]	1.30±0.06[b]
PBW 343	0d	1.48±0.03[a]	0.043±0.012[a]	0.080±0.007[a]	0.16±0.05[a]
	3d	2.21±0.07[a]	0.059±0.009[a]	0.532±0.005[b]	0.33±0.03[b]
	6d	1.26±0.01[b]	0.066±0.008[b]	0.120±0.001[b]	0.69±0.04[b]
	9d	0.90±0.06[b]	0.096±0.010[b]	0.060±0.003[a]	1.49±0.07[b]
SO	0d	1.51±0.06[a]	0.046±0.013[a]	0.085±0.002[a]	0.14±0.01[a]
	3d	1.39±0.03[a]	0.048±0.011[a]	0.890±0.007[b]	0.28±0.03[b]
	6d	1.11±0.05[b]	0.079±0.011[b]	0.310±0.006[b]	0.24±0.02[b]
	9d	0.75±0.02[b]	0.029±0.009[a]	0.232±0.005[b]	0.21±0.02[b]
CD Values in treatments =		0.29731	0.031118	0.136652	0.299057
CD Values in varieties =		0.445965	0.046677	0.204979	0.448586

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). CAT– Catalase (EU mg protein^{-1} min^{-1}); POX– Peroxidase (mmol o–dianisidine mg protein^{-1} min^{-1}); APOX Ascorbate peroxidase (mmol ascorbate mg protein^{-1} min^{-1}); GR– Glutathione reductase (μmol NADPH oxidized mg protein^{-1} min^{-1}).

In case of catalase (Table 67) and superoxide dismutase (Figure 51), activities decreased at all periods of drought stress in case of MW, GY, LV and SO whereas in KD, GN, KW, UP 2752 and PBW 343 activities of these enzymes increased initially before showing a continued decline. Activity of SOD showed an initial increase in case of KD, GN, KW, UP 2752 and PBW 343 however, with increase in periods of stress, the activities of superoxide dismutase decreased in these varieties.

Table 67 (a). ANOVA of data presented in table 67 for CAT

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	4.324486	3	1.441495	15.43684	8.34E–06	3.008787
Columns	2.144657	8	0.268082	2.870867	0.021581	2.355081
Error	2.241126	24	0.09338			
Total	8.710269	35				

Table 67 (b). ANOVA of data presented in table 67 for APOX

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.74907	3	0.583023	29.55392	3.15E–08	3.008787
Columns	0.484331	8	0.060541	3.068891	0.015771	2.355081
Error	0.473459	24	0.019727			
Total	2.706859	35				

Table 67 (c). ANOVA of data presented in table 67 for POX

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.022374	3	0.007458	7.290829	0.001218	3.008787
Columns	0.034532	8	0.004316	4.219647	0.002844	2.355081
Error	0.024551	24	0.001023			
Total	0.081457	35				

Table 67 (d). ANOVA of data presented in table 67 for GR

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2.333479	3	0.777826	8.23263	0.00061	3.008787
Columns	1.707472	8	0.213434	2.259018	0.058654	2.355081
Error	2.267541	24	0.094481			
Total	6.308493	35				

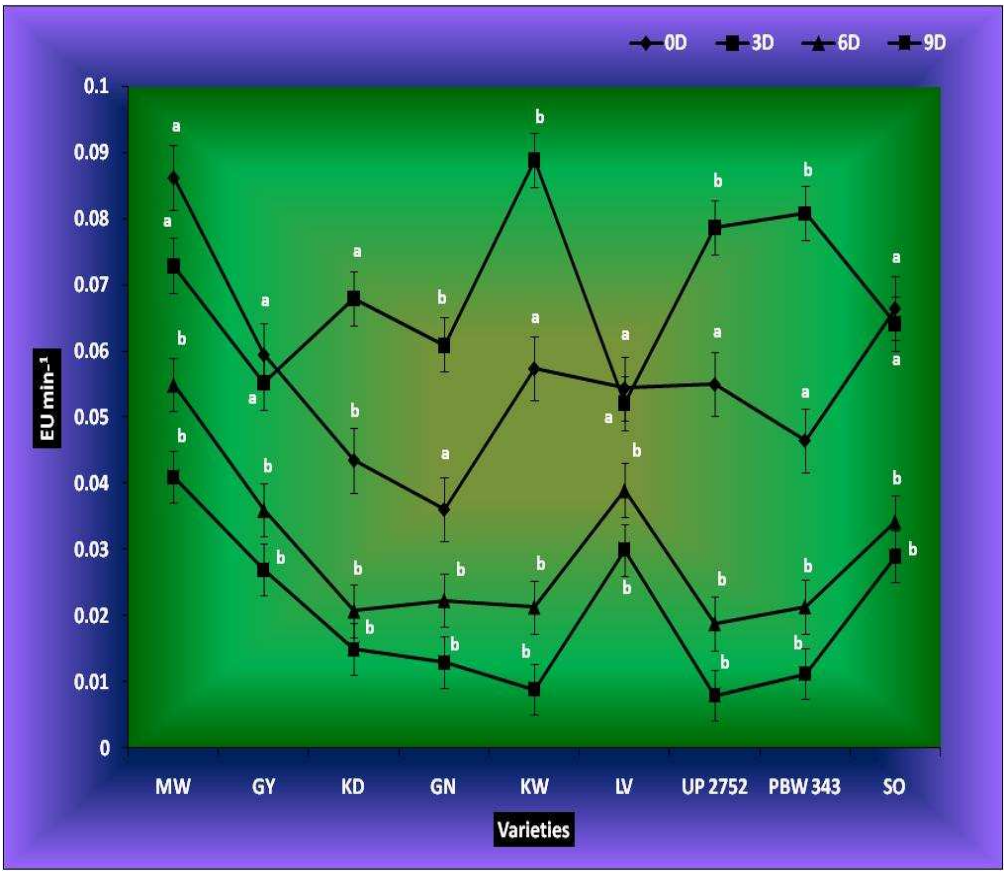

Figure 51. Superoxide dismutase activities in nine varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

4.9.2. Enzyme activities during salinity

Activity of peroxidase (POX) (Table 68) showed a continuous enhancement with increase in the concentration of salt in all varieties during the 1st and 3rd day of salt stress with respect to the control however in case of MW, LV, SO and GY the activity of peroxidase with prolonged stress showed a significant decline in the later period of stress and higher concentration of salt. In all other cases, the activity of peroxidase increased even during the highest concentration of salt in our study and during the 3rd day of salt stress.

The activity of catalase (CAT) (Table 68) following salinity stress showed a continued decline in case of MW, GY, LV and SO with the increase in the concentration and duration of salt stress however in case of KW, KD, GN, UP 2752 and PBW 343 there was an initial enhancement in the activity of CAT followed by decline in its activity with increasing concentration of salt; the activity of CAT increased slightly during 3rd day of 50mM salt stress than the 1st day of salt stress at the same salt concentration. The activity of ascorbate peroxidase (APOX) (Table 69) after a significant initial enhancement showed a general decrease with increasing concentration of salt and duration of salt stress in all the varieties.

Glutathione reductase (GR) (Table 69) activity declined after a significant initial increase in all the varieties with increase in the concentration and duration of salt stress however, in case of KD, GN, KW, UP 2752 and PBW 343 the activity of GR continued to increase with the increase in the concentration of salt and the duration of salt stress. In case of superoxide dismutase (SOD) (Figure 52), activities decreased at all periods and concentration of salt stress in case of MW, GY and whereas in KD, GN, KW, UP 2752, PBW 343 and SO activities of these enzymes increased initially before showing a continued decline. The response of antioxidative enzymes in the wheat varieties was slightly higher during the salt stress than during drought stress.

Table 68. Activities of antioxidative enzymes in control and salt stressed wheat varieties

Varieties	Treatment (mM)	CAT		POX	
		1D	3D	1D	3D
MW	0	1.67±0.05	1.67±0.001[a]	0.020±0.005[a]	0.021±0.01[a]
	50	1.51±0.01	1.42±0.011[a]	0.022±0.011[b]	0.023±0.02[b]
	100	1.41±0.02[b]	1.37±0.009[b]	0.035±0.004[b]	0.036±0.02[b]
	200	1.01±0.01[b]	0.98±0.010[a]	0.321±0.003[b]	0.028±0.02[b]
GY	0	1.77±0.01[a]	1.74±0.004[a]	0.023±0.004[a]	0.022±0.04[a]
	50	1.71±0.03[a]	1.54±0.003[b]	0.024±0.002[a]	0.025±0.02[a]
	100	1.38±0.05[b]	1.33±0.007[b]	0.034±0.001[b]	0.037±0.03[b]
	200	1.14±0.03[b]	0.98±0.011[b]	0.029±0.005[b]	0.026±0.02[a]
KD	0	1.77±0.06[a]	1.76±0.010[a]	0.045±0.008[a]	0.046±0.01[a]
	50	1.81±0.04[b]	1.82±0.007[b]	0.075±0.001[b]	0.084±0.03[b]
	100	1.83±0.08[b]	1.80±0.009[b]	0.110±0.003[b]	0.132±0.05[b]
	200	1.66±0.07[b]	1.61±0.009[b]	0.178±0.002[b]	0.190±0.06 [b]
GN	0	1.18±0.02[a]	1.19±0.010[a]	0.035±0.001[a]	0.036±0.02[a]
	50	1.45±0.04[b]	1.47±0.012[b]	0.088±0.006[b]	0.090±0.01[b]
	100	1.47±0.01[b]	1.38±0.010[b]	0.099±0.005[b]	0.110±0.01[b]
	200	1.27±0.02[b]	1.08±0.008[a]	0.165±0.004[b]	0.171±0.05[b]
KW	0	1.61±0.02[a]	1.60±0.011[a]	0.055±0.010[a]	0.055±0.08[a]
	50	1.80±0.06[b]	1.82±0.007[b]	0.078±0.010[b]	0.085±0.07[b]
	100	1.59±0.01[a]	1.55±0.011[a]	0.096±0.006[b]	0.099±0.05[b]
	200	1.43±0.02[b]	1.29±0.010[b]	0.173±0.007[b]	0.180±0.03[b]
LV	0	1.34±0.01[a]	1.34±0.008[a]	0.019±0.005[a]	0.020±0.01[a]
	50	1.29±0.06[b]	1.27±0.009[a]	0.031±0.011[b]	0.031±0.03[b]
	100	1.01±0.04[b]	0.98±0.010[b]	0.027±0.008[b]	0.025±0.02[b]
	200	0.61±0.02[b]	0.53±0.011[b]	0.020±0.005[b]	0.015±0.01[b]
UP 2752	0	1.53±0.01[a]	1.51±0.011[a]	0.049±0.001[a]	0.048±0.05[a]
	50	1.71±0.02[b]	1.75±0.010[a]	0.058±0.002[b]	0.060±0.02[b]
	100	1.82±0.03[a]	1.78±0.008[b]	0.081±0.003[b]	0.084±0.01[b]
	200	1.21±0.08[b]	1.01±0.012[b]	0.120±0.002[b]	0.132±0.03[b]
PBW 343	0	1.48±0.04[a]	1.48±0.008[a]	0.043±0.003[a]	0.044±0.01[a]
	50	1.59±0.05[a]	1.62±0.007[b]	0.049±0.004[b]	0.052±0.01[b]
	100	1.49±0.06[b]	1.38±0.006[b]	0.066±0.002[b]	0.068±0.02[b]
	200	1.01±0.01[b]	0.98±0.003[b]	0.079±0.001[b]	0.834±0.08[b]
SO	0	1.51±0.02[a]	1.52±0.008[a]	0.046±0.003[a]	0.046±0.09[a]
	50	1.49±0.01[a]	1.44±0.005[b]	0.047±0.006[a]	0.049±0.02[b]
	100	1.29±0.04[b]	1.23±0.010[b]	0.025±0.005[b]	0.025±0.01[b]
	200	1.03±0.05[b]	0.87±0.008[b]	0.017±0.004[b]	0.015±0.03[b]
CD Values between treatments =		0.138052	0.135743	0.026244	0.028692
CD Values between varieties =		0.207079	0.203614	0.039366	0.043038

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Catalase (EU mg protein^{-1} min^{-1}); POX– Peroxidase (mmol o–dianisidine mg protein^{-1} min^{-1})

Table 68 (a). ANOVA of data presented in table 68 for CAT during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.065992	3	0.355331	17.64856	2.88E–06	3.008787
Columns	1.283933	8	0.160492	7.971296	3.33E–05	2.355081
Error	0.483209	24	0.020134			
Total	2.833133	35				

Table 68 (b). ANOVA of data presented in table 68 for CAT during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.619017	3	0.539672	27.72426	5.71E–08	3.008787
Columns	1.320999	8	0.165125	8.48286	2.01E–05	2.355081
Error	0.467177	24	0.019466			
Total	3.407192	35				

Table 68 (c). ANOVA of data presented in table 68 for POX during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.013593	3	0.004531	6.227228	0.00279	3.008787
Columns	0.036546	8	0.004568	6.278363	0.000206	2.355081
Error	0.017463	24	0.000728			
Total	0.067601	35				

Table 68 (d). ANOVA of data presented in table 68 for POX during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.014937	3	0.004979	5.725121	0.004208	3.008787
Columns	0.044325	8	0.005541	6.370985	0.000185	2.355081
Error	0.020872	24	0.00087			
Total	0.080134	35				

Table 69. Activities of antioxidative enzymes in salt stressed wheat varieties

Varieties	Treatment (mM)	APOX		GR	
		1D	3D	1D	3D
MW	0	0.11±0.05[a]	0.12±0.01[a]	0.140±0.004[a]	0.14±0.01[a]
	50	0.68±0.01[b]	0.71±0.04[b]	0.400±0.011[b]	0.45±0.02[b]
	100	0.28±0.02[b]	0.26±0.05[b]	0.294±0.008[b]	0.28±0.03[b]
	200	0.15±0.01[b]	0.14±0.05[b]	0.195±0.002[b]	0.18±0.02[b]
GY	0	0.09±0.01[a]	0.09±0.02[a]	0.180±0.001[a]	0.18±0.01[a]
	50	0.29±0.03[b]	0.85±0.03[b]	0.387±0.002[b]	0.39±0.02[b]
	100	0.09±0.01[a]	0.39±0.02[b]	0.278±0.001[b]	0.26±0.02[b]
	200	0.05±0.02[b]	0.22±0.01[b]	0.174±0.005[b]	0.16±0.03[a]
KD	0	0.19±0.05[a]	0.19±0.06[a]	0.129±0.008[a]	0.13±0.06[a]
	50	0.29±0.04[b]	0.29±0.04[b]	0.510±0.001[b]	0.54±0.01[b]
	100	0.09±0.08[b]	0.08±0.02[b]	0.674±0.010[b]	0.68±0.03[b]
	200	0.05±0.06[b]	0.04±0.03[b]	1.100±0.002[b]	1.47±0.07[b]
GN	0	0.06±0.02[a]	0.06±0.01[a]	0.170±0.001[a]	0.16±0.02[a]
	50	0.11±0.05[b]	0.13±0.04[b]	0.530±0.002[b]	0.54±0.01[b]
	100	0.08±0.01[b]	0.09±0.02[b]	0.710±0.005[b]	0.77±0.06[b]
	200	0.05±0.06[a]	0.04±0.01[b]	0.974±0.004[b]	1.22±0.05[b]
KW	0	0.12±0.02[a]	0.11±0.02[a]	0.130±0.001[a]	0.13±0.08[a]
	50	0.14±0.06[b]	0.18±0.03[b]	0.500±0.003[b]	0.53±0.06[b]
	100	0.08±0.03[b]	0.07±0.01[b]	0.701±0.004[b]	0.72±0.09[b]
	200	0.53±0.01[b]	0.05±0.05[b]	1.100±0.005[a]	1.42±0.03[b]
LV	0	0.08±0.03[a]	0.08±0.01[a]	0.110±0.010[a]	0.12±0.02[a]
	50	0.57±0.04[b]	0.69±0.06[b]	0.387±0.011[b]	0.41±0.01[b]
	100	0.31±0.02[b]	0.29±0.04[b]	0.285±0.005[b]	0.26±0.01[b]
	200	0.21±0.01[b]	0.19±0.02[b]	0.187±0.004[b]	0.15±0.03[b]
UP 2752	0	0.09±0.02[a]	0.10±0.04[a]	0.140±0.003[a]	0.14±0.01[a]
	50	0.32±0.05[b]	0.39±0.02[b]	0.412±0.001[b]	0.43±0.06[b]
	100	0.12±0.04[b]	0.13±0.01[b]	0.490±0.005[b]	0.53±0.01[b]
	200	0.08±0.02[b]	0.07±0.01[b]	0.854±0.002[b]	0.98±0.09[b]
PBW 343	0	0.08±0.01[a]	0.08±0.01[a]	0.160±0.008[a]	0.16±0.04[a]
	50	0.37±0.06[a]	0.39±0.04[b]	0.405±0.006[b]	0.46±0.05[b]
	100	0.31±0.04[b]	0.31±0.02[b]	0.540±0.002[b]	0.56±0.06[b]
	200	0.13±0.03[b]	0.13±0.08[b]	0.886±0.002[b]	1.10±0.04[b]
SO	0	0.08±0.05[a]	0.08±0.04[a]	0.142±0.001[a]	0.14±0.02[a]
	50	0.58±0.02[b]	0.64±0.02[b]	0.398±0.004[b]	0.42±0.04[b]
	100	0.25±0.01[b]	0.22±0.01[b]	0.260±0.002[b]	0.24±0.01[b]
	200	0.16±0.06[b]	0.15±0.03[b]	0.165±0.007[b]	0.14±0.04[b]
CD Values between treatments =		0.109148	0.113747	0.190121	0.261039
CD Values between varieties =		0.163722	0.170621	0.285181	0.391558

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Ascorbate peroxidase (mmol ascorbate mg protein^{-1} min^{-1}); GR– Glutathione reductase (μmol NADPH oxidized mg protein^{-1} min^{-1}).

Table 69 (a). ANOVA of data presented in table 69 for APOX during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.606275	3	0.202092	16.05754	6.13E–06	3.008787
Columns	0.357595	8	0.044699	3.551663	0.007512	2.355081
Error	0.302051	24	0.012585			
Total	1.26592	35				

Table 69 (b). ANOVA of data presented in table 69 for APOX during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.813524	3	0.271175	19.83961	1.09E–06	3.008787
Columns	0.348422	8	0.043553	3.186393	0.013126	2.355081
Error	0.32804	24	0.013668			
Total	1.489986	35				

Table 69 (c). ANOVA of data presented in table 69 for GR during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.090041	3	0.363347	9.515377	0.000252	3.008787
Columns	0.904261	8	0.113033	2.960114	0.018723	2.355081
Error	0.916446	24	0.038185			
Total	2.910747	35				

Table 69 (d). ANOVA of data presented in table 69 for GR during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1.69731	3	0.56577	7.859483	0.000799	3.008787
Columns	1.454696	8	0.181837	2.526017	0.037719	2.355081
Error	1.727656	24	0.071986			
Total	4.879662	35				

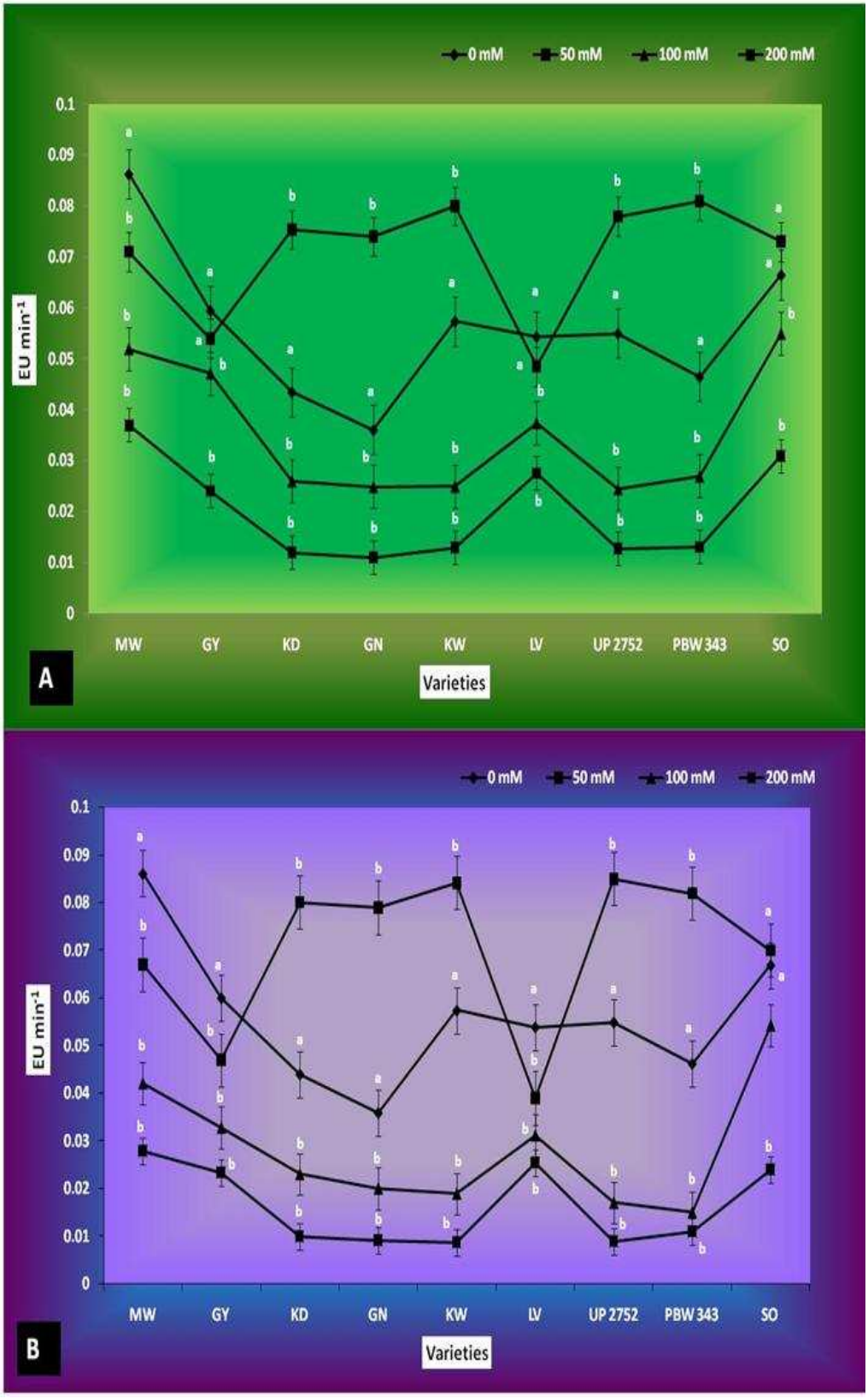

Figure 52. Superoxide dismutase activities in nine varieties of wheat subjected to salt (NaCl) stress treatments for 1 day (A) and 3 days (B). Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0mM, 50mM, 100mM, 200mM corresponds to the concentration of salt (NaCl)

4.10. Isozyme analysis of antioxidative enzymes under drought and salinity

4.10.1. Peroxidase

In the peroxidase isozyme analysis in NATIVE PAGE during drought and salinity stress in case of wheat varieties, significant differences was noticed among the varieties as well as during the different days of drought and different concentration of salt. In case of peroxidase isozyme analysis in NATIVE PAGE, new bands were observed in the stressed varieties with respect to control in case of almost all the varieties with highest number of new peroxizymes (Table 70–81) recorded in case of varieties like GN KW, KD followed by PBW 343 and UP 2752 than SO, LV, GY and MW with their respective control at 0 day of drought (Figure 53–56) and the same trend was observed during salt stress (Figure 57–64).

New bands of peroxidase isozyme were recorded throughout the analysis in NATIVE PAGE with respect to control with Rm value of about 0.99, 0.975, 0.85, 0.812, 0.712, 0.675, 0.60, 0.587, 0.55, 0.362 and 0.20 (approx.). However, a maximum of 6 bands for peroxidase isozyme was observed during both drought and salt stress. The value obtained for the activity of peroxidase (Table 75) for all these varieties could be correlated with the expression of catalase isozyme in NATIVE PAGE for all the tested varieties of wheat.

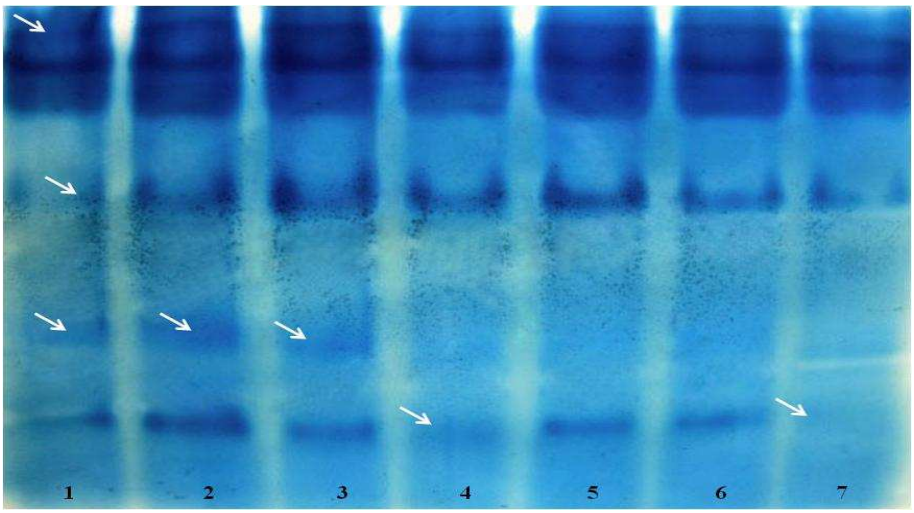

Figure 53. NATIVE PAGE of peroxidase isozyme follollowing drought stress in the leaves of wheat (MW and KD): 1 & 5 –0d, 2 & 6 – 3d, 3 & 7–6d, 4–9d respectively

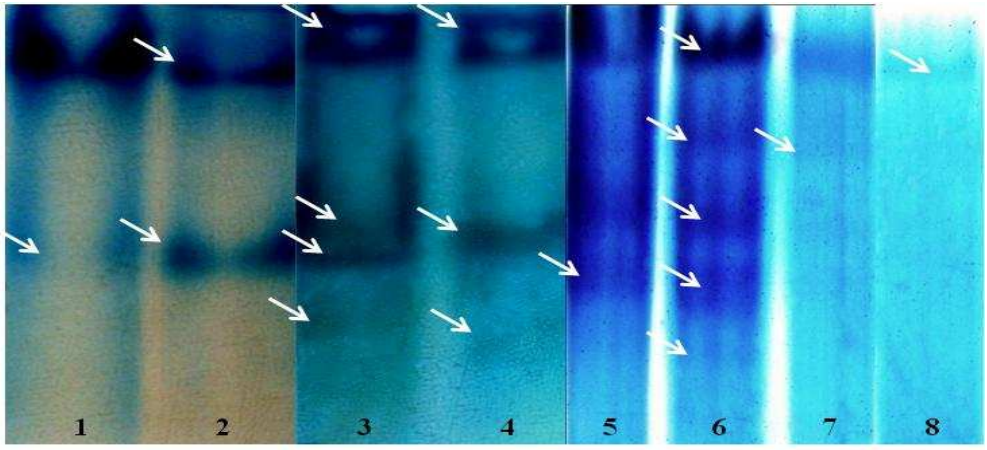

Figure 54. NATIVE PAGE of peroxidase isozyme following drought stress in the leaves of wheat (GY and LV): 1 & 5 –0d, 2 & 6 – 3d, 3 & 7–6d, 4 & 8–9d respectively

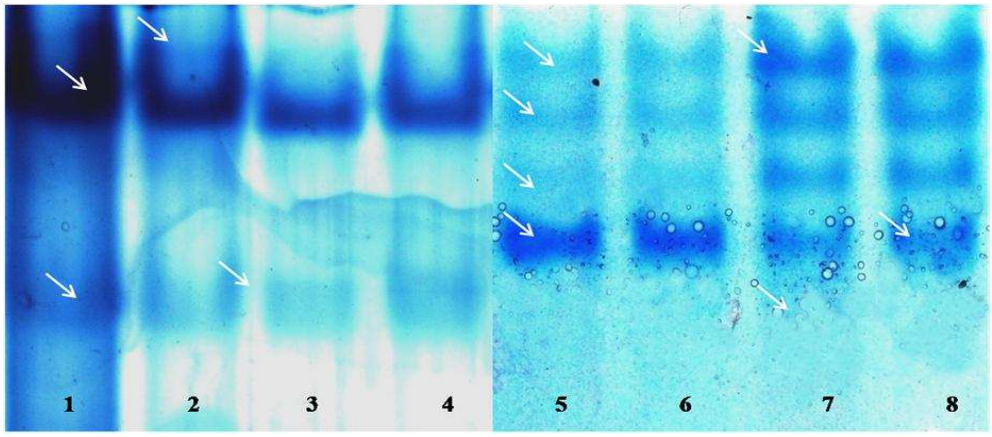

Figure 55. NATIVE PAGE of peroxidase isozyme following drought stress in the leaves of wheat (SO and PBW 343): 1 & 5 –0d, 2 & 6 – 3d, 3 & 7–6d, 4 & 8–9d respectively

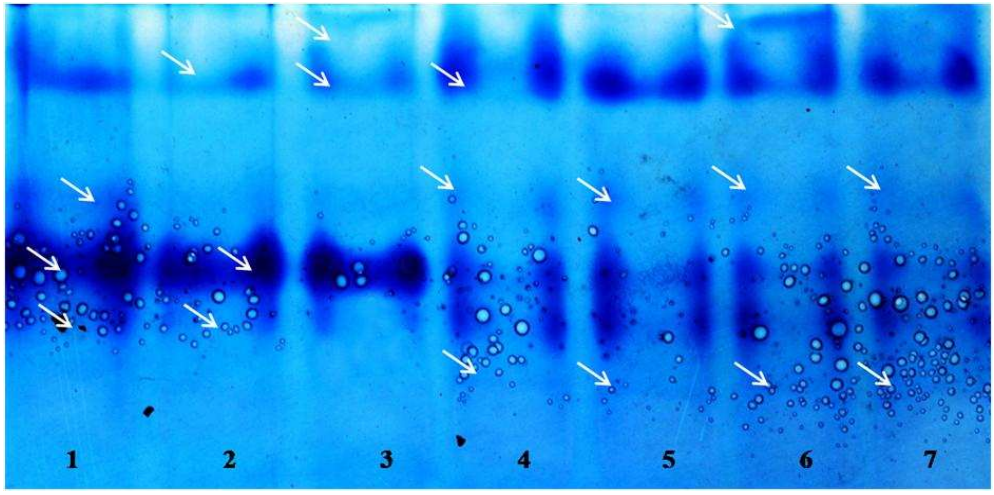

Figure 56. NATIVE PAGE of peroxidase isozyme following drought stress in the leaves of wheat (KW): 1–0d, 2 & 3 – 3d, 4 & 5–6d, 6 & 7–9d respectively

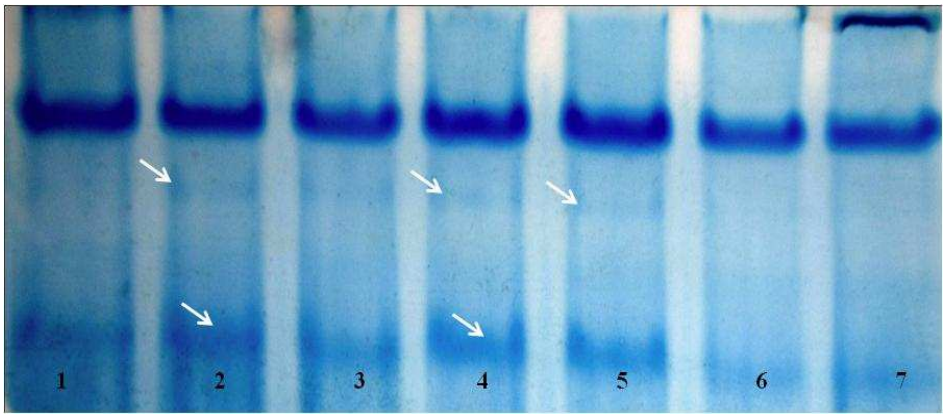

Figure 57. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (MW): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

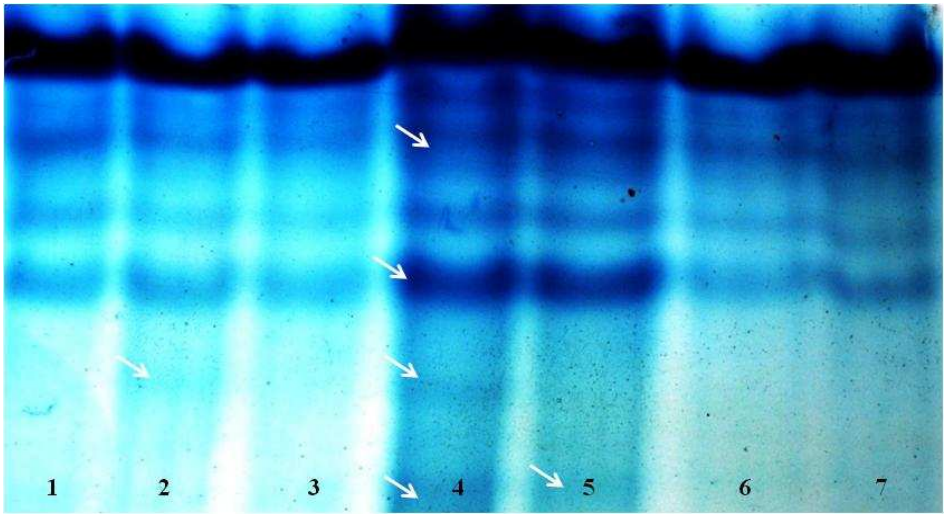

Figure 58. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (KD): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

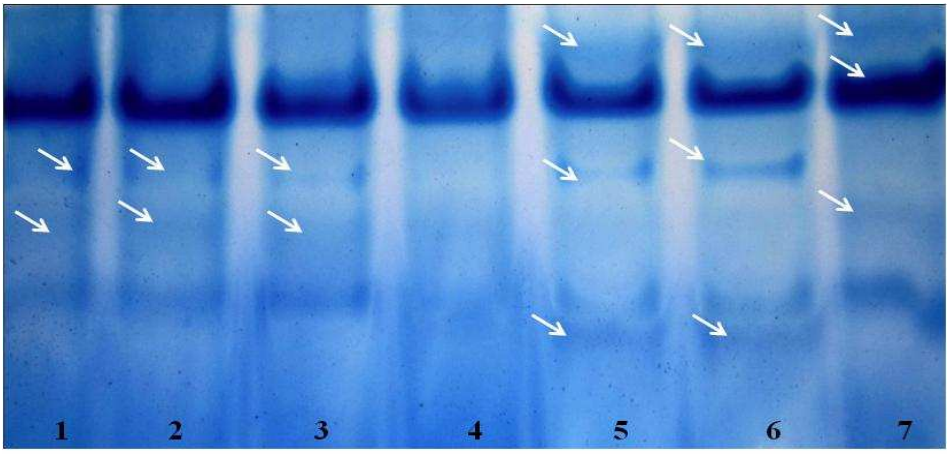

Figure 59. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (GY): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

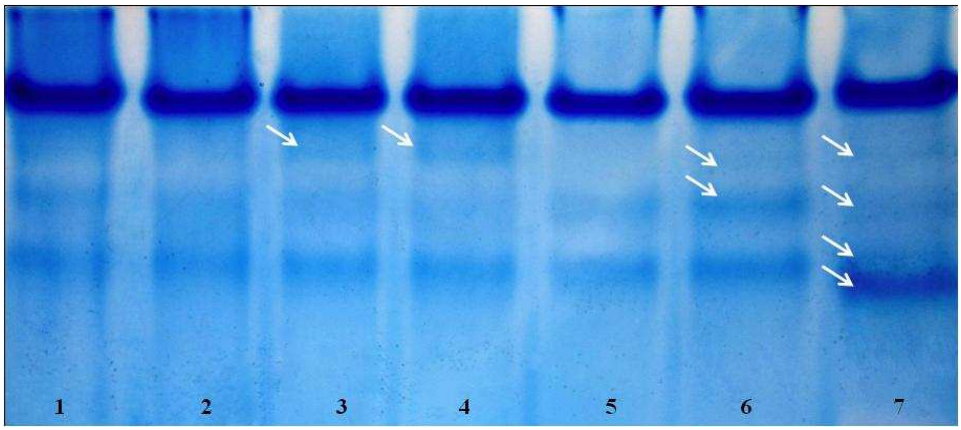

Figure 60. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (GN): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

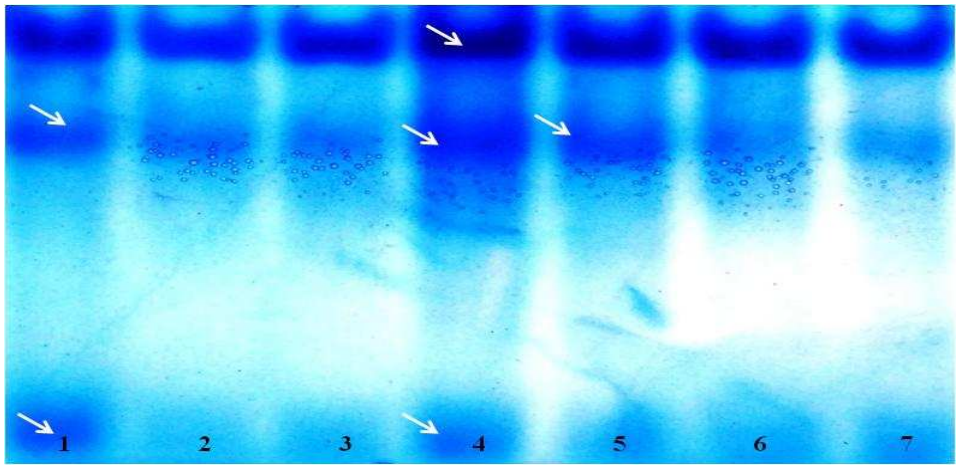

Figure 61. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (SO): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

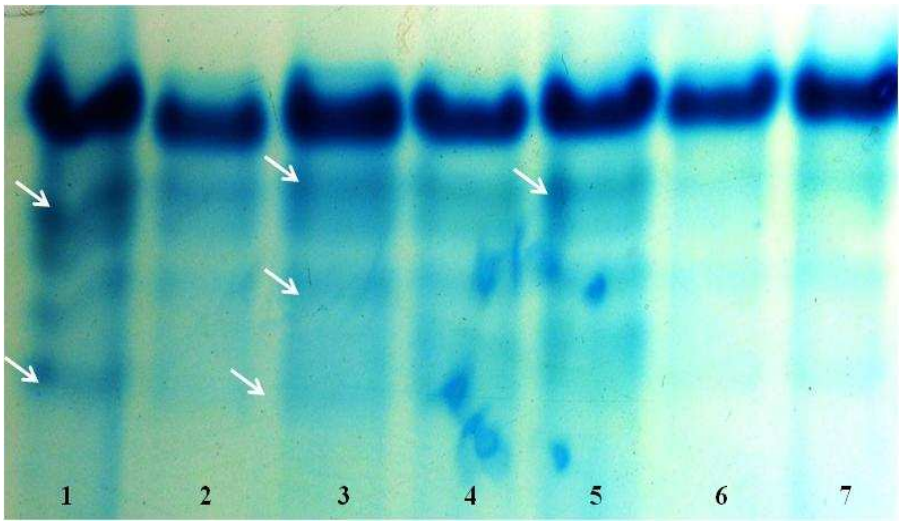

Figure 62. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (PBW 343): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

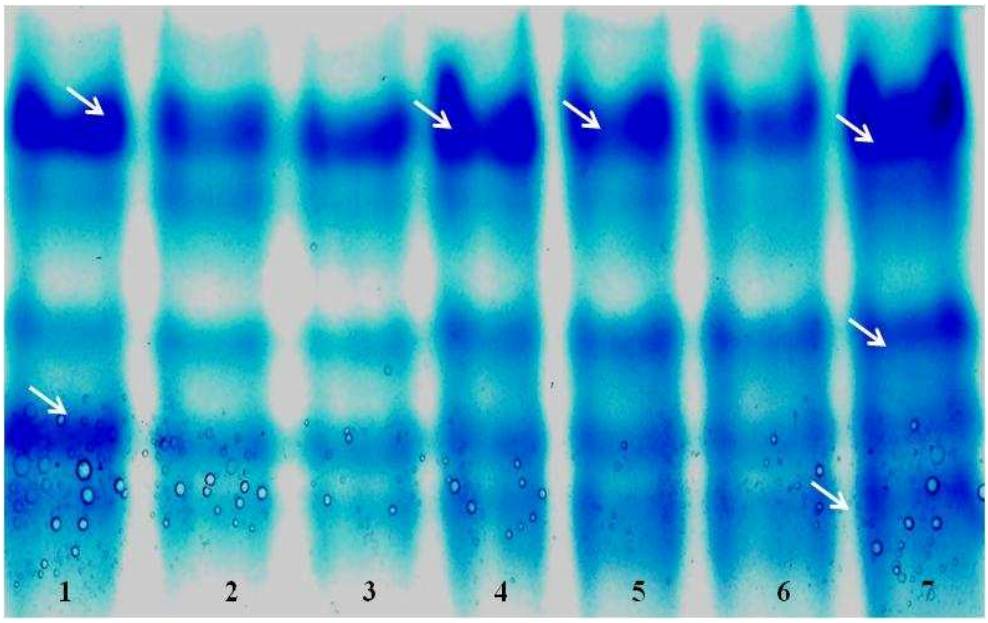

Figure 63. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (KW): 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

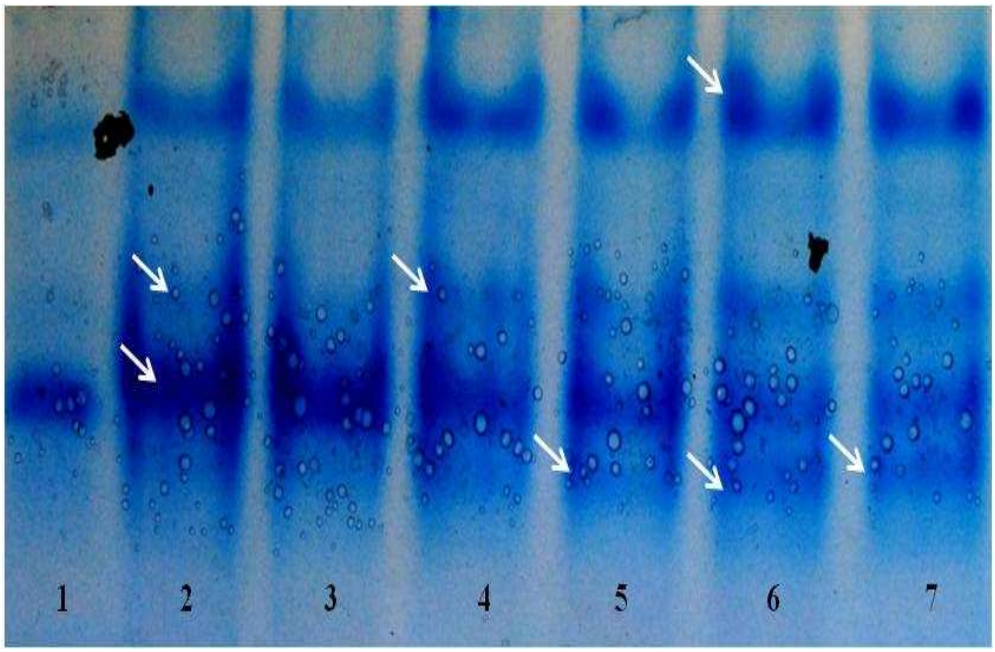

Figure 64. NATIVE PAGE of peroxidase isozyme following salt stress in the leaves of wheat (UP 2752: 1–0mM, 2 & 3 – 50mM (1d & 3d), 4 & 5–100mM (1d & 3d), 6 & 7–200mM (1d & 3d) respectively

Table 70. Rm value for peroxidase isozyme in Native PAGE in fig. 53

Variety with treatments	Lane No.	Rm value
MW 0D	1	0.951, 0.878, 0.621, 0.353, 0.195
MW 3D	2	0.951, 0.878, 0.621, 0.353, 0.195
MW 6D	3	0.951, 0.878, 0.621, 0.353, 0.195
MW 9D	4	0.951, 0.878, 0.621, 0.195
KD 0D	5	0.951, 0.878, 0.621, 0.195
KD 3D	6	0.951, 0.878, 0.621, 0.195
KD 6D	7	0.951, 0.878, 0.621, 0.353, 0.195

Table 71. Rm value for peroxidase isozyme in Native PAGE in fig. 54

Variety with treatments	Lane No.	Rm value
GY 0D	1	0.95, 0.675
GY 3D	2	0.95, 0.675
GY 6D	3	0.99*, 0.95, 0.712*, 0.675, 0.587*
GY 9D	4	0.99*, 0.95, 0.587*
LV 0D	5	0.95, 0.712, 0.675
LV 3D	6	0.95, 0.85*, 0.712, 0.675, 0.587*
LV 6D	7	0.95, 0.85*
LV 9D	8	0.95

*New band with respect to control

Table 72. Rm value for peroxidase isozyme in Native PAGE in fig. 55

Variety with treatments	Lane No.	Rm value
SO 0D	1	0.90, 0.65
SO 3D	2	0.975*, 0.90, 0.65
SO 6D	3	0.90, 0.65
SO 9D	4	0.90, 0.65
PBW 343 0D	5	0.90, 0.812, 0.725, 0.65
PBW 343 3D	6	0.90, 0.812, 0.725, 0.65
PBW 343 6D	7	0.90, 0.812, 0.725, 0.65, 0.60*
PBW 343 9D	8	0.90, 0.812, 0.725, 0.65

*New band with respect to control

Table 73. Rm value for peroxidase isozyme in Native PAGE in fig. 56

Variety with treatments	Lane No.	Rm value
KW 0D	1	0.90, 0.80, 0.712, 0.60
KW 3D	2	0.975*, 0.90, 0.712, 0.60
KW 3D	3	0.975*, 0.90, 0.712, 0.60
KW 6D	4	0.975*, 0.90, 0.80, 0.712, 0.60
KW 6D	5	0.975*, 0.90, 0.80, 0.712, 0.60
KW 9D	6	0.975*, 0.90, 0.80, 0.712, 0.60, 0.55*
KW 9D	7	0.975*, 0.90, 0.80, 0.712, 0.60, 0.55*

*New band with respect to control

Table 74. Rm value for peroxidase isozyme in Native PAGE in fig. 57

Variety with treatments	Lane No.	Rm value
MW 0mM	1	0.90, 0.60
MW 50mM 1D	2	0.90, 0.812*, 0.60
MW 50mM 3D	3	0.90, 0.812*, 0.60
MW 100mM 1D	4	0.90, 0.812*, 0.60
MW 100mM 3D	5	0.90, 0.812*, 0.60
MW 200mM 1D	6	0.90, 0.60
MW 200mM 3D	7	0.90, 0.60

*New band with respect to control

Table 75. Rm value for peroxidase isozyme in Native PAGE in fig. 58

Variety with treatments	Lane No.	Rm value
KD 0mM	1	0.90, 0.85, 0.812, 0.725, 0.65
KD 50mM 1D	2	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*
KD 50mM 3D	3	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*
KD 100mM 1D	4	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*, 0.20*
KD 100mM 3D	5	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*
KD 200mM 1D	6	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*
KD 200mM 3D	7	0.90, 0.85, 0.812, 0.725, 0.65, 0.362*

*New band with respect to control

Table 76. Rm value for peroxidase isozyme in Native PAGE in fig. 59

Variety with treatments	Lane No.	Rm value
GY 0mM	1	0.90, 0.812, 0.725, 0.65
GY 50mM 1D	2	0.90, 0.812, 0.725, 0.65
GY 50mM 3D	3	0.90, 0.812, 0.725, 0.65
GY 100mM 1D	4	0.90, 0.65
GY 100mM 3D	5	0.90, 0.812, 0.65, 0.60
GY 200mM 1D	6	0.90, 0.812, 0.65, 0.60
GY 200mM 3D	7	0.90, 0.812, 0.725, 0.65

Table 77. Rm value for peroxidase isozyme in Native PAGE in fig. 60

Variety with treatments	Lane No.	Rm value
GN 0mM	1	0.90, 0.725, 0.65
GN 50mM 1D	2	0.90, 0.725, 0.65
GN 50mM 3D	3	0.90, 0.85*, 0.725, 0.65
GN 100mM 1D	4	0.90, 0.85*, 0.725, 0.65
GN 100mM 3D	5	0.90, 0.725, 0.65
GN 200mM 1D	6	0.90, 0.85*, 0.725, 0.65
GN 200mM 3D	7	0.90, 0.85*, 0.725, 0.65, 0.60*

*New band with respect to control

Table 78. Rm value for peroxidase isozyme in Native PAGE in fig. 61

Variety with treatments	Lane No.	Rm value
SO 0mM	1	0.90, 0.725, 0.362
SO 50mM 1D	2	0.90, 0.725, 0.362
SO 50mM 3D	3	0.90, 0.725, 0.362
SO 100mM 1D	4	0.90, 0.725, 0.362
SO 100mM 3D	5	0.90, 0.725, 0.362
SO 200mM 1D	6	0.90, 0.725, 0.362
SO 200mM 3D	7	0.90, 0.725, 0.362

Table 79. Rm value for peroxidase isozyme in Native PAGE in fig. 62

Variety with treatments	Lane No.	Rm value
PBW 343 0mM	1	0.90, 0.812, 0.725, 0.65, 0.60
PBW 343 50mM 1D	2	0.90, 0.812, 0.725, 0.65, 0.60
PBW 343 50mM 3D	3	0.90, 0.812, 0.725, 0.65, 0.60
PBW 343 100mM 1D	4	0.90, 0.812, 0.725, 0.65, 0.60
PBW 343 100mM 3D	5	0.90, 0.812, 0.725, 0.65, 0.60
PBW 343200mM 1D	6	0.90, 0.812, 0.65, 0.60
PBW 343 200mM 3D	7	0.90, 0.812, 0.65, 0.60

Table 80. Rm value for peroxidase isozyme in Native PAGE in fig. 63

Variety with treatments	Lane No.	Rm value
KW 0mM	1	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 50mM 1D	2	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 50mM 3D	3	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 100mM 1D	4	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 100mM 3D	5	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 200mM 1D	6	0.975, 0.90, 0.85, 0.725, 0.65, 0.60
KW 200mM 3D	7	0.975, 0.90, 0.85, 0.725, 0.65, 0.60

Table 81. Rm value for peroxidase isozyme in Native PAGE in fig. 64

Variety with treatments	Lane No.	Rm value
UP 2752 0mM	1	0.90, 0.812, 0.725, 0.65
UP 2752 50mM 1D	2	0.90, 0.812, 0.725, 0.65
UP 2752 50mM 3D	3	0.90, 0.812, 0.725, 0.65
UP 2752 100mM 1D	4	0.90, 0.812, 0.725, 0.65
UP 2752 100mM 3D	5	0.90, 0.812, 0.725, 0.65
UP 2752 200mM 1D	6	0.975*, 0.90, 0.812, 0.725, 0.65
UP 2752 200mM 3D	7	0.975*, 0.90, 0.812, 0.725, 0.65

*New band with respect to control

4.10.2. Catalase

In the catalase isozyme analysis in NATIVE PAGE during drought and salinity stress in case of wheat varieties, significant differences was noticed among the varieties as well as during the different days of drought and different concentration of salt. Basically three bands were observed in the stressed varieties with respect to control in case of almost all the varieties (Table 82–87) with Rm value of about 0.853, 0.466 and 0.24 (approx.). All the three isozymes was expressed in NATIVE PAGE for GN, KD, KW during drought (Figure 65–66) and in KD and KW in case of salinity stress (Figure 67–70). Significantly, only two isozymes for catalase were observed in case of GN during salt stress. In case of MW, GY all the three isozyme for catalase was seen during the higher concentration of salt and prolonged period of water stress. The value obtained for the activity of catalase for all these varieties could be correlated with the expression of catalase isozyme in NATIVE PAGE for all the tested varieties of wheat for both drought and salt stress.

