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LEAF AGE AND SEASONALITY DETERMINES THE EXTENT OF OXIDATIVE STRESS AND INDUCTION OF ANTIOXIDANTS IN LEMONGRASS

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Oxidative stress with the enhanced production of reactive oxygen species (ROS) due to climatic perturbations is a critical factor in the plant survival and biomass yield. Various plant parts may have differential response to the environmental perturbations in various seasons and adopting strategies to scavenge the ROS. In the current work, penultimate, middle and bottom leaves of a population of lemongrass [*Cymbopogon citratus* (D.C.) Stapf] were investigated over monthly intervals for changes in the production of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) and the induction of enzymatic antioxidants including super oxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Moreover, correlations of these parameters with monthly changes in maximum and minimum temperature, relative humidity, evapotranspiration and average rainfall were drawn. The results revealed that production of MDA and H_2O_2 was significantly greater in the bottom leaf (15-26 and 79-107 nmol g^{-1} fresh weight MDA and H_2O_2, respectively) compared to penultimate (5-9 and 14-26 nmol g^{-1} fresh weight MDA and H_2O_2, respectively) and middle (6-11 and 59-77 nmol g^{-1} fresh weight MDA and H_2O_2, respectively) leaves. Results suggested that a higher induction of enzymatic antioxidants system in the scavenging of H_2O_2 and reduced production of MDA helped the penultimate and middle leaves to withstand adverse climatic conditions.
Keywords: Climate change, oxidative stress, antioxidants, leaf position, lemongrass

INTRODUCTION

The plants while growing in nature are subject to vagaries of environmental conditions in different seasons. Plants in nature are exposed to variety of stresses, which lead to the production of reactive oxygen species (ROS). There are various ROS, some are short lived e.g. hydroxyl ion while other are long lived e.g., hydrogen peroxide (Wahid *et al.*, 2013). Although low threshold levels of ROS are essential for normal cell functioning, their increased production under suboptimal conditions is deterrent to metabolic phenomena. The plants showing the production of ROS above the metabolic threshold said to be under oxidative stress (Hiner *et al.,* 2000). The production of ROS causes the disruption of membrane functions; the most obvious effect is the peroxidation of membrane lipids leading to the production of MDA (Gill and Tuteja, 2010; Sharma *et al.*, 2012).

In nature, the environmental factors are the key drivers in the production of an array of changes in plants starting from gene expression to the appearance of visible features. Among the environmental conditions, the changes in light, temperature and humidity are the most important ones. In order to encounter the excessive production of ROS and nullify their adverse effects, the plants induce the antioxidants machinery, both of enzymatic and non-enzymatic type (Wahid *et al.*, 2013). Among the enzymatic antioxidants, an increase in the activities of catalase, peroxidase, and super oxide dismutase are important (Sharma *et al.*, 2012). On the other hand, there is an array of non-enzymatic antioxidants including vitamins (Asensi-Fabado and Munné-Bosch, 2010), osmoprotectants (Shao *et al.*, 2008) and soluble phenolics (Michalak, 2006; Wahid and Tariq, 2008; Mahmood *et al.,* 2012; Lee *et al*., 2013).

Medicinal plants are considered economically important in view of their capability to synthesize the important metabolites, including antioxidants, which act as line of defense against stressing factors. Being a C_4 plant, lemongrass ([*Cymbopogon citratus* (D.C.) Stapf] responds fairly well to the prevailing conditions. Lemongrass extract shows the free radical inhibition and antimicrobial activities (Cheel *et al.*, 2005; Selim, 2011) and is good source of essential oils (de Guimaraes *et al.*, 2011). However, to our knowledge, prevalence of oxidative stress and induction of antioxidant system in different seasons in lemongrass has not been reported so far.

In the current work, the response of a selected lemongrass population was studied for the changes in the intensity of oxidative stress and the induction of enzymatic antioxidants on monthly basis and their possible association with the prevailing meteorological condition. Moreover, possible physiological advantage of such changes has been described.

MATERIALS AND METHODS

***Experimental details*:** For these studies, experimental field was selected in New Botanical Garden, University of Agriculture, and Faisalabad. Propagules of a selected lemongrass population were transplanted in plots measuring 2 × 2 m in the month of October, 2010 and 2011. In both the years, after sprouting 100 plants were retained in each plot with a plant-to-plant distance of 20 cm and row-to-row distance of 30 cm. The experimental design was randomized complete block design (RCBD). There were three blocks; each block with three replicates. All the plots were watered using irrigation water at fortnightly intervals during summer season at three weeks interval during winter season. From each replicate 500 g of the leaves were taken from three positions i.e., penultimate, middle and bottom on 10^{th} of each month during the years 2010 and 2011. The leaf samples were frozen at -50ºC for the measurements of oxidative stress and antioxidants.

Meteorological data during the years 2010 and 2011 were obtained from the Weather Observatory of the Department of Crop Physiology, University of Agriculture Faisalabad. These data were used to draw the correlations of various physiological and biochemical attributes with the environmental conditions prevailing during the course of experiment (Fig. 1).

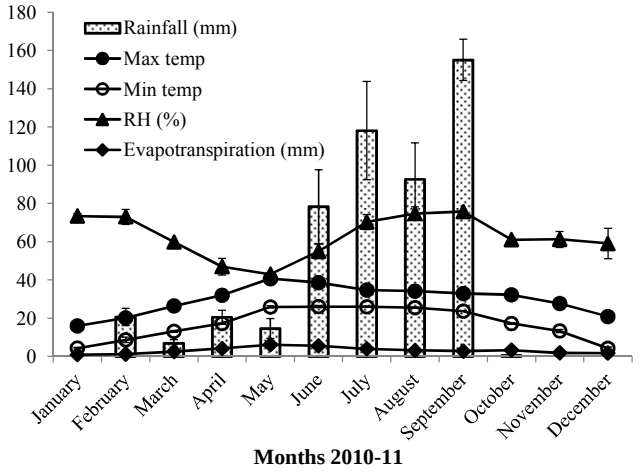

Figure 1. Average monthly data of meteorological conditions during the year 2010 and 2011. The error bars indicate the average variation in rainfall during both the years

***Analytical procedures*:** The determinations were made for oxidative stress parameters [hydrogen peroxide (H_2O_2) and malondialdehyde (MDA)] enzymatic [superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)].

***Oxidative stress parameters*:** Among the oxidative stress parameters, hydrogen peroxide (H_2O_2) was measured with the method of Velikova *et al*. (2000). Fresh leaf tissues (0.1 g) were extracted in 0.1% trichloroacetic acid (TCA) in ice bath. Then this extract was centrifuged at 12,000 × *g* and supernatant was collected. A 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide was added to 0.5 mL of supernatant. This mixture was vortexed and absorbance was noted at 390 nm. For malondialdehyde (MDA) estimation with the method of Heath and Packer (1968), 0.1 g of fresh leaf tissue was extracted in 1 mL of 5% (w/v) TCA and centrifuged at 12000 × *g* and supernatant was taken. To 1 mL of supernatant equal volume of thiobarbarturic acid [(TBA