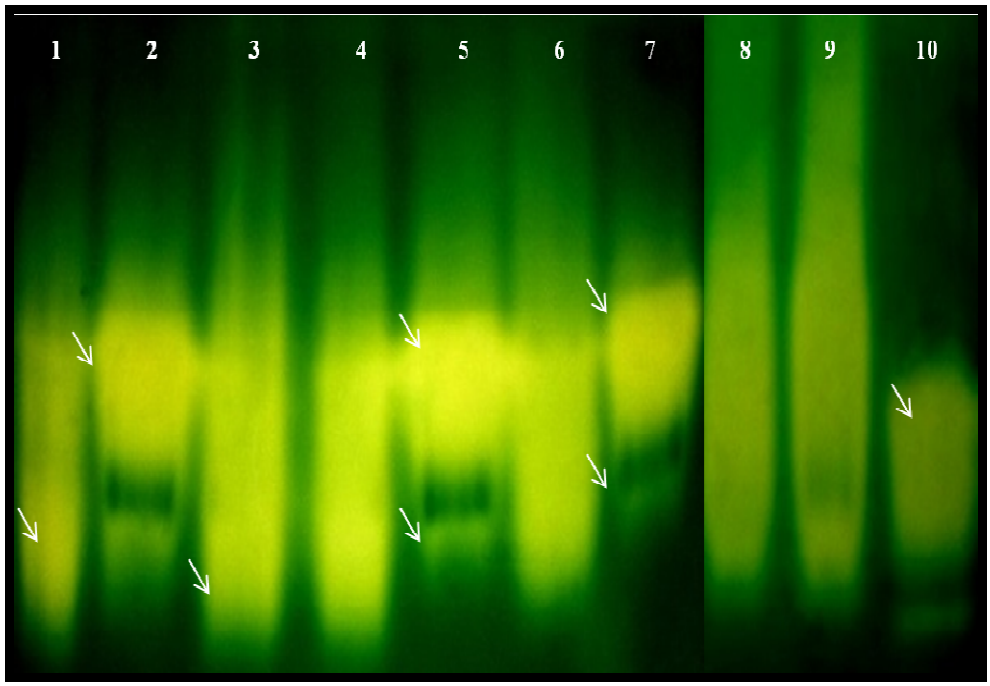

Figure 65. NATIVE PAGE of catalase isozyme following drought stress in the leaves of wheat (MW, GY and GN): 1, 4 & 8–0d; 2, 5 & 9–3d; 3, 6 & 10–6d respectively; 7–9d in GY

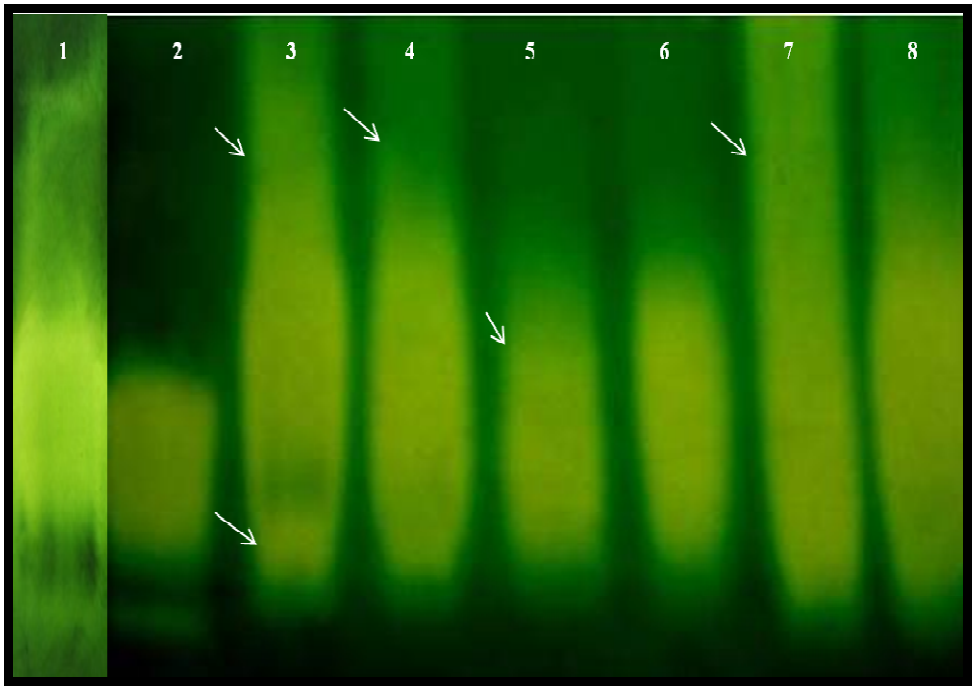

Figure 66. NATIVE PAGE of catalase isozyme following drought stress in the leaves of wheat (KD and KW): 1 & 5–0d; 2 & 6–3d; 3 & 7–6d; 4 & 8–9d respectively

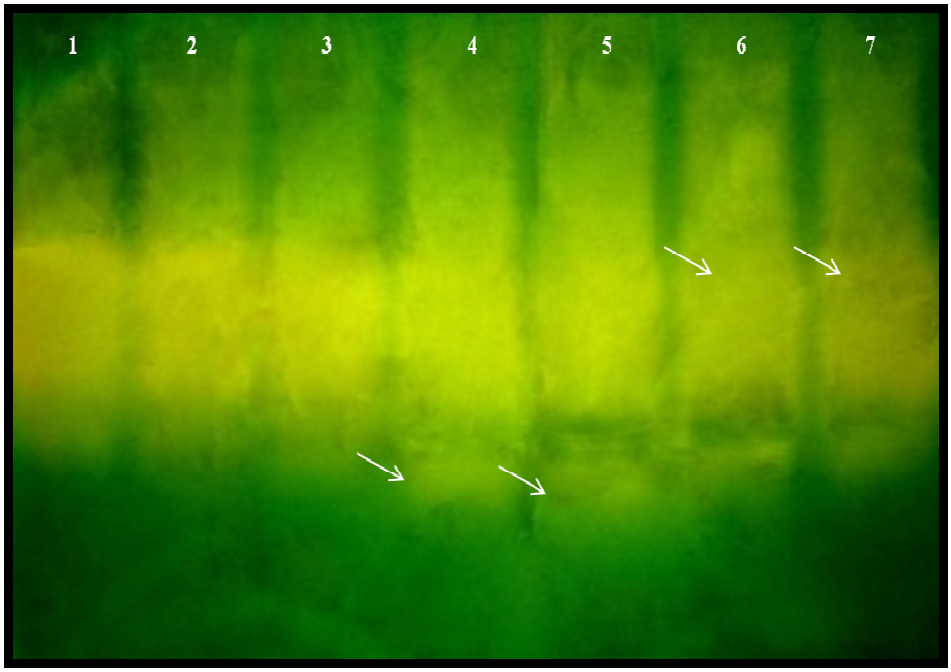

Figure 67. NATIVE PAGE of catalase isozyme following salt stress in the leaves of wheat (MW): 1–0mM; 2 & 3–50mM (1d & 3d); 4 & 5–100mM (1d & 3d); 6 & 7–200mM (1d & 3d) respectively

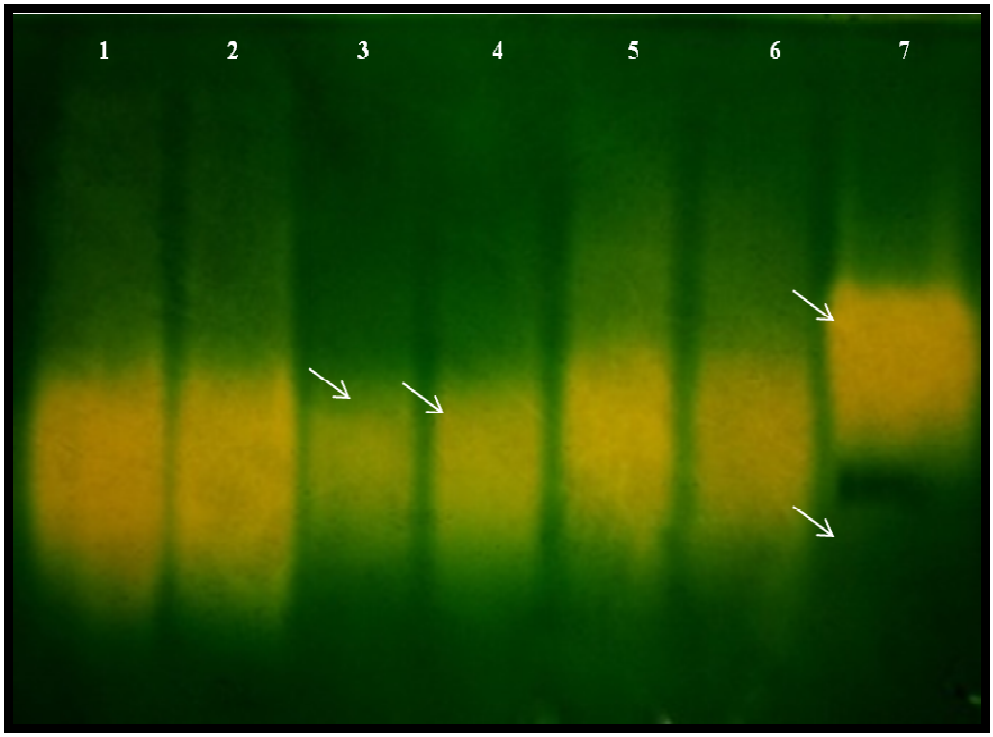

Figure 68. NATIVE PAGE of catalase isozyme following salt stress in the leaves of wheat (GN): 1–0mM; 2 & 3–50mM (1d & 3d); 4 & 5–100mM (1d & 3d); 6 & 7–200mM (1d & 3d) respectively

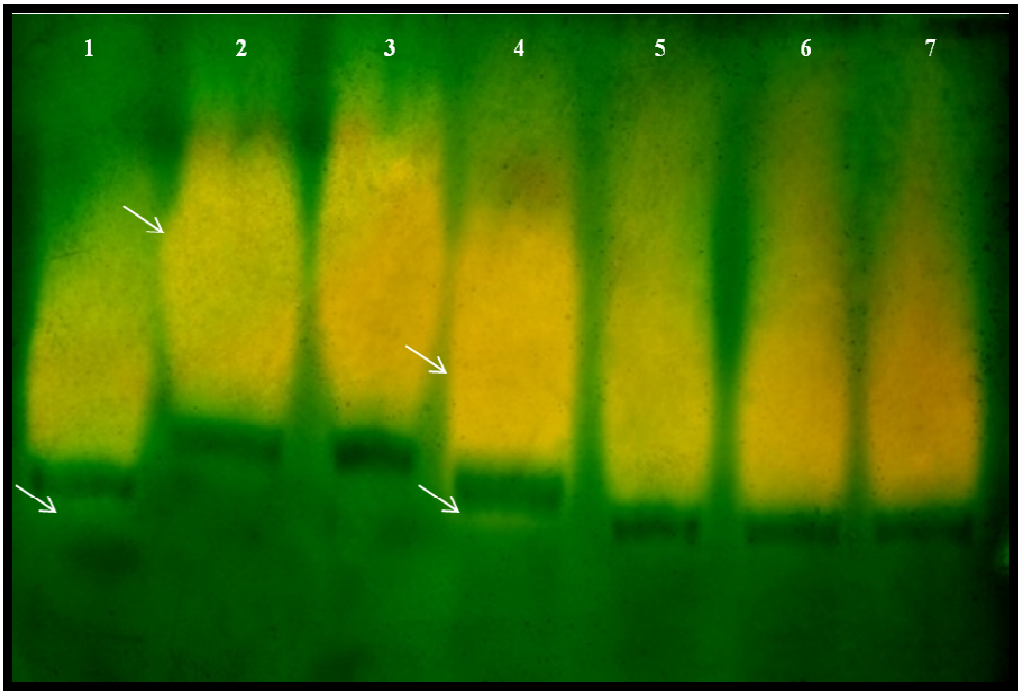

Figure 69. NATIVE PAGE of catalase isozyme following salt stress in the leaves of wheat (KW): 1–0mM; 2 & 3–50mM (1d & 3d); 4 & 5–100mM (1d & 3d); 6 & 7–200mM (1d & 3d) respectively

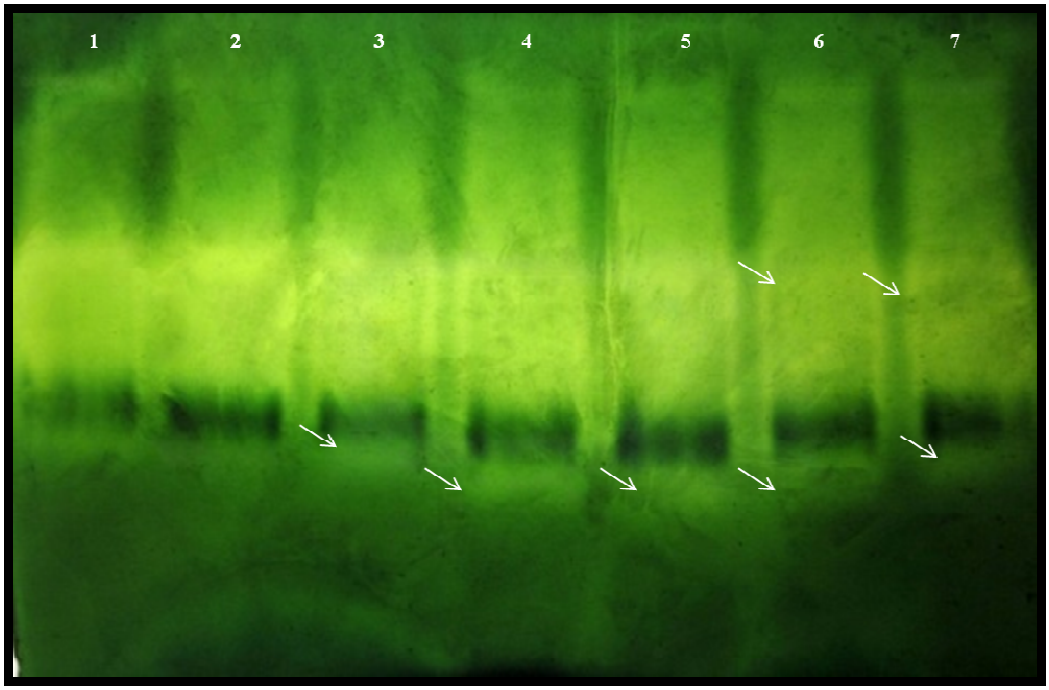

Figure 70. NATIVE PAGE of catalase isozyme following salt stress in the leaves of wheat (KD): 1–0mM; 2 & 3–50mM (1d & 3d); 4 & 5–100mM (1d & 3d); 6 & 7–200mM (1d & 3d) respectively

Table 82. Rm value for catalase isozyme in Native PAGE in fig. 65

Variety with treatments	Lane No.	Rm value
MW 0D	1	0.466, 0.24
MW 3D	2	0.466, 0.24
MW 6D	3	0.466, 0.24
GY 0D	4	0.466, 0.24
GY 3D	5	0.466, 0.24
GY 6D	6	0.466, 0.24
GY 9D	7	0.466, 0.24
GN 0D	8	0.853, 0.466, 0.24
GN 3D	9	0.853, 0.466, 0.24
GN 6D	10	0.466, 0.24

Table 83. Rm value for catalase isozyme in Native PAGE in fig. 66

Variety with treatments	Lane No.	Rm value
KD 0D	1	0.853, 0.466, 0.24
KD 3D	2	0.466, 0.24
KD 6D	3	0.853, 0.466, 0.24
KD 9D	4	0.853, 0.466, 0.24
KW 0D	5	0.466, 0.24
KW 3D	6	0.466, 0.24
KW 6D	7	0.853*, 0.466, 0.24
KW 9D	8	0.853*, 0.466, 0.24

*New band with respect to control

Table 84. Rm value for catalase isozyme in Native PAGE in fig. 67

Variety with treatments	Lane No.	Rm value
MW 0mM	1	0.466, 0.24
MW 50mM 1D	2	0.466, 0.24
MW 50mM 3D	3	0.466, 0.24
MW 100mM 1D	4	0.853*, 0.466, 0.24
MW 100mM 3D	5	0.853*, 0.466, 0.24
MW 200mM 1D	6	0.853*, 0.466, 0.24
MW 200mM 3D	7	0.853*, 0.466, 0.24

*New band with respect to control

Table 85. Rm value for catalase isozyme in Native PAGE in fig. 68

Variety with treatments	Lane No.	Rm value
GN 0mM	1	0.466, 0.24
GN 50mM 1D	2	0.466, 0.24
GN 50mM 3D	3	0.466, 0.24
GN 100mM 1D	4	0.466, 0.24
GN 100mM 3D	5	0.466, 0.24
GN 200mM 1D	6	0.466, 0.24
GN 200mM 3D	7	0.466, 0.24

Table 86. Rm value for catalase isozyme in Native PAGE in fig. 69

Variety with treatments	Lane No.	Rm value
KW 0mM	1	0.853, 0.466, 0.24
KW 50mM 1D	2	0.853, 0.466, 0.24
KW 50mM 3D	3	0.853, 0.466, 0.24
KW 100mM 1D	4	0.853, 0.466, 0.24
KW 100mM 3D	5	0.853, 0.466, 0.24
KW 200mM 1D	6	0.853, 0.466, 0.24
KW 200mM 3D	7	0.853, 0.466, 0.24

Table 87. Rm value for catalase isozyme in Native PAGE in fig. 70

Variety with treatments	Lane No.	Rm value
KD 0mM	1	0.853, 0.466, 0.24
KD 50mM 1D	2	0.853, 0.466, 0.24
KD 50mM 3D	3	0.853, 0.466, 0.24
KD 100mM 1D	4	0.853, 0.466, 0.24
KD 100mM 3D	5	0.853, 0.466, 0.24
KD 200mM 1D	6	0.853, 0.466, 0.24
KD 200mM 3D	7	0.853, 0.466, 0.24

4.11. Changes in H_2O_2 accumulation in wheat plants following stress

4.11.1. H_2O_2 content

In this study, although H_2O_2 accumulation (Table 88) increased during water stress, after a period of prolonged drought there was a decrease in H_2O_2 levels in varieties like KW, GN, KD, PBW 343 and UP 2752 indicating greater antioxidant activity whereas the accumulation of H_2O_2 continued to increase in LV, SO, GY and MW with the increase in the duration of withholding water from the plants. In our study it was observed that during salt stress (Table 89) the accumulation of H_2O_2 continued to increase in all the varieties with increasing concentration of salt and the duration of salt stress however, in case of KW, GN, KD, PBW 343 and UP 2752 the accumulation of H_2O_2 decreased whereas in LV, SO, GY and MW the content of H_2O_2 continued to increase. The accumulation of H_2O_2 during drought stress was higher in the wheat varieties in general than the content of H_2O_2 in the wheat varieties during salt stress.

Table 88. Effect of drought stress on accumulation of H_2O_2 in wheat

Varieties	Content of H_2O_2 Days of Stress			
	0D	**3D**	**6D**	**9D**
MW	271.9±1.81[a]	381.6±1.62[b]	406.2±1.32[b]	427.8±1.44[b]
GY	66.4±1.62[a]	359.4±1.55[b]	404.7±1.43[b]	449.7±1.21[b]
KD	175.9±1.32[a]	295.2±1.46[b]	338.8±1.84[b]	262.0±1.45[b]
GN	233.0±1.73[a]	351.6 ±1.29[b]	370.5±1.72[b]	295.3±1.94[b]
KW	185.3±1.85[a]	289.3±1.96[b]	344.0±1.91[b]	279.9±1.71[b]
LV	142.0±1.74[a]	243.1±1.86[b]	406.4±1.83[b]	450.2±1.66[b]
UP 2752	159.1±1.81[a]	248.8±1.94[b]	349.3±1.79[b]	330.6±1.84[b]
PBW 343	90.2±1.71[a]	326.0±1.87[b]	370.3±1.55[b]	350.0±1.62[b]
SO	154.9±1.32[a]	382.5±1.91[b]	391.4±1.95[b]	400.0±1.90[b]
CD Value between treatments				=50.9296
CD Value between varieties				=76.3944

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). H_2O_2 = expressed in terms of μ mol g tissue^{-1} (d.m.).

Table 88 (a). ANOVA of data presented in table 88 for H_2O_2

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	254118.2	3	84706.08	30.91281	2.07E–08	3.008787
Columns	35282.85	8	4410.356	1.609524	0.174395	2.355081
Error	65763.88	24	2740.161			
Total	355165	35				

Table 89. Effect of salt stress on accumulation of H_2O_2 in wheat

Varieties	**Day of sampling**	**Content of H_2O_2 Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	271.9±1.28[a]	379.5±1.23[b]	399.6±1.55[a]	419.9±1.11[b]
	3d	272.0±1.35[a]	382.6±1.25[b]	411.1±1.45[b]	431.2±1.52[b]
GY	**1d**	66.4±1.81[a]	361.1±1.63[b]	402.2±1.11[b]	422.1±1.51[b]
	3d	67.0 ±1.44[a]	363.6±1.95[b]	406.5±1.84[b]	452.6±1.65[b]
KD	**1d**	175.9±1.31[a]	301.1±1.41[b]	340.2±1.85[b]	270.1±1.44[a]
	3d	176.2±1.31[a]	322.0±1.81[b]	335.0±1.92[b]	264.5±1.86[b]
GN	**1d**	233.0±1.52[a]	355.1±1.16[a]	359.5±1.64[b]	264.3±1.78[a]
	3d	233.5±1.22[a]	363.3±1.14[b]	341.2±1.36[b]	245.5±1.42[a]
KW	**1d**	185.3±1.81[a]	297.7±1.93[a]	342.2±1.92[b]	257.7±1.29[b]
	3d	186.0±1.52[a]	368.8±1.22[b]	301.1±1.19[b]	244.4±1.59[b]
LV	**1d**	142.0±1.71[a]	405.5±1.85[b]	411.1±1.83[b]	462.0±1.65[b]
	3d	142.5±1.19[a]	412.2±1.41[b]	440.0±1.73[b]	465.5±1.19[b]
UP 2752	**1d**	159.1±1.78[a]	295.5±1.10[b]	340.1±1.34[b]	312.2±1.12[b]
	3d	160.2±1.15[a]	321.0±1.79[b]	336.4±1.16[b]	289.9±1.23[a]
PBW 343	**1d**	90.2±1.52[a]	314.0±1.96[b]	321.2±1.62[b]	279.2±1.52[b]
	3d	90.3±1.63[a]	325.8±1.62[b]	323.6±1.52[b]	269.0±1.31[b]
SO	**1d**	154.9±1.21[a]	298.8±1.12[a]	382.2±1.93[b]	400.0±1.87[b]
	3d	155.2±1.32[a]	345.5±1.98[b]	398.5±1.63[b]	415.0±1.54[b]
CD Value between treatments (1 day/3 day)					=48.15774 / 53.86688
CD Value between varieties (1 day/3 day)					=72.2366 / 161.6006

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). H_2O_2 = expressed in terms of μ mol g tissue^{-1} (d.m.)

Table 89 (a). ANOVA of data presented in table 89 during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	232586.2	3	77528.72	31.64424	1.66E–08	3.008787
Columns	49712.19	8	6214.024	2.536325	0.037087	2.355081
Error	58800.25	24	2450.01			
Total	341098.6	35				

Table 89 (b). ANOVA of data presented in table 89 during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	246046.4	3	82015.45	26.75569	7.91E–08	3.008787
Columns	58860.26	8	7357.533	2.400229	0.0464	2.355081
Error	73568.3	24	3065.346			
Total	378474.9	35				

4.11.2. Microscopic localization of H_2O_2

During microscopic studies of the leaf tissues in DAB staining test for the detection of H_2O_2, dark–brown spots were observed as big and small patches at the site of DAB polymerization. The leaf of SO, LV, GY and MW showed more darkly stained DAB–sites in the tissues than in the leaves from the other five varieties with respect to their control during both the drought (Figure 71, 72 and 73) and salt stress (Figure 74, 75 and 76). With increase in the duration of stress LV, MW, GY and SO showed darker staining in the leaf tissues particularly during the 6th and 9th day of water stress and at salt concentration of 200mM at 3rd day of salt stress; however, the other varieties also showed darker stained leaf tissues during the 9th day of drought and at salt stress of 200mM. Interestingly, DAB polymerization site was largely localized at the tip of the leaf, region surrounding the middle lamella and also the stomata of the leaf in the varieties under stress when compared to the leaf of the control set (Figure 77).

The transverse section of the leaf at the stained site showed that the DAB binding sites were localized mostly in the peripheral region of the cell (Figure 77). It was evident in our study that the accumulation of H_2O_2 as evident by the dark spots observed during DAB polymerization in our tested varieties of wheat under microscopic studies in case of wheat varieties during drought was higher than the accumulation during salt stress.

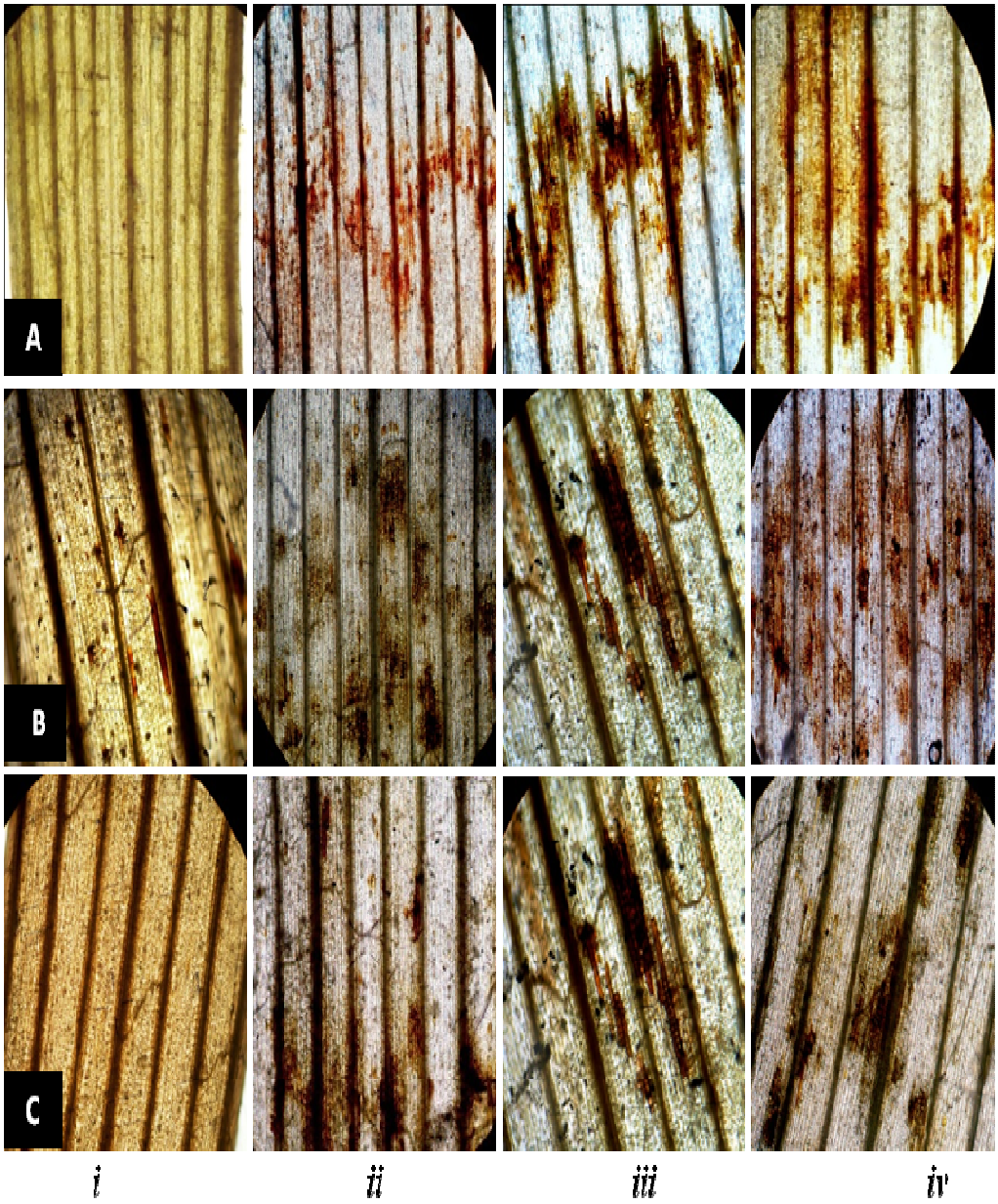

Figure 71. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following drought stress: A–KW, B–LV & C–UP 2752; i–0d, ii–3d, iii–6d & iv–9d respectively

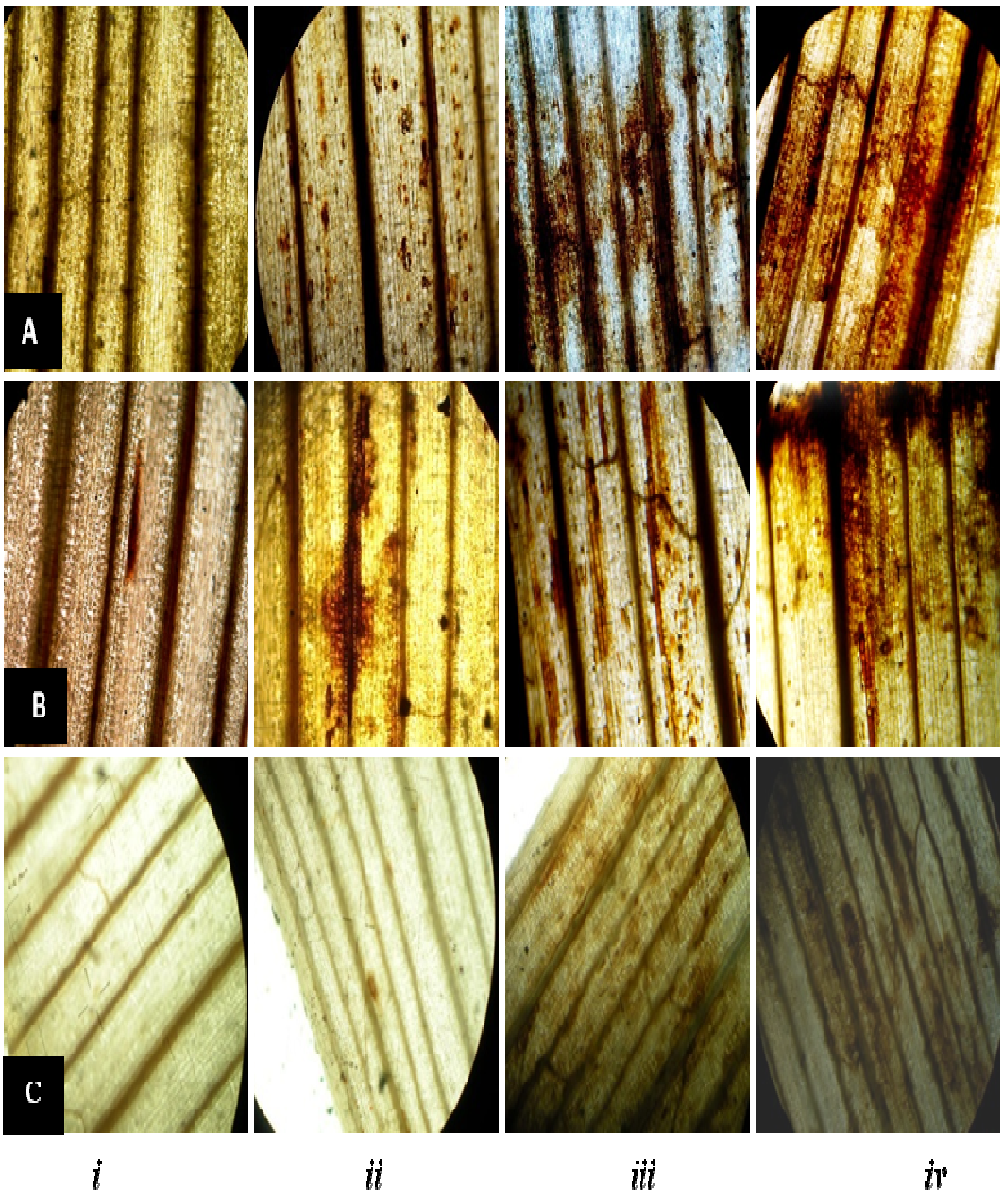

Figure 72. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following drought stress: A–PBW 343, B–SO & C–KD; i–0d, ii–3d, iii–6d & iv–9d respectively

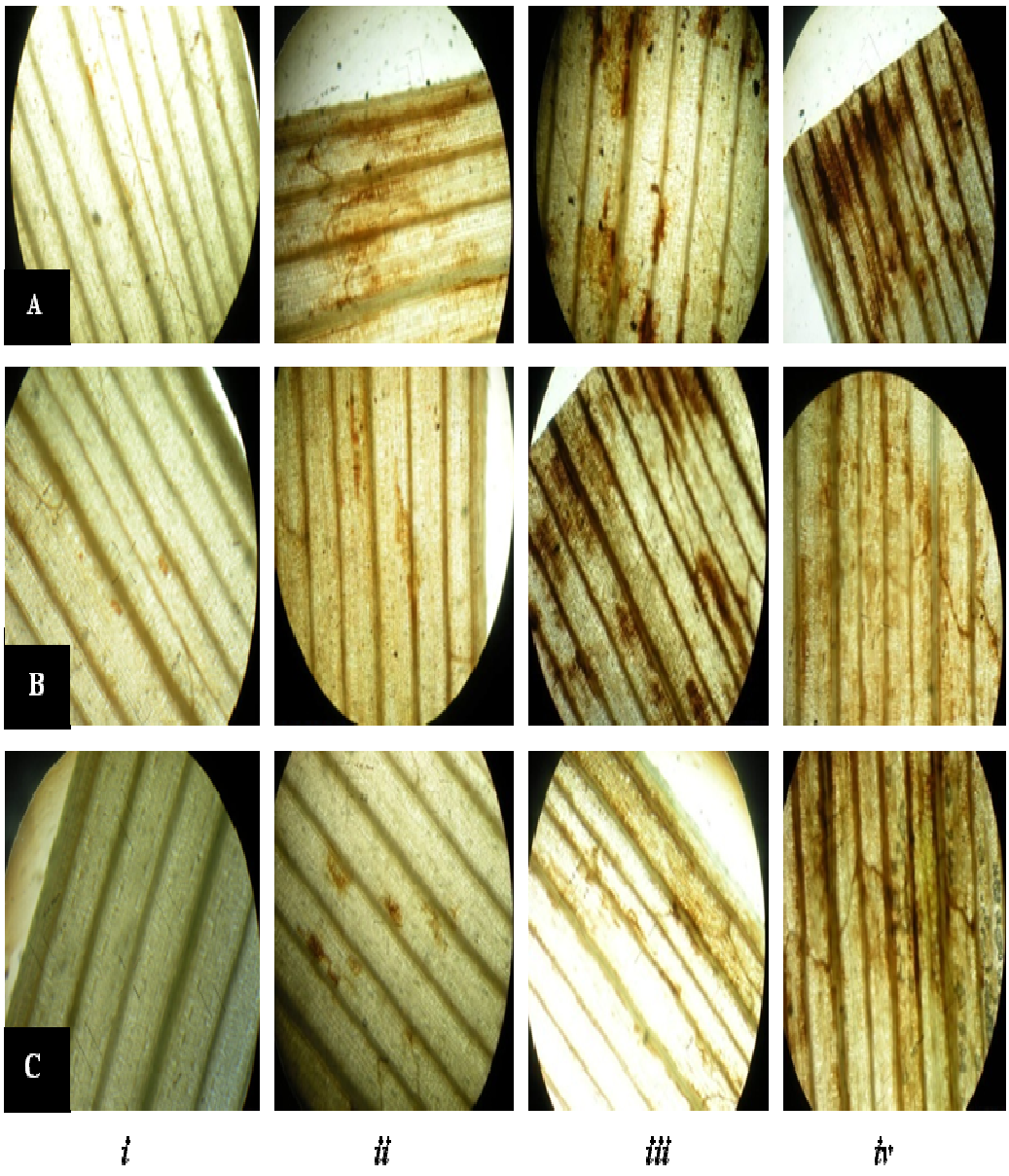

Figure 73. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following drought stress: A–MW, B–GY & C–GN; i–0d, ii–3d, iii–6d & iv–9d respectively

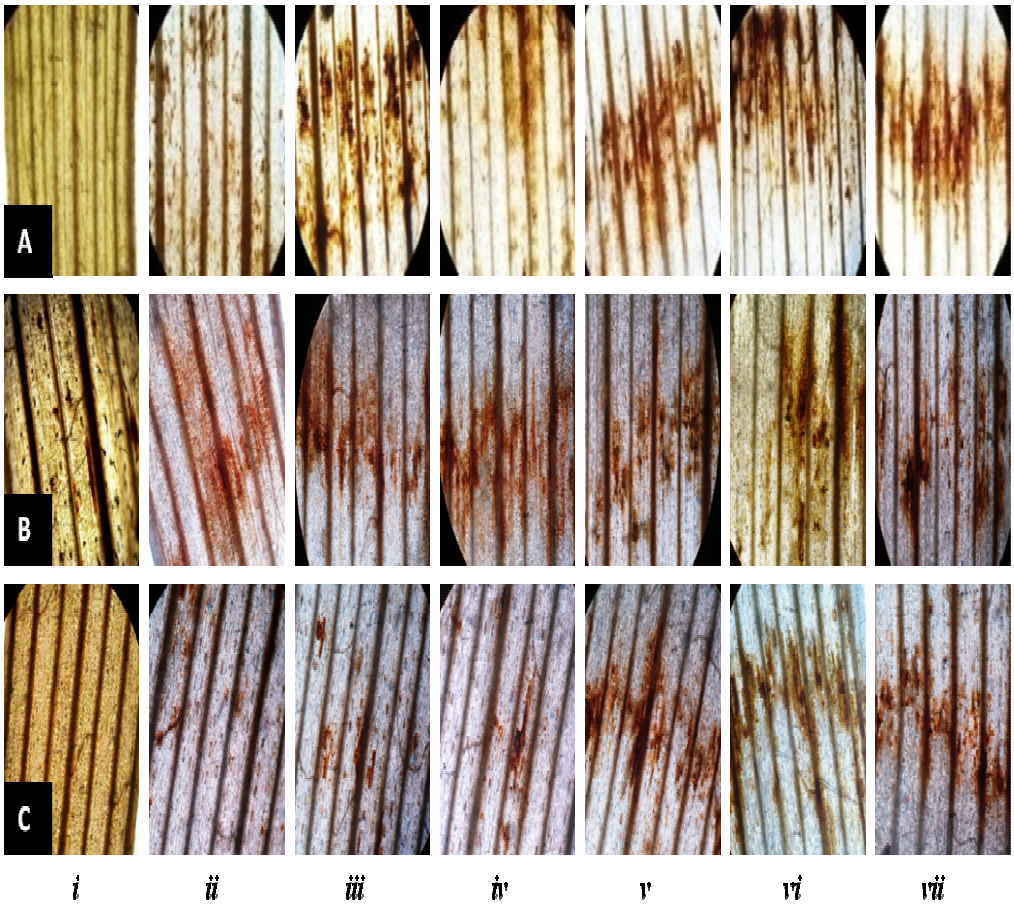

Figure 74. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following salt stress: A–KW, B–LV & C–UP 2752; i–0 mM, ii & iii–50mM (1d & 3d), iv & v–100mM (1d & 3d), vi & vii–200mM (1d & 3d) respectively

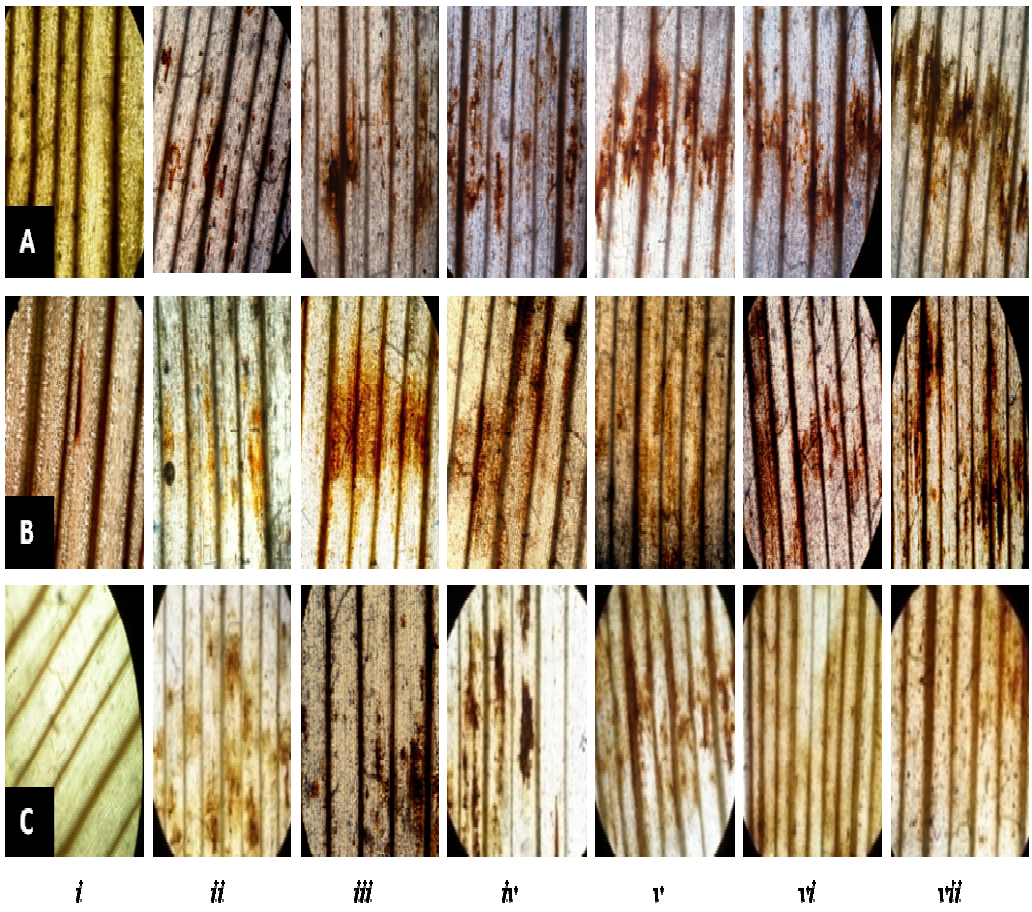

Figure 75. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following salt stress: A–PBW 343, B–SO & C–KD; i–0 mM, ii & iii–50mM (1d & 3d), iv & v–100mM (1d & 3d), vi & vii–200mM (1d & 3d) respectively

Figure 76. *In situ* detection of H_2O_2 in mid–portions of leaves of wheat following salt stress: A–MW 343, B–GY & C–GN; i–0 mM, ii & iii–50mM (1d & 3d), iv & v–100mM (1d & 3d), vi & vii–200mM (1d & 3d) respectively

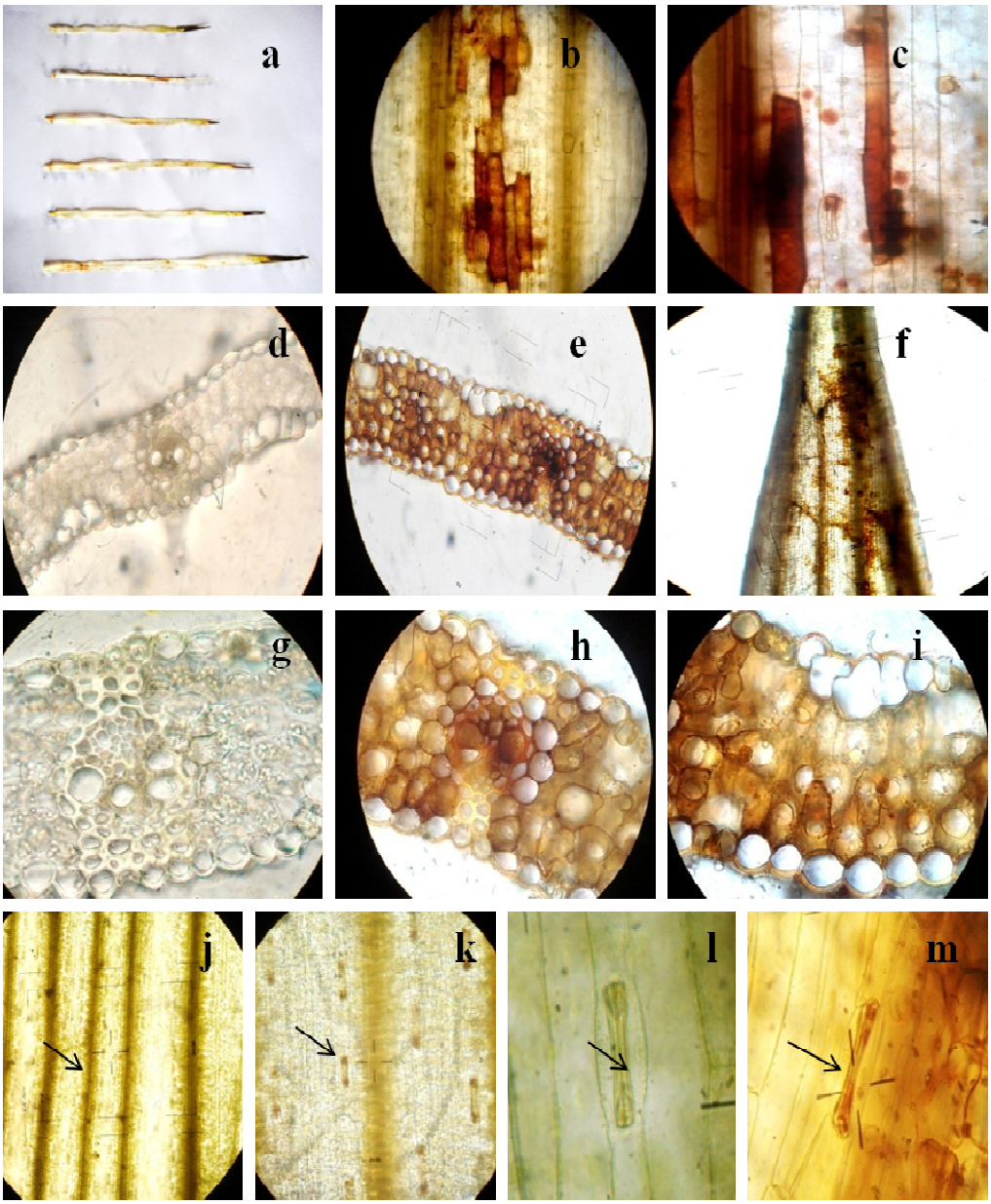

Figure 77. DAB stained sites shown as dark brown spots in: – a=Leaves; b & c= group of cells in 10X; d & e= t.s. of control leaf and drought stressed leaf respectively; f =tip portion of leaf; g & h = t.s. at the vascular bundle site of the leaf; i=t.s. of leaf showing region around bulliform cells; j& k= stomatal sites in 10X; l & m= stomatal sites showing DAB sites in 45X

4.12. Variations in levels of non–enzymatic antioxidants

4.12.1. Drought

Carotenoids, ascorbic acid and α–tocopherol (Vit E) three of the non–enzymatic antioxidants in plants increased significantly in all the nine varieties following drought stress. Accumulation of carotenoids in the leaves, during drought showed an initial enhancement in all the varieties followed by a decrease after 3 days in varieties MW, GY, LV and SO and after 6 days in KD, KW, GN, UP 2752 and PBW 343 (Table 90). However, ascorbate accumulation in the leaf was enhanced in all nine varieties even after 9 days of drought stress (Table 91). Accumulation of α–tocopherol in the leaves of the drought stressed plant in general increased during the 3rd day of drought except in case of GY where it decreased, however, in KW, LV, PBW 343 and SO the accumulation of Vit E decreased after 6 days of drought and after 9 days in MW, GY and KD (Table 92). In case of UP 2752 and GN however, the content of Vit E increased on the 9th day of stress.

Table 90. Carotenoids in the leaves of wheat varieties subjected to water stress

Varieties	Content of carotenoids			
	Days of Stress			
	0D	**3D**	**6D**	**9D**
MW	0.043±0.004^{a}	0.056±0.002^{a}	0.052±0.006^{a}	0.040±0.003^{a}
GY	0.044±0.006^{a}	0.067±0.002^{a}	0.041±0.002^{a}	0.030±0.001^{a}
KD	0.044±0.002^{a}	0.057±0.001^{b}	0.066±0.003^{b}	0.050±0.004^{a}
GN	0.042±0.003^{a}	0.063 ±.002^{b}	0.067±0.004^{b}	0.052±0.002^{a}
KW	0.048±0.002^{a}	0.051±0.001^{b}	0.061±0.005^{b}	0.055±0.002^{b}
LV	0.049±0.003^{a}	0.049±0.003^{a}	0.038±0.001^{b}	0.029±0.002^{b}
UP 2752	0.050±0.001^{a}	0.062±0.003^{b}	0.052±0.002^{a}	0.039±0.001^{b}
PBW 343	0.047±0.003^{a}	0.048±0.001^{a}	0.042±0.001^{a}	0.037±0.001^{b}
SO	0.041±0.002^{a}	0.049±0.001^{a}	0.042±0.005^{a}	0.033±0.002^{a}
CD Value between treatments				=0.00698
CD Value between varieties				=0.01047

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Carotenoids = mg g^{-1} (f.m.).

Table 90 (a). ANOVA of data presented in table 90 for carotenoids

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.001217	3	0.000406	7.889312	0.000782	3.008787
Columns	0.000995	8	0.000124	2.417682	0.045081	2.355081
Error	0.001234	24	5.14E–05			
Total	0.003447	35				

Table 91. Content of ascorbate in the leaves of wheat varieties subjected to water stress

Varieties	**Ascorbate Days of Stress**			
	0D	**3D**	**6D**	**9D**
MW	12.40±0.14[a]	13.30±0.12[b]	15.20±0.15[b]	16.90±0.18[b]
GY	9.40±0.17[a]	15.40±0.02[b]	16.60±0.03[b]	17.90±0.05[b]
KD	5.50±0.11[a]	9.20±0.22[b]	18.60±0.23[b]	21.80±0.25[b]
GN	9.40±0.31[a]	10.80±0.32[b]	11.20±0.13[b]	16.30±0.21[b]
KW	11.02±0.02[a]	12.19±0.02[b]	13.44±0.04[b]	14.78±0.09[b]
LV	9.98±0.06[a]	9.90±0.01[a]	10.10±0.03[a]	11.30±0.06[b]
UP 2752	12.11±0.02[a]	12.98±0.03[b]	14.90±0.05[b]	15.51±0.04[b]
PBW 343	11.96±0.02[a]	13.15±0.02[b]	13.51±0.02[b]	15.81±0.03[b]
SO	11.07±0.04[a]	12.14±0.05[b]	13.89±0.01[b]	14.65±0.03[b]
CD Value between treatments				=2.19148
CD Value between varieties				=3.28722

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Ascorbate (mg g^{-1} d.m).

Table 91 (a). ANOVA of data presented in table 91 for ascorbate

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	169.6303	3	56.54344	11.14477	8.95E–05	3.008787
Columns	61.00421	8	7.625526	1.502999	0.208204	2.355081
Error	121.7649	24	5.073539			
Total	352.3995	35				

Table 92. Content of Vit E in the leaf of nine water stressed varieties of wheat

Varieties	Vit E (*α*–tocopherol) Days of Stress			
	0D	**3D**	**6D**	**9D**
MW	0.314±0.12[a]	0.449±0.12[b]	0.469±0.13[b]	0.468±0.18[b]
GY	0.549±0.19[a]	0.393±0.19[b]	0.520±0.09[a]	0.495±0.09[b]
KD	0.511±0.12[a]	0.578±0.21[b]	0.630±0.14[b]	0.588±0.18[b]
GN	0.370±0.20[a]	0.577±0.11[b]	0.371±0.11[a]	0.407±0.09[b]
KW	0.397±0.13[a]	0.456±0.13[b]	0.398±0.09[a]	0.381±0.10[b]
LV	0.392±0.15[a]	0.401±0.14[a]	0.343±0.17[b]	0.319±0.16[b]
UP 2752	0.295±0.10[a]	0.390±0.15[b]	0.366±0.10[b]	0.410±0.15[b]
PBW 343	0.318±0.15[a]	0.423±0.17[b]	0.410±0.12[b]	0.397±0.13[b]
SO	0.341±0.17[a]	0.389±0.16[b]	0.352±0.18[a]	0.334±0.11[a]
CD Value between treatments				=0.05347
CD Value between varieties				=0.08021

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Vit E = mg g^{-1} (d.m).

Table 92 (a). ANOVA of data presented in table 92 for Vit E

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.018544	3	0.006181	2.046303	0.134157	3.008787
Columns	0.164413	8	0.020552	6.803613	0.000114	2.355081
Error	0.072497	24	0.003021			
Total	0.255453	35				

4.12.2. Salinity

Carotenoid content showed a general increase in its accumulation with the increase in the concentration and duration of salt stress in all the varieties but with prolonged days of salt stress and increasing concentration of salt the accumulation declined at 100mM concentration in case of MW, GY, LV and SO and at 200mM concentration in case of KD, GN, KW, UP 2752 and PBW 343 (Table 93). Accumulation of ascorbic acid increased at all periods of salt stress and enhanced with increasing concentration of salt in all the varieties (Table 94)

with the highest accumulation noted in case of GN, KW, KD, UP 2752 and PBW 343 at 200mM concentration of salt. Vit E content in the salt stressed leaf increased with the increase in the days and concentration of salt stress initially but declined at higher concentration and duration of salt stress in case of MW, GY, LV and SO and in the other five varieties it declined at the salt concentration of 200mM after a steady increase (Table 95).

Table 93. Carotenoids in the leaves of nine wheat varieties subjected to salt stress

Varieties	Day of sampling	Carotenoid Concentration of Salt			
		0mM	50mM	100mM	200mM
MW	**1d**	0.043±0.004[a]	0.051±0.002[b]	0.049±0.001[b]	0.042±0.004[a]
	3d	0.043±0.004[a]	0.053±0.001[b]	0.051±0.002[b]	0.038±0.008[b]
GY	**1d**	0.044±0.006[a]	0.061±0.004[b]	0.042±0.010[b]	0.032±0.003[b]
	3d	0.044±0.006[a]	0.062±0.005[b]	0.043±0.009[a]	0.030±0.005[b]
KD	**1d**	0.044±0.002[a]	0.058±0.005[b]	0.069±0.008[b]	0.052±0.004[b]
	3d	0.045±0.001[a]	0.059±0.001[b]	0.072±0.002[b]	0.049±0.005[b]
GN	**1d**	0.042±0.002[a]	0.065±0.009[a]	0.069±0.003[b]	0.053±0.007[b]
	3d	0.043±0.003[a]	0.066±0.006[b]	0.071±0.004[b]	0.050±0.002[b]
KW	**1d**	0.044±0.002[a]	0.057±0.004[a]	0.064±0.006[b]	0.052±0.006[b]
	3d	0.045±0.001[a]	0.061±0.003[b]	0.065±0.006[b]	0.051±0.002[b]
LV	**1d**	0.048±0.002[a]	0.049±0.002[a]	0.039±0.004[b]	0.028±0.004[b]
	3d	0.049±0.003[a]	0.052±0.001[b]	0.037±0.009[b]	0.025±0.008[b]
UP 2752	**1d**	0.050±0.001[a]	0.058±0.006[b]	0.060±0.005[b]	0.041±0.002[b]
	3d	0.050±0.001[a]	0.059±0.007[b]	0.061±0.008[b]	0.043±0.003[b]
PBW 343	**1d**	0.047±0.003[a]	0.049±0.007[b]	0.056±0.006[b]	0.042±0.005[b]
	3d	0.048±0.002[a]	0.052±0.004[b]	0.041±0.001[b]	0.028±0.001[b]
SO	**1d**	0.041±0.002[a]	0.048±0.003[b]	0.043±0.009[b]	0.034±0.006[b]
	3d	0.041±0.002[a]	0.052±0.004[b]	0.041±0.001[b]	0.028±0.009[b]
CD Value between treatments (1 day/3 day)				=0.00622 / 0.0065	
CD Value between varieties				=0.00932 / 0.00975	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Carotenoids = mg g^{-1} (f.m.).

Table 93 (a). ANOVA of data presented in table 93 for carotenoids during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.001268	3	0.000423	10.35678	0.000146	3.008787
Columns	0.001196	8	0.00015	3.663511	0.006356	2.355081
Error	0.00098	24	4.08E–05			
Total	0.003445	35				

Table 93 (b). ANOVA of data presented in table 93 for carotenoids during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.001971	3	0.000657	14.73359	1.19E–05	3.008787
Columns	0.001388	8	0.000173	3.890144	0.004556	2.355081
Error	0.00107	24	4.46E–05			
Total	0.004429	35				

Table 94. Content of ascorbate in the leaves of nine wheat varieties subjected to salt stress

Varieties	**Day of sampling**	**Ascorbate Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	12.40±0.06[a]	12.90±0.04[b]	15.80±0.02[b]	16.50±0.03[b]
	3d	12.50±0.05[a]	13.50±0.06[b]	13.80±0.03[b]	17.80±0.02[b]
GY	**1d**	9.40±0.08[a]	13.10±0.03[b]	15.50±0.02[b]	17.10±0.03[b]
	3d	9.30±0.05[a]	14.90±0.08[b]	15.60±0.01[b]	17.50±0.01[b]
KD	**1d**	5.50±0.04[a]	15.20±0.05[b]	16.80±0.03[b]	19.20±0.05[b]
	3d	5.40±0.05[a]	17.40±0.04[b]	18.50±0.05[b]	24.10±0.06[b]
GN	**1d**	9.40±0.05[a]	15.90±0.06[a]	20.10±0.08[b]	23.00±0.05[b]
	3d	9.50±0.08[a]	17.80±0.05[b]	22.20±0.08[b]	23.90±0.06[b]
KW	**1d**	11.02±0.09[a]	14.20±0.02[a]	19.40±0.09[b]	23.00±0.04[b]
	3d	11.40±0.06[a]	18.10±0.03[b]	21.00±0.05[b]	23.50±0.02[b]
LV	**1d**	9.98±0.07[a]	11.20±0.01[b]	13.90±0.05[b]	17.20±0.01[b]
	3d	10.00±0.03[a]	11.32±0.02[b]	14.50±0.01[b]	18.10±0.09[b]
UP 2752	**1d**	12.11±0.02[a]	13.50±0.05[b]	16.60±0.01[b]	21.20±0.04[b]
	3d	12.00±0.05[a]	14.50±0.04[b]	17.90±0.04[b]	23.00±0.04[b]
PBW 343	**1d**	11.96±0.03[a]	15.00±0.03[b]	19.20±0.05[b]	20.00±0.06[b]
	3d	12.00±0.04[a]	15.60±0.01[b]	19.40±0.04[b]	22.10±0.02[b]
SO	**1d**	11.40±0.01[a]	13.40±0.05[a]	17.10±0.08[b]	17.80±0.07[b]
	3d	11.35±0.08[a]	13.90±0.08[b]	18.50±0.02[b]	18.80±0.01[b]
CD Value between treatments (1 day/3 day)				=1.65055 / 2.03666	
CD Value between varieties (1 day/3 day)				=2.47582 / 2.1602	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Ascorbate (mg g^{-1} d.m).

Table 94 (a). ANOVA of data presented in table 94 for ascorbate during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	425.0115	3	141.6705	49.22507	2.1E–10	3.008787
Columns	68.27354	8	8.534192	2.965305	0.01857	2.355081
Error	69.07237	24	2.878015			
Total	562.3574	35				

Table 94 (b). ANOVA of data presented in table 94 for ascorbate during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	545.3745	3	181.7915	41.48591	1.18E–09	3.008787
Columns	103.7564	8	12.96955	2.959729	0.018735	2.355081
Error	105.1682	24	4.382006			
Total	754.2991	35				

Table 95. Content of Vit E (α–tocopherol) in the leaf of nine salt stressed varieties of wheat

Varieties	**Day of sampling**	**Vit E (α–tocopherol) Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	0.314±0.14^{a}	0.430±0.13^{b}	0.421±0.11^{b}	0.406±0.12^{b}
	3d	0.320±0.10^{a}	0.415±0.12^{b}	0.385±0.12^{b}	0.377±0.10^{b}
GY	**1d**	0.549±0.11^{a}	0.561±0.12^{a}	0.434±0.11^{b}	0.411±0.11^{b}
	3d	0.550±0.12^{a}	0.542±0.19^{a}	0.412±0.10^{b}	0.394±0.10^{b}
KD	**1d**	0.511±0.15^{a}	0.595±0.13^{b}	0.641±0.19^{b}	0.595±0.11^{b}
	3d	0.515±0.16^{a}	0.602±0.13^{b}	0.632±0.11^{b}	0.584±0.10^{b}
GN	**1d**	0.370±0.10^{a}	0.551±0.11^{b}	0.589±0.12^{b}	0.521±0.16^{b}
	3d	0.381±0.11^{a}	0.562±0.10^{b}	0.551±0.11^{b}	0.499±0.18^{b}
KW	**1d**	0.397±0.19^{a}	0.498±0.11^{b}	0.587±0.15^{b}	0.556±0.19^{b}
	3d	0.410±0.15^{a}	0.521±0.12^{b}	0.564±0.14^{b}	0.502±0.12^{b}
LV	**1d**	0.392±0.11^{a}	0.405±0.12^{a}	0.371±0.16^{a}	0.354±0.11^{b}
	3d	0.385±0.12^{a}	0.411±0.15^{a}	0.356±0.14^{a}	0.314±0.10^{b}
UP 2752	**1d**	0.295±0.11^{a}	0.445±0.18^{b}	0.526±0.12^{b}	0.468±0.19^{b}
	3d	0.310±0.11^{a}	0.465±0.16^{b}	0.505±0.11^{b}	0.455±0.16^{b}
PBW 343	**1d**	0.318±0.15^{a}	0.431±0.10^{b}	0.499±0.10^{b}	0.410±0.13^{b}
	3d	0.320±0.10^{a}	0.442±0.10^{b}	0.478±0.19^{b}	0.397±0.14^{b}
SO	**1d**	0.341±0.10^{a}	0.366±0.19^{a}	0.351±0.14^{a}	0.322±0.11^{b}
	3d	0.343±0.19^{a}	0.389±0.12^{b}	0.329±0.17^{a}	0.298±0.12^{b}
CD Value between treatments (1 day/3 day)				=0.05478 / 0.05069	
CD Value between varieties (1 day/3 day)				=0.08217 / 0.07604	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Vit E = mg g^{-1} (d.m).

Table 95 (a). ANOVA of data presented in table 95 for Vit E during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.05637	3	0.01879	5.927749	0.003559	3.008787
Columns	0.189428	8	0.023678	7.469881	5.56E–05	2.355081
Error	0.076077	24	0.00317			
Total	0.321875	35				

Table 95 (b). ANOVA of data presented in table 95 for Vit E during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	0.046055	3	0.015352	5.655056	0.004461	3.008787
Columns	0.197342	8	0.024668	9.086781	1.14E–05	2.355081
Error	0.065152	24	0.002715			
Total	0.308549	35				

4.13. Effect of water stress and salinity on total antioxidative activities of wheat

The total antioxidative activity in the leaf following water stress increased with the increase in the duration of withholding water in all varieties with a slight decline observed during the 3rd day in case of MW (Table 96). In GN, KD, UP 2752 and PBW 343 the total antioxidative activity continued to increase even after 9 days of drought however, it declined on the 9th day of water stress in case of KW, MW, GY, LV and SO. In KD, GN, UP 2752 and PBW 343 the total antioxidative activity was significantly higher than the other varieties. The percent inhibition of DPPH i.e. the total antioxidant activity in the leaf of salt stressed plants increased with the increase in the concentration and duration of stress but decreased at the concentration of 100mM in case of MW, LV, SO and GY (Table 97). In KD, GN, KW, UP 2752 and PBW 343 the total antioxidative activity was significantly high even at 200mM

concentration and the highest value for the content of DPPH were observe in these varieties.

The total antioxidant activity following both drought and salt was compared and it was evident that total antioxidant activity during salt stress in the tested varieties in our study was higher than during drought stress.

Table 96. Total antioxidant activity in wheat varieties during water stress

Varieties	DPPH (Total antioxidant activity) Days of Stress			
	0D	**3D**	**6D**	**9D**
MW	8.434±0.01[a]	4.494±0.06[b]	10.526±0.06[b]	3.330±0.05[b]
GY	4.558±0.05[a]	5.470±0.03[b]	9.540±0.08[b]	4.110±0.04[b]
KD	8.805±0.03[a]	21.429±0.05[b]	29.710±0.04[b]	56.369±0.09[b]
GN	4.828±0.06[a]	23.611±0.09[b]	26.829±0.06[b]	36.021±0.07[b]
KW	8.920±0.08[a]	15.245±0.06[b]	11.630±0.08[b]	9.638±0.06[b]
LV	5.210±0.06[a]	10.400±0.05[b]	7.890±0.06[b]	6.881±0.01[a]
UP 2752	6.878±0.09[a]	19.220±0.07[b]	21.430±0.04[b]	39.530±0.01[b]
PBW 343	5.758±0.06[a]	11.501±0.03[b]	15.300±0.03[b]	22.240±0.02[b]
SO	7.120±0.04[a]	10.820±0.05[b]	9.887±0.09[a]	4.023±0.08[b]
CD Value between treatments				=8.13063
CD Value between varieties				=12.1959

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Total antioxidant activity = % inhibition of DPPH absorbance

Table 96 (a). ANOVA of data presented in table 96 for DPPH (total antioxidant activity)

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2274.272	8	284.284	4.070697	0.003513	2.355081
Columns	859.1479	3	286.3826	4.100747	0.017508	3.008787
Error	1676.081	24	69.8367			
Total	4809.501	35				

Table 97. Total antioxidant activity in wheat varieties during salt stress

Varieties	Day of sampling	DPPH (Total antioxidant activity) Concentration of Salt			
		0mM	50mM	100mM	200mM
MW	**1d**	8.43±0.02[a]	9.20±0.02[b]	7.45±0.05[b]	4.66±0.01[b]
	3d	8.42±0.03[a]	9.50±0.02[b]	6.11±0.05[b]	3.36±0.01[b]
GY	**1d**	4.56±0.02[a]	6.56±0.01[b]	5.59±0.09[b]	4.21±0.09[a]
	3d	4.61±0.01[a]	7.78±0.03[b]	8.20±0.09[b]	5.22±0.08[a]
KD	**1d**	8.81±0.06[a]	29.36±0.05[b]	36.65±0.05[b]	55.32±0.06[b]
	3d	8.82±0.03[a]	39.55±0.06[b]	42.21±0.06[b]	50.01±0.03[b]
GN	**1d**	4.83±0.09[a]	28.88±0.04[b]	37.87±0.08[b]	54.95±0.03[b]
	3d	4.84±0.05[a]	35.36±0.08[b]	43.65±0.04[b]	48.64±0.05[b]
KW	**1d**	8.92±0.06[a]	19.98±0.02[b]	29.80±0.06[b]	36.60±0.04[b]
	3d	8.91±0.04[a]	21.20±0.01[b]	33.30±0.04[b]	38.90±0.08[b]
LV	**1d**	5.21±0.02[a]	8.50±0.06[b]	6.99±0.02[b]	4.60±0.09[a]
	3d	5.19±0.03[a]	8.21±0.06[b]	4.87±0.03[a]	2.98±0.09[b]
UP 2752	**1d**	6.88±0.01[a]	16.98±0.04[b]	19.50±0.05[b]	29.78±0.02[b]
	3d	6.92±0.05[a]	18.90±0.01[b]	23.50±0.06[b]	33.30±0.01[b]
PBW 343	**1d**	5.76±0.09[a]	15.68±0.08[b]	21.20±0.04[b]	28.80±0.03[b]
	3d	5.76±0.08[a]	19.60±0.07[b]	22.74±0.04[b]	32.70±0.02[b]
SO	**1d**	7.12±0.08[a]	11.80±0.07[b]	8.78±0.05[a]	5.30±0.02[b]
	3d	6.99±0.01[a]	13.35±0.01[b]	9.10±0.03[b]	3.65±0.05[b]
CD Value between treatments (1 day/3 day)				=8.61954 / 8.59014	
CD Value between varieties (1 day/3 day)				=12.9293 / 12.8852	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in't' test. Results are expressed as the mean of three replicates (10 plants each). Total antioxidant activity = % inhibition of DPPH absorbance

Table 97(a). ANOVA of data presented in table 97 for DPPH during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1565.217	3	521.7388	6.647364	0.001998	3.008787
Columns	3698.57	8	462.3213	5.890337	0.000325	2.355081
Error	1883.714	24	78.48809			
Total	7147.501	35				

Table 97(b). ANOVA of data presented in table 97 for DPPH during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1629.62	3	543.2066	6.968337	0.001557	3.008787
Columns	4421.348	8	552.6685	7.089716	8.32E–05	2.355081
Error	1870.885	24	77.95354			
Total	7921.853	35				

4.14. Effect of stresses on Na^+ and K^+ content

Na^+ and K^+ content in case of both water and salt stress increased significantly with the onset of stress treatments. Following water and salt stress treatments the content of Na^+ in case of roots was much higher than that of leaf in all varieties whereas K^+ content was higher in the leaf than the roots during the stress.

Table 98. Effect of drought on sodium (Na^+) contents in wheat varieties

Varieties	**Part used**	**Content of Na^+ Days of Stress**			
		0D	**3D**	**6D**	**9D**
MW	Leaf	6.21±0.11 [a]	8.11±0.22 [a]	9.22±0.25 [b]	10.14±0.22 [b]
	Root	4.24±0.21 [a]	25.63±0.32 [b]	61.11±0.23 [b]	70.22±0.23 [b]
GY	Leaf	7.11±0.33 [a]	8.52±0.15 [a]	9.98±0.31 [a]	12.12±0.24 [b]
	Root	5.12±0.15 [a]	35.58±0.14 [b]	51.34±0.28 [b]	68.97±0.19 [b]
KD	Leaf	4.51±0.36 [a]	6.12±0.22 [b]	8.74±0.31 [b]	9.01±0.18 [b]
	Root	3.52±0.25 [a]	15.14±0.23 [b]	39.14±0.28 [b]	59.61±0.11 [b]
GN	Leaf	4.88±0.12 [a]	6.32±0.33 [b]	8.99±0.19 [b]	9.25±0.22 [b]
	Root	3.87±0.32 [a]	16.96±0.36 [b]	37.64±0.17 [b]	58.68±0.29 [b]
KW	Leaf	5.01±0.12 [a]	7.11±0.31 [b]	9.31±0.31 [b]	10.21±0.26 [b]
	Root	4.11±0.21 [a]	16.32±0.19 [b]	41.02±0.23 [b]	60.13±0.31 [b]
LV	Leaf	7.01±0.31 [a]	10.22±0.21 [a]	15.64±0.31 [a]	17.99±0.28 [b]
	Root	6.88±0.21 [a]	36.84±0.22 [b]	55.94±0.29 [b]	75.31±0.19 [b]
UP 2752	Leaf	5.99±0.22 [a]	6.55±0.23 [a]	8.85±0.28 [b]	12.41±0.19 [b]
	Root	4.52±0.33 [a]	19.98±0.31 [b]	49.98±0.18 [b]	65.24±0.21 [b]
PBW 343	Leaf	6.47±0.16 [a]	6.58±0.32 [a]	9.21±0.16 [b]	12.33±0.22 [b]
	Root	5.13±0.25 [a]	20.01±0.26 [b]	50.11±0.19 [b]	66.56±0.23 [b]
SO	Leaf	5.89±0.27 [a]	7.98±0.29 [a]	11.34±0.21 [b]	15.91±0.19 [b]
	Root	5.31±0.19 [a]	21.56±0.28 [b]	57.34±0.23 [b]	62.32±0.28 [b]
CD Value between treatments (leaf/root)				=1.24397 / 4.30572	
CD Value between varieties (leaf/root)				=1.86595 / 6.45857	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in't' test. Results are expressed as the mean of three replicates (10 plants each). Na^+ contents = mg g^{-1} (d.w.).

Table 98(a). ANOVA of data presented in table 98 for content of Na^+ (leaf)

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	95.37465	8	11.92183	7.292735	6.7E–05	2.355081
Columns	207.793	3	69.26435	42.36988	9.59E–10	3.008787
Error	39.23411	24	1.634754			
Total	342.4018	35				

Table 98(b). ANOVA of data presented in table 98 for content of Na^+ (root)

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	870.1735	8	108.7717	5.553788	0.000489	2.355081
Columns	19558.51	3	6519.504	332.8802	1.11E–19	3.008787
Error	470.0432	24	19.58513			
Total	20898.73	35				

In case of water stress the increase in the days of withholding water resulted in the increase in the content of Na^+ in both leaf and root (Table 98). In MW, GY, LV and SO the increase in the Na^+ content was more than the increase in the other varieties following stress and the content of Na^+ in the roots were higher. In case of salt stress in the leaf (Table 99) and root (Table 100) of wheat varieties similar results were obtained as in the case of water stress. The increase in the content of Na^+ was much more in the roots than the leaf and four varieties i.e. MW, GY, LV and SO showed the highest content of Na^+ in both the leaf and roots with increasing concentration and duration of salt stress.

In case of KW, GN, KD, UP 2752 and PBW 343 Na^+ content increased but comparatively it was lesser than the other varieties. K^+ content in the water stressed leaf was more than that of the roots. K^+ content in both leaf and root increased significantly during the initial phase of water stress but later with prolonged stress, the content of K^+ in both leaf and root declined; the decline in case of MW, GY, LV and SO was much more than compared to the other five varieties where the decrease in the content of K^+ was lesser (Table 101). Similar trend was observe during salt stress where the K^+ content of the leaf (Table 102) and root (Table 103) initially showed an increase which

declined in the higher concentration of salt and prolonged period of salt stress. The decline in K^+ content of the leaf and root during salinity stress in case of MW, GY, LV and SO was much more pronounced than the varieties GN, KD, KW, UP 2752 and PBW 343. The K^+ content in case of leaf was more than that of root during the stress treatments.

Table 99. Effect of salt stress on sodium (Na^+) contents in the leaf of wheat varieties

Varieties	Days of sampling	Content of Na^+ Concentration of Salt			
		0mM	50mM	100mM	200mM
MW	**1d**	6.21±0.21 [a]	7.11±0.29 [b]	10.13±0.19 [a]	14.24±0.21 [b]
	3d	6.19±0.11 [a]	8.97±0.18 [a]	15.65±0.25 [b]	19.98±0.22 [b]
GY	**1d**	7.11±0.32 [a]	6.97±0.25 [a]	11.51±0.16 [a]	17.64±0.31 [b]
	3d	7.10±0.11 [a]	8.97±0.19 [a]	16.52±0.31 [b]	18.99±0.18 [b]
KD	**1d**	4.51±0.21 [a]	6.89±0.31 [a]	11.11±0.25 [b]	13.21±0.15 [b]
	3d	4.49±0.22 [a]	7.32±0.32 [b]	11.99±0.26 [b]	15.22±0.28 [b]
GN	**1d**	4.88±0.19 [a]	6.12±0.24 [a]	9.94±0.32 [b]	10.99±0.35 [b]
	3d	4.89±0.23 [a]	6.25±0.25 [b]	10.01±0.18 [b]	12.02±0.24 [b]
KW	**1d**	5.01±0.31 [a]	7.01±0.36 [a]	12.21±0.12 [b]	13.99±0.16 [b]
	3d	5.09±0.12 [a]	7.22±0.11 [b]	12.52±0.22 [b]	14.32±0.18 [b]
LV	**1d**	7.01±0.19 [a]	7.85±0.22 [a]	18.84±0.34 [b]	21.01±0.19 [b]
	3d	6.99±0.25 [a]	7.96±0.24 [a]	19.98±0.17 [b]	22.10±0.24 [b]
UP 2752	**1d**	5.99±0.32 [a]	6.95±0.21 [a]	12.64±0.23 [b]	15.33±0.25 [b]
	3d	6.01±0.24 [a]	7.23±0.18 [a]	14.65±0.16 [b]	16.64±0.12 [b]
PBW 343	**1d**	6.47±0.31 [a]	7.12±0.17 [a]	12.32±0.35 [b]	15.97±0.33 [b]
	3d	6.45±0.28 [a]	7.56±0.22 [a]	13.99±0.22 [b]	17.52±0.23 [b]
SO	**1d**	5.89±0.24 [a]	7.02±0.24 [a]	14.33±0.35 [b]	18.85±0.12 [b]
	3d	5.91±0.12 [a]	7.22±0.16 [a]	15.22±0.31 [b]	18.91±0.16 [b]
CD Value between treatments (1 day/3 day)				=1.48128 / 1.37217	
CD Value between varieties (1 day/3 day)				=2.22192 / 2.05825	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at $P \leq 0.01$, in't' test. Results are expressed as the mean of three replicates (10 plants each). Na^+ contents = mg g^{-1} (d.w.).

Table 99(a). ANOVA of data presented in table 99 for content of Na^+ during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	87.06095	8	10.88262	4.694859	0.00148	2.355081
Columns	579.7651	3	193.255	83.37194	7.84E–13	3.008787
Error	55.63167	24	2.317986			
Total	722.4577	35				

Table 99(b). ANOVA of data presented in table 99 for content of Na^+ during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	109.0096	8	13.6262	6.850532	0.000108	2.355081
Columns	799.5327	3	266.5109	133.9876	4.02E–15	3.008787
Error	47.73773	24	1.989072			
Total	956.2801	35				

Table 100. Effect of salt stress on sodium (Na^+) contents in the root of wheat varieties

Varieties	**Days of sampling**	**Content of Na^+ Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	4.24±0.15 [a]	28.63±0.25 [b]	59.98±0.28 [b]	65.66±0.19 [b]
	3d	4.21±0.14 [a]	29.88±0.32 [b]	62.32±0.19 [b]	66.54±0.18 [b]
GY	**1d**	5.12±0.25 [a]	30.01±0.21 [b]	58.85±0.19 [b]	66.53±0.22 [b]
	3d	5.16±0.16 [a]	32.21±0.13 [b]	60.13±0.23 [b]	68.83±0.15 [b]
KD	**1d**	3.52±0.24 [a]	18.55±0.12 [b]	38.65±0.24 [b]	43.52±0.32 [b]
	3d	3.48±0.38 [a]	19.99±0.31 [b]	40.22±0.18 [b]	47.77±0.21 [b]
GN	**1d**	3.87±0.25 [a]	16.68±0.15 [b]	27.73±0.17 [b]	33.32±0.16 [b]
	3d	3.82±0.34 [a]	17.89±0.23 [b]	32.42±0.21 [b]	36.64±0.15 [b]
KW	**1d**	4.11±0.15 [a]	21.21±0.12 [b]	39.96±0.32 [b]	44.41±0.22 [b]
	3d	4.09±0.25 [a]	23.39±0.36 [b]	42.24±0.31 [b]	47.89±0.33 [b]
LV	**1d**	6.88±0.34 [a]	35.44±0.24 [b]	66.45±0.23 [b]	71.34±0.28 [b]
	3d	6.91±0.22 [a]	38.88±0.12 [b]	69.19±0.19 [b]	75.64±0.19 [b]
UP 2752	**1d**	4.52±0.15 [a]	29.99±0.11 [b]	40.88±0.38 [b]	44.41±0.29 [b]
	3d	4.53±0.23 [a]	31.32±0.25 [b]	48.84±0.34 [b]	53.53±0.18 [b]
PBW 343	**1d**	5.13±0.36 [a]	28.85±0.29 [b]	39.89±0.25 [b]	43.52±0.15 [b]
	3d	5.11±0.29 [a]	30.01±0.35 [b]	47.88±0.28 [b]	51.11±0.26 [b]
SO	**1d**	5.31±0.34 [a]	28.58±0.14 [b]	55.54±0.25 [b]	59.23±0.34 [b]
	3d	5.29±0.19 [a]	29.98±0.25 [b]	57.87±0.32	61.23±0.3 [b]
CD Value between treatments (1 day/3 day)					=6.32031/16.6382
CD Value between varieties (1 day/3 day)					=9.48046/8.31911

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Na^+ contents = mg g^{-1} (d.w.).

Table 100(a). ANOVA of data presented in table 100 for content of Na^+ during 1st day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	2068.075	8	258.5094	6.125817	0.000246	2.355081
Columns	12876.58	3	4292.193	101.7108	8.79E–14	3.008787
Error	1012.8	24	42.19998			
Total	15957.45	35				

Table 100(b). ANOVA of data presented in table 100 for content of Na^+ during 3rd day of salt stress

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	1940.385	8	242.5482	7.464334	5.59E–05	2.355081
Columns	15224.61	3	5074.87	156.1773	7.06E–16	3.008787
Error	779.8628	24	32.49428			
Total	17944.86	35				

Table 101. Effect of drought on potassium (K^+) contents in wheat varieties

Varieties	Day of sampling	Content of K+ Days of Stress			
		0D	3D	6D	9D
MW	Leaf	19.87±0.22[a]	22.31±0.28[a]	16.84±0.22[b]	16.02±0.28[b]
	Root	5.55±0.23[a]	3.11±0.19[b]	3.01±0.15[b]	2.97±0.15[b]
GY	Leaf	21.11±0.12[a]	19.84±0.25[a]	18.88±0.26[b]	14.44±0.26[b]
	Root	6.15±0.25[a]	3.41±0.36[b]	3.22±0.12[b]	2.85±0.18[b]
KD	Leaf	22.45±0.19[a]	26.48±0.28[b]	23.22±0.24[a]	21.34±0.35[b]
	Root	6.87±0.39[a]	7.01±0.15[a]	6.81±0.35[a]	6.54±0.24[b]
GN	Leaf	23.01±0.25[a]	28.5±0.23[b]	25.64±0.24[a]	22.32±0.26[a]
	Root	7.01±0.18[a]	7.21±0.36[b]	6.86±0.11[b]	6.74±0.35[a]
KW	Leaf	25.14±0.32[a]	26.12±0.25[a]	22.11±0.22[b]	19.94±0.12[b]
	Root	5.97±0.25[a]	6.11±0.12[b]	5.74±0.13[a]	5.68±0.34[a]
LV	Leaf	18.97±0.36[a]	18.1±0.21[a]	16.12±0.26[b]	9.97±0.26[b]
	Root	6.01±0.25[a]	2.35±0.35[b]	2.25±0.35[b]	1.89±0.22[b]
UP 2752	Leaf	23.13±0.13[a]	24.11±0.23[a]	16.46±0.24[b]	14.54±0.35[b]
	Root	6.14±0.24[a]	6.11±0.36[a]	5.87±0.31[b]	5.64±0.29[b]
PBW 343	Leaf	19.64±0.18[a]	20.01±0.25[a]	18.21±0.22[b]	11.25±0.16[b]
	Root	5.94±0.36[a]	6.01±0.32[a]	5.89±0.15[b]	5.19±0.18[a]
SO	Leaf	19.64±0.29[a]	17.24±0.21[a]	17.01±0.28[b]	12.31±0.25[b]
	Root	5.88±0.15[a]	4.97±0.14[a]	4.23±0.39[b]	3.16±0.33[b]
CD Value between treatments (leaf/root)					=1.37195 / 0.74842
CD Value between varieties (leaf/root)					=2.52409 / 1.12263

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are the mean of three replicates (10 plants each). Potassium (K^+) contents = mg g^{-1} (d.w.).

Table 101(a). ANOVA of data presented in table 101 for content of K^+ in leaf

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	346.1905	8	43.27381	14.46643	1.79E–07	2.355081
Columns	237.038	3	79.01265	26.41393	8.89E–08	3.008787
Error	71.79181	24	2.991325			
Total	655.0203	35				

Table 101(b). ANOVA of data presented in table 101 for content of K^+ in root

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	62.92191	8	7.865238	13.2919	3.96E–07	2.355081
Columns	13.59376	3	4.531255	7.657617	0.000927	3.008787
Error	14.20156	24	0.591732			
Total	90.71723	35				

Table 102. Effect of salt stress on potassium (K^+) contents in the leaf of wheat varieties

Varieties	**Day of sampling**	**Content of K+ Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	19.87±0.12 [a]	16.31±0.23 [b]	12.32±0.19 [b]	6.63±0.12 [b]
	3d	20.01±0.21 [a]	14.51±0.21 [b]	11.21±0.18 [b]	5.65±0.24 [b]
GY	**1d**	21.11±0.32 [a]	16.55±0.25 [b]	13.66±0.27 [b]	7.22±0.16 [b]
	3d	20.08±0.31 [a]	13.96±0.30 [b]	11.35±0.11 [b]	5.99±0.31 [b]
KD	**1d**	22.45±0.25 [a]	19.25±0.26 [a]	15.35±0.26 [a]	14.55±0.28 [b]
	3d	21.02±0.19 [a]	17.55±0.17 [a]	14.85±0.25 [b]	11.35±0.11 [b]
GN	**1d**	23.01±0.15 [a]	21.03±0.14 [a]	17.41±0.16 [a]	15.64±0.19 [a]
	3d	22.98±0.24 [a]	18.91±0.12 [a]	15.42±0.13 [b]	13.63±0.21 [b]
KW	**1d**	25.14±0.29 [a]	18.96±0.22 [a]	16.55±0.19 [b]	13.64±0.26 [b]
	3d	25.03±0.32 [a]	16.52±0.31 [b]	13.25±0.26 [b]	11.05±0.24 [b]
LV	**1d**	18.97±0.31 [a]	14.52±0.15 [b]	8.64±0.25 [b]	4.36±0.14 [b]
	3d	18.94±0.25 [a]	11.01±0.14 [b]	6.67±0.23 [b]	2.99±0.16 [b]
UP 2752	**1d**	23.13±0.26 [a]	17.82±0.21 [a]	12.32±0.21 [b]	10.32±0.13 [b]
	3d	23.23±0.11 [a]	15.66±0.27 [a]	10.55±0.24 [b]	9.22±0.21 [b]
PBW 343	**1d**	19.64±0.18 [a]	16.98±0.26 [a]	12.66±0.28 [b]	10.8±0.30 [b]
	3d	19.61±0.22 [a]	14.22±0.32 [a]	11.52±0.30 [b]	9.99±0.25 [b]
SO	**1d**	19.64±0.20 [a]	15.55±0.33 [b]	12.02±0.23 [b]	8.85±0.14 [b]
	3d	19.81±0.32 [a]	13.25±0.24 [b]	11.51±0.14 [b]	8.23±0.16 [b]
CD Value between treatments (1 day/3 day)					=1.27759 / 3.80077
CD Value between varieties (1 day/3 day)					=1.91638 / 1.90038

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Potassium (K^+) contents = mg g^{-1} (d.w.).

Table 102(a). ANOVA of data presented in table 102 for content of K^+ in leaf during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	202.3759	8	25.29699	14.67075	1.57E–07	2.355081
Columns	639.7098	3	213.2366	123.6645	9.91E–15	3.008787
Error	41.38357	24	1.724315			
Total	883.4693	35				

Table 102(b). ANOVA of data presented in table 102 for content of K^+ in leaf during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	180.2946	8	22.53683	13.29098	3.96E–07	2.355081
Columns	772.1498	3	257.3833	151.7905	9.76E–16	3.008787
Error	40.69556	24	1.695648			
Total	993.14	35				

Table 103. Effect of salt stress on potassium (K^+) contents in the root wheat varieties

Varieties	**Day of sampling**	**Content of K+ Concentration of Salt**			
		0mM	**50mM**	**100mM**	**200mM**
MW	**1d**	5.55±0.21 [a]	4.21±0.21 [b]	3.98±0.22 [b]	3.21±0.13 [b]
	3d	5.61±0.23 [a]	3.89±0.28 [b]	3.52±0.23 [b]	3.02±0.19 [b]
GY	**1d**	6.15±0.32 [a]	4.13±0.31 [b]	3.87±0.16 [b]	3.11±0.25 [b]
	3d	6.22±0.30 [a]	3.85±0.25 [b]	3.09±0.21 [b]	2.99±0.16 [b]
KD	**1d**	6.87±0.22 [a]	6.72±0.30 [a]	5.98±0.31 [a]	5.13±0.25 [b]
	3d	6.91±0.26 [a]	6.65±0.19 [a]	5.45±0.29 [a]	4.98±0.31 [b]
GN	**1d**	7.01±0.27 [a]	7.12±0.18 [a]	6.55±0.26 [a]	5.81±0.20 [a]
	3d	6.99±0.24 [a]	7.06±0.26 [a]	6.25±0.27 [a]	5.56±0.11 [b]
KW	**1d**	5.97±0.19 [a]	6.15±0.27 [a]	5.87±0.15 [a]	5.32±0.29 [b]
	3d	5.92±0.15 [a]	6.30±0.32 [a]	5.52±0.24 [a]	4.96±0.27 [b]
LV	**1d**	6.01±0.21 [a]	4.52±0.31 [b]	3.25±0.18 [b]	2.85±0.26 [b]
	3d	6.11±0.28 [a]	4.26±0.29 [b]	3.11±0.31 [b]	2.71±0.25 [b]
UP 2752	**1d**	6.14±0.25 [a]	5.55±0.16 [a]	5.21±0.29 [b]	4.88±0.31 [b]
	3d	6.21±0.16 [a]	5.42±0.28 [b]	5.19±0.16 [b]	4.65±0.28 [b]
PBW 343	**1d**	5.94±0.25 [a]	5.64±0.19 [a]	5.12±0.24 [a]	4.56±0.26 [b]
	3d	5.91±0.14 [a]	5.12±0.31 [b]	4.98±0.26 [a]	4.43±0.19 [b]
SO	**1d**	5.88±0.31 [a]	4.15±0.26	3.78±0.17	3.21±0.22
	3d	5.82±0.26 [a]	4.07±0.11	3.56±0.23	3.14±0.33
CD Value between treatments (1 day/3 day)				=1.35069 / 0.48413	
CD Value between varieties (1 day/3 day)				=0.67534 / 0.72620	

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each).

Table 103(a). ANOVA of data presented in table 103 for content of K^+ in root during 1st day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	28.4324	8	3.55405	16.59665	4.78E–08	2.355081
Columns	18.15278	3	6.050926	28.25653	4.79E–08	3.008787
Error	5.139422	24	0.214143			
Total	51.7246	35				

Table 103(b). ANOVA of data presented in table 103 for content of K^+ in root during 3rd day

Source of Variation	*SS*	*df*	*MS*	*F*	*P–value*	*F crit*
Rows	29.1291	8	3.641138	14.70531	1.53E–07	2.355081
Columns	23.22841	3	7.742803	31.27054	1.85E–08	3.008787
Error	5.942567	24	0.247607			
Total	58.30008	35				

4.15. Effect of chemical pre–treatments on amelioration of drought in wheat

The various effects of drought stress were ameliorated with the use of chemicals like ABA, SA and proline. Previous reports are available where the ill–effects of temperatures were ameliorated by the use of certain chemicals. In the present study abscisic acid (ABA), salicylic acid (SA) and proline were selected as chemicals for the pre–treatments for the amelioration of drought in case of wheat.

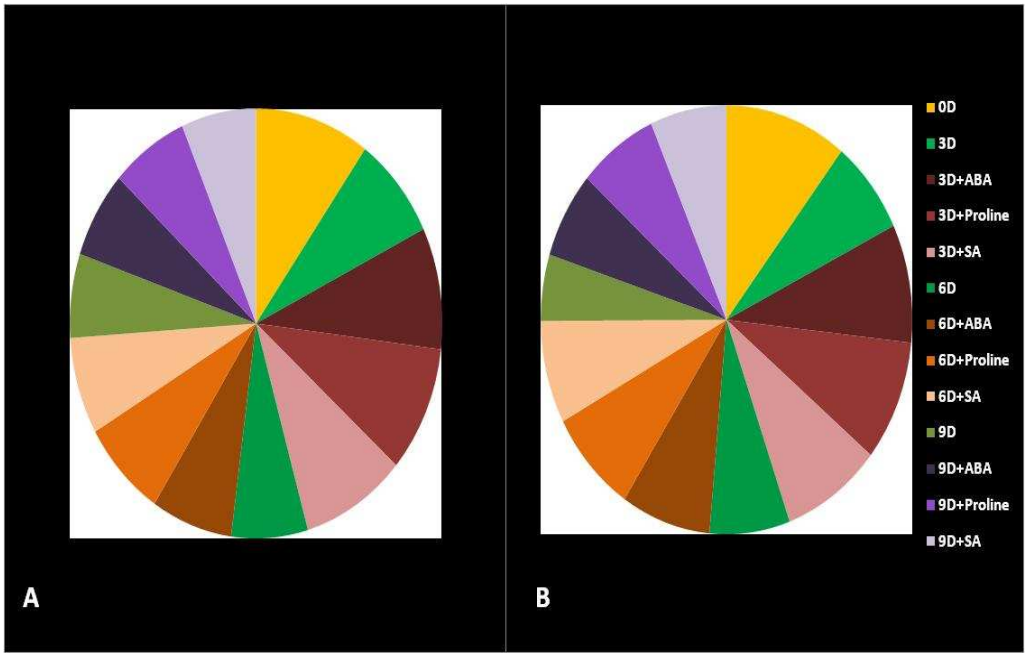

Figure 78. Relative water content (%) in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). A–GN and B–LV respectively

Two varieties where the effect of both drought and salt stress was highest and lowest were selected i.e. GN where the effect of drought stress was comparatively lowest or the variety was most tolerant and LV where the effect of drought stress was maximum amongst the tested varieties of wheat. Pre–treatments of seedlings with solutions of ABA, SA and proline forwarded by drought stress for 3, 6 and 9 days revealed that all three chemicals could provide protection against oxidative stress due to water stress in the wheat varieties. The RWC and CMS of the seedlings during drought for subsequent days after chemical pre–treatments showed an increase in both GN and LV.

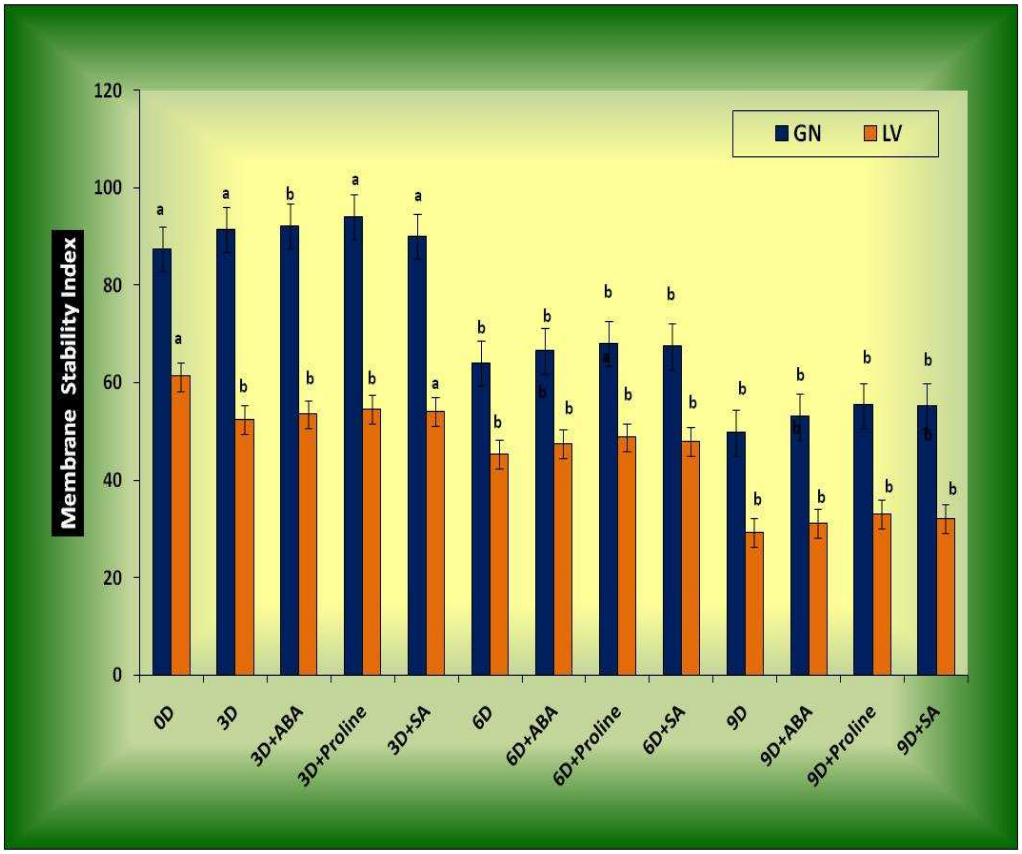

Figure 79. Cell membrane stability index in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

The increase in RWC (Figure 78) and CMS (Figure 79) of GN was higher after pre–treatment than that of LV during drought however; the RWC and CMS of LV was significantly higher after pre–treatments by the three

chemicals than without pre–treatment with the highest values observed in case of pre–treatment by proline followed by pre–treatments by ABA and SA. So, the application of pre–treatment by proline had the best effect in the amelioration of drought in case of both tolerant and susceptible variety of wheat in the present study. Lipid peroxidation after pre–treatments in GN and LV followed by drought stress showed a decrease in its value (Table 104) than the values obtained during drought without pre–treatments with the chemicals.

Table 104. Lipid peroxidation after pre–treatments in wheat following drought stress

Days of stress + Chemical treatments	Lipid Peroxidation	
	GN	LV
0D	0.40 ± 0.01^{a}	0.41 ± 0.06^{a}
3D	0.45 ± 0.05^{a}	1.54 ± 0.07^{b}
3D+ABA	0.42 ± 0.02^{a}	1.51 ± 0.05^{b}
3D+Proline	0.38 ± 0.08^{a}	1.45 ± 0.02^{b}
3D+SA	0.39 ± 0.06^{a}	1.49 ± 0.03^{b}
6D	0.67 ± 0.03^{b}	1.72 ± 0.06^{b}
6D+ABA	0.61 ± 0.03^{b}	1.68 ± 0.01^{b}
6D+Proline	0.58 ± 0.05^{b}	1.55 ± 0.01^{b}
6D+SA	0.60 ± 0.04^{b}	1.63 ± 0.02^{b}
9D	0.81 ± 0.04^{b}	2.82 ± 0.09^{b}
9D+ABA	0.77 ± 0.06^{b}	2.41 ± 0.02^{b}
9D+Proline	0.65 ± 0.03^{b}	2.01 ± 0.05^{b}
9D+SA	0.72 ± 0.03^{b}	2.33 ± 0.01^{b}

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at $P\leq0.01$, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Lipid peroxidation= μ mol g tissue^{-1} (d.m.).

The pre–treatment by the use of proline, ABA and SA could reduce lipid peroxidation in case of both GN and LV. There was a significant decrease in the peroxidation of membrane lipid as evident by the reduced value of MDA content in case of LV and this susceptible variety was able to combat the

deleterious effect of water stress. After the induction of drought treatments preceded by pre–treatments in the seedlings it was found that the content of proline (Table 105), total soluble carbohydrates (Figure 80), reducing carbohydrate and starch content (Table 106) showed a markedly lower value than the values obtained without chemical pre–treatments. Seedlings of GN and LV where no pre–treatment was done had shown gradual increase in its value with the increasing days of drought but after pre–treatments the values were significantly lower in both GN and LV.

The content of proline, total soluble sugars, reducing sugars and starch in case of LV was significantly lower during increasing days of drought following pre–treatments with chemicals and both GN and LV were more tolerant to drought and therefore the effect of drought stress was ameliorated by the use of chemicals. The effect of pre–treatment by proline showed the best result followed by ABA and SA in both the varieties.

Table 105. Proline content after chemical pre–treatment in wheat

Days of stress + Chemical treatments	Content of Proline			
	Leaf		Root	
	GN	LV	GN	LV
0D	2.00±0.07[a]	1.70±0.05[a]	0.35±0.03[a]	1.46±0.02[a]
3D	2.60±0.04[a]	1.60±0.06[a]	0.53±0.02[b]	0.31±0.01[a]
3D+ABA	1.80±0.04[a]	1.20±0.01[a]	0.31±0.05[a]	0.30±0.09[a]
3D+Proline	1.20±0.06[a]	0.87±0.06[b]	0.25±0.01[b]	0.15±0.06[b]
3D+SA	1.40±0.05[a]	1.11±0.08[b]	0.30±0.08[a]	0.25±0.03[a]
6D	6.00±0.07[b]	5.00±0.10[b]	0.24±0.04[a]	0.26±0.02[a]
6D+ABA	5.00±0.05[b]	3.87±0.06[b]	0.22±0.04[b]	0.25±0.05[a]
6D+Proline	3.50±0.02[b]	1.98±0.04[a]	0.16±0.03[b]	0.12±0.05[b]
6D+SA	4.10±0.03[b]	2.54±0.02[b]	0.18±0.03[b]	0.22±0.01[a]
9D	10.6±0.20[b]	5.10±0.02[b]	0.71±0.02[b]	0.40±0.03[b]
9D+ABA	8.70±0.03[b]	3.98±0.01[b]	0.51±0.05[b]	0.38±0.07[b]
9D+Proline	5.60±0.06[b]	2.50±0.01[b]	0.38±0.06[b]	0.32±0.05[b]
9D+SA	6.80±0.02[b]	2.03±0.05[b]	0.40±0.07[b]	0.29±0.06[b]

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in‘t’ test. Results are expressed as the mean of three replicates (10 plants each). Proline content= mg g^{-1} (d.w.).

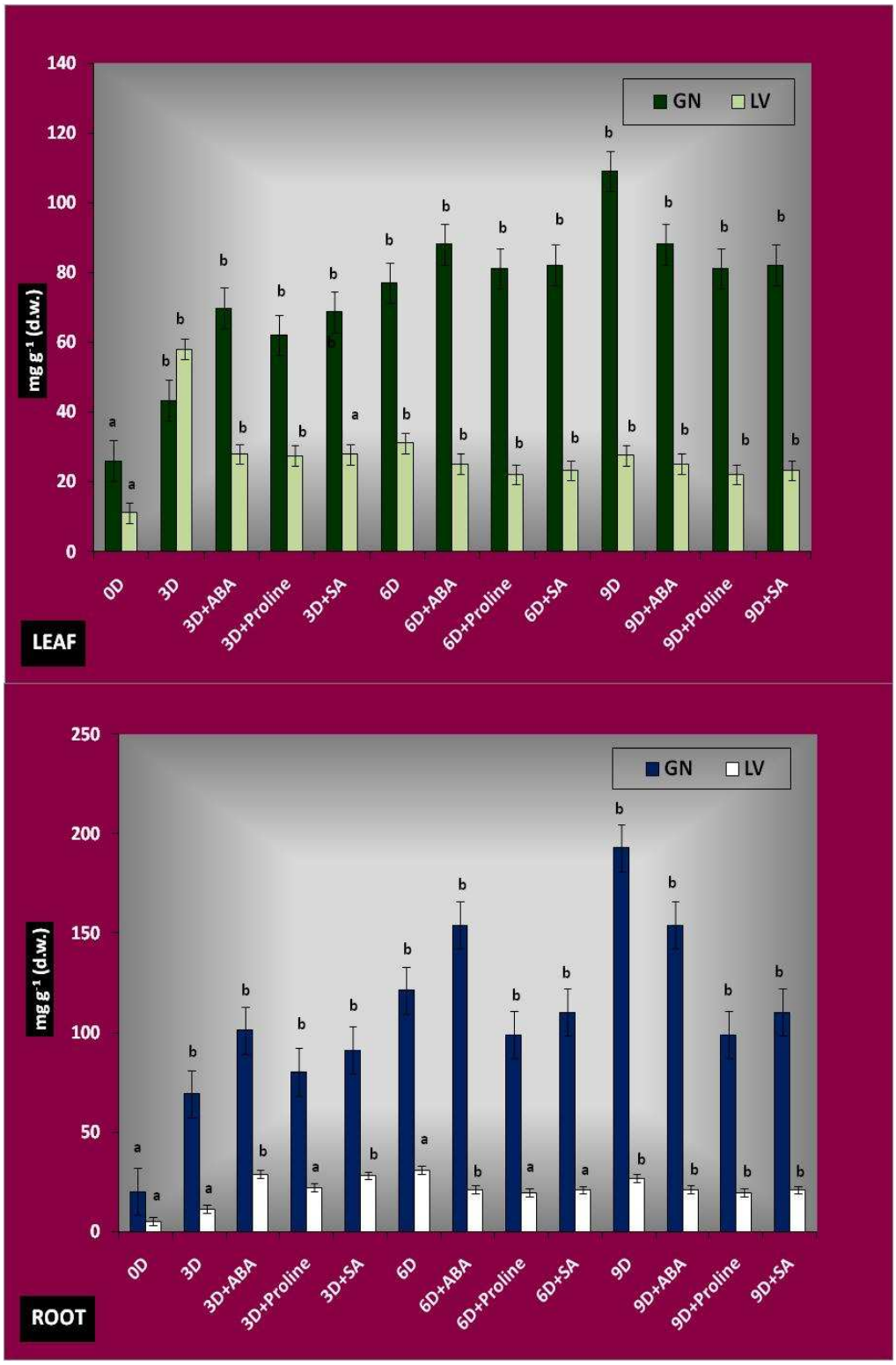

Figure 80. Content of total soluble sugar in the leaf and roots of two drought stressed wheat varieties (GN & LV) following chemical pre–treatments in leaf and root. Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

Table 106. Content of reducing sugar and starch after chemical pre–treatment in wheat

Days of stress+ Chemical treatments	Leaf		Root	
	GN	LV	GN	LV
		Reducing Sugar		
0D	1.76±0.04[a]	3.20±0.04[a]	0.11±0.06[a]	0.60±0.04[a]
3D	10.56±0.08[b]	1.07±0.05[b]	7.26±0.07[b]	0.63±0.02[a]
3D+ABA	9.50±0.02[a]	0.99±0.02[a]	5.20±0.02[a]	0.58±0.02[a]
3D+Proline	7.50±0.03[a]	0.87±0.03[b]	4.90±0.05[b]	0.51±0.02[b]
3D+SA	8.50±0.01[a]	0.81±0.03[a]	5.20±0.04[b]	0.52±0.01[a]
6D	28.70±0.07[a]	3.06±0.04[a]	23.71±0.06[a]	1.22±0.01[b]
6D+ABA	21.10±0.07[b]	2.80±0.04[b]	21.10±0.06[b]	1.10±0.07[a]
6D+Proline	19.50±0.03[b]	1.98±0.09[b]	19.80±0.05[a]	0.88±0.01[b]
6D+SA	19.10±0.02[b]	2.20±0.05[b]	19.10±0.05[b]	0.91±0.06[a]
9D	36.11±0.06[b]	4.10±0.03[b]	52.22±0.08[b]	2.15±0.06[b]
9D+ABA	30.10±0.02[b]	3.90±0.08[b]	48.80±0.05[b]	1.98±0.05[b]
9D+Proline	28.40±0.02[b]	2.99±0.07[b]	44.50±0.04[b]	1.85±0.04[b]
9D+SA	29.80±0.05[b]	3.50±0.05[b]	42.20±0.03[b]	1.89±0.01[b]
		Starch		
0D	11.52±0.02[a]	7.19±0.03[a]	11.59±0.06[a]	9.37±0.02[a]
3D	21.14±0.05[b]	14.66±0.05[b]	47.56±0.02 [b]	20.79±0.01[b]
3D+ABA	19.80±0.01[b]	11.20±0.03[a]	42.20±0.05[b]	17.50±0.01[b]
3D+Proline	17.80±0.03[b]	9.80±0.07[a]	39.90±0.06[b]	15.40±0.10[b]
3D+SA	18.10±0.04[b]	12.10±0.05[b]	40.10±0.02[b]	16.60±0.09[b]
6D	9.88±0.06[a]	8.14±0.11[b]	24.14±0.02 [b]	11.34±0.07[b]
6D+ABA	8.80±0.10[b]	7.40±0.05[a]	21.10±0.07[b]	9.90±0.05[a]
6D+Proline	6.98±0.11[b]	6.90±0.04[a]	18.90±0.03[b]	8.90±0.07[a]
6D+SA	7.80±0.08[b]	7.11±0.06[a]	19.80±0.03[b]	9.50±0.03[a]
9D	7.50±0.03[b]	7.39±0.08[a]	16.50±0.01[b]	9.67±0.02[a]
9D+ABA	6.20±0.03[b]	6.80±0.02[b]	13.2±0.08[b]	8.70±0.01[a]
9D+Proline	5.89±0.05[b]	5.80±0.03[b]	11.1±0.10[b]	8.20±0.04[b]
9D+SA	6.09±0.06[b]	6.50±0.05[b]	12.8±0.09[a]	8.40±0.03[b]

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Reducing sugar=mg g^{-1} (d.w.) and starch=mg g^{-1} (d.w.).

Total phenol content and ortho phenol content in drought stressed GN and LV following chemical pre–treatments showed a comparatively lower value than the seedlings which were untreated with chemicals (Figure 81). The lower value of total phenol and ortho phenol in case of LV with respect to the fact that LV was the most susceptible variety in the present study was very significant. The antioxidative profile of the LV and GN with prolonged drought after pre–treatment with ABA, proline and SA was better than values obtained in GN and LV seedlings were no chemical pre–treatments was done

with the chemicals. Pre–treatment with proline increased the antioxidative profile in both the varieties more than that of SA and ABA with increasing days of drought stress.

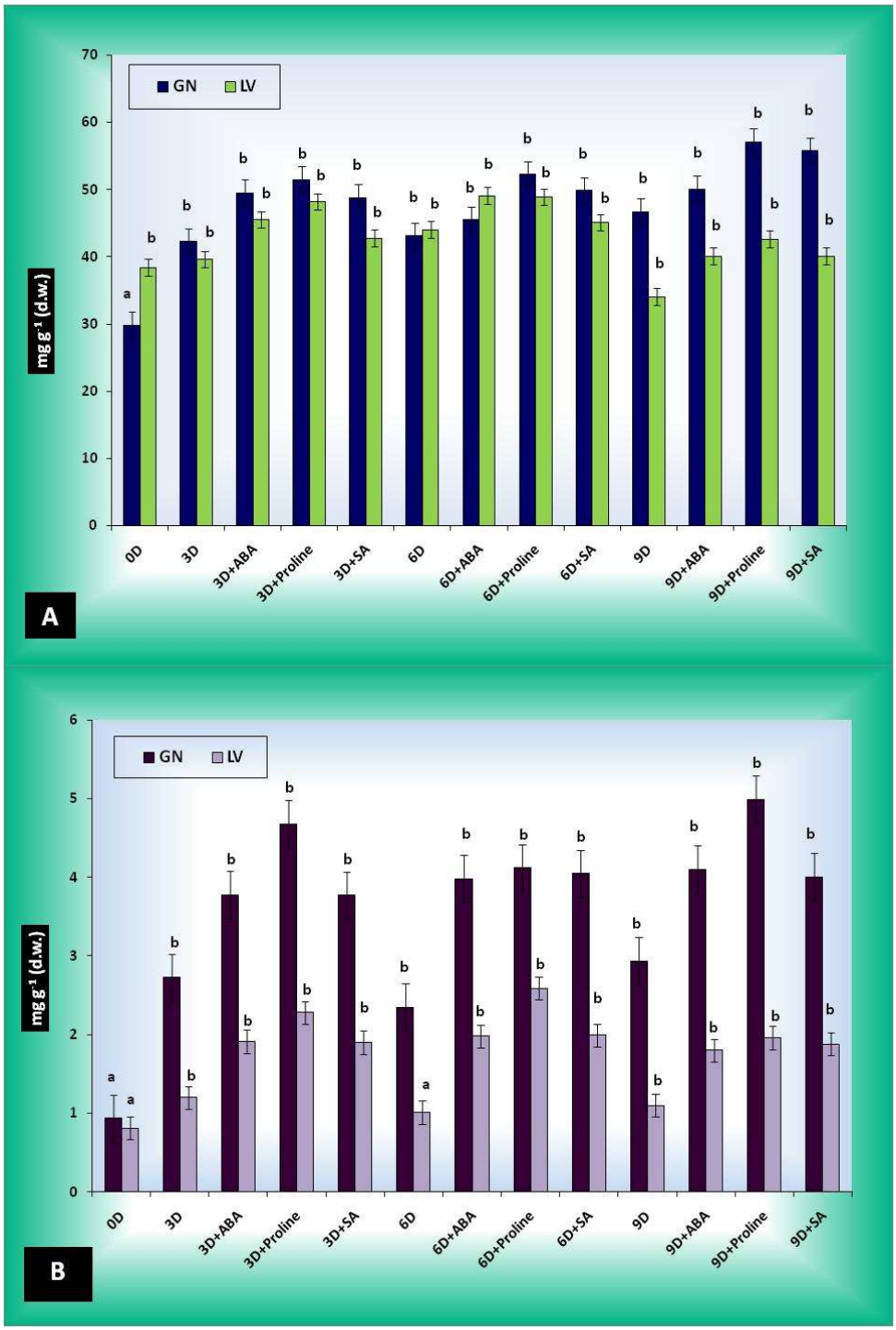

Figure 81. Total phenol content (A) and ortho phenol content (B) in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

Protein content of GN and LV following drought stress after pre–treatment with ABA, proline and SA decreased significantly during all days of water stress when compared to the corresponding values obtained in GN and LV where no treatment was done. The protein content was comparatively lower in case of LV for the initial days of stress however; on the 9th day of drought stress the change in the content of protein was significant (Table 107) during water stress following chemical pre–treatments than the LV seedlings where no such treatment was done. Similarly in GN, the protein content without chemical pre–treatments was higher during the 9th day and decreased after the chemical pre–treatments followed by water stress. The content of protein in the seedlings treated with proline showed a significant difference in both the varieties and therefore was more efficient.

Table 107. Protein content after chemical pre–treatment in wheat

Days of stress+ Chemical treatments	Protein Content			
	Leaf		Root	
	GN	LV	GN	LV
0D	288.83±0.05^{a}	129.5±0.02^{a}	55.67±0.09^{a}	62.0±0.06^{a}
3D	69.61±0.04^{a}	66.0±0.01^{b}	72.33±0.07^{b}	48.5±0.03^{b}
3D+ABA	65.10±0.04^{b}	60.20±0.05^{b}	62.40±0.01^{a}	48.0±0.09^{a}
3D+Proline	60.50±0.06^{b}	55.90±0.01^{b}	55.20±0.06^{b}	47.6±0.06^{b}
3D+SA	62.50±0.05^{b}	58.90±0.08^{b}	58.90±0.08^{a}	50.1±0.03^{a}
6D	66.78±0.02^{b}	53.2±0.04^{b}	64.33±.08^{a}	52.0±0.02^{b}
6D+ABA	57.40±0.05^{b}	55.50±0.04^{b}	60.10±0.06^{b}	50.4±0.05^{a}
6D+Proline	49.40±0.02^{b}	51.90±0.03^{b}	53.60±0.04^{a}	48.2±0.05^{b}
6D+SA	59.20±0.03^{b}	53.20±0.03^{b}	53.00±0.02^{a}	51.3±0.01^{b}
9D	68.03±0.03^{b}	51.12±0.07^{b}	71.11±0.05^{b}	61.12±0.03^{a}
9D+ABA	54.50±0.03^{b}	49.20±0.05^{b}	65.50±0.01^{b}	59.1±0.07^{b}
9D+Proline	49.40±0.06^{b}	48.70±0.06^{b}	50.60±0.01^{b}	55.4±0.05^{b}
9D+SA	56.00±0.02^{b}	50.90±0.07^{b}	61.20±0.05^{b}	58.39±0.06^{b}

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). Protein content =mg g^{-1} (d.w.).

The activity of antioxidative enzymes following drought stress where the seedlings were pre–treated separately with ABA, proline and SA enhanced remarkably in both GN and LV. The increase in the activity of the antioxidative enzymes in the leaves of these varieties enabled the plant to combat the deleterious effect of drought in regards of better scavenging of the ROS from the system. Catalase activity in case of pre–treated plants was significantly high in both GN and LV following drought stress however the increase in CAT activity was higher in GN than LV and the seedling pre–treated with proline in both the cases showed higher CAT activity than the seedling treated with SA and ABA (Figure 82). Better results were obtained in the activity of catalase during drought which enhanced significantly in GN following chemical pre–treatments than the seedlings of GN where no pre–treatment was done. Similarly enhance catalase activity was observed in LV seedlings pre–treated with chemicals than the seedlings which were not treated with increasing days of drought stress. Highest activity was observed in case of seedlings where chemical pre–treatment was done.

Figure 82. Activity of catalase in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

The activity of peroxidase increased significantly in both GN and LV with the onset of drought after pre–treatment where the POX activity increased with prolonged period of drought (Figure 83). The increase in the POX activity was significant in case of LV where the antioxidative profile of the leaves after pre–treatment enhanced with increasing days of withholding water which was significantly higher in the wheat seedling pre–treated with proline followed by SA and ABA. The activity of POX during drought enhance in both GN and where the chemical pre–treatment was done than the seedlings where no such treatment was done.

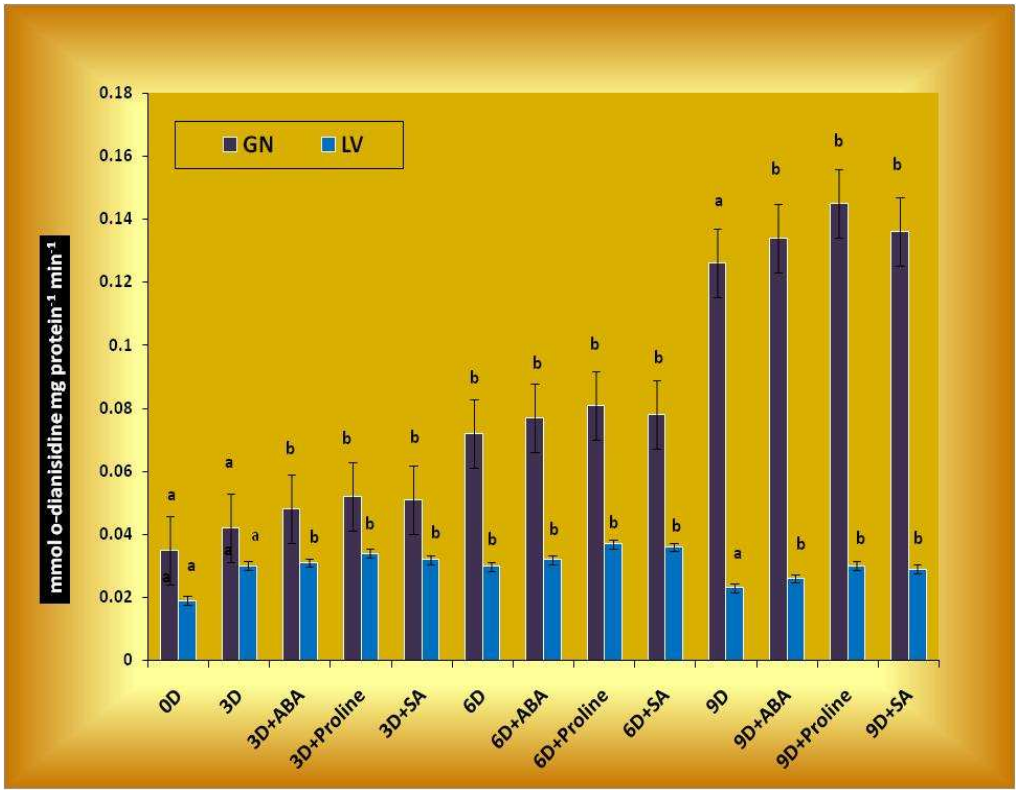

Figure 83. Activity of peroxidase in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

APOX activity in the pre–treated seedlings in LV showed a very significant increase than the increase in case of pre–treated seedlings of GN following drought stress however in both the varieties pre–treatment with ABA, proline and SA followed by drought stress ameliorated the effect of drought stress (Figure 84). The corresponding values for the activity of APOX

obtained during the increasing days of drought in case of both GN and LV was lower without the chemical pre–treatment so it could be concluded that the APOX activity enhance in the seedlings during drought when they were pre–treated with chemicals. Here also, pre–treatment of seedlings with proline increased the activity of APOX in both the varieties during the amelioration of drought than by ABA and SA.

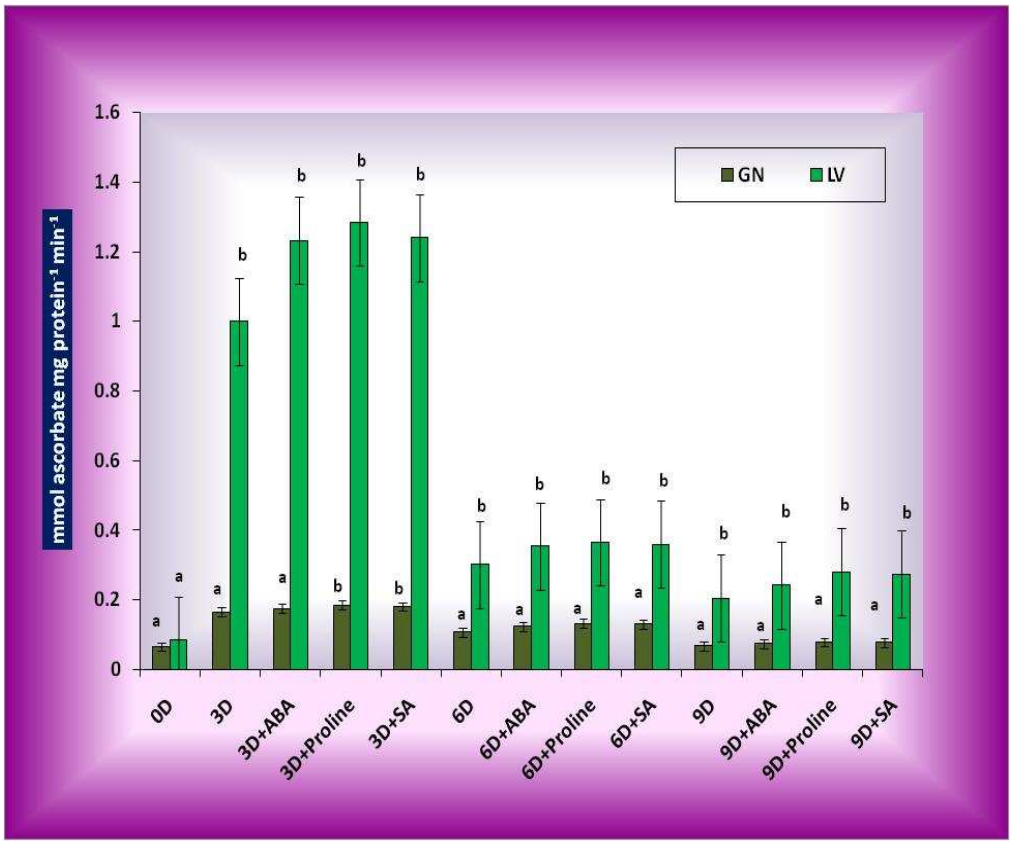

Figure 84. Activity of ascorbate peroxidase in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

The activity of GR (Figure 85) and SOD (Figure 86) following pre–treatments in the seedlings of GN and LV followed by drought stress enhanced with the increase in the periods of drought when compared to the untreated seedlings of GN and LV during drought respectively. The enhancement in the activity of GR in GN was much more than the increase in case of LV with increase in the days of water stress following pre–treatments with chemicals.

However, drought stress in both the varieties following pre–treatments increased the activity of GR in both the cases which was significantly higher in the pre–treated seedlings than the seedlings of GN and LV which was not chemically pre–treated. Similarly, the activity of SOD increased in both GN and LV following pre–treatments followed by the induction of drought than the seedlings which was not pre–treated by chemicals. The increase in the activity of the entire antioxidative enzyme was much more pronounced in case of pre–treatments with proline followed by SA and ABA. Significant change with respect to the enhancement of activity of antioxidative enzyme during drought was observed in the seedlings of GN and LV where the plants were pre–treated with chemicals than the plants were no such pre–treatment was done. LV considered as the most susceptible variety in our study too showed a better performance in combating drought evident by the increased activity of antioxidative enzymes when it was pre–treated with chemicals.

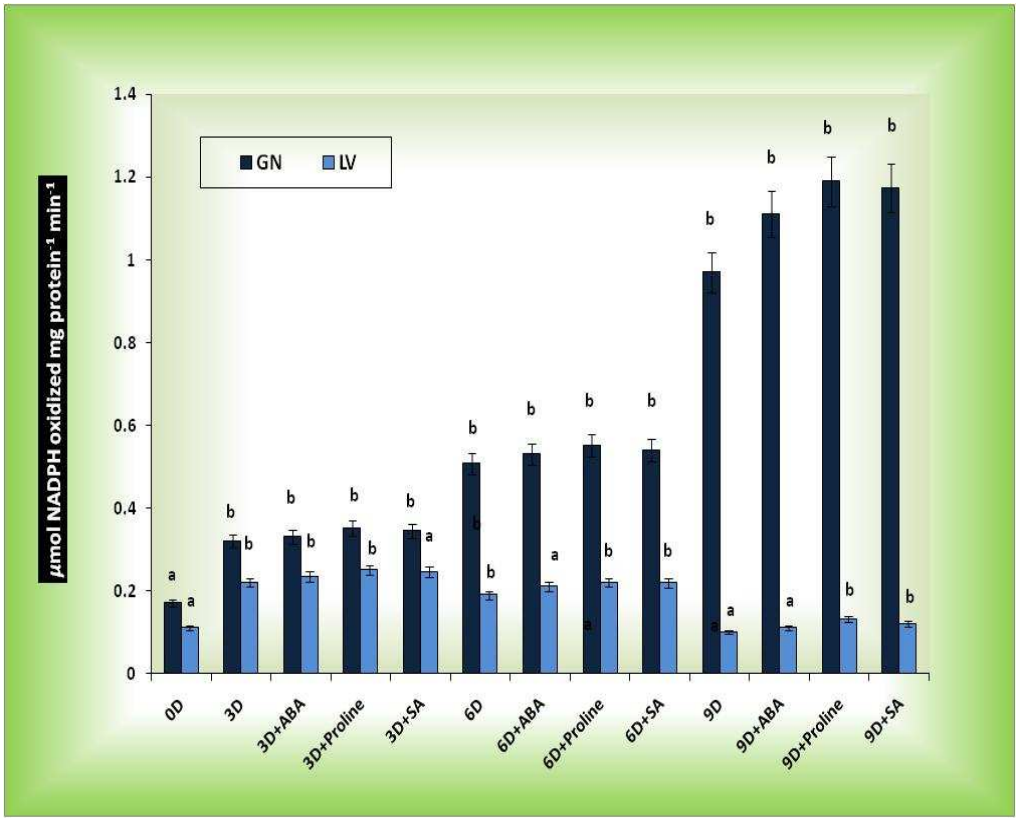

Figure 85. Activity of glutathione reductase in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

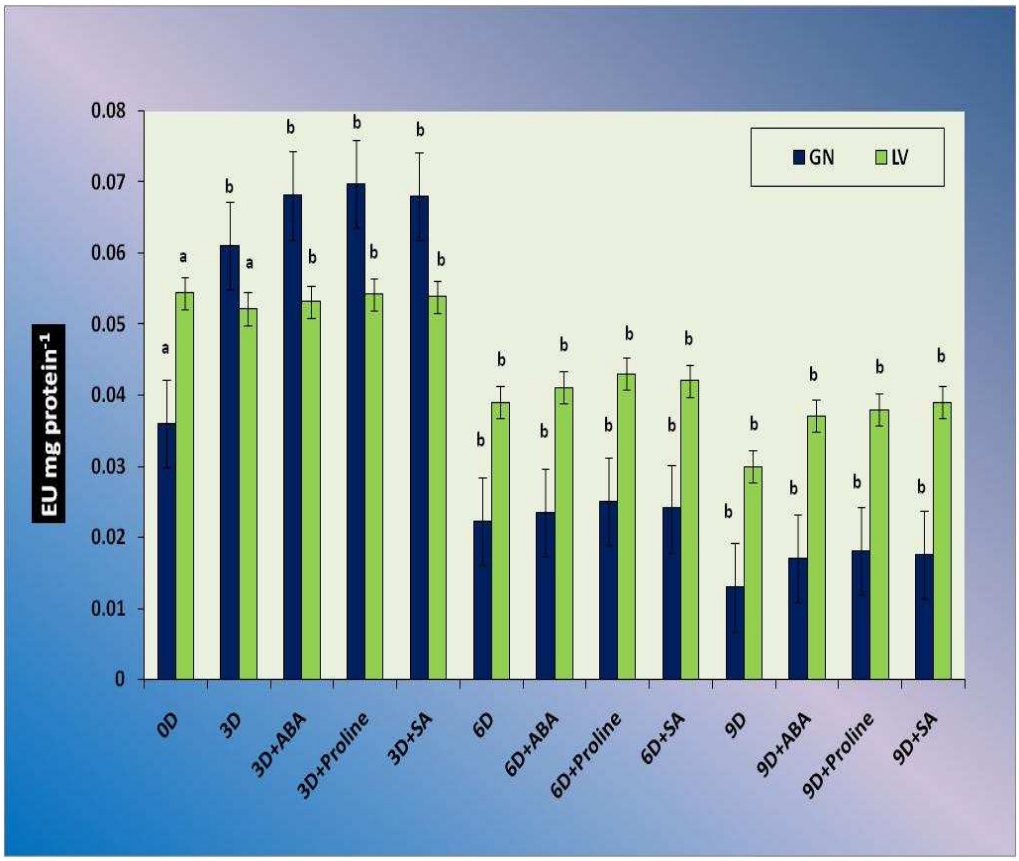

Figure 86. Activity of superoxide dismutase in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

In this study, H_2O_2 accumulation during water stress after the pre–treatment of seedling of GN and LV with chemicals decreased in GN and LV indicating greater antioxidant activity following pre–treatments with chemicals (Table 108). The accumulation of H_2O_2 in the leaf of both GN and LV where no pre–treatment was done in the seedlings before the onset of drought stress was much higher during the increasing days of drought than the seedlings were pre–treatment with ABA, proline and SA was done.

The leaf of GN showed lesser H_2O_2 accumulation than that of LV both with and without chemical pre–treatments however, pre–treatment with chemicals showed better results. During microscopic studies of the leaf tissues in DAB staining test for the detection of H_2O_2, dark–brown spots were observed as big and small patches at the site of DAB polymerization. The leaf of LV and GN showed lesser darkly stained DAB–sites in the tissues respect to

their control and also when compared to the DAB–sites in the leaf of untreated GN and LV respectively following pre–treatments with chemicals (Figure 87). The accumulation of H_2O_2 significantly decreased in case of LV which was remarkable as the LV was considered as the most susceptible variety in our case.

Table 108. H_2O_2 content in wheat after pre–treatment following drought stress

Days of stress+	**Content of H_2O_2**	
Chemical treatments	**GN**	**LV**
0D	233.00±1.73[a]	142.00±1.74[a]
3D	351.6 ±1.29[b]	243.1±1.86[b]
3D+ABA	301.80±1.82[b]	222.10±1.79[b]
3D+Proline	295.81±1.78[a]	218.90±1.68[b]
3D+SA	291.30±1.86[a]	220.10±1.84[b]
6D	370.5±1.72[b]	406.4±1.83[b]
6D+ABA	289.30±1.66[b]	387.50±1.95[b]
6D+Proline	222.30±1.98[b]	375.60±1.48[b]
6D+SA	222.30±1.77[b]	389.90±1.57[b]
9D	295.3±1.94[b]	450.2±1.66[b]
9D+ABA	202.20±1.65[a]	421.10±1.86[b]
9D+Proline	178.51±1.69[b]	400.00±1.94[b]
9D+SA	189.50±1.73[b]	419.90±1.64[b]

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in 't' test. Results are expressed as the mean of three replicates (10 plants each). H_2O_2 content= μ mol g tissue^{-1} (d.m.).

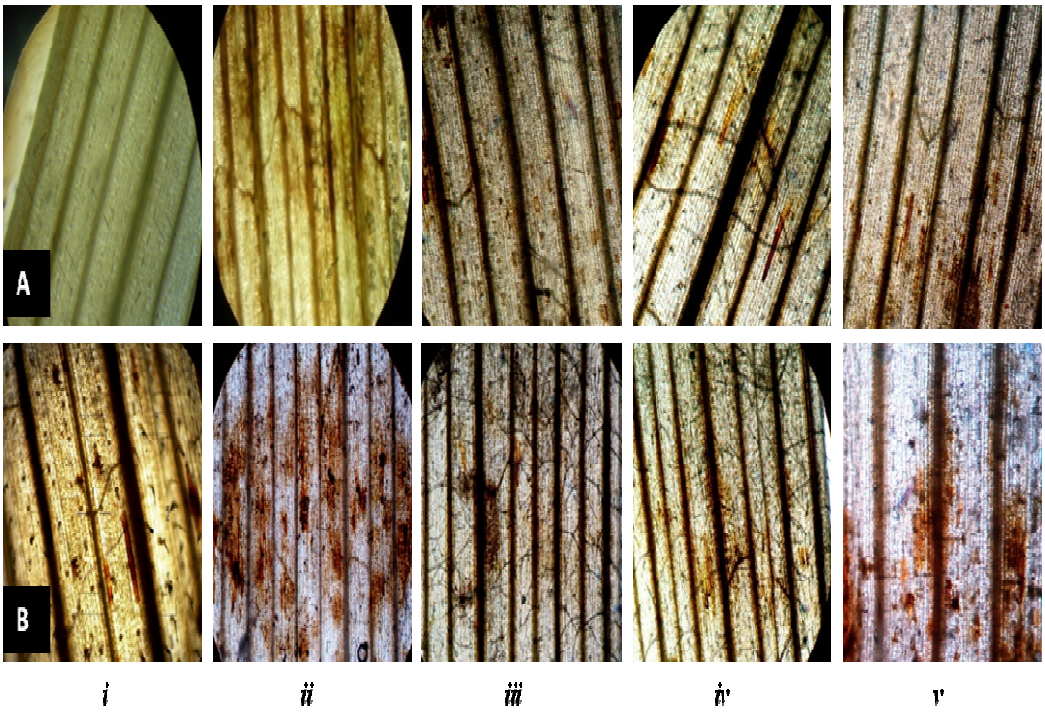

Figure 87. *In situ* detection of H_2O_2 in mid–portions of leaves of two drought stressed wheat varieties (A–GN & B–LV) following chemical pre–treatments: i & ii – 0 day & 9th day of drought stressed (untreated with chemicals) leaf respectively; iii, iv & v – leaves from plant under 9 days of drought stress (pre–treated with chemicals ABA, proline and SA respectively

Total antioxidant activity in drought stressed GN and LV following chemical pre–treatments increased significantly during all days of stress. The total antioxidant activity in case of proline pre–treated seedlings of both GN and LV showed the best results in terms of better antioxidative profile during drought (Figure 88). LV which was considered the most susceptible variety in our study showed a decreased total antioxidant activity with the increase in the duration of withholding water and continued to show higher values when compared to the LV seedlings in drought without pre–treatment respectively. The increase in the total antioxidant activity of drought stressed GN and LV after pre–treatment with chemicals proved that the use of chemicals prior to the stress conditions could render the plant more vigor to tolerate drought stress.

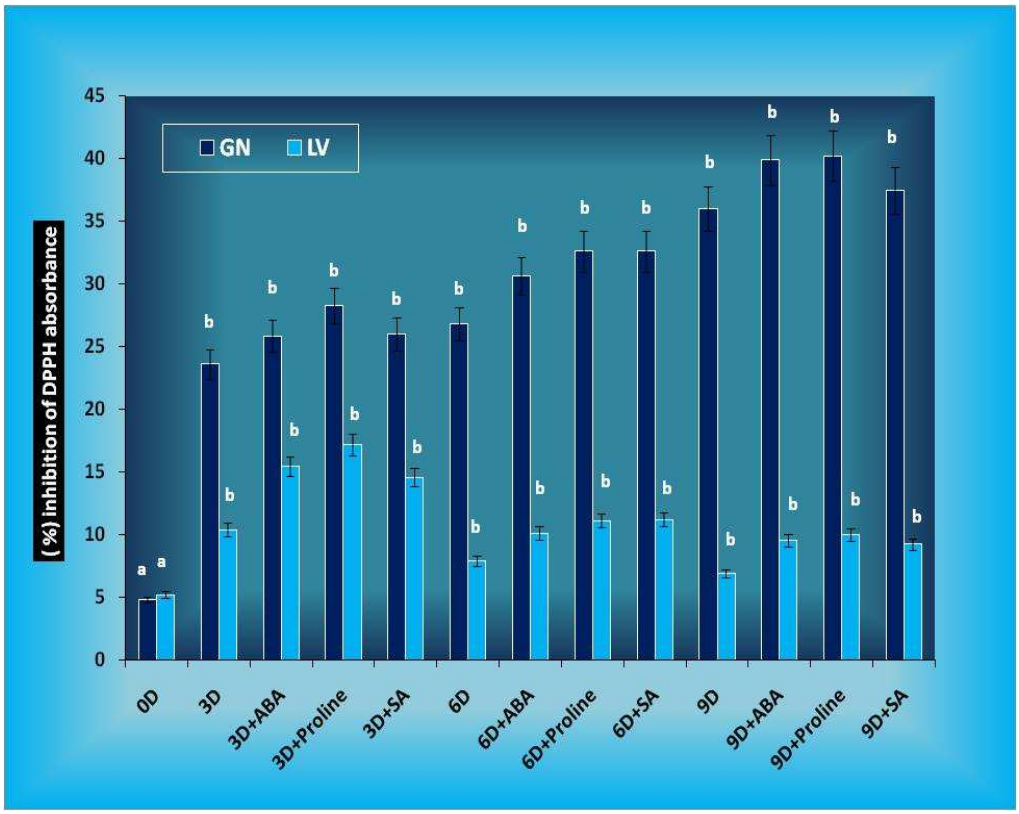

Figure 88. Total antioxidant activity in two drought stressed wheat varieties (GN & LV) following chemical pre–treatments. Results are expressed as the mean of three replicates (10 plants each). Bar represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). 0D– 0 day, 3D– 3 days, 6D– 6 days, 6D– 9 days of drought treatment

Carotenoids and ascorbic acid two of the non–enzymatic antioxidants in plants increased significantly in both GN and LV following drought stress after the seedlings where pre–treated with chemicals (Table 109) than the seedlings of GN and LV where drought was induced without chemical pre–treatments. Accumulation of carotenoids in the leaves, during drought showed an initial enhancement previously without any pre–treatment in case of GN however the pre–treated plants of GN after the induction of drought showed significantly higher values of both the antioxidants in their leaves. LV which was considered as the most susceptible variety in our study where the carotenoids and ascorbate value was the least during prolonged period of drought stress increased significantly in the leaves of pre–treated plants of LV even during the 9^{th} day of drought.

Table 109. Content of carotenoid and ascorbate in wheat after pre–treatments with chemicals following drought stress

Days of stress+ Chemical treatments	GN	LV
	Carotenoids	
0D	0.042±0.003[a]	0.048±0.003[a]
3D	0.063 ±.002[b]	0.049±0.003[a]
3D+ABA	0.068±0.002[a]	0.051±0.002[a]
3D+Proline	0.071±0.003[a]	0.054±0.005[b]
3D+SA	0.073±0.006[a]	0.053±0.001[b]
6D	0.067±0.004[b]	0.038±0.001[b]
6D+ABA	0.069±0.001[b]	0.040±0.003[b]
6D+Proline	0.069±0.004[b]	0.045±0.001[a]
6D+SA	0.071±0.008[b]	0.048±0.004[a]
9D	0.052±0.002[a]	0.029±0.002[b]
9D+ABA	0.055±0.002[a]	0.030±0.003[b]
9D+Proline	0.057±0.001[b]	0.032±0.007[b]
9D+SA	0.058±0.005[b]	0.037±0.004[b]
	Ascorbate	
0D	9.40±0.31[a]	9.98±0.06[a]
3D	10.80±0.32[b]	9.90±0.01[a]
3D+ABA	12.81±0.42[a]	12.11±0.11[a]
3D+Proline	13.54±0.44[b]	12.54±0.25[b]
3D+SA	12.99±0.51[a]	11.98±0.33[a]
6D	11.20±0.13[b]	10.10±0.03[a]
6D+ABA	13.21±0.55[b]	11.23±0.25[a]
6D+Proline	13.64±0.39[b]	12.65±0.17[b]
6D+SA	13.56±0.46[b]	11.99±0.28[a]
9D	16.30±0.21[b]	11.30±0.06[b]
9D+ABA	17.41±0.35[b]	12.31±0.18[b]
9D+Proline	18.23±0.47[b]	12.51±0.36[b]
9D+SA	17.65±0.38[b]	12.48±0.37[b]

Means ± S.E., n=10. Different superscripts in each column express significant difference with control at P≤0.01, in't' test. Results are expressed as the mean of three replicates (10 plants each). Content of carotenoid=mg g (f.w.) tissue^{-1} and ascorbate=mg g^{-1} (d.m.).

Accumulation of both carotenoids and ascorbate in the pre–treated leaf of both tolerant and least tolerant or susceptible variety increased with pre–treatments with ABA, SA and proline and the highest values for the accumulation of both the antioxidants was observed in case of plants pre–treated with proline solution than the plants pre–treated with ABA and SA.

CHAPTER 5
DISCUSSION

The ability of plants to survive under severely stressed condition is due to various metabolic adaptations, which, if successful trigger cascades of signaling pathways leading to tolerance of the plant. Drought and salinity are two of the most important abiotic stresses of the present age. Feeding of ever growing population with lesser and lesser arable land will have to depend on increasing plant productivity. For this, selection of plants with ability to tolerate stress conditions, on the basis of knowledge of the mechanisms involved in tolerance is essential. The present study was undertaken to test differential responses of different wheat varieties to drought and salt stress.

For this, initially drought was imposed on nine wheat varieties, up to 9 days, while salinity stress was imposed by adding 200mM NaCl to the rhizosphere. The nine tested varieties did not show severe wilting symptoms morphologically even after 7 days of drought stress, but during the ninth day the plants showed morphological signs of wilt. The salt stress resulted in the yellowing of leaf during the higher concentration of salt and with the increase in the days of salt, i.e., during the 3rd day in our study, wilting symptoms were more visible in MW, GY, SO and LV than the other five varieties. Drought and salt stress had deleterious effect with increase in the duration and concentration respectively (Chakraborty and Pradhan, 2012, 2011). Workers like Patade *et al* (2011) have suggested that shoot growth is more affected than root growth, and continuation of root growth under stress is an adaptive mechanism that facilitates water uptake from deeper soil layers and the emergence of new leaves is slower and the older leaves show early senescence. Similarly Chaves *et al* (2009) have reported that drought and salinity share a physiological water deficit that attains, more or less intensely, all plant organs; however, under prolonged salt stress plants respond in addition to dehydration to hyper–ionic and hyper–osmotic stress.

Results of the influence of water stress in water relations of wheat varieties showed that decrease in the RWC on the 9th day of stress with respect to zero day, i.e., the control plants was lower in KD, GN, PBW 343, UP 2752 and KW compared to MW, GY, SO and LV. In other words KD, GN, PBW 343, UP 2752 and KW could retain comparatively higher amount of water in their leaf with the increase in the days of drought. It has also been reported in previous studies that drought–resistant cultivars maintain higher RWC during drought stress, whereas in susceptible cultivars RWC shows greater decrease (Farooqui *et al*. 2000; Chakraborty *et al*. 2002; Iqbal and Bano 2009). Drought was found to decrease the RWC of plant leaves (Sánchez–Blanco *et al*., 2002). Previous studies have also confirmed that the ability to maintain higher RWC is one of the mechanisms of drought tolerance in plants (Farooqui *et al*., 2000; Chakraborty *et al*., 2002; Iqbal and Bano, 2009). Similarly the influence of salt stress in wheat varieties in the present study showed that RWC decreased with increase in the days of stress in case of SO, MW, GY and LV compared to the decrease in case of KD, GN, PBW 343, UP 2752 and KW where the decrease was comparatively lesser. Extent of salt–induced effects on relative water content has been used as one of the vital water relation parameters for assessing degree of salt tolerance in plants and our results are in accordance with the results obtained by various authors like Asfaw (2011), Ghogdi *et al* (2012), Hossain *et al* (2006), Win *et al* (2011) and Saleh (2013) during salt stress. According to the works of Saleh (2013) on leaf relative water content (RWC) during salt stress RWC in leaf was found to be an alternative measure of plant water status, reflecting the metabolic activity in plant tissues (Asfaw 2011, Ghogdi *et al* 2012, Hossain *et al* 2006, Win *et al* 2011). The extent of salt–induced effects on relative water content has been used as one of the vital water relation parameters for assessing degree of salt tolerance in plants (Asfaw 2011, Ghogdi *et al* 2012, Hossain *et al* 2006, and Win *et al* 2011). Among several methods used to characteristics internal plant water status, RWC is an integrative indicator (Parsons and Howe, 1984) and was used successfully to identify drought resistant cultivars (Matin *et al*, 1989). Sinclair and Ludlow (1985) proposed that leaf relative water content (RWC) was a better indicator of water status than water potential. Singh *et al*, (1990)

observed significant differences in water potential among wheat genotypes under drought stress. Other workers like Sharada and Naik (2011) have also shown in their studies that there was a decrease in relative water content in stressed plants as observed in case of groundnut and under severe drought stress in groundnut genotypes respectively.

Results obtained on cell membrane stability index were in accordance with the result of many authors. In case of MW, GY, SO and LV the cell membrane stability was visibly disrupted during both drought and salinity stress and was found to be most affected in case of LV out of all tested varieties. In the other five varieties, GN, KW, PBW 343, KD and UP 2752 the cell membrane stability index was highest and thus these varieties could tolerate the stress conditions imposed on them and showed higher values for cell membrane stability index. Hence, it is safe to say that out of all the nine tested varieties, GN followed by KW, PBW 343, KD and UP 2752 showed the highest membrane stability during both drought and salt stress and with the increase in the duration of stress, the change in the values for cell membrane stability index was comparatively lower than the other four varieties where high degree of change in their CMS was observed. Bajji *et al* (2002) in their work on the use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat suggested that the extent of the cell membrane damage not only correlated well with the growth responses of wheat seedlings belonging to various cultivars to withholding water but also with the recognised field performances of these cultivars. According to their study, varying the stress conditions influenced both the percent and the kinetics of electrolyte leakage during rehydration and electrolyte leakage exhibited a characteristic pattern reflecting the condition of cellular membranes (repair and hardening). Enhanced water retention and membrane stability in tolerant wheat genotypes have also been observed in other studies (Gupta and Gupta, 2005). Helal and Abdel–Aziz (2008) suggested that lower membrane stability index reflects the extent of lipid peroxidation, which in turn is a consequence of higher oxidative stress due to water stress conditions according to their research findings. The CMS has been extensively used as selection criterion for different abiotic stresses including

drought and high temperature in sorghum (Premachandra *et al,* 1992), mustard (Hashem *et al,* 1998), rice (Tripathy *et al,* 2000), wheat (Blum *et al,* 2001; Rahman *et al,* 2006a) and cotton (Ullah *et al,* 2006; Rahman *et al,* 2006b).The change in biological membranes stability is a key indicator of cellular damage. Drought and other stresses always results in cellular membrane injures including the increase of membrane permeability (Esfandiari *et al* 2011, Gomathi and Rakkiyapan 2011, Senadheera *et al* 2012) and therefore serves as an important parameter for testing the plant for its capacity to avoid or resist the stress related changes.

A significantly low tolerance index was observed in case of the leaf of LV, MW, GY and SO which in case of KD, GN, KW, UP 2752 and PBW 343 was higher during the 9th day of drought and also during the salt concentration of 200mM on the 3rd day. The tolerance index of all the nine varieties was higher during the salt stress than during drought.

The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to drought stress (Thankamani *et al*. 2003) and the amount of lipid peroxidation is considered to be one of the determinants which indicate the extremity of stress experienced by a plant. It was observed that although MDA content, a measure of lipid peroxidation, increased in all varieties during drought stress, after 9 days, MDA content in susceptible varieties in our study i.e., MW, GY, SO and LV was more than three times that of tolerant varieties i.e., GN, KD, KW, UP 2752 and PBW 343. Tatar and Gevrek (2008) also reported that MDA content in wheat increased with severity of drought stress. Similarly, MDA content following salt stress increased significantly in all the nine varieties with the increase in salt concentration and the duration of salt stress with the highest value observed during the 3rd day of salt stress of 200mM. As observed in water stress, here too MDA content in case of MW, GY, LV and SO was more than three to four times that of KW, GN, KD, UP 2752 and PBW 343 during both the 1st and 3rd day of stress. The highest peroxidation of lipids in the membranes in the leaf was observed in case of LV in our tested varieties and the least value for MDA content was obtained in case of GN. According to Esfandiari (2007) lipid peroxidation is linked to the activity of antioxidant

enzymes e.g. with the increase of SOD, APX, GPX, CAT, etc, oxidative stress tolerance is enhanced and MDA is decreased. Our data are in accordance with various workers who determined that the increase in the concentration of MDA in higher salt levels due to the low activity of SOD and GR or CAT was not a critical factor for the damage of oxidative stress. Workers like Pandey *et al* (2010) have also reported increased levels of MDA content in *Avena* during drought stress and thus lipid peroxidation determination of a plant or a cultivar serves as important criteria for the stress studies in plants. Tatar and Gevrek (2008) have also reported increase in MDA content with increase in the degree of stress in wheat. In addition, Türkan *et al*. (2005) found that MDA content was lower in the leaves of drought–tolerant *Phaseolus acutifolius* Gray. than that in drought–sensitive *P. vulgaris* L. Sairam and Srivastava (2001) reported that the drought–tolerant genotypes of wheat showed lower lipid peroxidation level and higher CMS value than the susceptible ones which is in conformity with the present findings.

Free proline accumulation was enhanced in all nine varieties during prolonged drought stress and salt stress after an initial non–significant decrease. After 9 days of drought, proline content in KW, GN, UP2752, PBW 343 and KD was about 2.5 times higher than the other four varieties and about 3 times during both 1d and 3d of salt stress, although in control plants all varieties had more or less similar amounts. It is quite clear that proline accumulation during stress is one of the mechanisms of tolerance. During salinity stress the accumulation of proline in the salt stressed plant increased with the increase in concentration of salt and the duration of salt stress. This study revealed that in GN, KD, KW, UP 2752 and PBW 343 the accumulation of free proline in both leaf and root was almost 3times higher than that of LV, GY, MW and SO in all concentration of salt stress during the 3rd day however, the during the first day of salt stress this trend varied in some varieties. Proline, which is usually considered as an osmoprotectant, may also be involved in reducing oxidative damage by scavenging the free radicals (Vendruscolo *et al*., 2007; Tatar and Gevrek, 2008). The effect of water stress on *Triticum aestivum* L. was investigated by many researchers such as Tatar and Gevrek (2008) who concluded that proline accumulation increased after lipid peroxidation content

became higher and RWC content of leaves became lower, furthermore, they concluded that proline was mainly involved in protection against oxidative stress than osmotic adjustment during initial steps of water stress. Munns and Tester (2008), Lokhande *et al.* (2010) also suggested that the plants exposed to salt stress may use saline ions as an osmoticum; however, synthesis of compatible solute is also required to prevent ion toxicity, while plants exposed to dehydration stress solely rely on synthesis of compatible solutes for maintenance of cell turgor. Modulation of intracellular proline levels affects flowering time and inflorescence architecture in *Arabidopsis* (Mattioli *et al*, 2008).

The beneficial roles of proline in conferring osmotolerance have been widely reported by workers like Kishor *et al.* (1995), Bajji *et al.* (2000), Yancey (1994) and Errabii *et al.* (2006). Zlatev and Stoyanov (2005) suggested that proline accumulation of plants could be only useful as a possible drought injury sensor instead of its role in stress tolerance mechanism. However, Vendruscolo *et al.* (2007) point that proline is involved in tolerance mechanisms against oxidative stress and this was the main strategy of plants to avoid detrimental effects of water stress. Tatar and Gevrek (2008) and Kameli and Losel (1996) showed that wheat dry mater production, relative water content (RWC) decreased and proline content increased under drought stress. Higher proline content in wheat plants after water stress has been reported by Vendruscolo *et al.* (2007) and Patel and Vora (1985). Increasing amount of proline was also established in several stress conditions such as salinity (Poustini *et al..*, 2007), cold (Charest and Phan, 1990) and UV (Tian and Lei, 2007) in wheat. In our study higher content of proline was obtained in case of GN, KW, KD, UP 2752 and PBW 343 with increase in the duration of drought as well as during increasing concentration of salt suggesting that these five varieties were able to accumulate higher amount of proline in both leaf and root than the other four varieties in our study. The content of proline was higher in the leaf tissue than the tissues of the roots in our study.

Free amino acids detected in HPLC following drought stress in case of wheat (GN and LV) showed different peaks of amino acids which either appeared or disappeared with increasing days of drought stress. There was a

marked difference in the number of peaks, peak height and area in both the varieties following drought stress and also between the duration of stresses in each variety respectively. Several amino acids could be identified in HPLC in both GN and LV such as follows aspartic acid, glutamic acid, serine, threonine, DL alanine, arginine, proline, hydroxyl proline, tyrosine, valine, leucine, cysteine, methionine, isoleucine, alanine, DL–phenyl–alanine, arginine, lysine etc. Some peaks remain unidentified. Out of all the peaks the peak for proline was undoubtedly the highest peak obtained in both the varieties in accordance with the value obtained during its quantification in GN and LV with significant differences among the varieties as well as between the treatments in each variety. Aspartic acid, glutamic acid, serine, threonine, proline, hydroxyl proline, tyrosine, valine, methionine, leucine, cysteine, isoleucine, DL–phenyl alanine and lysine were found to be most significant during drought treatments in both GN and LV with the peak height and area being comparatively greater in case of GN than LV where it was smaller and some peaks were absent during prolonged stress. Increased levels of amino acids identified in HPLC studies was correlated with the increase in the accumulation of proline and also increased acclimation of GN during drought stress than in LV. The reason for the increase in the amino acid might stem from enhanced amino acid synthesis and/or from enhanced stress–induced protein breakdown and while the overall accumulation of amino acids upon stress might indicate cell damage in some species (Widodo *et al.*., 2009), increased levels of specific amino acids have a beneficial effect during stress acclimation. Krasensky and Jonak (2012) have suggested that the accumulation of amino acids has been observed in many studies on plants exposed to abiotic stress which has also been earlier suggested by many early and contemporary authors like Barnett and Naylor (1966), Draper (1972), Handa *et al.* (1983), Rhodes *et al.* (1986), Fougere *et al.* (1991), Kaplan *et al.* (2004), Brosche *et al.* (2005), Zuther *et al.* (2007), Kempa *et al.* (2008), Sanchez *et al.* (2008), Usadel *et al.* (2008), Lugan *et al.* (2010). Similarly, Gill and Tuteja (2012) have elaborately discussed the role of polyamines in abiotic stress tolerance in plants.

Chlorophylls, which are one of the first molecules to be affected by any kind of abiotic stress, showed a significant decrease in the leaf of all

varieties during both drought and salt treatment. However, in tolerant varieties such as GN, KD, KW, UP 2752 and PBW 343 there was an initial non significant increase. Interestingly, the chl. a/b ratio showed an initial increase in all varieties before declining, and this decrease was greater in the less–tolerant varieties which in our study was LV, SO, MW and GY. Havaux (1998) and Kiani *et al.* (2008) suggests that water stress, among other changes, has the ability to reduce the tissue concentrations of chlorophylls and carotenoids, primarily with the production of ROS in the thylakoids (Niyogi, 1999; Reddy *et al..*, 2004); however, reports dealing with the strategies to improve the pigment contents under water stress are entirely scarce. Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing powers and both the chlorophyll a and b are prone to soil drying (Farooq *et al..*, 2009). Anjum *et al.* (2003) and Farooq *et al*. (2009) have reported that drought stress produced changes in the ratio of chlorophyll 'a' and 'b' and carotenoids during drought stress. The ratio of chl. a to chl b in our study was considered as the best method to determine the effect of stress on the content of chl. a and chl. b during stress and the increase of decrease in their ratio could explain the fate of photosynthetic pigments during stress (Chakraborty and Pradhan, 2011, 2012). Decline in the chlorophyll content of leaves during abiotic stress was reported by several workers (Ramanjulu *et al,* 1998). According to Estill *et al.* (1991) and Ashraf *et al.* (1994) chlorophyll b content increased in two lines of okra, whereas chlorophyll a remained unaffected resulting in a significant reduction in Chl. a: b ratio in both cultivars under water limiting regimes. According to Mafakheri *et al.* (2010) a decrease of total chlorophyll content with drought stress implies a lowered capacity for light harvesting and since the production of reactive oxygen species is mainly driven by excess energy absorption in the photosynthetic apparatus, this might be avoided by degrading the absorbing pigments. Erdei and Taleisnik (1993), Huang and Redmann (1995) suggested that the reduction in growth of salinized plants may be related to salt–induced disturbance of the plant water balance, and in the extreme to a loss of leaf turgor which can reduce leaf expansion and therefore, photosynthetic leaf area. The imbalance caused by salinity affects the nutrients involved in protein synthesis and those involved in

photosynthesis, which can lead to inhibition of these processes (Vieira–Santos *et al.*, 2001), as well as to the degradation of pigments chlorophyll *a* and *b* (Di Martino *et al.*, 2003).

The content of total carbohydrates, i.e., soluble and reducing sugar in case of drought and salt stress in all the nine varieties for both leaf and root showed a significant initial increase with the increase in the duration and severity of stress which after an initial increase declined in case of MW, GY, LV and SO. However, in case of other five varieties the accumulation of soluble carbohydrates and reducing sugars continued to increase even after the 9^{th} day of water stress and after 3^{rd} day of salt stress for all the salt concentration. Increase in the duration of both water and salt stress till 9 and 3 days respectively resulted in increase in the accumulation of carbohydrates in KW, GN, KD, UP 2752 and PBW 343 with the highest values observed during 200mM of salt concentration. The accumulation of sugars both total and reducing in our study was significantly highest in case of GN in both root and leaf than all the other varieties with the lowest value observed in case of LV. In comparison, the accumulation of sugars following both water and salt stress in case of leaf and root in MW, GY, LV and SO was not very significant however, in the other five varieties the induction of stress and the increase in the severity of stress resulted in a significant difference in the accumulation of sugars in the leaf and root. The accumulation of carbohydrates in case of leaf was much more than the accumulation in case of leaf in KW, GN and KD. Almodares *et al* (2008) has reported the effect of salt stress on growth and carbohydrate accumulation in case of sweet sorghum. Morsy *et al* (2007) reports the alteration of oxidative and carbohydrate metabolism under abiotic stress in two rice (*Oryza sativa* L.) genotypes contrasting in chilling tolerance. Earlier worker like Martin *et al.* (1993) have reported that drought stress is a decrease of soil water potential so plants reduce their osmotic potential for water absorption by congestion of soluble carbohydrates and proline and in other words osmotic regulation is performed (Martin *et al.*, 1993). The amount of total soluble sugar/embryonic axes fresh weight increase rapidly answering to the increasing concentrations of NaCl, this result agree with the result of some researchers that indicate that salinity stress induce soluble sugar

accumulation (Prado *et al.*,2000). The accumulation of starch in the leaf and roots of water stressed and salt stressed wheat varieties showed a general decline after an initial increase in all the cases. As explained by Giorgini and Suda (1990), the higher level of soluble sugars detected is probably necessary for the turgor and growth of embryonic axes during emergence. Gill *et al* (2001) have reported the effect of various abiotic stresses on the growth, soluble sugars and water relations of sorghum seedlings in light and darkness. Singh (2004) proved that a greater accumulation of sugar lowers the osmotic potential of cells and reduces loss of turgidity in tolerant genotypes. The other possible role of sugar may be as a readily available energy source Dkhil and Dendon (2010).

The content of starch during water stress increased significantly during the 3rd day of stress followed by a decrease after 6 days in case of leaf. However the accumulation of starch in case of root was lower than that of the leaf in all the wheat varieties. Accumulation of starch in both roots and leaf was found to be lowest in case of LV and SO. Our result showed that the increase in the days of salt stress resulted in the increase in the accumulation of starch in case of both leaf and root; however for the higher concentration of salt the accumulation decreased during the 3rd day. The accumulation of starch decreased with the increasing concentration of salt after a steep rise for the initial concentration, this decrease was more pronounced in case of leaf than that of roots. The highest accumulation of starch was observed for GN and KD following both the stresses and the lowest was observed in LV and SO. Thakur and Sharma (2005) also reported similar observation in sorghum seeds where the stress caused a decrease in starch content and an increase in sugar content (Thakur and Sharma, 2005). Work of several authors like Todaka *et al.* (2000), Kaplan and Guy (2004), Basu *et al.* (2007).), Kempa *et al.* (2008), Madden *et al.* (1985) and Kaplan and Guy (2004) have suggested in details that the main carbohydrate store in most plants is starch which can be rapidly mobilized to provide soluble sugars and the metabolism of carbohydrate is very sensitive to changes in the environment. In addition to diurnal fluctuations in starch levels, salt and drought stress generally leads to a depletion of starch content and to the accumulation of soluble sugars in leaves as suggested by Krasensky and

Jonak (2012) sugars that accumulate in response to stress can function as osmolytes to maintain cell turgor and have the ability to protect membranes and proteins from stress damage. The increase in sugar levels accompanied by decrease in starch content in embryos and cotyledons was directly linked to the activity of α and ß–amylases, which is in agreement with the existing reports of Monerri *et al.*, 1986; Gupta *et al.*, 1993). Kameli and Losel (1995) confirmed that this increase might be considered to play an important role in osmotic adjustment, which is widely regarded as an adaptive response to water deficit conditions.

The potential of phenolics to act as antioxidants is also suggested by earlier workers like Rice–Evans *et al* (1997) mainly due to their properties to act as hydrogen donators, reducing agents and quenchers of singlet O_2. The accumulation of total phenol and ortho phenol was greatly enhanced during both the stress with the increase being higher with increase of duration of water stress and concentration of salt in case of KW, GN, UP2752, PBW 343 and KD whereas in other varieties such as LV, MW, SO and GY, a decrease was observed during the later stages of stress and higher concentration of salt. The accumulation of total and ortho phenol was most marked in case of GN among all varieties and the least was observed in case of LV in our study. Leinhos and Bergman (1995) had studied the plant defense system against various types of stress with respect to the involvement of polyphenols as a response to stress and the results were in accordance with their findings.

The profile of total phenols in HPLC in the leaf of GN and LV during drought and salinity stress showed that the total phenols with respect to the number, height and area of the peaks in HPLC was much more in case of GN than LV during prolonged drought as well as increasing concentration of salt. The highest content was recorded in case of GN in our study and the least in case of LV following both water and salt stress separately which was confirmed by HPLC analyses. The main peaks which were identified during the total phenol analysis of GN and LV in the leaf during drought and salt stress were ferulic acid, salicylic acid, chlorogenic acid and caffeic acid. In the present study, the most prominent peak was found to be ferulic acid which served as the most important compound expressed during drought and salinity

stress in our study and the antioxidative action of phenols in GN rendering it the most tolerant variety in our study was evident since, the accumulation of total phenol both quantitatively and qualitatively was greater in case of GN that the least tolerant variety LV in our study. Chakraborty *et al*. (2002), Chakraborty and Pradhan (2011, 2012) has also reported earlier that drought stress–induced accumulation of phenols was much higher in tolerant cultivars of tea. The involvement of polyphenols in plant defense against various stresses has been reported previously (Leinhos and Bergmann 1995).

The synthesis of phenolics is generally affected in response to different biotic/ abiotic stresses including salinity as suggested by Parida *et al.* (2004) and Singh (2004) who in their study determined that tolerant genotypes of chickpea showed a higher level of total phenols, whereas a significant reduction was observed in susceptible genotypes. In our study the specific analysis of phenolic acid in the leaf of GN and LV done in HPLC revealed that one of the most prominent peaks observed in case of GN and LV was identified as ferulic acid, followed by vanillic acid, cinnamic acid, chlorogenic acid and also salicylic acid. Rondini *et al.* (2004) and Manach *et al.* (2004) have reported that highest amounts of FA are present in cereals with up to 90 % and more of total phenols. The differential response of plants in phenolic accumulation at different growth stages has been well documented in the work by Choi *et al.* (2006) and Barros *et al.*, (2007). Several reports are available where reduced phenolic contents were observed such as in *Cynara cardunculus* leaves under saline conditions by Falleh *et al.* (2008). Hichem *et al.*, (2009) reported that such variation in concentration of leaf phenolics within a plant under salt stress in relation to leaf age may be due to the reflection of different requirements for counteracting abiotic stresses at different growth stages. Kabiri *et al.* (2012) suggest that the results obtained in the last few years strongly prove that salicylic acid could be a very promising and protective compound for the reduction of biotic and abiotic stresses in crops, because under certain conditions, it has been found to mitigate the damaging effects of various stress factors in plants. In their study, salicylic acid was used in control, and drought stressed plants, and the role of this compound in reduction of oxidative damages in *Nigella* plant was investigated

(Kabiri *et al.*, 2012). Leaf phenolic contents are also important protective components of plant cells Ashraf *et al.* (2010). Hydroxycinnamic acids are the most widespread group of phenolic acids with four major phenolic acids in plants: ferulic acid (FA), sinapic acid (SA), caffeic acid (CA) and p–coumaric acid (PCA). CA is the most abundant PA in fruits with more than 75 % of total phenols and is found in all parts of the fruit (Manach *et al..*, 2004). Engert (2011) from his studies observed that different studies proved that phenolic acids as strong antioxidants are present in wheat. He suggests that hydroxycinnamic acids provide the major part of phenolic acids in cereals with FA as the abundant PA, followed by SA, PCA, CA and VA as a hydroxybenzoic acid (Adom and Liu, 2002, Adom *et al*., 2003, Slavin, 2003, Zhou and Yu, 2004). Further, it has been suggested that the antioxidative ability of PAs is to inhibit lipid oxidation by trapping peroxyl radicals (Engert, 2011).

Soluble protein contents both in leaf and roots in case of wheat varieties decreased following water stress and the decrease continued with the increase in the days of drought. The decrease in the soluble protein content in case of GN, KW, UP 2752 and KD was more or less similar and did not show much significant difference during the later stages of drought. However, the protein content changed significantly in case of MW, GY, LV, PBW 343 and SO with the increase in the days of withholding water from the plant. Similarly the accumulation of soluble protein in the leaves during salt stress for 1st and 3rd day showed a general decline with the increase in the severity of salt stress whereas the content of soluble protein in the roots for 1st and 3rd day showed a difference. Simova–Stoilova (2008) has postulated that the data on leaf protein basis reflect the relative proportion of the enzyme in the total protein content. Several stress response proteins, protein–protein interaction and post–translation modification have been also identified (Salekdeh *et al..*, 2002). SDS–PAGE analyses of proteins in the leaf of wheat plants in the present study has shown different band patterns with the enhanced accumulation of proteins and expression of new proteins in case of tolerant varieties and increase in the number of bands during both drought and salt treatments. Fazeli *et al.* (2007) have reported reduction of protein content in leaves and roots in

case of sesame and suggest that water stress may cause generation of ROS. They obtained new and colourful protein bands in SDS–PAGE of protein in leaves during stress; however, in roots the proteins did not show any important differences among the treatments. Further the analysis of total soluble proteins in GN and LV in FPLC has shown significant difference among them and it was observed that the increase in the days of drought in case of LV changed the profiles of peaks in FPLC. The number, height and the area of peak showed a significant difference during the increasing days of water stress with respect to control plant. It was seen that the change in height and number of peak in case of GN during increasing days of withholding water was comparatively lower than LV; however the total content of protein as determined by the peak height and area was higher in case of GN than LV which was in accordance with the content of total soluble protein in the leaf of GN and LV. A significant difference in the number and height of peak in FPLC analysis was observed in case of LV during the 9^{th} day.

Soluble protein contents in the roots in case of MW, GY, GN, LV and SO increased during water stress during the later stages of drought while in case of all the other varieties it showed a general decline. In case of KD, GN, KW, UP 2752, PBW 343 and SO the accumulation of soluble protein in the roots decreased with the increase in the concentration of salt whereas in case of MW, GY and LV the content of soluble proteins increased significantly at higher concentrations on the initial day of salt stress but the accumulation again showed a decline on the 3^{rd} day of salt stress. The accumulation of soluble proteins in case of leaf was higher than the root. As suggested by Saleh (2013) salinity promotes the synthesis of salt stress–specific proteins; many of these proteins were suggested to protect the cell against the adverse effect of salt stress and the accumulation of these proteins is a common response to salt stress (Kong–ngern *et al.* 2005, Mahmoodzadeh 2009, Meratan *et al.* 2008, Metwali *et al.* 2011, Mohamed 2005). High salt concentrations inhibit enzymes by impeding the balance of forces controlling the protein structure (Serrano *et al.*, 1999). Some experimental evidence suggests that drought–sensitive species and varieties have higher proteolytic activity compared to the resistant ones (Hieng *et al..*, 2004). However, data on proteolytic activity

relation to drought sensitivity or resistance are still quite limited. According to Yordanova *et al.* (2004) changes in proteins can result from a variety of environmental stresses such as water stress.

Water stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly O_2^- and H_2O_2 in chloroplasts, mitochondria, and peroxisomes. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stresses (Foyer and Noctor, 2003). Activity of antioxidative enzymes like POX, CAT, APOX, GR and SOD following osmotic stress i.e. water stress and salinity stress showed a difference in during different stages of water stress and salinity stress. The activity of all the antioxidative enzymes seemed to be correlated with each other during the stress response of the plant. Previous studies have also reported differential responses of genotypes to drought stress with respect to antioxidant enzymes (Dhanda *et al*., 2004; Nair *et al*., 2008). It has been suggested that the coordinated activity of the different H_2O_2–scavenging enzymes play a part in the plant redox homeostasis (Foyer and Noctor, 2005).

During drought, activities of APOX and GR increased significantly in all nine varieties initially after 3 days of drought stress. With prolonged water stress, the activities of ascorbate peroxidase decreased in all varieties and the activity of glutathione reductase decreased in varieties MW, GY, LV and SO whereas its activity continued to increase in case of KD, GN, KW, UP 2752 and PBW 343. The activity of APOX after a significant initial enhancement showed a general decrease with increasing concentration of salt and duration of salt stress in all the varieties. It is clear that this enzyme showed a steep rise with the onset of stress followed by a decline when the duration and severity of stress increased i.e., 9th day in case of drought and at 200 mM during the 3rd day in case of salt stress. The potential of APOX to metabolize H_2O_2 depends on the redox state of such compounds. APOX and GR are believed to act in conjunction for H_2O_2 scavenging during environmental stresses (Sairam and Saxena, 2000). Mandhania *et al*. (2006) found that activities of CAT and APX increased with increasing the salt stress in both salt tolerant and salt sensitive wheat cultivars.

GR activity declined after a significant initial increase in all the varieties with increase in the concentration and duration of salt stress however, in case of KD, GN, KW, UP 2752 and PBW 343 the activity of GR continued to increase with the increase in the concentration of salt and the duration of salt stress. Mandhania *et al*. (2006) reported that GR may play a vital role in the glutathione cycle in the eukaryotic cells and through his work he indicated that GR activity increased with increasing salt stress in both salt tolerant and salt sensitive wheat cultivars. Kim *et al* (2005) have reported significantly increased GR activity in the roots in response to the NaCl treatment. GR activity and behaviour of its isoforms were analyzed in wheat (*Triticum aestivum* L.) leaves and roots exposed to a chronic treatment with a toxic cadmium (Cd) concentration by Yannarelli *et al* (2007) and reported that up–regulation of GR activity by the induction of distinctive isoforms occurs as a defense mechanism against Cd–generated oxidative stress in roots and they reported that wheat leaves did not show any change in their GR activity over time, whereas roots presented a remarkable increase. They suggested that GR activity is not only related to different organs and plant species but also to Cd concentration and/or a given period of exposure. Sairam and Srivastava (2002) reported that chloroplastic fraction showed higher total GR activity, followed by mitochondrial fraction in case of total GR. Enhancement of GR activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate–glutathione cycle than the less tolerant cultivars. This cycle has been implicated in mitigating the effects of ROS (Molina *et al*., 2002; Mandhania *et al*., 2006). Enhancement of GR activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate–glutathione cycle than the less–tolerant cultivars. GR, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), is an important endogenous antioxidant (McKersie and Leshem 1994). Chai *et al*. (2005) obtained increased glutathione reductase activities in two cultivars of banana subjected to drought stress. Similarly, both catalase and peroxidase are actively involved in detoxification of ROS by breaking down H_2O_2.

It was noted that the activity of POX enhanced greatly with increase in the period of water stress in case of GN, KD, KW, UP 2752 and PBW 343

whereas in LV, GY, MW and SO, the activity declined. Its acitivity showed a continuous enhancement with increase in the concentration of salt in all varieties during the 1st and 3rd day of salt stress with respect to the control; however in case of MW, LV, SO and GY the activity of POX with prolonged stress showed a significant decline in the later period of stress and higher concentration of salt. In all other cases, the activity of POX increased even during the highest concentration of salt in our study and during the 3rd day of salt stress. Other workers like Nayar and Kaushal (2002) also reported that the increased activity of POX enzymes constitute potential defense mechanism against chilling induced oxidative damage in germinating wheat grains. Increase in the POX enzymes to alleviate ROS has been also been reported by Sergi and Alegre (2003) and Agarwal and Pandey (2004). Chakraborty and Pradhan (2011, 2012) reported that POX seems to have greater role in tolerance than CAT during prolonged drought stress. Chakraborty *et al.* (2002) also reported that POX activities increased initially in all tea cultivars following drought stress, but in tolerant cultivars it increased even with prolonged periods. Iqbal and Bano (2009) obtained greater increase in activities of POX and CAT in wheat accessions that were tolerant to drought stress than those that were less tolerant. Previous workers have also reported differential responses of genotypes to drought stress with respect to antioxidant enzymes (Dhanda *et al*. 2004; Nair *et al*. 2008).

In the POX isozyme analysis in NATIVE–PAGE during drought and salinity stress in case of wheat varieties, significant differences were noticed among the varieties as well as during the different days of drought and different concentration of salt. In case of peroxidase isozyme analysis in NATIVE–PAGE, new bands were observed in the stressed varieties with respect to control in case of almost all the varieties with highest number of new peroxizymes recorded in case of varieties like GN KW, KD followed by PBW 343 and UP 2752 than SO, LV, GY and MW with their respective control and the same trend was observed during salt stress. High peroxidase isozyme content was reported by Kumari *et al* (2006) in peanut leaves during treatment of the plant with jasmonic acid and in barley seedlings by Popova *et al* (2003). El–beky (2003) has reported different electrophoretic bands of peroxidase

isozyme of different onion cultivars during salt stress and has suggested the use of NATIVE–PAGE of POX as biochemical marker for selection of salt tolerance in onion plants.

In case of CAT, activities decreased at all periods of drought stress in case of MW, GY, LV and SO whereas in KD, GN, KW, UP 2752 and PBW 343 activities of these enzymes increased initially before showing a continued decline. In this study a general decrease was seen in the activity of CAT during both drought and salt stress in case of MW, GY, LV and SO whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. The activity of CAT following salinity stress showed a continued decline in case of MW, GY, LV and SO with the increase in the concentration and duration of salt stress; however in case of KW, KD, GN, UP 2752 and PBW 343 there was an initial enhancement in the activity followed by decline with increasing concentration of salt. Dat *et al* (2000) has suggested that peroxisomes and glyoxysomes produce large amounts of H_2O_2 during photorespiration and fatty acid oxidation, respectively and this H_2O_2 is rapidly scavenged by catalases. Other workers like Nayar and Kaushal (2002) also reported that the increased activity of CAT and POX enzymes constitute potential defense mechanism against chilling induced oxidative damage in germinating wheat grains. They further suggested that catalase activity increased under water stress conditions in both tolerant and susceptible genotypes.

In the catalase isozyme analysis in NATIVE–PAGE during drought and salinity stress, significant differences were noticed among the varieties as well as during the different days of drought and different concentration of salt. CAT isozyme was more expressed in case of leaf in GN, KD, KW during drought and in KD and KW in case of salinity stress. In case of MW, GY isozyme for catalase was expressed during the higher concentration of salt and prolonged period of water stress. Different electrophoretic bands of CAT isozyme have been reported in case of onion cultivar during salt stress by El–beky (2003) and have suggested that the analysis of CAT isozyme in NATIVE–PAGE can serve as an important biochemical marker during salt stress.

Activity of SOD showed an initial increase in case of KD, GN, KW, UP 2752 and PBW 343; however, with increase in periods of stress, the activities of superoxide dismutase decreased in these varieties. Activities decreased at all periods of drought and concentration of salt stress in case of MW, GY and whereas in KD, GN, KW, UP 2752, PBW 343 and SO activities of these enzymes increased initially before showing a continued decline. With prolonged drought and salinity activity of SOD in MW, GY, LV and SO showed a continued decline whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. SOD is the first enzyme which is expressed in the antioxidant mechanism and it increased initially in the more tolerant varieties and was also involved in contribution to the initial accumulation of H_2O_2. However, following increase in the severity or days of water stress, SOD activity declined even in the tolerant varieties and POX metabolized the H_2O_2 produced; thus, a synergic activity of various enzymes is required for the attainment of tolerance (Chakraborty and Pradhan, 2011). Patade *et al* (2011) in their study have shown that the salt stressed plants showed an increase in the activities of SOD and APOX, while PEG stress led to an increase in SOD but not APOX activity as compared to the control. In a study by Pompelli *et al*. (2010) they have also reported that although the rate of superoxide formation may increase considerably in N–deficient coffee plants under high light conditions; however, no corresponding increase in the activity of SOD per unit mass could be found. Under drought stress, enhanced SOD activity was found in pea, tobacco and bean (Moran *et al*. 1994; Van Rensburg and Kruger 1994; Zlatev *et al*. 2006), decreased superoxide dismutase activity in sunflower seedlings and banana (Quartacci and Navari–Izzo 1992; Chai *et al*. 2005) and unaffected superoxide dismutase activity in maize (Luna *et al*. 1985). In wheat, SOD activity increased or remained unchanged in the early phase of drought, but decreased with prolonged drought stress (Zhang and Kirkham 1995), as also obtained in this study. Feng *et al* (2004) reported an increased SOD and CAT activities for a mild water deficit, whereas Guo *et al* (2006) pointed that severe or prolonged drought stress caused a decline in activities of this enzyme. Simova–Stoilova *et al* (2008) in their study on antioxidative protection in wheat varieties under severe

recoverable drought at seedling stage found that SOD activity only slightly changed and in recovery CAT activity became significantly higher; they were able to reveal three isoforms of SOD, one of catalase and three of GPX. They also determined that SOD activity was little changed as a consequence of drought stress and CAT activity was very low in drought–treated plants and after recovery it was significantly higher than that of control.

The over–expression of SOD, if accompanied by enhanced H_2O_2 scavenging mechanisms, like CAT and POX enzyme activities, has been considered as an important anti–drought mechanism to cope with oxidative stress during water deficit conditions. It was observed in our study that though all antioxidative enzymes increased initially, POX and GR activities could be maintained at higher levels in the tolerant varieties and hence contributed to the defense response. In the present study, POX showed a much pronounced or greater role in mechanism for imparting tolerance compared to CAT following increase in the degree of water stress. This was in accordance with the results obtained by Chakraborty *et al*. (2002) in their study on tea cultivars. However, in the study of Iqbal and Bano (2009) a greater increase in POX and CAT activities in wheat accessions was reported in both tolerant and susceptible plants following water stress. This result is similar to the results as obtained by Abedi and Pakniyat (2010) who reported enhanced activities of SOD and decreased CAT activity. To evaluate the degree of tolerance to NaCl, changes in growth parameters and activities of the antioxidant enzymes (SOD, CAT, APX and GR) were monitored by El–Bastawisy (2010) whose studies point to an enhanced degradation of H_2O_2 in tolerant wheat either directly from the oxidative stress or as a result of SOD activity suggesting faster elimination of ROS in tolerant variety than in the susceptible one. Moreover, he suggested the decreased APOX activity in susceptible wheat would result in higher accumulation of H_2O_2 than in the tolerant variety. He further suggested that such accumulation could result from a decrease in CAT activity with a consequence shortage in H_2O_2 degradation and/or a decrease in APOX activity with inefficiency in H_2O_2 scavenging by ascorbic acid. Sharada and Naik (2011) in their study on drought stress in groundnut also determined that the activities of antioxidant enzyme such as SOD, CAT, POX and GR increased

considerably with the progression of drought stress. Our data is also in accordance with work of authors like Hameed *et al* (2013), Kranner *et al* (2006) who have suggested that plants have evolved both enzymatic and nonenzymatic systems to scavenge the ROS where enzymes, including SOD, CAT, APOX, non–specific (guaiacol), POX, GR etc., work in concert with non–enzymatic antioxidants such as glutathione and ascorbate to detoxify ROS and have conclude that the antioxidant defense system may have a crucial role in signaling and execution of plant programmed cell death.

H_2O_2, resulting from the action of SOD, is toxic to cells. Therefore, it is important that H_2O_2 be scavenged rapidly by the antioxidative defense system to water and oxygen (Guo *et al*., 2006). The decline in CAT activity was in correlation with the increase in the accumulation of H_2O_2 following water stress as well as increased lipid peroxidation in all varieties. H_2O_2 accumulation and lipid peroxidation were significantly higher in susceptible varieties in comparison with tolerant ones. Our results are in conformity with those of several previous workers (Chai *et al*. 2005; Zlatev *et al*. 2006). Increased concentrations of H_2O_2, a strong oxidant, cause localized oxidative damage, disruption of metabolic functions and lipid peroxidation (Foyer *et al*. 1997; Velikova *et al*. 2000; Zlatev *et al*. 2006). However, besides being an ROS, H_2O_2 is also a signal molecule, which is involved in signal transduction mechanisms for several processes in plants such as stomatal closure, root growth and responses to pathogen challenge (Neill *et al*. 2002; Laloi *et al*. 2004; Desikan *et al*. 2005). Varieties which were less tolerant accumulated higher amount of H_2O_2. Various previous workers reported similar results (Chai *et al*., 2005; Zlatev *et al*., 2006) as those of the present study. The enhanced H_2O_2 levels under water deficit would be alleviated through the combined action of CAT and APOX. Thus, levels of H_2O_2 are efficiently controlled to maintain balance between production and breakdown. In this study, although H_2O_2 accumulation increased during drought and salinity stress, after a period of prolonged drought there was a decrease in H_2O_2 levels in tolerant varieties, indicating greater antioxidant activity. Similar results have been reported by Godfray *et al* (2011) in their study where they have compared

the peroxidation of lipid, leaf membrane thermostability and antioxidant system in four sugarcane genotypes differing in salt tolerance.

Foyer and Noctor (2012) have suggested that serious problems persist in our ability to actively extract, assay and accurately quantify H_2O_2 in tissues or extracts which are fraught with ambiguities. Such problems, according to him may be addressed by the development of biochemically suitable staining techniques. *In situ* detection of H_2O_2 in leaf tissues and microscopic observations revealed darker staining in tissues subjected to prolonged drought and salt stress, especially in the less–tolerant varieties such as LV, MW, SO and GY. In our studies the detection of cellular levels of H_2O_2 was done by DAB staining method and our results shows a clear difference in the degree of staining achieved in the control and the stressed plant in both drought and salt stressed varieties. The leaf of SO, LV, GY and MW showed more darkly stained DAB–sites in the tissues than in the leaves from the other five varieties with respect to their control during both drought and salt stress. Interestingly, DAB polymerization site was largely localized at the tip of the leaf, region surrounding the middle lamella and also the stomata of the leaf in the varieties under stress when compared to the leaf of the control set of GY. The transverse section of the leaf at the stained site showed that the DAB binding sites were localized mostly in the peripheral region of the cell.

Ascorbic acid, carotenoids and α–tocopherol (Vit E), non–enzymatic antioxidants in plants, increased significantly in all nine varieties. Accumulation of ascorbate was enhanced in all nine varieties even after 9 days of drought stress and even at 200 mM of salt stress on the 3rd day; carotenoids, however, decreased after 3 days of drought and at 100 mM of salt stress (3d) in varieties MW, GY, LV and SO and after 6 days of drought and at 200 mM (3d) in case of the other five varieties. Accumulation of α–tocopherol in the leaves of the drought stressed plant in general increased during the 3rd day of drought except in case of GY where it decreased, however, in KW, LV, PBW 343 and SO the accumulation of Vit E decreased after 6days of drought and after 9days in MW, GY and KD. In case of UP 2752 and GN however, the content of α–tocopherol increased on the 9th day of stress. Jaleel (2009) reported enhanced accumulation of ascorbic acid during drought stress in winter cherry (*Withania*

somnifera). In this study, the increase in ascorbate, along with glutathione reductase, indicates involvement of the ascorbate–glutathione cycle as a predominant mechanism of oxidative stress detoxification. Nair *et al*. (2008) reported that ascorbic acid contents in cowpea decreased with severity of drought stress, but tolerant cultivars had higher ascorbic acid contents during severe stress in comparison with susceptible cultivars. L–Ascorbic acid is a strong antioxidant but also performs several other functions in the plant (Noctor and Foyer 1998). Shalata and Neumann (2001) in their study concluded that there appears to have been no quantitative investigations of the effects of an additional supply of ascorbic acid on plant resistance to severe salt stress. Dalmia and Savhney (2004) observed an involvement of antioxidant metabolites in ROS detoxification under drought with increased pools of ascorbate and glutathione at the beginning of the water stress and diminution when the stress becomes more severe. Simova–Stoilova (2008) suggested that the ratio between reduced and oxidised ascorbate was more or less conserved. They suggest participation of the low–molecular antioxidative compounds in the defense against ROS under severe drought and rather good functionality of the ascorbate/glutathione cycle, which allowed wheat plants to maintain a low hydrogen peroxide level. Plant tissues also contain substantial amounts of carotenoids that serve as non–enzymatic oxygen radical scavengers (Young and Britton, 1990). Havaux (1998) and Kiani *et al.* (2008) suggest that water stress, among other changes, has the ability to reduce the tissue concentrations of carotenoids, primarily with the production of ROS in the thylakoids (Niyogi, 1999; Reddy *et al..*, 2004); however, reports dealing with the strategies to improve the pigments contents under water stress are entirely scarce.

The total antioxidative activity in the leaf following water stress increased with the increase in the duration of withholding water in all varieties with a slight decline observed during the 3rd day in case of MW. In GN, KD, UP 2752 and PBW 343 the total antioxidative activity continued to increase even at prolonged period of drought however; it declined in case of KW, MW, GY, LV and SO. In KD, GN, UP 2752 and PBW 343 the total antioxidative activity was significantly higher than the other varieties. The percent inhibition

of DPPH i.e. the total antioxidant activity in the leaf of salt stressed plants increased with the increase in the concentration and duration of stress but decreased at higher salt concentration in MW, LV, SO and GY.. In KD, GN, KW, UP 2752 and PBW 343 the total antioxidative activity was significantly high even at 200mM concentration and the highest value for the content of DPPH were observed in five of these varieties. Farooq and Azam (2002) have reported the co–existence of salt and drought tolerance in Triticaceae.

Na^+ and K^+ content in case of both water and salt stress increased significantly with the onset of stress treatments. Following water and salt stress treatments the content of Na^+ in case of roots was much higher than that of leaf in all varieties whereas K^+ content was higher in the leaf than the roots during the stress. It seems that the salt overly sensitive (SOS) pathway also regulates the vacuolar Na^+/H^+ exchange activity and contributes to Na^+ compartmentalization. (Qiu *et al*, 2004). SOS pathway co–ordinately regulates plasma membrane and tonoplast Na^+/H^+ antiporter activity which leads to Na^+ homeostasis and as a consequence salt tolerance. The transport of K^+ and Na^+ are regulated by *Saccharomyces cerevisiae* cation transport systems, such as HAL1 and HAL3, respectively. The transgenic tomato lines overexpressing the HAL1 gene were more salt–tolerant than the wild type plants in both callus and plant growth besides exhibiting better fruit yield under salt stress (Gisbert *et al*. 2000; Rus *et al*. 2001). Salt tolerance has been well studied and similar results have been obtained by workers such as Garcia–Sanchez (2006) on orange trees on rootstocks. Ghogdi *et al* (2012) have reported that salinity stress decreased K^+ content, K^+/Na^+ ratio and grain yield; however Na^+ content in all the genotypes and in both stages were increased and have reported that higher accumulation of Na^+ content was related with reduction in yield in case of salt sensitive genotype and higher K^+/Na^+ in case of tolerant genotype than the salt sensitive genotype. Serrano *et al* (1999) had also suggested that ion transporters selectively transport ions in order to maintain physiologically relevant concentrations whereas Na^+/H^+ antiporter play a vital role in sustaining cellular ion homeostasis, thus allow plant survival and growth under saline conditions through regulation of cytoplasmic pH, sodium levels, and cell turgor. In their study Bartels and Sunkar (2005) suggested that there exists

three types of mechanisms to prevent excess Na^+ accumulation in the symplast of plant cells which are firstly by restricting the Na^+ permeation and entry into plant cytosol by Na^+ transporters, secondly, the compartmentalization of Na^+ in to vacuole via, Na Na^+/H^+ antiporter and the third by the transport of cytosolic Na^+ back to the external medium or to the apoplast by plasma membrane Na^+/H^+ antiporter. Even though leaves accumulated high sodium concentrations, fruits displayed very low sodium content, demonstrating the potential to maintain fruit yield and quality at high salt levels (Jan *et al.*, 2013). According to Tester and Davenport (2003) the high levels of Na^+ or Na^+ K^+ ratios can disrupt various enzymatic processes in the cytoplasm. Blaha *et al.* (2000) suggest that protein synthesis requires high concentrations of K^+, owing to the K^+ requirement for the binding of tRNA to ribosomes and probably disruption of protein synthesis by elevated concentrations of Na^+ appears to be an important cause of damage by Na^+. Our data is in accordance with the results obtained by Ghogdi *et al.* (2012) who have documented that a greater of salt tolerance in plants is associated with a more efficient system for selective uptake of K^+ over Na^+ (Wenxue *et al..*, 2003) and under salt stress, plants maintain high concentration of K^+ and low concentration of Na^+ in the cytosol. They do this by regulation the expression and activity of K^+ and Na^+ transporters and H^+ pumps that generate the driving force for transport (Zhu, 2003) and the regulation of K^+ uptake, prevention of Na^+ influx, promotion of Na^+ efflux from the cell and utilization of Na^+ for osmotic adjustment are the strategies commonly used by plants to maintain desirable K^+/Na^+ ratio in cytosol.

The various effects of drought stress were ameliorated with the use of chemicals like ABA, SA and proline. Ali *et al.* (2008) have reported in their extensive study that the role of different compatible solutes in plant tolerance to drought stress is significant because they regulate multitude of metabolic processes including ion transport and have hypothesized that the exogenous application of proline might regulate uptake of mineral nutrients in plants subjected to water deficit conditions. Various workers have reported that the use of certain exogenous chemicals on the plants before stress treatment can induce tolerance to a much surprising level. In particular, induced

thermotolerance has been reported when plants were pre–treated with salicylic acid (SA) (Aldesuquy *et al*. 2012, Dat *et al.*. 1998, Larkindale and Knight 2002), abscisic acid (ABA) (Bonham–Smith *et al.*. 1998, Bray 1991, Jiang and Huang 2001, Larkindale and Knight 2002), calcium (Gong *et al.*. 1997) and ethylene (Larkindale and Knight 2002). Larkindale and Huang (2004) have 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 pre–treatment's was associated with the regulation of antioxidant regenerating enzymes. Different reports suggests that the exogenous application of proline induces abiotic stress tolerance in plants (Claussen, 2005; Ali *et al..*, 2007) although much attention has been paid on the role of proline in stress tolerance as a compatible osmolyte (McCue & Hanson, 1990; Samras *et al..*, 1995), little attention has been given to its role in affecting the uptake and accumulation of inorganic nutrients in plants (Okuma *et al..*, 2000; Khedr *et al..*, 2003).

In the present study abscisic acid (ABA), salicylic acid (SA) and proline were selected as pre–treatment chemicals for the amelioration of drought. Pre–treatments of seedlings with solutions of ABA, SA and proline followed by drought stress for 3, 6 and 9 days revealed that all three chemicals could provide protection against oxidative stress due to water stress in the wheat varieties. The RWC and CMS of the seedlings during drought for subsequent days after chemical pre–treatments showed an increase in both GN and LV. The increase in RWC and CMS of GN was higher after pre–treatment than that of LV during drought. The application of pre–treatment by proline had the best effect in the amelioration of drought in case of both tolerant and susceptible variety of wheat in the present study. Lipid peroxidation after pre–treatments in GN and LV followed by drought stress showed a decrease in its value than the values obtained during drought without pre–treatments with the chemicals. The pre–treatment by the use of proline, ABA and SA could reduce lipid peroxidation in case of both GN and LV. There was a significant decrease in the peroxidation of membrane lipid as evident by the reduced value of MDA content in case of LV and this susceptible variety was able to combat the

deleterious effect of water stress. After the induction of drought treatments preceded by pre–treatments in the seedlings it was found that the content of proline, total soluble carbohydrate, reducing carbohydrate and starch content showed a markedly lower value than the values obtained without chemical pre–treatments. Seedlings of GN and LV where no pre–treatment was done had shown gradual increase in its value with the increasing days of drought but after pre–treatments the values were significantly lower in both GN and LV. The content of proline, total soluble sugars, reducing sugars and starch in case of LV was significantly lower during increasing days of drought following pre–treatments with chemicals and both GN and LV were more tolerant to drought and therefore the effect of drought stress was ameliorated by the use of chemicals. Travaglia *et al* (2007) have also reported that the exogenous abscisic acid increases carbohydrate accumulation and redistribution to the grains in wheat grown under field conditions of soil water restriction. The effect of pre–treatment by proline showed the best result followed by ABA and SA in both the varieties. Fang *et al* (2008) reported that mutations of genes in synthesis of the carotenoid precursors of ABA lead to pre–harvest sprouting and photooxidation in rice. Freeman *et al* (2005) have reported that constitutively elevated salicylic acid signals glutathione–mediated nickel tolerance in Thlaspi nickel hyper–accumulators in their study. Somasundaram *et al* (2009) have also reported the role of paclobutrazol and ABA in drought stress amelioration in *Sesamum indicum* L.

The antioxidative profile of the LV and GN with prolonged drought after pre–treatment with ABA, proline and SA was better than values obtained in GN and LV seedlings were no chemical pre–treatments was done with the chemicals. Pre–treatment with proline increased the antioxidative profile in both the varieties more than that of SA and ABA with increasing days of drought stress. Total phenol content and ortho phenol content in drought stressed GN and LV following chemical pre–treatments showed a comparatively lower value than the seedlings which were untreated with chemicals. The lower value of total phenol and ortho phenol in case of LV with respect to the fact that LV was the most susceptible variety in the present study was very significant. Total antioxidant activity in drought stressed GN

and LV following chemical pre–treatments increased significantly during all days of stress. The total antioxidant activity in case of proline pre–treated seedlings of both GN and LV showed the best results in terms of better antioxidative profile during drought. LV which was considered the most susceptible variety in our study showed an increased total antioxidant activity (% inhibition of DPPH) with the increase in the duration of withholding water and continued to show higher values when compared to the LV seedlings in drought without pre–treatment respectively. The increase in the total antioxidant activity of drought stressed GN and LV after pre–treatment with chemicals proved that the use of chemicals prior to the stress conditions could render the plant more vigorous to tolerate drought stress. Carotenoids and ascorbic acid – two of the non–enzymatic antioxidants in plants increased significantly in both GN and LV following drought stress after the seedlings where pre–treated with chemicals than the seedlings of GN and LV where drought was induced without chemical pre–treatments. Accumulation of both carotenoids and ascorbate in the pre–treated leaf of both tolerant and least tolerant or susceptible variety increased with pre–treatments with ABA, SA and proline and the highest values for the accumulation of both the antioxidants was observed in case of plants pre–treated with proline solution than the plants pre–treated with ABA and SA.

The activity of antioxidative enzymes following drought stress where the seedlings were pre–treated separately with ABA, proline and SA enhanced remarkably in both GN and LV. The increase in the activity of the antioxidative enzymes in the leaves of these varieties enabled the plant to combat the deleterious effect of drought in regard of better scavenging of the ROS from the system. CAT activity in case of pre–treated plants was significantly high in both GN and LV following drought stress; however the increase in CAT activity was higher in GN than LV and the seedling pre–treated with proline in both the cases showed higher CAT activity than the seedling treated with SA and. The activity of POX increased significantly in both GN and LV with the onset of drought after pre–treatment where the POX activity increased with prolonged period of drought. The increase in the POX activity was significant in case of LV where the antioxidative profile of the

leaves after pre–treatment enhanced with increasing days of withholding water which was significantly higher in the wheat seedling pre–treated with proline followed by SA and ABA. APOX activity in the pre–treated seedlings in LV showed a very significant increase than the increase in case of pre–treated seedlings of GN following drought stress however in both the varieties pre–treatment with ABA, proline and SA followed by drought stress ameliorated the effect of drought stress. The activity of GR and SOD following pre–treatments in the seedlings of GN and LV followed by drought stress enhanced with the increase in the periods of drought when compared to the untreated seedlings of GN and LV during drought respectively. The enhancement in the activity of GR in GN was much more than the increase in case of LV with increase in the days of water stress following pre–treatments with chemicals. The increase in the activity of the entire antioxidative enzyme was much more pronounced in case of pre–treatments with proline followed by SA and ABA. Significant change with respect to the enhancement of activity of antioxidative enzyme during drought was observed in the seedlings of GN and LV where the plants were pre–treated with chemicals than the plants were no such pre–treatment was done. LV considered as the most susceptible variety in our study too showed a better performance in combating drought evident by the increased activity of antioxidative enzymes when it was pre–treated with chemicals.

Protein content of GN and LV following drought stress after pre–treatment with ABA, proline and SA decreased significantly during all days of water stress when compared to the corresponding values obtained in GN and LV where no treatment was done. The protein content was comparatively lower in case of LV for the initial days of stress however; on the 9^{th} day of drought stress the change in the content of protein was significant during water stress following chemical pre–treatments than the LV seedlings where no such treatment was done. Similarly in GN, the protein content without chemical pre–treatments was higher during the 9^{th} day and decreased after the chemical pre–treatments followed by water stress. The content of protein in the seedlings treated with proline showed a significant difference in both the varieties and therefore was more efficient.

In this study, H_2O_2 accumulation during water stress after the pre–treatment of seedling of GN and LV with chemicals decreased in GN and LV indicating greater antioxidant activity following pre–treatments with chemicals. The accumulation of H_2O_2 in the leaf of both GN and LV where no pre–treatment was done in the seedlings before the onset of drought stress was much higher during the increasing days of drought than the seedlings were pre–treatment with ABA, proline and SA was done. The leaf of GN showed lesser H_2O_2 accumulation than that of LV both with and without chemical pre–treatments however, pre–treatment with chemicals showed better results. During microscopic studies of the leaf tissues in DAB staining test for the detection of H_2O_2, dark–brown spots were observed as big and small patches at the site of DAB polymerization. The leaf of LV and GN showed lesser darkly stained DAB–sites in the tissues respect to their control and also when compared to the DAB–sites in the leaf of untreated GN and LV respectively following pre–treatments with chemicals. The accumulation of H_2O_2 significantly decreased in case of LV which was remarkable as the LV was considered as the most susceptible variety in our case. Our data is in accordance with the data of several workers such as Xue–Xuan *et al* (2010) have reported that biotechnological implications from abscisic acid (ABA) roles in cold stress and leaf senescence as an important signal for improving plant sustainable survival under abiotic–stressed conditions, free proline accumulation under drought (Yamada *et al*, 2005), responses of plants to drought and stress tolerance (Yordanov and Christova, 1997), pattern of antioxidant enzyme system in wheat exposed to water deficit conditions (Zhang *et al*, 2003).

Our results indicate that both drought and salt stress induced oxidative damage in wheat varieties could be overcome by enhanced activities of antioxidative enzymes. The activity of all the antioxidative enzymes seemed to be correlated with the each other during the stress response of the plant which can be used as biochemical markers for the identification of tolerant and susceptible varieties. Accumulation of compatible solutes like sugars, phenol, protein, proline and free amino acid increased in response to both drought and salt stress and their quantification was markedly different in the tolerant and

the susceptible varieties. RWC, CMS, tolerance index, chlorophyll content, lesser peroxidation of membranes, lesser accumulation of H_2O_2 was much more pronounced in the tolerant varieties which may have led to more protection from oxidative damage and hence these parameters are important in stress studies. The adaptation of wheat varieties to salt stress was comparatively better than during drought stress and it was concluded in our study that wheat varieties were able to withstand moderate salt stress of about 50mM or lesser. However, only the tolerant varieties were able to combat drought stress and showed better results in terms of their tolerance level whereas the susceptible variety at higher severe condition of drought were prone to poor growth and development and eventually death. Pre-treatments with some chemicals could induce tolerance to a certain degree and in our study pre-treatment with proline showed the best result in the amelioration of drought.

CHAPTER 6
CONCLUSION

- The seedling stage of the plants, i.e., one month old plant was the best stage to study the effects of drought and salinity stress on the plant growth and development.
- The morphology of the plant with the onset of drought salt stress did not show immediate effect on the plant. With prolonged water stress GN, KD, KW, UP 2752 and PBW 343 showed better tolerance when compared to LV, MW, SO and GY which showed wilting and yellowing of the leaf starting from the 3rd day of drought, however, the former wheat varieties did not show such symptoms almost till the 7th day.
- Similarly, during higher concentration of salt varieties such as LV, MW, SO and GY showed yellowing of the leaf and this yellowing and wilting increased with increase in the days of salt stress when compared to the other varieties.
- RWC in the leaf tissues was found to be an important parameter to study the plants' ability to retain water in their leaf during drought and salinity stress. Out of the nine varieties studied, it was evident that the highest amount of water was retained in the varieties like GN, KD, KW, UP 2752 and PBW 343 during drought treatments as well as during different salt concentrations. The RWC in case LV, MW, GY and SO was significantly lower than the other five varieties. However, with the onset of both drought and salt stress, the RWC in the leaves of all varieties was comparatively lower with respect to their respective control set.
- A significantly low tolerance index was observed in case of the leaf of LV, MW, GY and SO which in case of KD, GN, KW, UP 2752 and PBW 343 was higher during the 9th day of drought and also during the salt concentration

of 200mM on the 3rd day. The tolerance index of all the nine varieties was higher during the salt stress than during drought.

❖ CMS of the leaf served as another important physiological parameter which was used to identify the most tolerant genotype among the tested variety. The cell membrane stability decreased in all the varieties with the onset of stress treatments with respect to their respective control set. CMS index was comparatively higher in case of GN, KD, KW, UP 2752 and PBW 343 than LV, MW, SO and GY during drought and salt treatment. Highest CMS value was obtained in case of GN and the least was observed in case of LV.

❖ Lipid peroxidation in the membranes increased or was higher in the stressed plants in case of all the varieties with the onset of drought and salt treatments. Lipid peroxidation in the membranes of the leaf tissue was found to be the least in case of GN during drought and salt stress followed by KD, KW, UP 2752 and PBW 343 than the other four varieties where LV showed the highest degree of peroxidation of their membranes of the leaf tissues as evident by the MDA content of their leaf followed by GY, MW and SO.

❖ Decrease in RWC, CMS and lower tolerance index during drought and salinity stress could be correlated with higher degree of lipid peroxidation and therefore it served as an important physiological parameter to test the tolerance level among the tested nine varieties of wheat.

❖ Higher proline content in both leaf and root was recorded with the onset of drought and salt in all the varieties. The accumulation of free proline was higher in the leaves than their accumulation in the roots in all nine tested varieties. Proline content in the leaf tissues of GN, KD, KW, UP 2752 and PBW 343 was significantly higher than the other four. However, in the other four varieties, the increase was comparatively lower and in case of LV, lowest accumulation of proline was observed and the highest value for proline was obtained in GN.

❖ The profile of free amino acids as observed in the HPLC study supported our findings that the amount of free proline along with other important free amino acid was significantly higher with the increase in the

stress treatment. This increase was much more in case of GN than LV as observed in HPLC during drought treatments.

❖ Chlorophyll content decreased in almost all varieties with the induction of drought and salt treatments. However in case of varieties such as GN and KD there was an initial increase. The lowest chlorophyll content was observed in case of LV with prolonged periods of drought and increase in the concentration and days of salt stress when compared the their respective control. The decrease in the content of chlorophyll was higher in LV, MW, GY and SO than the decrease in case of GN, KD, KW, UP 2752 and PBW 343.

❖ Significantly, the data obtained for the ratio of chlorophyll a and chlorophyll b suggested that the trend obtained in the ratio of these two pigments could better explain response of plants to drought and salt stress with increasing days of water stress and increasing concentration of salt stress. The ratio of chlorophyll a to chlorophyll b increased initially in all varieties and decline with increase in the severity of stress. However, in LV, GY, SO and MW, the decrease in the ratio of chl. a/b was greater than the decrease in case of other five varieties in our study. The decrease in the ratio was attributed to the fact that the content of chl. a decreased significantly in the varieties during stress.

❖ The accumulation of total soluble sugar in both leaf and root of all the nine varieties during both drought and salt stress showed significant increase with the increase in the severity of stress treatment in case of GN, KW, KD, UP 2752 and PBW 343 which in case of LV, MW, GY and SO decreased after an initial increase. The accumulation of total soluble sugar in both leaf and root was highest in GN than all the tested varieties and the least accumulation was observed in LV with respect to their control set. The content of total soluble carbohydrates in the leaf was found to be higher than the roots in all cases including the control plants.

❖ Similarly the accumulation of reducing sugars in both leaf and root of all the tested nine varieties in our study during stress treatments showed significant increase with the increase in the severity of stress treatments. The content of reducing sugar in the leaf and root in all varieties continued to increase during stress treatments with the increase in case of GN, KW, KD, UP

2752 and PBW 343 being much higher that of the increase in its content LV, MW, GY and SO decreased after an initial increase. Reducing sugars in both leaf and root was highest in GN than all the tested varieties and the least accumulation was observed in LV with respect to their control set and the leaf reducing sugars was much more than that in the roots in both control and stressed plants.

❖ Accumulation of total soluble starch increased significantly in the leaf and roots of all the nine varieties with a slight decrease with the increase in the severity of both drought and salt treatments. This decrease was higher in the leaf than the roots in all cases. GN and KD accumulated more starch in their tissues than all the other varieties in both leaf and root and the lowest accumulation was observed in case of LV.

❖ The increase in the accumulation of total and o–dihydroxy phenols was evident in all the nine varieties with the induction of drought stress. In MW, GY, LV, PBW 343 and UP 2752 the accumulation of total phenol decreased at higher duration of drought. In case of GN, KD and KW the content of total phenol was found to be the higher and the highest value obtained in the leaf of GN during drought stress. However, the accumulation of total phenol in the tested nine varieties during salt stress increased in GN, KD, KW and UP 2752 only and in other varieties total phenol content was higher only at lower concentration of salt treatment. GN leaf showed the highest content of total phenols and in LV lowest content of phenol accumulation was observed in our findings.

❖ HPLC analysis of phenols revealed that during both drought and salt stress, the accumulation of ferulic acid was most significant in GN and LV and the change in the accumulation of ferulic acid as observed in the HPLC data was found to be significant with increase in the days of drought and the concentration of salt. Along with ferulic acid, salicylic acid, chlorogenic acid, caffeic acid was also found to play a significant change during stress. These phenols in case of GN were significantly accumulated in much more concentration than in case of LV during drought and salt stress. Phenolic acid analysis by HPLC also revealed that during both drought and salt stress again, the accumulation of ferulic acid was most significant in GN and LV and the

change in the accumulation of ferulic acid as observed in the HPLC data was found to be significant. Along with ferulic acid, vanillic acid, cinnamic acid, chlorogenic acid and salicylic acid were also very significant during drought and salt treatments and the accumulation of these Phenolic acid was higher in GN than in LV.

❖ The accumulation of total soluble proteins in the leaf and roots in the case of all the tested wheat varieties showed a general decline with the increase in the days of withholding water and the increase in the concentration of salt. In case of LV, GY, MW, SO and PBW 343 the decrease in the accumulation of protein in the leaf was higher than the decrease in the other varieties while in roots there was a slight increase in the protein content in MW, GY, GN, LV and SO. During salt stress, the content of soluble protein in roots slightly increased however, it decreased with higher concentration of salt and increase in the days of salt stress.

❖ At least a total of about 34 new bands were observed in SDS PAGE of proteins during drought and salinity. Seedlings of wheat subjected to drought and salt stress showed an expression of new protein bands in the SDS PAGE analysis of protein in the leaf in some cases and suppression of certain existing bands at different duration of withholding water and at increasing concentration of salt. More number of new bands was observed in case of GN, KD, KW, UP 2752 and PBW 343 during the SDS PAGE analysis of leaf proteins during both drought and salinity stress which in case of other four varieties was comparatively lesser in number.

❖ The total soluble protein profile in the leaf of two varieties of wheat (GN and LV) during drought stress was determined in Fast protein liquid chromatography (FPLC) where it was observed that the number, height and the area of peak showed a significant difference during the increasing days of water stress with respect to control plant.

❖ The accumulation of metabolites during stress such as total soluble sugar, reducing sugar, starch, free amino acids could be correlated with the protein content in the leaf and root and could serve as important parameters to study the metabolism of the plants under stress.

❖ APOX activity increased significantly in all nine varieties however decreased with prolonged water stress in all cases. Similarly during salt stress the activity enhanced with increase in the concentration of salt however decreased on increasing days of salt stress in all the tested varieties. Increase in the activity of APOX was significantly much more pronounced in case of MW, GY, LV and SO. Thus, the enhancement in the activity of APOX suggested a possible underlying mechanism during drought and salt stress in case of these varieties.

❖ With prolonged period of drought stress and with increase in the concentration of salt the activity of GR increased significantly in all varieties but in MW, GY, LV and SO it decreased with increase in the severity of stress however, in case of KD, GN, KW, UP 2752 and PBW 343 the activity increased significantly.

❖ It was noted that the POX activity enhanced greatly with increase in the period of water stress in case of GN, KD, KW, UP 2752 and PBW 343 whereas in LV, GY, MW and SO, the activity of POX declined. Activity of peroxidase (POX) showed a continuous enhancement with increase in the concentration of salt in all varieties however in case of MW, LV, SO and GY the activity of peroxidase with prolonged stress showed a significant decline in the later period of stress and higher concentration of salt.

❖ In case of peroxidase isozyme analysis in NATIVE–PAGE, new bands were observed in the stressed varieties with respect to control in case of almost all the varieties with highest number of new peroxizymes recorded in case of varieties like GN KW, KD followed by PBW 343 and UP 2752 than SO, LV, GY and MW with their respective control at 0days of drought and the same trend was observed during salt stress. The activity of peroxidase and the occurrence of bands in NATIVE PAGE for peroxidase isozyme were correlated.

❖ A general decrease was seen in the activity of CAT during both drought and salt stress in case of MW, GY, LV and SO whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. The activity of CAT following salinity stress showed a continued decline in case of MW, GY, LV and SO with the increase in the concentration and duration of

salt stress however in case of KW, KD, GN, UP 2752 and PBW 343 there was an initial enhancement in the activity of CAT followed by decline in its activity with increasing concentration of salt.

❖ In the catalase isozyme analysis in NATIVE–PAGE during drought and salinity stress, significant differences was noticed among the varieties as well as during the different days of drought and different concentration of salt. The occurrence of catalase isozyme was more expressed in case of NATIVE PAGE of leaf in GN, KD, KW during drought and in KD and KW in case of salinity stress.

❖ During drought and salt stress, SOD activities decreased at all periods of drought and concentration of salt stress in case of MW, GY and whereas in KD, GN, KW, UP 2752, PBW 343 and SO activities of these enzymes increased initially before showing a continued decline. With prolonged stress the activity of SOD during both drought and salt stress in case of MW, GY, LV and SO showed a continued decrease whereas in case of KW, GN, UP2752, PBW 343 and KD an initial enhancement was seen. SOD is the first enzyme which is expressed in the antioxidant mechanism and it increased initially in the more tolerant varieties and was also involved in contribution to the initial accumulation of H_2O_2. Although H_2O_2 accumulation increased during water and salt stress, after a period of prolonged drought and with the increase in the concentration of salt in the medium there was a decrease in H_2O_2 levels in varieties like KW, GN, KD, PBW 343 and UP 2752 indicating greater antioxidant activity whereas the accumulation of H_2O_2 continued to increase in LV, SO, GY and MW with the increase in the duration of withholding water from the plants and increase in the concentration of salt and days of salt stress. Highest H_2O_2 accumulation was observed in case of LV and lowest was observed in case of GN with increase in the severity of stress.

❖ In microscopic studies, the leaf of SO, LV, GY and MW showed more darkly stained DAB–sites in the tissues than in the leaves from the other five varieties with respect to their control during both the drought and salt stress. DAB polymerization site was largely localized at the tip of the leaf, region surrounding the middle lamella and also the stomata of the leaf in the varieties under stress when compared to the leaf of the control set. The

transverse section of the leaf at the stained site showed that the DAB binding sites were localized mostly in the peripheral region of the cell.

❖ The decreased activity of CAT in case of MW, SO, LV and GY was correlated with higher concentration of H_2O_2 in these varieties and similarly the higher activity of CAT was correlated with lower concentration of H_2O_2 in these varieties during both drought and salt stress respectively. This was also evident in the microscopic detection of H_2O_2 in the respective varieties during drought and salt treatments.

❖ Accumulation of carotenoids in the leaves, during drought showed an initial enhancement in all the varieties followed by a decrease after 3 days in varieties MW, GY, LV and SO and after 6 days in KD, KW, GN, UP 2752 and PBW 343. Carotenoid content showed an increase in its accumulation with the increase in the concentration and duration of salt stress in all the varieties but with prolonged days of salt stress and increasing concentration of salt the accumulation declined at in case of MW, GY, LV and SO and at higher concentration of salt in case of KD, GN, KW, UP 2752 and PBW 343.

❖ The accumulation of ascorbate in the leaf was enhanced in all nine varieties even after 9 days of drought stress and increased at all periods of salt stress and enhanced with increasing concentration of salt in all the varieties. The highest accumulation of ascorbate was noted in case of GN, KW, KD, UP 2752 and PBW 343.

❖ Accumulation of α–tocopherol in the leaves of the drought stressed plant in general increased but decreased after 3^{rd} day in case of GY, after 6^{th} day in KW, LV, PBW 343 and SO and after 9^{th} day in MW, GY and KD however, in case of UP 2752 and GN it increased during the 9^{th} day of water stress. α–tocopherol content in the salt stressed leaf increased with the increase in the days and concentration of salt stress initially but declined at higher concentration and duration of salt stress in case of MW, GY, LV and SO and in the other five varieties it declined at higher salt concentration.

❖ Total antioxidant activity increased in case of GN, KD, UP 2752 and PBW 343 even after nine days of drought with a slight initial decline in MW during drought and in KW, MW, GY, LV and SO it decline at prolonged periods of drought. The total antioxidant activity declined at higher

concentration of salt in LV, MW, SO and GY however it continued to increase in the other five varieties.

❖ Carotenoids, ascorbic acid and α–tocopherol three of the non–enzymatic antioxidants in plants increased significantly in all the nine varieties following drought stress and were correlated with the total antioxidant activity following drought and salt stress which was again correlated with the activity of antioxidative enzyme activities.

❖ Ionic imbalance with respect to the content of Na+ and K+ content was observed during drought and salt stress. Na^+ content in case of both water and salt stress increased significantly with the onset of stress treatments. Following water and salt stress treatments the content of Na^+ in case of roots was much higher than that of leaf in all varieties. In MW, GY, LV and SO the increase in the Na^+ content was more than the increase in the other varieties following stress and the content of Na^+ in the roots were higher. The increase in the content of Na^+ was much more in the roots than the leaf and four varieties i.e. MW, GY, LV and SO showed the highest content of Na^+ in both the leaf and roots with increasing concentration and duration of salt stress.

❖ K^+ content was higher in the leaf than the roots during the stress. K^+ content in both leaf and root increased significantly during the initial phase of water stress but later with prolonged stress, the content of K^+ in both leaf and root declined; the decline in case of MW, GY, LV and SO was much more than compared to the other five varieties where the decrease in the content of K^+ was lesser. The decline in K^+ content of the leaf and root during salinity stress in case of MW, GY, LV and SO was much more pronounced than the varieties GN, KD, KW, UP 2752 and PBW 343.

❖ The effect of ABA, SA and proline pre–treatments on amelioration of drought in GN and LV showed a better response of the plant to drought treatments in both the varieties after ABA, SA and proline pre–treatment especially in the case of LV which was considered as the most susceptible variety or in other words the least tolerant variety in our study.

❖ The effect of proline pre–treatment in GN and LV showed better results than the pre–treatment with ABA followed by pre–treatment with SA.

❖ Pre–treatments of seedlings of GN and LV with solutions of ABA, SA and proline forwarded by drought stress for 3, 6 and 9 days revealed that all three chemicals could provide protection against oxidative stress due to water stress in these varieties with respect to enhanced enzyme activities like CAT, POX, APOX, SOD and GR, decreased accumulation of phenols and of non–enzymatic antioxidants like carotenoids and ascorbate suggesting enhanced antioxidative mechanisms in the two varieties during drought stress.

❖ Pre–treatments by these chemicals could also enhance the physiological parameters such as RWC, CMS and was able to decrease the peroxidation of membranes in the leaf and lesser accumulation of H_2O_2 was observed in both GN and LV which was responsible for maintaining a better morphological and physiological property of the plants under drought.

❖ Both GN and LV seedlings when pre–treated with solutions of ABA, SA and proline before the induction of drought showed lesser accumulation of protein and the content of compatible solutes like proline, total soluble carbohydrate, reducing sugars showed a decrease in the seedlings of both the varieties in both leaf and roots suggesting the lesser need of plant to produce these compatible solutes as these chemicals could ameliorate the effect of drought stress and therefore lesser compatible solutes was needed by the plants under stress to combat water stress.

❖ The results of this study clearly indicate that both drought and salt stress induced oxidative damage in wheat varieties could be overcome by enhanced activities of antioxidative enzymes. The activity of all the antioxidative enzymes seemed to be correlated with the each other during the stress response of the plant.

❖ The higher accumulation of compatible solutes like sugars, phenol, protein, proline and free amino acid, higher RWC, high values for CMS, greater tolerance index along with increased antioxidative profile, lower chlorophyll content, lesser peroxidation of membranes, lesser accumulation of H_2O_2 was much more pronounced in five varieties KW, GN, UP2752, PBW 343 and KD than in MW, GY, LV and SO, the former being probably more tolerant and therefore more protected from oxidative damage. Taking into consideration all the available data, it is concluded that whereas KW, GN,

UP2752, PBW 343 and KD could be considered as tolerant, MW, GY, LV and SO were susceptible to both drought and salt stress.

- ❖ According to the data, GN was found to be most tolerant variety and LV was found to be least tolerant or susceptible variety.
- ❖ It was evident the adaptation of wheat varieties to salt stress was comparatively better than during drought stress and it was concluded in our study that wheat varieties were able to withstand moderate salt stress of about 50mM or lesser. However, only the tolerant varieties were able to combat drought stress and showed better results in terms of their tolerance level whereas the susceptible variety at higher severe condition of drought were prone to poor growth and development and eventually death. However, pre–treatments with some chemicals could induce tolerance to a certain degree.

CHAPTER 7
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APPENDICES

APPENDIX A: List of Thesis related publications

Publications in journals/Books:

1. **Chakraborty U and Pradhan B**. Oxidative stress in five wheat varieties (*Triticum aestivum* L.) exposed to water stress and study of their antioxidant enzyme defense system, water stress responsive metabolites and H_2O_2 accumulation. *Braz. J. Plant Physiol.* **24**(2): 117-130, 2012.

2. **Pradhan B and Chakraborty U**. ROS production, H_2O_2 detection and biochemical characterization of water stressed wheat (*Triticum aestivum* L.) varieties. *North Bengal University J. Plant Sci.* **6**(1):63-70, 2012.

3. **Chakraborty U and Pradhan B** Drought stress-induced oxidative stress and antioxidative responses in four wheat (*Triticum aestivum* L.) varieties. *Archiv. Agro. Soil Science.* **58**(6):617-630, 2011.

4. **Chakraborty U, Pradhan D, Lama R, Pradhan B, De U and Chakraborty BN**. Abiotic stress induced biochemical response in cereals and legumes and associated changes in arbuscular mycorrhizal population. *Microbial resources for crop improvement*. Satish Serial Publishing house. ISBN 978-93-81226-39-1.183-198, 2013.

APPENDIX B: List of Abbreviations

α–toc	α–tocopherol or vitamin E
n	number
t test	students' t test
°C	degree Celsius
μ	micro
μL	micro litre
μm	micro meter
ACC	1–aminocyclopropane–1–carboxylic acid
A	absorbance
ABA	abscisic acid
AO*	active oxygen species
AOH	active hydroxyl radical
APOX	ascorbate peroxidase
Approx	approximately
APS	ammonium per sulphate
AsA	ascorbate
AA	ascorbic acid
ATP	adenosine tri phosphate
Bha	billion hectares
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
C/N ratio	carbon to nitrogen ration
CA	cinnamic acid
Ca^{2+}	calcium divalent cation
$CaCl_2$	calcium chloride
CAT	catalase
Cd	cadmium
CL	chemi line
Chl a/b	chlorophyll *a* to chlorophyll *b*
Cl^-	chloride ion
cm	centimetre
CMS	cell membrane stability
$CuSO_4$	copper sulphate
d	day
d.m.	dry mass
d.w.	dry weight
DAB	diaminobenzidine
D	Day (or days)
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DL	dextro–laevorotatory
DNA	deoxyribonucleic acid
DNPH	2, 4, dinitrophenylhydrazine
DPPH	2,2–diphenyl–1–picrylhydrazyl
dSm^{-1}	deciSiemens per metre
EC	enzyme commission
EDTA–Na_2	ethylene diamine tetra acetic acid–di sodium salt
C1	electrical conductivity
et al	*et* alii (and others)
EU	enzyme unit

f.m.	fresh mass
f.w.	fresh weight
Fd	ferredoxin
FA	ferulic acid
Fig	figure
FPLC	fast protein liquid chromatography
FW_c	fresh weight control
FW_t	fresh weight treatment
g	gram
gr	gram
gm	gram
GN	Gandhari
GY	Gayetri
GB	glycinebetaine
GPX	glutathione peroxidase
GR	glutathione reductase
GSH	Glutathione reductase
GSSG	Oxidised glutathione
H_2O_2	hydrogen peroxide
H_2SO_4	sulphuric acid
Ha	hectare
HCl	hydrochloric acid
$HgCl_2$	mercury chloride
HPLC	high performance liquid chromatography
h	hour
ha	hectare
hr	hour
hrs	hours
(HO^-)	hydroxyl radical
(HR)	hypersensitive response
K	potassium
K+	potassium monovalent cation
KW	Kaweri
KDa	kilo dalton
KD	Kedar
L	litre
LV	Local Variety
m	metre
M	molar
mA	milli ampere
MDA	malondialdehyde
mg	milli gram
mg g^{-1}	milli gram per gram
Mg^{2+}	magnesium divalent cataion
$MgCl_2$	magnesium chloride
mha	million hectare
min	minute
mL	milli litre
mM	milli molar
mmol	milli molar
MW	Mohan Wonder
mol	molar
MDA^-	monodehydroascorbate radical
$MDHAR^-$	monodehydroascorbate reductase
MPa	mega pascal

mV	milli volt
mVs	milli volt second
N	nitrogen
Na	sodium
Na+	sodium monovalent cation
Na_2CO_3	sodium carbonate
Na_2MoO_4	sodium molybdate
Na_2SO_4	sodium sulphate
NADPH	nicotinamide adenine dinucleotide phosphate reduced
NADPH–Na_2	nicotinamide adenine dinucleotide phosphate reduced tetrazolium salt
$NaHCO_3$	sodium bicarbonate
$NaNO_2$	sodium nitrite
NaOH	sodium hydroxide
Na_2PO_4	sodium phosphate
PAGE	polyacrylamide gel electrophoresis
NBT	nitrobluetetrazolium chloride
nm	nanometre
No.	number
O_2	oxygem
OD	optical density
OA	osmotic adjustment
ψP	osmotic potential
p	probability
PA	phenolic acid
PCD	programmed cell death
PCA	p–coumaric acid
PITC	phenylisothiocyanate
PMSF	phenylmethylsulfonylfluoride
PPO	polyphenol oxidase
PS II	photosystem ii
PVPP	polyvinylpolypyrrolidone
POX	peroxidase
QTL	quantitative trait loci
R* or ROO	reactive species
GSH	reduced glutathione
RH	relative humidity
Rm	relative mobility
ROS	reactive oxygen species
rpm	rotation per minute
RWC	relative water content
S.E	standard error
s	second
SA	salicylic acid
SDS	sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH	sulphhydrl containing enzymes
$^1O_2$	singlet oxygen
SOD	superoxide dismutase
SO	Sonalika
$O_2{}^{*-}$	superoxide radical
O_2^-	superoxide anion
SAA	systemic acquired acclimation
SAR	systemic acquired resistance

t.s.	transverse section
TCA	trichloroacetic acid
TEMED	N,N,N',N'–tetramethylethylenediamine
TBARS	thiobarbituric acid reactive substances
TI	tolerance index
$TiSO_4$	titanium sulphate
TE	tracheary elements development
TEA	triethylamine
UBKV	uttar banga krishi vishwavidyalaya
UV	ultra violet
v/v	volume by volume
VIS	visible
vit E	vitamin E
w/v	weight by volume
µS	micro siemen

APPENDIX C: List of Chemicals and solutions

1. 0.001M di sodium salt– Ethylenediaminetetraacetic acid (Na_2EDTA)
2. 0.001M Ethylene diamine tetra acetic acid (EDTA) (pH 8)
3. 0.001M Phenylmethylsulfonylfluoride (PMSF)
4. 0.0025 % of DPPH (2,2–diphenyl–1–picrylhydrazyl)
5. 0.01M Sodium bicarbonate ($NaHCO_3$)
6. 0.001M di sodium salt– Ethylenediaminetetraacetic acid (Na_2EDTA)
7. 0.001M Ethylene diamine tetra acetic acid (EDTA) (pH 8)
8. 0.001M Phenylmethylsulfonylfluoride (PMSF)
9. 0.0025 % of DPPH (2,2–diphenyl–1–picrylhydrazyl)
10. 0.01M Sodium bicarbonate ($NaHCO_3$)
11. 0.01M ß–Mercaptoethanol
12. 0.025 % Coomasie Brilliant Blue R–250
13. 0.04% Riboflavin
14. 0.05M of potassium phosphate (KPO_4) buffer (pH 7.6)
15. 0.05M of sodium phosphate (Na_2PO_4) buffer (pH 7)
16. 0.05M Potassium Phosphate (pH 6.5)
17. 0.05M sodium chloride (NaCl)
18. 0.05M sodium phosphate (Na_2PO_4) (pH 7.2)
19. 0.05M sodium phosphate (Na_2PO_4) buffer (pH 6.8)
20. 0.05M sodium phosphate buffer (pH 6.8)
21. 0.05M Tris–HCl
22. 0.05M Tris–HCl buffer (pH 7)
23. 0.06M Tris buffer
24. % Ascorbic acid
25. 0.1% (w/v) Trichloroacetic acid (TCA)
26. 0.1% Bromophenol Blue
27. 0.1% Titanium sulphate ($TiSO_4$)
28. 0.15% Ammonium persulphate (APS)
29. 0.06% Tris in 300mL of distilled water
30. 0.29% glycine in 300mL of distilled water
31. 0.1M Ammonium Acetate (pH 6.5)
32. 0.1M Ethylenediaminetetraacetic acid (EDTA)
33. 0.1M potassium phosphate (K_2PO_4) buffer (pH 7.6)
34. 0.1M sodium chloride (NaCl)
35. 0.1M Tris–HCl (pH 8)
36. 0.1mM Nicotinamideadeninedinucleotide phosphate reduced tetrazolium salt–(NADPH)
37. 0.1N Sodium hydroxide (NaOH)
38. 0.2% Anthrone
39. 0.2% Ferric chloride ($FeCl_3$)
40. 0.2M Methionine
41. 0.2M sodium phosphate (Na_2PO_4) (pH 5.4)
42. 0.4 % Copper sulphate ($CuSO_4$)
43. 0.4% Hydrogen peroxide (H_2O_2)

44. 0.5% (w/v) Thiobarbituric acid (TBA)
45. 0.5% o–dianisidine
46. 0.5% of 2, 2'– dipyridyl solution
47. 0.5N Hydrochloric acid (HCl)
48. 0.67% Sodium hydroxide (NaOH)
49. 1.0M Tris Buffer (pH 6.8)
50. 1% Bromophenol Blue
51. 1% Copper sulphate ($CuSO_4$)
52. 1% Ferric chloride
53. 1% Polyvinylpolypyrrolidone (PVPP)
54. 1% Potassium Ferricyanide
55. 1.04 % Benzidine
56. 1.5% Polyvinylpolypyrrolidone (PVPP)
57. 1.5M Sodium carbonate (Na_2CO_3)
58. 1.5M Tris buffer (pH 8.8)
59. 1.6 % Sodium Potassium tartarate
60. 10 % Sodium dodecyl sulphate (SDS)
61. 10 % Sodium molybdate (Na_2MoO_4)
62. 10 % Sodium nitrite ($NaNO_2$)
63. 10% Ammonium per sulphate (APS)
64. 10% Ammonium per sulphate (APS)
65. 10% Glycerol
66. 10% Sodium dodecyl sulphate (SDS
67. 10% Thiourea
68. 12.5 % Acrylamide stock (10% Acrylamide and 2.5 % bis acrylamide)
69. 12.5% Glycerol
70. 18% Sodium sulphate (Na_2SO_4)
71. 1M Hydrochloric acid (HCl)
72. 1N Sodium hydroxide (NaOH)
73. 1 mol/ L Sodium hydroxide (NaOH)
74. 2% DNPH (2, 4–dinitrophenylhydrazine)
75. 10% Thiourea
76. 12.5 % Acrylamide stock (10% Acrylamide and 2.5 % bis acrylamide)
77. 12.5% Glycerol
78. 18% Sodium sulphate (Na_2SO_4)
79. 1M Hydrochloric acid (HCl)
80. 1N Sodium hydroxide (NaOH)
81. 1 mol/ L Sodium hydroxide (NaOH)
82. 2% DNPH (2, 4–dinitrophenylhydrazine)
83. 2% Sodium carbonate (Na_2CO_3)
84. 2% Sodium dodecyl sulfate (SDS)
85. 2% Sodium Potassium tartarate
86. 2% ß–Mercaptoethanol
87. 2.25mM Nitrobluetetrazolium chloride (NBT)
88. 2.4 % Sodium carbonate (Na_2CO_3)
89. 20 % Sodium carbonate (Na_2CO_3)
90. 20% Sulphuric acid (H_2SO_4)

91. 20% Trichloroacetic acid (TCA)
92. 200mM sodium chloride (NaCl)
93. 20mM Magnesium chloride ($MgCl_2$)
94. 20mM ß–Mercaptoethanol
95. 250mM Glycine
96. 25mM Tris Base
97. 28.74 % Acrylamide Stock (28% Acrylamide and 0.74 % bis acrylamide)
98. 2mM Phenylmethanesulfonyl Fluoride (PMSF)
99. 3% Hydrogen peroxide (H_2O_2)
100. 3% Sulfosalicylic acid.
101. 3.3mM Hydrogen peroxide (H_2O_2)
102. 30% Acrylamide (29% acrylamide and 1% N'N'–methylene bis–acrylamide),
103. 30% Hydrogen peroxide (H_2O_2)
104. 3mM Ethylenediaminetetraacetic acid (EDTA)
105. 4 % Ninhydrin solution
106. 40% Sucrose
107. 5% Sucrose
108. 50 % Folin–ciocalteu's phenol reagent
109. 50μM solution of Abscisic acid (ABA)
110. 50μM solution of Proline
111. 50μM solution of Salicylic acid (SA)
112. 52% Perchloric acid ($HClO_4$)
113. 6% Trichloro acetic acid (TCA)
114. 60μM Riboflavin
115. 6mM glutathione
116. 6mol / L Hydrochloric acid (HCl)
117. 7% Acetic acid
118. 70% Ethanol
119. 80 % Acetone
120. 80% Ethanol
121. 9% Glacial Acetic acid
122. 90% Ethanol
123. 95% Ethanol
124. Absolute Ethanol
125. Acetic acid (HPLC Grade)
126. Acetonitrile (HPLC Grade)
127. Alanine
128. Arginine
129. Ascorbic acid (Vit C)
130. Aspartic acid
131. α –Tocopherol (Vitamin E)
132. Buffer capsules for pH (4, 7 and 9)
133. Bovine serum albumin (BSA)
134. Caffeic acid
135. Chlorogenic acid

136. Cinnamic acid
137. Concentrated sulphuric acid (H_2SO_4)
138. Cysteine
139. D–glucose.
140. Diaminobenzidine (DAB) (pH 3.8).
141. DL alanine
142. DL–Phenyl–Alanine
143. Double distilled water
144. Ethylenediaminetetraacetic acid (EDTA)
145. Ferulic acid
146. Folin–ciocalteu's phenol reagent
147. Diaminobenzidine (DAB) (pH 3.8).
148. DL alanine
149. DL–Phenyl–Alanine
150. Double distilled water
151. Ethylenediaminetetraacetic acid (EDTA)
152. Ferulic acid
153. Folin–ciocalteu's phenol reagent
154. Glacial Acetic acid
155. Glutamic acid
156. Glycerol
157. Hexane
158. HPLC Grade Water
159. Hydroxyl Proline
160. Isoleucine
161. Leucine
162. Liquid Nitrogen
163. Lysine
164. Membrane Filter (pore size 0.45μm)
165. Methanol
166. Methanol (HPLC Grade)
167. Methionine
168. Millipore membrane (0.45μm) filter
169. N,N,N',N'–Tetramethylethylenediamine (TEMED)
170. N'N'–Methylene Bis–Acrylamide
171. Nelson's Arsenomolybdate
172. Phenylisothiocyanate (PITC)
173. Potassium hydroxide (KOH)
174. Proline
175. Pre–stained protein molecular marker (Genei)
176. PVPP (Polyvinyl Polypyrrolidone)
177. Salicylic acid
178. Sea sand
179. Serine
180. ß–Mercaptoethanol,
181. Threonine
182. Toluene

183. Triethylamine (TEA)
184. Tris Buffer (6.8)
185. 36.6 % Tris buffer (pH 8.9)
186. 5.98% Tris buffer (pH 6.7)
187. Tyrosine
188. Valine
189. Vanillic acid
190. Whatman No.1 Filter Paper

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Drought stress-induced oxidative stress and antioxidative responses in four wheat (*Triticum aestivum* L.) varieties

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Drought stress was imposed on four varieties of wheat (*Triticum aestivum* L.), Mohan Wonder (MW), Kedar (K), Gayetri (GY) and Gandhari (GN), for 3, 6 and 9 days. The activities of all five tested antioxidative enzymes, peroxidase, ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase, were enhanced initially in varieties K and GN, whereas in MW and GY, catalase and superoxide dismutase showed a decrease in activity at all periods of drought stress. Peroxidase and glutathione reductase activities increased even on the ninth day of stress in K and GN, but all other activities showed a decrease after 3 days of stress. H_2O_2 accumulation increased with drought stress, but in K and GN there was decrease during prolonged drought stress. Lipid peroxidation increased significantly due to drought stress, which was higher in the case of MW and GY. Proline, phenol and ascorbate content increased with period of drought stress. Carotenoid accumulation also increased initially. Total chlorophylls showed a general decrease during drought stress. The results of this study indicate that two of the varieties, MW and GY, are susceptible to drought stress, whereas the other two, K and GN, are tolerant, with peroxidase and glutathione reductase being most important in conferring tolerance.

Keywords: *Triticum aestivum* L.; drought; lipid peroxidation; antioxidative enzymes; antioxidants

Introduction

Drought stress not only affects cell water potential, induces closure of stomata and a decrease in photosynthesis, nitrate assimilation and various anabolic enzyme reactions (Sairam 1994; Zlatev et al. 2006), but also induces the generation of active oxygen species, such as superoxide radical, hydrogen peroxide and hydroxyl radical, causing lipid peroxidation and consequently membrane injury, protein degradation, enzyme inactivation, pigment bleaching and disruption of DNA strands (Pompelli, Barata-Luis et al. 2010). These reactive oxygen species (ROS) include superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Asada 1999), which mediate the degradation of membrane components, the oxidation of protein sulfydryl groups, the formation of gel-phase domains and the loss of membrane function (Quartacci et al. 1995; Sgherri et al. 1996; Navari-Izzo et al. 1999). The detoxification of superoxide radical and hydrogen peroxide is consequently of prime importance in any defense mechanism.

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Because plants have limited mechanisms of drought stress avoidance, they require flexible means of adapting to changing drought conditions. Various tolerance mechanisms have been suggested on the basis of biochemical and physiological changes related to drought (Quartacci et al. 1994, 1995; Sgheeri et al. 2000). Plants also possess several tissue antioxidants for protection against the potentially cytotoxic forms of activated oxygen species, such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, ascorbic acid, α-tocopherol and carotenoids (Vranova et al. 2002; Dalmia and Sawhney 2004; Pompelli, Martins et al. 2010). Antioxidative enzymes are the most important components in the scavenging system of ROS. Superoxide dismutase is a major scavenger of O_2^-; it catalyzes the dismutation reaction of superoxide radical anions into O_2 and H_2O_2. Removal of the highly toxic H_2O_2 produced during dismutation is essential for the cell to avoid the inhibition of enzymes such as those controlling the Calvin cycle in the chloroplast (Creissen et al. 1994), and it can be scavenged by catalase and a variety of peroxidases. Catalase, which is only present in peroxisomes, dismutates H_2O_2 into water and molecular oxygen, whereas peroxidase decomposes H_2O_2 by oxidation of co-substrates such as phenolic compounds and/or antioxidants.

Wheat is one of the most important cultivated cereals of the world. In different parts of India, productivity is affected by drought stress conditions and the selection of drought-resistant varieties becomes essential. This study was undertaken to determine drought stress-induced oxidative stress in four varieties of wheat with special emphasis on the role of antioxidants in protective mechanisms.

Materials and methods

Seeds of four varieties of wheat (*Triticum aestivum* L.), Mohan Wonder (MW), Kedar (K), Gayetri (GY) and Gandhari (GY), which are commercially relevant lines, were selected for experimental purposes. Their tolerance to drought has not yet been worked out. For planting, these seeds were initially surface sterilized with 0.1% (w/v) $HgCl_2$ for 3–4 minutes, washed with sterile distilled water and then transferred to petriplates under aseptic conditions. The seeds were allowed to germinate in the petriplates for one week and then the seedlings were transferred to earthen pots. Plants were maintained in growth chamber at a temperature of 20–25°C, relative humidity 65–70%, 16 h photoperiod and irradiance of 400 μmol m^{-2} s^{-1}. Drought stress was induced in one-month-old plants by withholding water completely for the required period. Sampling was carried out after 3, 6 and 9 days of each period of drought stress, morphological changes were noted and the relative water content (RWC) of leaves was determined as described by Farooqui et al. (2000), calculated by the following formula:

$$\mathrm{RWC}(\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fully turgid weight} - \text{Dry weight}} \times 100$$

Various biochemical assays were then performed as given below.

Extraction and antioxidant enzyme assay

For extraction of enzymes, leaves from wheat seedlings were homogenized in 5 mL of ice-cold 50 mM sodium phosphate buffer, pH 7.2, containing 1% (w/v)

polyvinylpolypyrrolidone using liquid nitrogen in a chilled mortar and pestle. The homogenate was then centrifuged at 6700 *g* for 20 min at 4°C. The supernatant was used directly as crude extract for enzyme assays. Peroxidase (EC 1.11.17) activity was assayed spectrophotometrically in 4802 UV VIS spectrophotometer (Cole Parmer, USA) at 460 nm by monitoring the oxidation of *o*-dianisidine in the presence of H_2O_2 (Chakraborty et al. 1993). Specific activity was expressed as mmol *o*-dianisidine mg protein^{-1} min^{-1}. Ascorbate peroxidase (EC 1.11.1.11) activity was assayed as a decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada and Takahashi (1987) with some modification. Enzyme activity was expressed as mmol ascorbate mg protein^{-1} min^{-1}. Catalase (EC 1.11.1.6) activity was assayed as described by Chance and Machly (1955) by estimating the breakdown of H_2O_2, which was measured at 240 nm. The enzyme activity was expressed as μmol H_2O_2 mg protein^{-1} min^{-1}. Glutathione reductase (EC 1.6.4.2) activity was determined by the oxidation of NADPH at 340 nm as described by Lee and Lee (2000). Enzyme activity was expressed as μmol NADPH oxidized mg protein^{-1} min^{-1}. Superoxide dismutase (EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of nitro blue tetrazolium according to the method of Dhindsa et al. (1981) with some modification. The absorbance of the samples was measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the nitro blue tetrazolium reduction rate in the controls containing no enzymes. Protein contents in extracts were quantified following the method of Lowry et al. (1951), using bovine serum albumin as standard.

Quantification of H_2O_2 and in situ detection of H_2O_2

H_2O_2 levels in the leaves were estimated according to Jena and Choudhuri (1981). Leaf tissue (500 mg) was homogenized with 12 mL of 50 mM potassium phosphate buffer (pH 6.5), centrifuged at 2415 *g* for 25 min and the supernatant was used for H_2O_2 determination. The intensity of the yellow color was measured at 410 nm in the spectrophotometer and H_2O_2 levels were calculated using extinction coefficient 0.28 μmol^{-1} cm^{-1}. In situ detection of H_2O_2 was carried out following the method of Thordal-Christensen et al. (1997) with minor modifications using diaminobenzidine. Cut leaf discs of 2 cm diameter were vacuum infiltrated with diaminobenzidine (1 mg mL^{-1}, pH 3.8). The leaf discs were then incubated at 30°C in the dark for 24 h under gentle stirring of 150 rpm; they were then transferred to 90% ethanol at 70°C until the chlorophyll was removed. H_2O_2 was visualized as reddish-brown color at the site of diaminobenzidine polymerization. Diaminobenzidine polymerizes instantly and locally at sites of peroxidase activity into a reddish-brown polymer.

Determination of lipid peroxidation

Lipid peroxidation was measured as accumulation of malondialdehyde (MDA) determined by the thiobarbituric acid reaction. Cells (0.25 g) were homogenized in 2 mL of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 6700 *g* for 10 min. To 0.5 mL of the aliquot of the supernatant, 2 mL of 20% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid were added. The mixture was heated at 95°C for 30 min and then quickly cooled on ice. The

absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of 155 $mmol^{-1}$ cm^{-1} (Heath and Packer 1968).

Antioxidants

Carotenoids were extracted and estimated following the method described by Lichtenthaler (1987). Extraction was carried out in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480 nm in a VIS spectrophotometer and the carotenoid content was calculated using standard formula. Ascorbic acid was extracted and estimated following the method described by Mukherjee and Choudhuri (1983).

Extraction and estimation of biochemical components

Total phenols were extracted from the leaves following the method described by Mahadevan and Sridhar (1982) and quantified (Bray and Thorpe 1954). Proline was extracted from the leaves using 3% sulfosalicylic acid and free proline was estimated following the method of Bates et al. (1973). Chlorophyll was extracted with methanol and total, chl *a* and chl *b* contents were estimated after measurement of absorbance at 663 and 645 nm as described by Harborne (1973).

Results and discussion

Changes in morphological characteristic and the RWC content

The four tested varieties did not show severe wilting symptoms morphologically even after 7 days of drought stress, but during the ninth day the plants showed morphological signs of wilt. The RWC decreased significantly with induction of drought stress and duration of drought stress in all varieties (Figure 1). However, the decrease in RWC after 9 days in relation to control was less in K and GN (35.7 and 36% respectively) compared with MW and GY (53 and 53.4% respectively). It has also been reported in previous studies that drought-resistant cultivars maintain higher RWC during drought stress, whereas in susceptible cultivars RWC shows greater decrease (Farooqui et al. 2000; Chakraborty et al. 2002; Iqbal and Bano 2009).

Effect of drought stress on antioxidant enzyme activities in wheat varieties

In this study, activities of ascorbate peroxidase (Figure 2), peroxidase (Figure 3) and glutathione reductase (Figure 4) increased significantly in all four varieties initially after 3 days of drought stress. With prolonged stress, the activities of peroxidase, ascorbate peroxidase and glutathione reductase decreased in varieties MW and GY, whereas peroxidase and glutathione reductase increased with duration of drought stress in varieties K and GN. In these two varieties activities of ascorbate peroxidase, however, decreased after 3 days. In case of catalase (Figure 5) and superoxide dismutase (Figure 6), activities decreased at all periods of drought stress in MW and GY, whereas in K and GN activities increased initially before decreasing. Our results thus reveal that, in K and GN, which are more tolerant than the other two varieties, the activities of all antioxidant enzymes increased initially and activities of

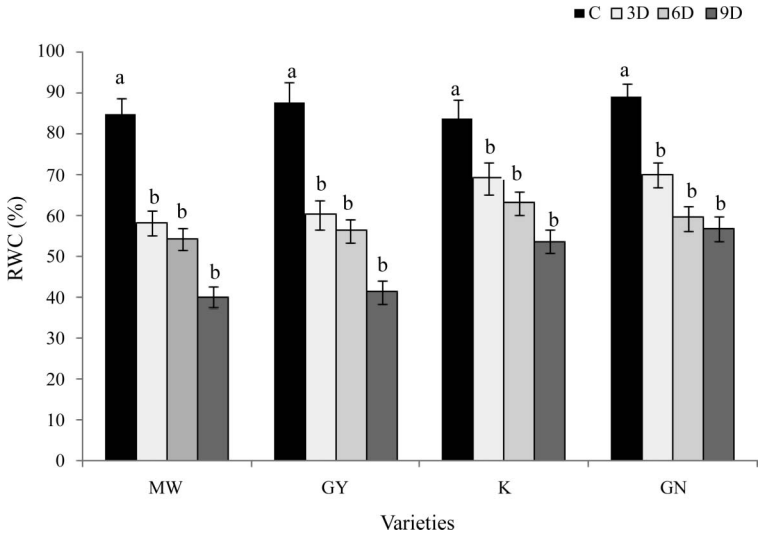

Figure 1. Relative water content of four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

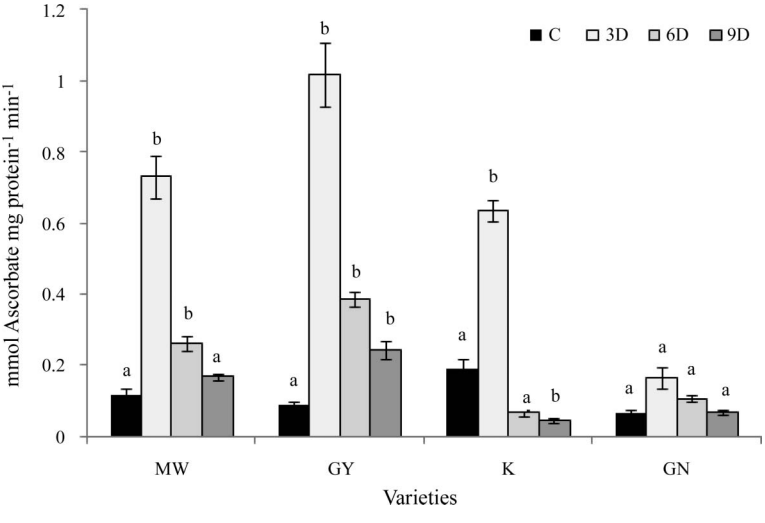

Figure 2. Ascorbate peroxidase activities in four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

peroxidase and glutathione reductase continued to increase, indicating their involvement in tolerance, whereas ascorbate peroxidase, catalase and superoxide dismutase did not contribute directly to tolerance. Superoxide dismutase, being the first enzyme in the antioxidant pathway, increases initially in the tolerant varieties,

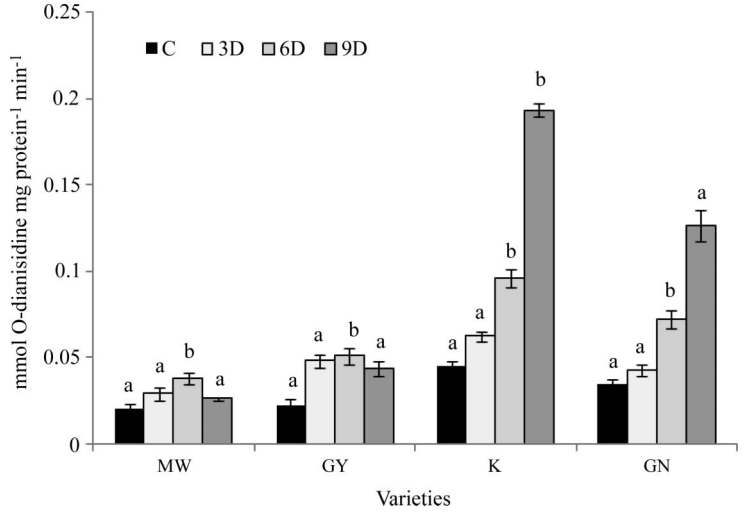

Figure 3. Peroxidase activities in four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

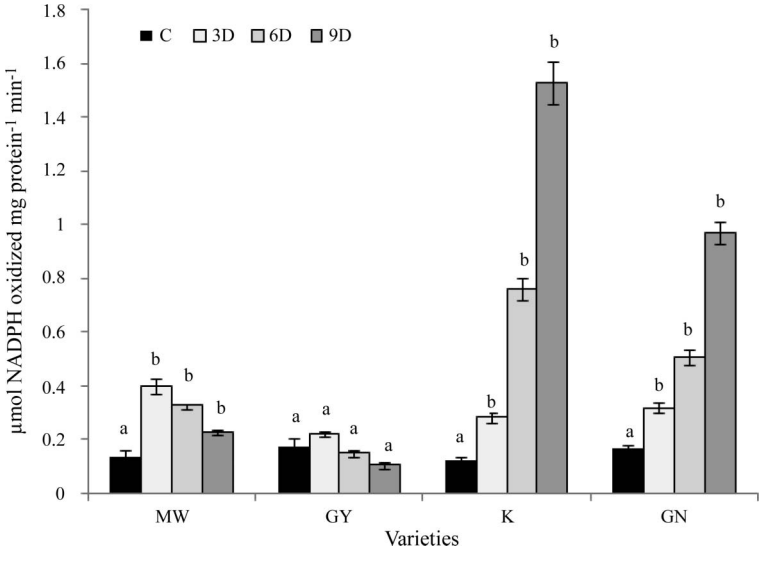

Figure 4. Glutathione reductase activities in four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

and contributes to the initial accumulation of H_2O_2. However, with increase in periods of stress, the activities of superoxide dismutase decrease even in tolerant varieties and the H_2O_2 produced is metabolized by peroxidase. Thus, a combination

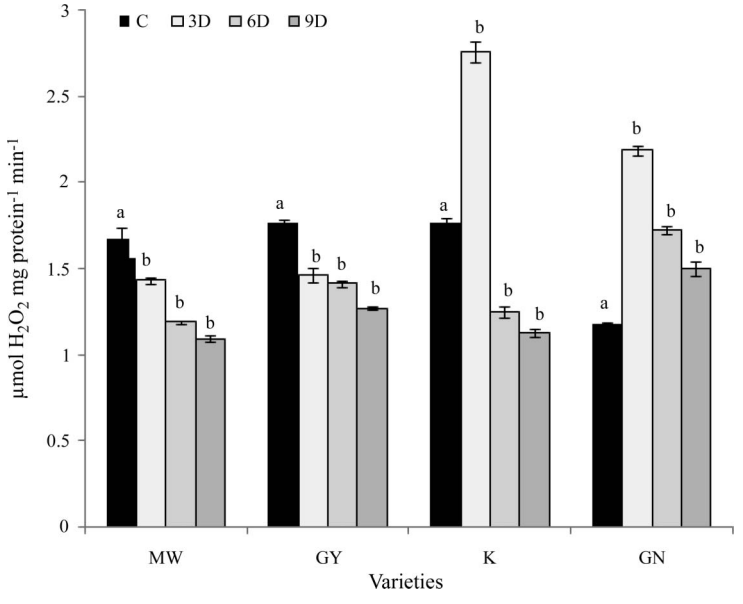

Figure 5. Catalase activities in four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

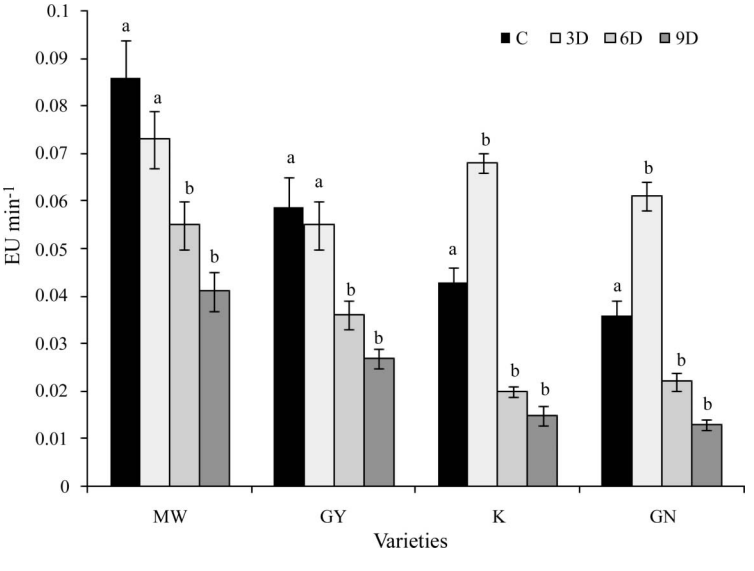

Figure 6. Superoxide dismutase activities in four varieties of wheat subjected to drought stress treatments. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

of activities of the different enzymes is essential for tolerance. In a study by Pompelli, Martins et al. (2010), they also reported that although the rate of superoxide formation may increase considerably in N-deficient coffee plants under high light conditions, no corresponding increase in the activity of superoxide dismutase per unit mass could be found. Enhancement of glutathione reductase activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate–glutathione cycle than the less-tolerant cultivars. Glutathione reductase, which catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), is an important endogenous antioxidant (McKersie and Leshem 1994). This cycle has been implicated in mitigating the effects of ROS (Molina et al. 2002; Mandhania et al. 2006). Chai et al. (2005) obtained increased glutathione reductase activities in two cultivars of banana subjected to drought stress. Similarly, both catalase and peroxidase are actively involved in detoxification of ROS by breaking down H_2O_2. In this study, peroxidase seems to have greater role in tolerance than catalase during prolonged drought stress. Chakraborty et al. (2002) also reported that peroxidase activities increased initially in all tea cultivars following drought stress, but in tolerant cultivars it increased even with prolonged periods. Iqbal and Bano (2009) obtained greater increase in activities of peroxidase and catalase in wheat accessions that were tolerant to drought stress than those that were less tolerant. Previous workers have also reported differential responses of genotypes to drought stress with respect to antioxidant enzymes (Dhanda et al. 2004; Nair et al. 2008). Under drought stress, enhanced superoxide dismutase activity was found in pea, tobacco and bean (Moran et al. 1994; Van Rensburg and Kruger 1994; Zlatev et al. 2006), decreased superoxide dismutase activity in sunflower seedlings and banana (Quartacci and Navari-Izzo 1992; Chai et al. 2005) and unaffected superoxide dismutase activity in maize (Luna et al. 1985). In wheat, superoxide dismutase activity increased or remained unchanged in the early phase of drought, but decreased with prolonged drought stress (Zhang and Kirkham 1995), as also obtained in this study.

H_2O_2 accumulation, in situ detection of H_2O_2 and lipid peroxidation in leaves of wheat varieties following drought stress

A decrease in catalase activity following drought stress was correlated with increased accumulation of H_2O_2 (Figure 7) in all varieties. H_2O_2 accumulation and lipid peroxidation were significantly higher in susceptible varieties in comparison with tolerant ones. Our results are in conformity with those of several previous workers (Chai et al. 2005; Zlatev et al. 2006). Increased concentrations of H_2O_2, a strong oxidant, cause localized oxidative damage, disruption of metabolic functions and lipid peroxidation (Foyer et al. 1997; Velikova et al. 2000; Zlatev et al. 2006). However, besides being an ROS, H_2O_2 is also a signal molecule, which is involved in signal transduction mechanisms for several processes in plants such as stomatal closure, root growth and responses to pathogen challenge (Neill et al. 2002; Laloi et al. 2004; Desikan et al. 2005). Thus, levels of H_2O_2 are efficiently controlled to maintain balance between production and breakdown. In this study, although H_2O_2 accumulation increased during drought stress, after a period of prolonged drought there was a decrease in H_2O_2 levels in tolerant varieties, indicating greater antioxidant activity. In situ detection of H_2O_2 in leaf tissues and microscopic observations revealed darker staining in tissues subjected to prolonged drought stress, especially in the less-tolerant varieties (Figure 8).

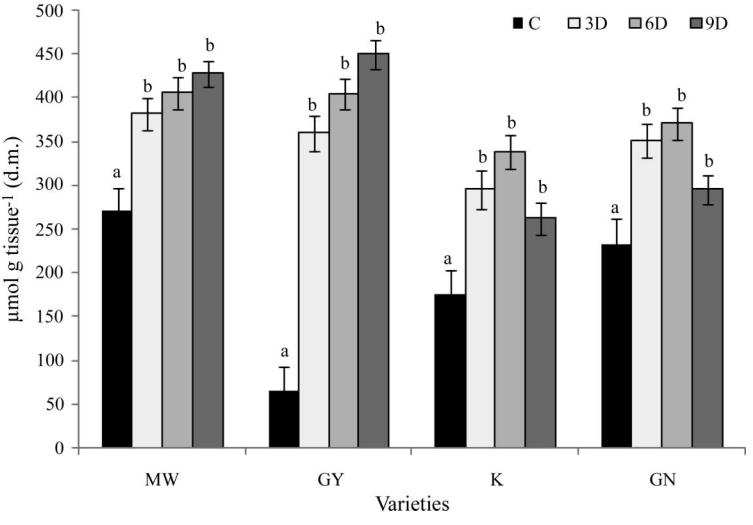

Figure 7. Effect of drought stress on accumulation of H_2O_2 in four varieties of wheat. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

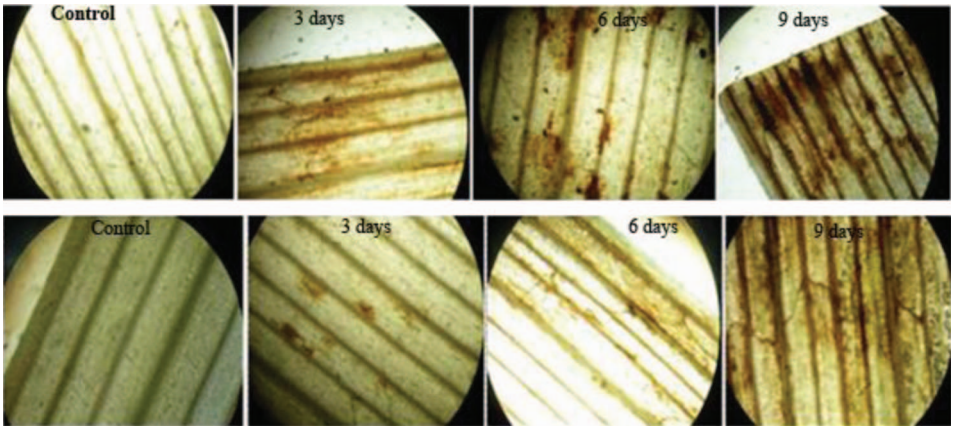

Figure 8. *In situ* detection of H_2O_2 in mid-portions of leaves of two wheat varieties (upper row = variety MW and lower row = variety GN) of wheat following drought stress.

The peroxidation of lipids in the cell membrane is one of the most damaging cellular responses observed in response to drought stress (Thankamani et al. 2003) and the amount of lipid peroxidation is considered to be one of the determinants which indicate the extremity of stress experienced by a plant. It was observed that although MDA content, a measure of lipid peroxidation, increased in all varieties during drought stress, after 9 days, MDA content in susceptible varieties was more than three times that of tolerant varieties (Figure 9). Tatar and Gevrek (2008) also reported that MDA content in wheat increased with severity of drought stress.

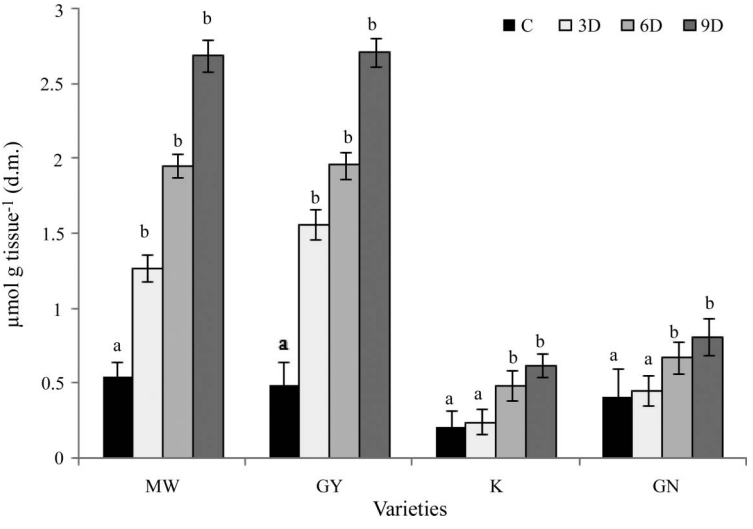

Figure 9. Effect of drought stress on lipid peroxidation (expressed as MDA content) in four varieties of wheat. Results are expressed as the mean of three replicates (10 plants each). Bars represent SE. Different letters indicate significant differences with respect to control ($p \leq 0.01$). C, control; 3, 6 and 9 D, days after withholding water.

Change in ascorbate, carotenoids and chlorophyll contents of leaves

Ascorbic acid and carotenoids, two of the antioxidants in plants, increased significantly in all four varieties. Ascorbate accumulation was enhanced in all four varieties even after 9 days of drought stress (Table 1); carotenoids, however, decreased after 3 days in varieties MW and GY and after 6 days in K and GN (Table 2). Nair et al. (2008) reported that ascorbic acid contents in cowpea decreased with severity of drought stress, but tolerant cultivars had higher ascorbic acid contents during severe stress in comparison with susceptible cultivars. Jaleel (2009) reported enhanced accumulation of ascorbic acid during drought stress in winter cherry (*Withania somnifera*). L-Ascorbic acid is a strong antioxidant but also performs several other functions in the plant (Noctor and Foyer 1998). In this study, the increase in ascorbate, along with glutathione reductase, indicates involvement of the ascorbate–glutathione cycle as a predominant mechanism of oxidative stress detoxification. Chlorophylls, which are one of the first molecules to be affected by drought stress, showed a significant decrease in all varieties. However, in tolerant varieties, there was an initial nonsignificant increase. Interestingly, the chl *a*/*b* ratio showed an initial increase in all varieties before declining, and this decrease was greater in the less-tolerant varieties (Table 2).

Water-stress responsive metabolites

Free proline accumulation was enhanced in all four varieties during prolonged drought stress (Table 1) after an initial nonsignificant decrease. After 9 days, proline content in tolerant varieties was $\sim$2.5 times higher than those of the less-tolerant ones, although in control plants all varieties had more or less similar amounts. It is quite clear that proline accumulation during drought stress is one of the mechanisms

Table 1. Contents of proline, phenol and ascorbate in the leaves of four wheat varieties subjected to drought stress (3, 6 and 9 days after withholding water).

Variety	Treatment	(g kg^{-1} DM) Proline	Phenol	Ascorbate
MW	Control	2.3 ± 0.03[a]	23.6 ± 0.06[a]	12.4 ± 0.14[a]
	3d	1.9 ± 0.02[a]	19.2 ± 0.05[b]	13.3 ± 0.12[b]
	6d	3.6 ± 0.04[b]	21.1 ± 0.07[b]	15.2 ± 0.15[b]
	9d	6.9 ± 0.08[b]	22.5 ± 0.05[b]	16.9 ± 0.18[b]
GY	Control	2.2 ± 0.06[a]	28.3 ± 0.20[a]	9.4 ± 0.17[a]
	3d	2.0 ± 0.01[a]	17.3 ± 0.14[b]	15.4 ± 0.02[b]
	6d	4.8 ± 0.14[b]	20.9 ± 0.07[b]	16.6 ± 0.03[b]
	9d	5.8 ± 0.01[b]	21.3 ± 0.01[b]	17.9 ± 0.05[b]
K	Control	1.9 ± 0.07[a]	15.7 ± 0.03[a]	5.5 ± 0.11[a]
	3d	1.4 ± 0.08[a]	22.0 ± 0.08[b]	9.2 ± 0.22[b]
	6d	11.1 ± 0.03[b]	45.0 ± 0.05[b]	18.6 ± 0.23[b]
	9d	20.3 ± 0.50[b]	49.4 ± 0.05[b]	21.8 ± 0.25[b]
GN	Control	2.0 ± 0.07[a]	29.8 ± 0.08[a]	9.4 ± 0.31[a]
	3d	2.6 ± 0.04[a]	42.3 ± 0.11[b]	10.8 ± 0.32[b]
	6d	6.0 ± 0.07[b]	43.1 ± 0.08[b]	11.2 ± 0.13[b]
	9d	10.6 ± 0.20[b]	46.7 ± 0.12[b]	16.3 ± 0.21[b]

DM, dry matter; ±, standard error. Mean of three replicates, with 10 plants in each replicate. Different superscripts in each column express significant difference from control at $p = 0.01$, in t-test.

Table 2. Contents of total chlorophyll and carotenoids and chlorophyll a/b ratio in the leaves of four wheat varieties subjected to drought stress (3, 6 and 9 days after withholding water).

Variety	Treatment	Total chlorophyll (g kg^{-1} FM)	Chl a/b ratio	Carotenoids (g kg^{-1} FM)
MW	C	0.93 ± 0.08[a]	1.54	0.043 ± 0.004[a]
	3d	0.56 ± 0.01[b]	1.95	0.055 ± 0.002[a]
	6d	0.36 ± 0.03[b]	1.20	0.052 ± 0.006[a]
	9d	0.28 ± 0.01[b]	0.95	0.040 ± 0.003[a]
GY	C	1.01 ± 0.06[a]	1.25	0.044 ± 0.006[a]
	3d	0.56 ± 0.08[b]	1.99	0.067 ± 0.002[a]
	6d	0.45 ± 0.01[b]	1.33	0.041 ± 0.002[a]
	9d	0.41 ± 0.02[b]	0.76	0.030 ± 0.001[a]
K	C	0.93 ± 0.06[a]	1.45	0.044 ± 0.002[a]
	3d	0.98 ± 0.07[a]	1.65	0.057 ± 0.001[b]
	6d	0.48 ± 0.02[b]	1.45	0.066 ± 0.003[b]
	9d	0.27 ± 0.01[b]	1.04	0.050 ± 0.004[a]
GN	C	1.05 ± 0.07[a]	1.45	0.042 ± 0.003[a]
	3d	1.21 ± 0.04[a]	1.94	0.063 ± 0.002[b]
	6d	0.65 ± 0.07[b]	1.86	0.067 ± 0.004[b]
	9d	0.57 ± 0.20[b]	1.67	0.052 ± 0.002[a]

FM, fresh matter; ±, standard error. Mean of three replicates, with 10 plants in each replicate. Different superscripts in each column express significant difference with control at $p = 0.01$, in t-test.

of tolerance. Proline, which is usually considered as an osmoprotectant, may also be involved in reducing oxidative damage by scavenging the free radicals (Vendruscolo et al. 2007; Tatar and Gevrek 2008). This study revealed that in tolerant varieties – K

and GN – phenol content increased at all periods of drought stress, although in the other two varieties there was an initial decrease (Table 1). The involvement of polyphenols in plant defense against various stresses has been reported previously (Leinhos and Bergmann 1995). Chakraborty et al. (2002) also reported earlier that drought stress-induced accumulation of phenols was much higher in tolerant cultivars of tea.

Conclusion

The results of this study clearly indicate that although drought stress induced oxidative damage in wheat varieties as evidenced by decrease in RWC, increased lipid peroxidation, accumulation of H_2O_2 and chlorophyll degradation, antioxidative mechanisms including enhanced activities of antioxidative enzymes, accumulation of other antioxidants, proline and phenols were also induced. Antioxidative mechanisms were much more pronounced in two of the varieties, Kedar (K) and Gandhari (GN), and hence, these were protected from oxidative damage to a great extent. Taking into consideration all the available data, it is concluded that whereas Kedar and Gandhari could be considered as tolerant, Mohan Wonder and Gayetri were susceptible to drought stress.

Acknowledgements

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RESEARCH ARTICLE

Oxidative stress in five wheat varieties (*Triticum aestivum* L.) exposed to water stress and study of their antioxidant enzyme defense system, water stress responsive metabolites and H_2O_2 accumulation

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ABSTRACT

Five varieties of wheat (*Triticum aestivum* L.) — KW, UP 2752, PBW 343, SO and LV — were subjected to water stress and sampling was done on the 3rd, 6th and 9th day of stress. RWC decline in KW, UP 2752 and PBW 343 (36.65, 42.34 and 40.75% respectively) was comparatively lesser than in LV and SO (52.93 and 52.67% respectively). In all varieties tested, three antioxidant enzymes (POX, APOX and GR) showed an initial increase. The activity of POX and GR increased with the increase in the duration of stress in KW, UP 2752 and PBW 343, while the activity of APOX declined. However, CAT and SOD showed an initial increase in these varieties, whereas it declined in SO and LV with increase in the period of stress. Accumulation of H_2O_2 declined during prolonged water stress in KW, UP 2752 and PBW 343, while it increased in LV and SO. The accumulation of MDA content was three times higher in susceptible varieties than in tolerant varieties. The content of proline, phenol and ascorbate increased during water stress whereas the accumulation of carotenoid showed a significant decrease after showing an initial increase in the tested varieties. Higher values of total antioxidant and MSI were recorded in KW, UP 2752 and PBW 343 during stress while after 6 days MSI declined in LV and SO. During water stress there was a general decline in the total chlorophyll content. Analyzing the data, the present work suggested that out of the five varieties, KW, UP 2752 and PBW 343 showed more tolerance to water stress than SO and LV.

Keywords: antioxidants, carotenoids, drought, lipid peroxidation.

Abbreviations: APOX: ascorbate peroxidase (EC.1.11.1.11); CAT: catalase (EC.1.11.1.6); CMS: cell membrane stability; DAB: diaminobenzidine; EC: electrical conductivity; GR: glutathione reductase (EC 1.6.4.2); H_2O_2: hydrogen peroxide; KW: Kaweri; LV: local variety; MDA: malondialdehyde; MSI: membrane stability index; NBT: nitro blue tetrazolium; PPO: polyphenol oxidase; POX: peroxidase (EC. 1.11.17); RH: relative humidity; ROS: reactive oxygen species; RWC: relative water content; SO: Sonalika; SOD: superoxide dismutase (EC 1.15.1.1); TBA: thiobarbituric acid; TCA: trichloroacetic acid.

INTRODUCTION

Tas and Tas (2007) have defined drought as one of the environmental stresses, which is the most significant factor restricting plant growth and crop productivity in the majority of agricultural fields of the world. In general, drought is responsible for several metabolic processes of plants, with photosynthetic apparatus being one of the most important (Nayyar and Gupta, 2006). Besides changes in photosynthesis, such adverse effects on metabolism lead to growth inhibition, stomata closure with consecutive reduction of transpiration, which are considered necessary for coping with osmotic changes in their tissues (Lawlor and Cornic, 2002; Yordanov et al., 2003; Zhu, 2002). Water stress leads to the formation of ROS, which are extremely harmful to the plants. Gratão et al. (2005) suggested that to prevent or alleviate injuries from ROS, plants have evolved an antioxidant defense system that includes non-enzymatic compounds, like ascorbate, glutathione, tocopherol, carotenoids, flavonoids and enzymes such as SOD, CAT, POX, APOX, GR and PPO. Similar views were also expressed earlier by authors as Mittler (2002) and Gechev et al. (2002). According to Imlay (2003), water-stress conditions may trigger an increased formation of the superoxide radical and H_2O_2 which can inactive SH-containing enzymes and directly attack membrane lipids. Gratão et al. (2005) suggested that in higher plants GR is involved in defense against oxidative stress, being responsible for the reduction of oxidized glutathione for the chain reactions of scavenging H_2O_2 by APX and GPX, that might be completed and continued. They further postulated that SOD acts as the first line of defense against ROS and that it is responsible for the dismutation of O_2^- into H_2O_2. The H_2O_2 liberated in the peroxisome is metabolized by CAT following the conversion of glycolate to glyoxylate during photorespiration into H_2O and O_2, while NADPH-dependent reduction of oxidized GSSG to the reduce form GSH is catalyzed by GR (Gratão et al., 2005). The utilization of multiple isoforms of enzymes is one of the primary control mechanisms of cellular metabolism in plants (Sang et al., 2005).

Generation of ROS also leads to lipid peroxidation (Chen et al., 2000). Foyer and Noctor (2000) suggested that the central factor in both biotic and abiotic stress, which occurs during imbalance in any cell compartment between the production of ROS and antioxidant defense system is oxidative stress which leads to a varied degree of physiological challenges. They further suggest that since ROS have multiple functions therefore in spite of being a harmful molecule, the cells have developed mechanisms to control the concentrations of ROS and they are not completely eliminated (Foyer and Noctor, 2000). Foyer and Noctor (2003) suggested that ROS can be viewed as cellular indicators of stress and secondary messengers involved in all aspects of plant biology from gene expression and translation to enzyme chemistry.

H_2O_2 is a non-radical ROS produced in a two-electron reduction of molecular oxygen. Several sites have been recognized as H_2O_2 sources, including organelles (mitochondria, peroxisomes and chloroplasts), the apoplastic and the plasma membrane as well as cell-wall associated enzymes (various NADPH oxidases and PERs). According to Upadhyaya et al. (2007) environmental stresses are known to induce H_2O_2 and other toxic oxygen species production in cellular compartments, resulting in acceleration of leaf senescence through lipid peroxidation and other oxidative damage. As H_2O_2 is a strong oxidant, it can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity, actions that result in senescence promotion. The role of H_2O_2 in stress-induced damage has long been recognized, but it is now also generally accepted that H_2O_2 is an integral component of cell signaling cascades (Mittler, 2002; Vranova et al., 2002) and an indispensable second messenger in biotic and abiotic stress situations (Pastori and Foyer, 2002).

The overproduction of H_2O_2 has been observed in plants exposed to a number of stress conditions and is considered as one of the factors causing oxidative stress (Snyrychova, 2009). According to Foyer and Noctor (2012), out of the various forms of ROS, the central role in plant signaling, regulating plant development and adaptation to abiotic and biotic stresses, is played by H_2O_2. Increased availability of H_2O_2 is a commonly observed feature of plant stress response signature. The physiological context involves a continuous supply of environmental stimuli that can trigger intracellular H_2O_2 accumulation or modulate the response to such accumulation.

With physiological parameters as CMS, differences in the tolerance for compound stresses, such as salinity and water deficiency, can be detected. Measurement of CMS is one technique that has often been used for screening against drought tolerance in various crops, as rice (Tripathy et al., 2000), wheat and wild relatives of wheat (Farooq and Azam, 2002), for example. Suzuki et al. (2012) has studied the networks of ROS/redox signaling in the chloroplast and mitochondria and suggested that they play essential roles in the acclimation of plants to abiotic stresses, these signals contributing to a delicate balance of homeostasis within each organelle, as well as to cross-talk between different cellular components by regulating important biological pathways such as gene expression, energy metabolism and protein phosphorylation under stress condition.

The present work was undertaken to evaluate the oxidative stress and the antioxidant response system in 1-month-old wheat plant subjected sequentially to water stress with a detailed study on the accumulation of H_2O_2 and its *in situ* detection in the leaf of the stressed and control plants.

MATERIAL AND METHODS

Plant material and experimental conditions: Seeds of commercially relevant lines of five wheat (*Triticum aestivum* L.) varieties (KW, LV, UP 2752, PBW 343 and SO) were selected for experimental purposes. LV and KW are popularly grown in the fields of this region. UP 2752, PBW 343 and SO were obtained from the research station of Uttar Banga Krishi Vishwavidyalaya (UBKV). They were selected as all are locally grown and a comparison of their responses could give a better understanding of the susceptibility/tolerance to the different varieties to drought. The seeds were surface sterilized for 3 to 5 min with 0.1% (w/v) $HgCl_2$ solution, washed twice with sterile double-distilled water. The seeds were then transferred to petriplates maintaining aseptic conditions to avoid contamination. One-week-old seedlings were selected and transferred to earthen pots with soil containing a suitable amount of manure and the pots were labeled. The transferred seedlings in the pots were then maintained in growth chamber at a favorable temperature of 20 to 25°C, 65 to 70% of RH, standard photoperiod of 16 hours, and 400 μmol m^{-2} s^{-1} irradiance. To impart water stress, watering of the plant was completely withheld for the test period, when the plants were 1-month-old. Drought stress was induced in 1-month-old plants by withholding water completely for specific period. Sampling of the plant was done on the 3rd, 6th and 9th day to study the response of plants to varied days of drought. After the 9th day, i.e. from 10th day, the leaves showed severe wilting symptoms in all varieties so the sampling was done to till 9th day. In all estimations, sampling was also done at zero day of drought, which was considered as control. RWC, which is considered one of the important test to assess the water content during stress, was calculated by the formula as given by Farooqui et al. (2000):

$$RWC\ (\%) = \frac{\text{fresh weight} - \text{dry weight}}{\text{fully turgid weight} - \text{dry weight}} \times 100$$

The biochemical assays of the plant were done as given below.

Extraction and assays of antioxidant enzyme

Preparation of extracts: The extraction of enzymes from the plant was done by homogenizing the leaves of 1-month-old plant using liquid nitrogen in an ice-cold 50-mM sodium phosphate buffer of pH 7.2 along with 1% (w/v) polyvinylpolypyrrolidone by a pre-chilled mortar and pestle. Centrifugation of the homogenized mixture was done at 6,708 g^n for 20 min at 4°C. The supernatant was directly used for various enzymatic assays as crude enzyme extract. The quantification of soluble protein present in the extract was done by Lowry's method (Lowry et al., 1951) utilizing bovine serum albumin (BSA) as standard.

Assay of antioxidant enzymes

POX (EC. 1.11.17): its activity was assayed spectrophotometrically as described by Chakraborty et al. (1993) with some modifications using 4802 UV VIS spectrophotometer (Cole Parmer, USA). Specific activity was expressed as mmol o-dianisidine oxidized mg protein^{-1} min^{-1}.

APOX (EC.1.11.1.11): its assay was done by using the method described by Asada and Takahashi (1987) with some minor modification. Enzyme activity was expressed as mmol ascorbate oxidized mg protein^{-1} min^{-1}.

CAT (EC.1.11.1.6): its activity was measured by estimating the breakdown of H_2O_2, which was determined at 240 nm as described by Beers and Sizer (1952). The enzyme activity was expressed as μmol H_2O_2 mg protein^{-1} min^{-1}.

GR (C 1.6.4.2): the activity of GR was determined by using the method described by Lee and Lee (2000) by measuring the oxidation of NADPH at 340 nm. Enzyme activity was expressed as µM NADPH oxidized mg protein^{-1} min^{-1}.

SOD (EC 1.15.1.1): after some minor modification, the method as proposed by Dhindsa et al. (1981) was used for the assay of the activity of SOD, in which the inhibition of the photochemical reduction of NBT was monitored. One 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.

H_2O_2 quantification and its *in situ* detection: The H_2O_2 in the leaf samples were quantified and the estimation was done following the method described by Jena and Choudhuri (1981). A specific amount of the leaf samples were weighed and homogenized in 50 mM potassium phosphate buffer (pH 6.5), which was then centrifuged at 2,415 g^n for 25

min, and the supernatant was used for H_2O_2 determination. All the extractions and estimations were done in minimal light conditions (Chakraborty and Pradhan, 2011).

The reaction was completed as an intense yellow color started developing. This was was monitored at 410 nm spectrophotometrically. The levels of H_2O_2 in the samples were determined by the use of extinction coefficient 0.28 μmol^{-1} cm^{-1} in the calculation. DAB staining technique described by Thordal-Christensen et al. (1997) was used with some minor modifications (Chakraborty and Pradhan, 2011) to detect the *in situ* levels of H_2O_2 in the leaf samples. Vacuum infiltration of the leaf discs (20 mm in diameter) was done with DAB (pH 3.8) at a rate of 1 mg/mL. Incubation of the leaf was done in dark for 24 hours at 30°C with a continuous stirring of the leaf at 150 rpm followed by their transfer to 90% ethanol at 70°C in water bath for the removal of chlorophyll. The polymerization of DAB at the site of activity of the enzyme POX was achieved locally and instantly in the treated leaf samples. The development of reddish-brown color was the result of polymerization of DAB with H_2O_2 at the site of their production.

Determination of lipid peroxidation: The accumulation of MDA content, a measure of lipid peroxidation was monitored by the TBA reaction. A sample of 0.25 g of the leaf was homogenized in 0.1% (w/v) TCA. Centrifuge was done for 10 min at 6,708 g^n and 2 mL of 20% TCA containing 0.5% (w/v) TBA were added to 0.5 mL of the supernatant obtained followed by heating of the mixture for 30 min at 95°C followed by cooling it on ice. The absorbance of the sample was determined at 600 and 532 nm. Using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968) the concentration of MDA was calculated.

Extraction and estimation of non-enzymatic antioxidants: Carotenoids were extracted in methanol and, for the estimation, the method described by Lichtenthaler (1987) was used. Extraction was done in methanol and the extract was filtered. Absorbance of the filtrate was noted at 480, 663 and 645 nm in a VIS spectrophotometer (Systronics, India, Model 101) and the content of carotenoid was determined by the formula as given below:

$$A_{480} - (1.144 \times A_{663} - 0.638\ A_{645})$$

For the extraction and estimation of ascorbic acid (ascorbate) the method as given by Mukherjee and Chaudhuri (1983) was used.

Total antioxidant activity or free radical scavenging activity was measured by following the method of (Blois, 1958) and expressed as percent (%) inhibition of DPPH absorbance, which was measured at 515 nm. The percentage inhibition of the absorbance of the solution of DPPH was determined by the formula as described by Chakraborty and Pradhan (2011). Total antioxidant activity was thus measured as free radical scavenging ability in terms of inhibition of absorbance by DPPH.

MSI (Membrane stability index): Leaf MSI was determined according to the method of Premchandra et al. (1990), as modified by Sairam (1994). Leaf discs (100 mg) were thoroughly washed in running tap water followed by washing with double distilled water thereafter the discs were heated in 10 mL of double distilled water at 40°C for 30 min. Then EC (C1) was recorded by EC meter. Subsequently the same samples were place in a boiling water bath (100°C) for 10 min and their EC was also recorded (C2) in a conductivity meter (Labindia) with K=0.946, cell constant=1, solution condition=84 μS, coefficient-1 at 25°C. The MSI was calculated as:

$$MSI=[1-(C1/C2)]x100$$

Extraction and estimation of biochemical components: The sample leaf was extracted by the method as given by Mahadevan and Sridhar (1982) and, for the quantification, the method of Bray and Thorpe (1954) was used. 3% sulphosalicylic acid was used for the extraction of free proline in the leaf sample from the plants and the estimation was done by using the method of Bates et al. (1973). The extraction of chlorophyll was done in 80% acetone and, for the estimation of total chlorophyll, chlorophyll a (chl a) and chlorophyll b (chl b), the method given by Harborne (1973) was used.

RESULTS

Morphological responses and relative water content of leaves: The plants of all the five varieties under test showed less or no symptoms of wilting till the 6 to 7th day of stress, however severe wilting occurred in the plants after 9th days of stress more pronounced in varieties LV and SO. RWC in the leaf of all the varieties declined remarkably as the severity of stress in terms of days was increased (Figure 1). Our data showed that the decrease in RWC was significantly lower in case of PBW 343, UP 2752 and KW on the 9th day of stress when compared to their respective controls (40.75, 42.34 and 36.65%, respectively) however the decline in RWC was much more in LV and SO with respect to their controls (52.93 and 52.67%, respectively).

Antioxidative enzyme activities in wheat after water stress: In the present study, activities of APOX (Figure 2) and GR (Figure 3) showed a significant increase in all the tested wheat varieties during the initial 3 days of stress. As the severity or the duration of stress was increased, the activity of APOX declined in all the five varieties; however activity of GR continued to increase with the increase in the days of stress in KW, UP 2752 and PBW 343 but declined in case of SO and LV. In case of CAT (Figure 4) and SOD (Figure 5), activities showed an increase in the initial stages of stresses, i.e. during the 3rd day of water stress in three varieties. However, in case of LV and SO, the activity of CAT and SOD showed a general decline with increase in the severity of water stress, while in the other three varieties the activity of these two enzymes declined during the later period of water stress. It was noted that the activity of POX enhanced greatly with increase in the period of stress in KW, UP 2752 and PBW 343 whereas in LV and SO, the activity declined (Figure 6).

H_2O_2 accumulation and its *in situ* levels, and change in MDA content in the leaf of wheat during water stress: The accumulation of H_2O_2 and lipid peroxidation increased with increase in the days of stress. The accumulation was higher in case of LV, SO following

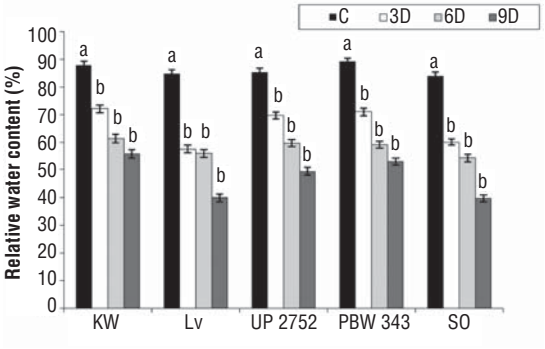

Figure 1. Relative water content of five varieties of wheat subjected to water stress treatments. Results are expressed as the mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

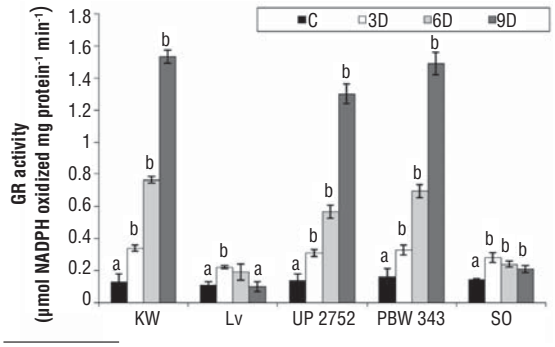

Figure 3. Glutathione reductase (GR) activities in five varieties of wheat subjected to water stress treatments. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

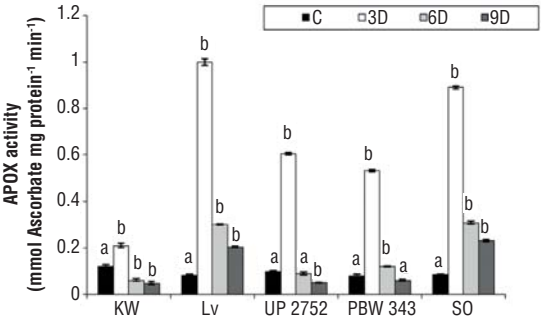

Figure 2. Ascorbate peroxidase activities in five varieties of wheat subjected to water stress treatments. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

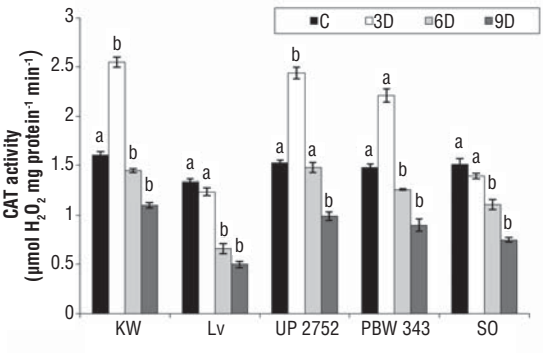

Figure 4. Catalase activities in five varieties of wheat subjected to water stress treatments. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

water stress and lower in case of KW, UP 2752 and PBW 343 (Figure 7A). Lipid peroxidation is measured in terms of MDA content which during water stress was found to be thrice in SO and LV than in KW, UP 2752 and PBW 343 (Figure 7B). It also showed a general increase with the increase in the severity of water stress with respect to their respective controls.

During microscopic studies of the leaf tissues in DAB staining test, dark-brown spots were observed as big and small patches at the site of DAB polymerization. The leaf of SO and LV showed more darkly stained DAB-sites in the tissues than in the leaves from the other three varieties with respect to their control. With increase in the duration of stress LV and SO showed darker staining in the leaf tissues particularly during the 6th and 9th day of stress; however, the other varieties also showed darker stained leaf tissues during the 9th day of water stress (Figure 8). Interestingly, DAB polymerization site was largely localized at the tip of the leaf, region surrounding the middle lamella and also the stomata of the leaf in the varieties under stress when compared to the leaf of the control set of GY (Figure 9). The transverse section of the leaf at the stained site showed that the DAB binding sites were localized mostly in the peripheral region of the cell (Figure 9).

Changes in ascorbate, carotenoids, total antioxidants and chlorophyll content of the leaf: Following water stress there was a significant increase in the two antioxidants,

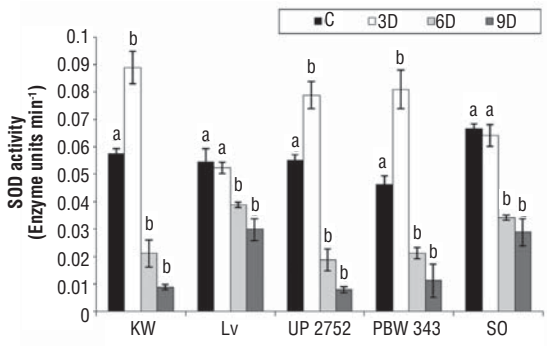

Figure 5. Superoxide dismutase activities in five varieties of wheat subjected to water stress treatments. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

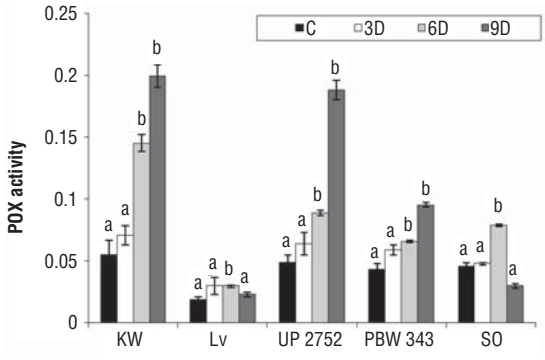

Figure 6. Peroxidase activities in five varieties of wheat subjected to water stress treatments. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

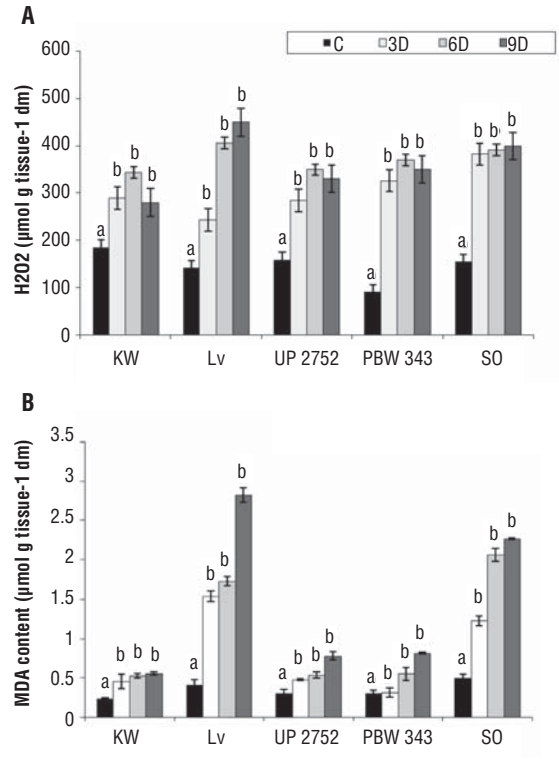

Figure 7. Effect of water stress on accumulation of H_2O_2 (A) and lipid peroxidation (B) (expressed as MDA content) in five varieties of wheat. Results are expressed as mean of three replicates (ten plants each). Bars represent standard error. Different letters indicate significant differences with respect to control ($p<0.01$). C: control; 3, 6 and 9 D: days after withholding water.

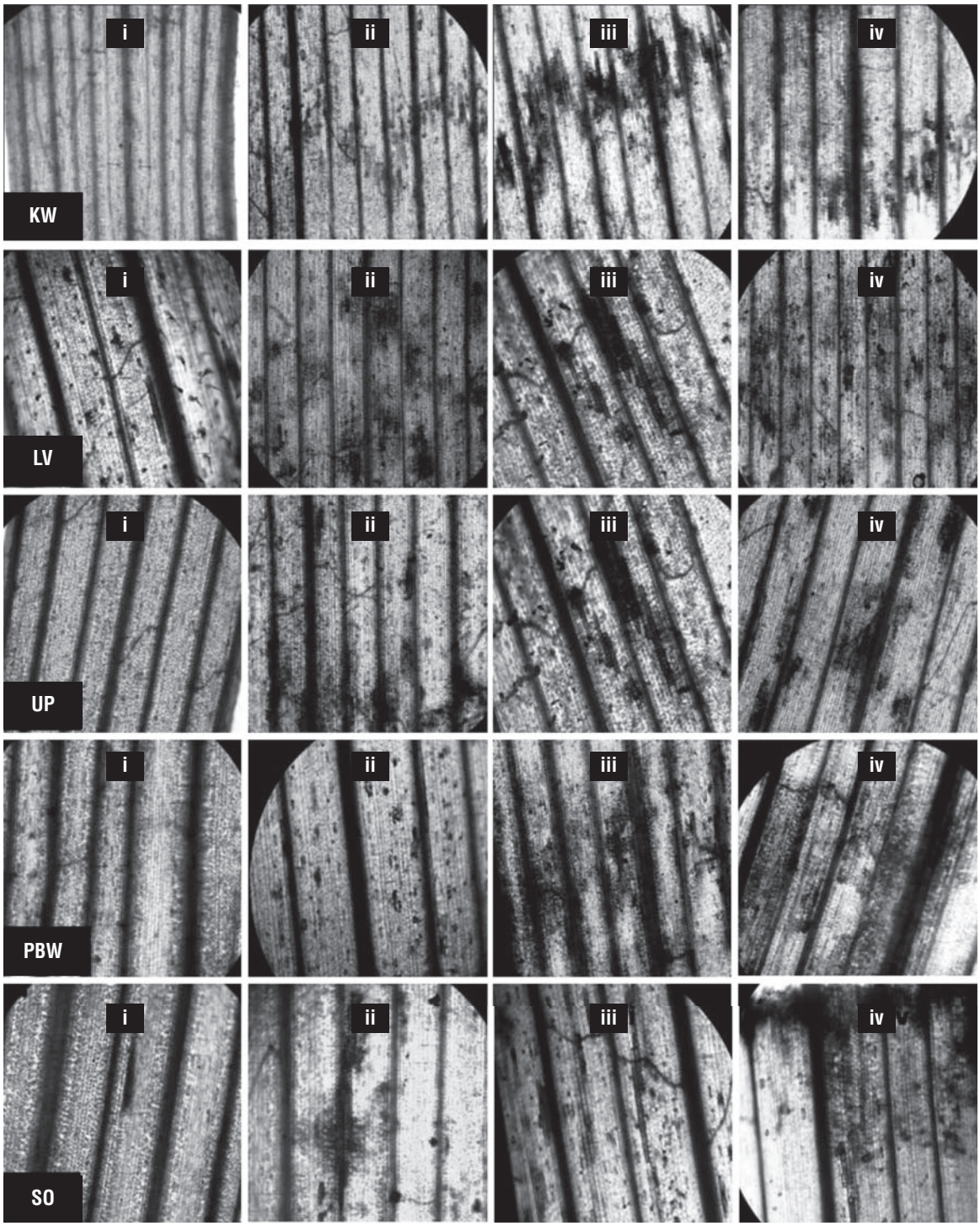

Figure 8. *In situ* detection of H_2O_2 in mid-portions of leaves of five varieties (KW, LV, UP 2,752, PBW 343 and SO) of wheat following water stress. i: control; ii: 3 days of water stress; iii: 6 days of water stress; iv: 9 days of water stress.

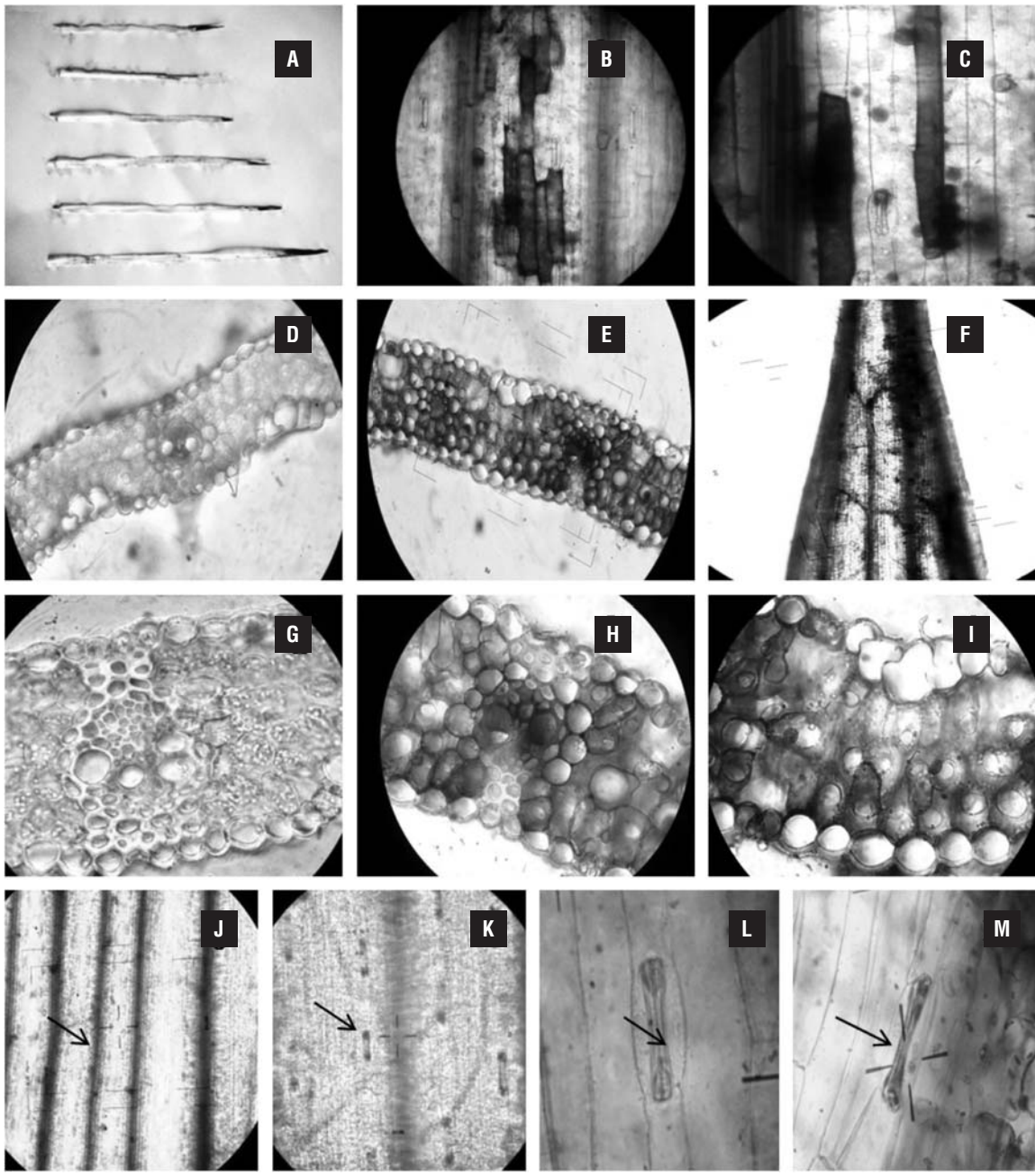

Figure 9. Diaminobenzidine stained sites shown as dark brown spots in the GY variety of leaf. (A) full length leaves; (B–C) 10x view of group of cells in the stressed leaf of 6th day; (D–E) t.s. of control leaf and drought stressed leaf respectively; (F) tip portion of leaf; (G–H) t.s. at the vascular bundle site of the leaf in control and stressed leaf respectively; (I) t.s. of leaf showing diaminobenzidine stained region around bulliform cells; (J–K) stomatal sites in 10x; (L–M) stomatal sites showing diaminobenzidine sites in 45x.

Table 1. Content of proline, phenol and ascorbate in the leaves of four wheat varieties subjected to water stress.

Varieties	Treatment	Proline (mg g^{-1} dm)	Phenol (mg g^{-1} dm)	Ascorbate (mg g^{-1} dm)
KW	C	2.0±0.02[a]	29.9±0.05[a]	11.02±0.02[a]
	3d	2.9±0.02[b]	29.6±0.02[b]	12.19±0.02[b]
	6d	8.1±0.03[b]	39.9±0.06[b]	13.44±0.04[b]
	9d	10.8±0.02[b]	40.1±0.03[b]	14.78±0.09[b]
LV	C	1.7±0.05[a]	38.4±0.10[a]	9.98±0.06[a]
	3d	1.6±0.06[a]	39.6±0.10[b]	9.90±0.01[a]
	6d	5.0±0.10[b]	44.0±0.01[b]	10.10±0.03[b]
	9d	5.1±0.02[b]	34.0±0.01[b]	11.30±0.06[b]
UP 2752	C	2.5±0.04[a]	33.6±0.02[a]	12.11±0.02[a]
	3d	2.3±0.05[a]	31.0±0.05[b]	12.98±0.03[b]
	6d	9.8±0.01[b]	46.8±0.02[b]	14.90±0.05[b]
	9d	11.2±0.05[b]	30.1±0.04[b]	15.51±0.04[b]
PBW 343	C	1.7±0.06[a]	36.8±0.05[a]	11.96±0.02[a]
	3d	2.3±0.05[b]	33.6±0.09[b]	13.15±0.01[b]
	6d	7.5±0.06[b]	40.8±0.05[b]	13.51±0.02[b]
	9d	11.9±0.10[b]	29.1±0.10[b]	15.81±0.03[b]
SO	C	1.5±0.02[a]	21.1±0.04[a]	11.07±0.04[a]
	3d	2.6±0.05[b]	29.4±0.10[b]	12.14±0.05[b]
	6d	4.9±0.01[b]	37.5±0.05[b]	13.89±0.01[b]
	9d	6.1±0.08[b]	38.0±0.07[b]	14.65±0.03[b]

Means±standard error, n=10; Different superscripts in each column express significant difference with control at p=0.01, in *Student´s t-test.*
C: control; d: days.

ascorbate and carotenoids in all the five wheat varieties in comparison to their respective control sets. According to the data there was an enhancement in the ascorbic acid accumulation in all the varieties under stress which continued after the 9th day of stress (Table 1); on the other hand, it was observed that carotenoids showed an initial increase followed by a general decline after 3rd day in the varieties under study. The highest decline was recorded in case of SO, LV and also to some extent in case of PBW 343 (Table 2).

The total antioxidant value showed an enhancement in its accumulation in the all varieties, but in the more tolerant varieties KW, UP 2752 and PBW 343, with the increase in the days of stress also this activity was maintained whereas in LV and SO the total antioxidant initially increased and then declined (Table 3).

There was a significant decrease in the total chlorophyll content in the varieties taken for study, the decline being much pronounced in case of the susceptible varieties. However, in case of the tolerant varieties, the decline was lesser. The ratio of chl a/b after an initial enhancement declined in all the varieties with the decrease being much more pronounced in case of SO and LV (Table 2).

Effect on leaf Membrane stability index: Membrane stability was expressed as % relative injury and results revealed that water stress had significant effect on MSI (Table 3). Linear decrease occurred in MSI with the increase in the duration of stress. Higher MSI value was observed in the cultivar KW, UP 2752 and PBW 343 during the 6th and 9th day of stress. While after 6 and 9 days of water stress, maximum decrease was recorded in LV and SO.

Accumulation of water-stress responsive metabolites: Free proline content increased with the increase in the days and severity of stress in general with respect to the control (Table 1). The accumulation of free proline in KW, UP 2752 and PBW 343 on the onset of 9th day of water stress was twice than that of free proline content found to be accumulated in LV and SO.

DISCUSSION

Results of the influence of water stress in water relations of wheat varieties showed that decrease in the RWC on the 9th day of stress with respect to zero day (control) was lower in PBW 343, UP 2752 and KW (40.75,

Table 2. Content of total chlorophyll, chlorophyll a/b ratio and carotenoids in the leaves of five wheat varieties subjected to water stress.

Varieties	Treatment	Total chlorophyll (mg g^{-1} fm)	Chlorophyll a/b ratio	Carotenoids (mg g^{-1} fm)
KW	C	0.90±0.04[a]	1.57	0.044±0.002[a]
	3d	0.77±0.02[b]	1.66	0.051±0.001[b]
	6d	0.49±0.02[b]	1.54	0.061±0.005[b]
	9d	0.31±0.01[b]	1.78	0.055±0.002[b]
LV	C	1.22±0.05[a]	0.70	0.048±0.003[a]
	3d	0.41±0.04[b]	1.44	0.049±0.003[a]
	6d	0.31±0.02[b]	1.55	0.038±0.001[b]
	9d	0.29±0.01[b]	0.89	0.029±0.002[b]
UP 2752	C	2.30±0.07[a]	1.47	0.050±0.001[a]
	3d	0.75±0.06[b]	1.60	0.062±0.003[b]
	6d	0.63±0.01[b]	1.59	0.052±0.002[a]
	9d	0.62±0.03[b]	1.66	0.038±0.001[b]
PBW 343	C	1.73±0.05[a]	1.19	0.047±0.003[a]
	3d	0.86±0.03[b]	1.24	0.048±0.001[a]
	6d	0.57±0.07[b]	1.97	0.042±0.001[a]
	9d	0.39±0.11[b]	1.93	0.037±0.001[b]
SO	C	0.96±0.09[a]	1.34	0.041±0.002[a]
	3d	0.62±0.05[b]	1.43	0.049±0.001[a]
	6d	0.49±0.04[b]	1.38	0.042±0.005[a]
	9d	0.31±0.01[b]	1.12	0.033±0.002[a]

Means±standard error, n=10; different superscripts in each column express significant difference with control at p=0.01, in *Student´s t-test*.
C: control; d: days.

Table 3. Effect of water stress on Cell membrane stability and total antioxidant activity.

Varieties	Treatment	Relative injury (%)*	Total antioxidant activity**
KW	C	69.49	8.92
	3d	56.25	15.25
	6d	46.49	11.63
	9d	38.24	9.64
LV	C	61.32	5.21
	3d	52.52	10.40
	6d	45.33	7.89
	9d	29.32	6.88
UP 2752	C	86.29	6.88
	3d	79.91	19.22
	6d	59.66	21.43
	9d	40.41	39.53
PBW 343	C	67.22	5.76
	3d	81.23	11.50
	6d	67.58	15.30
	9d	32.39	22.24
SO	C	69.44	7.12
	3d	54.56	10.82
	6d	49.64	9.88
	9d	30.33	4.02

*CMS expressed as percent (%) relative injury; **free radical scavenging activity (total antioxidant activity) expressed as percent (%) inhibition of DPPH absorbance.
C: control; d: days.

42.34 and 36.65% respectively) compared to SO and LV (52.67 and 52.93%, respectively). Drought was found to decrease the RWC of plant leaves (Sánchez-Blanco et al., 2002). Previous studies have also confirmed that the ability to maintain higher RWC is one of the mechanisms of drought tolerance in plants (Farooqui et al., 2000; Chakraborty et al., 2002; Iqbal and Bano, 2009).

Water stress is inevitably associated with increased oxidative stress due to enhanced accumulation of ROS, particularly O_2^- and H_2O_2 in chloroplasts, mitochondria, and peroxisomes. As a result, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stresses (Foyer and Noctor, 2003). The potential of APOX to metabolize H_2O_2 depends on the redox state of such compounds. APOX and GR are believed to act in conjunction for H_2O_2 scavenging during environmental stresses (Sairam and Saxena, 2000). It has been suggested that the coordinated activity of the different H_2O_2-scavenging enzymes play a part in the plant redox homeostasis (Foyer and Noctor, 2005).

Results of the present study reveal that in case of KW, PBW 343 and UP 2752 activities of POX and GR showed an increase with the increase in the duration of water stress whereas activities of CAT, SOD and APOX declined

after an initial increase. When compared to these varieties SO and LV showed either a decrease in activities at all days of water stress (CAT and SOD) or a lower increase in the activity of these antioxidative enzymes, following water stress and the severity of stress. These results indicate that, among the five varieties, antioxidant mechanisms in terms of antioxidative enzymes are enhanced in three of the varieties which can be considered potentially tolerant.

H_2O_2, resulting from the action of SOD, is toxic to cells. Therefore, it is important that H_2O_2 be scavenged rapidly by the antioxidative defense system to water and oxygen (Guo et al., 2006). The over-expression of SOD, if accompanied by enhanced H_2O_2 scavenging mechanisms, like CAT and POD enzyme activities, has been considered as an important anti-drought mechanism to cope with oxidative stress during water deficit conditions. SOD is the first enzyme which is expressed in the antioxidant mechanism and it increased initially in the more tolerant varieties and was also involved in contribution to the initial accumulation of H_2O_2. However, following increase in the severity or days of water stress, SOD activity declined even in the tolerant varieties and POX metabolized the H_2O_2 produced, thus, a synergic activity of various enzymes is required for the attainment of tolerance (Chakraborty and Pradhan, 2011). It was observed in our study that though all antioxidative enzymes increased initially, POX and GR activities could be maintained at higher levels in the tolerant varieties and hence contributed to the defense response. This result is similar to the results as obtained by Abedi and Pakniyat (2010) who reported enhanced activities of SOD and decreased CAT activity. Enhancement of GR activity in tolerant varieties indicated that tolerant plants exhibit a more active ascorbate-glutathione cycle than the less tolerant cultivars. This cycle has been implicated in mitigating the effects of ROS (Molina et al., 2002; Mandhania et al., 2006). In the present study, POX showed a much pronounced or greater role in mechanism for imparting tolerance compared to CAT following increase in the degree of water stress. This was in accordance with the results obtained by Chakraborty et al. (2002) in their study on tea cultivars. However, in the study of Iqbal and Bano (2009) a greater increase in POD and CAT activities in wheat accessions was reported in both tolerant and susceptible plants following water stress.

Varieties which were less tolerant accumulated higher amount of H_2O_2 and the increase in the lipid peroxidation were remarkably higher in case susceptible varieties than the tolerant ones. Various previous workers reported similar results (Chai et al., 2005; Zlatev et al., 2006) as we obtained. The decline in CAT activity was in correlation with the increase in the accumulation of H_2O_2 following water stress as well as increased lipid peroxidation in all varieties. The enhanced H_2O_2 levels under water deficit would be alleviated through the combined action of CAT and APX. Foyer and Noctor (2012) have suggested that serious problems persist in our ability to actively extract, assay and accurately quantify H_2O_2 in tissues or extracts which are fraught with ambiguities. Such problems, according to him may be addressed by the development of biochemically suitable staining techniques. In our studies the detection of cellular levels of H_2O_2 was done by DAB staining method and our results shows a clear difference in the degree of staining achieved in the control and the stressed plant.

Lipid peroxidation in the cell membranes is said to be one of the most challenging and detrimental effect of water stress in the membranes of all the cells exposed to varied degree of stress as quoted by Thankamani et al. (2003) and the degree of lipid peroxidation measured in terms of MDA content is one of the determinants which indicates the severity of stress experienced by any plant. Other workers like Tatar and Gevrek (2008) have also reported increase in MDA content with increase in the degree of stress in wheat. In addition, Türkan et al. (2005) found that MDA content was lower in the leaves of drought-tolerant *Phaseolus acutifolius* Gray. than that in drought-sensitive *P. vulgaris* L. Sairam and Srivastava (2001) reported that the drought-tolerant genotypes of wheat showed lower lipid peroxidation level and higher MSI value than the susceptible ones which is in conformity with the present findings.

Small antioxidants such as ascorbate and carotenoids also increased in tolerant varieties. According to Nair et al. (2008) there was a decrease in ascorbate content in susceptible varieties of cowpea when the degree of water stress increased. Increased ascorbate accumulation was reported by Jaleel (2009) in his study in *Withania somnifera* following water stress. Enhancement in the accumulation of ascorbic acid and increase in GR hints to the occurrence of ascorbate-glutathione cycle as a predominant mechanism of ROS detoxification. The overall antioxidant activity of all varieties showed an initial increase in all varieties, which was still high even after 9 days of stress in the tolerant varieties but, in the other two varieties, the total antioxidant activity declined after 6 days.

Decrease in chlorophyll content and an increase in proline accumulation was observed in the present study. Several workers have reported higher proline levels in

plants subjected to water stress conditions. Hsu et al. (2003) studied the effect of PEG-induced water stress at -1.5 MPa and observed accumulation of proline and its precursor's glutamic acid, ornithine and arginine in rice leaves. The high correlation between proline accumulation and drought-tolerance increase has also been described; however, such accumulation can be only a stress effect (Parida and Das, 2005). Several workers like Vendruscolo et al. (2007), Tatar and Gevrek (2008) have considered proline as an osmotolerant whose accumulation in the cell system suggests its active involvement the scavenging of free radicals thus by reducing damage caused by various kinds of oxidative stress. In our study it was determines that in KW, UP 2752 and PBW 343, phenol contents increased with increase in the duration of the stress, though in the other two varieties the accumulation of total phenol decline or remained unchanged during the 9th day. Leinhos and Bergman (1995) had studied the plant defense system against various types of stress with respect to the involvement of polyphenols as a response to stress, also other workers such as Chakraborty et al. (2002) had studied and reported that the accumulation of phenols was greater in case of tolerant cultivars of tea.

It was indicated by the results of our study that water stress was responsible for the induction of oxidative stress and thus related damage as shown by RWC decrease, increase in the MDA content (i.e., lipid peroxidation), H_2O_2 accumulation, degradation of chlorophyll molecule, enhanced antioxidative responses as evident in the differential or varied levels of antioxidative enzyme activities, accumulation of different antioxidants like carotenoids, ascorbate, phenols and osmolytes such as soluble sugars. Out of the five varieties taken for the study, KW, UP 2752 and PBW 343 showed a much more pronounced antioxidative mechanisms after the induction of water stress and hence, they seem to be protected from the detrimental effects or damage caused by oxidative stress as a result of water stress even at the longer duration and increased severity of stress. CMS as well as difference in the percent relative injury among the varieties served as important criteria to study the effects of water stress and also to determine the susceptible and tolerant variety. Considering all the above data our study showed that KW, Up 2752 and PBW 343 were more tolerant varieties, whereas LV and SO were less tolerant or, in other words, more susceptible to water stress.

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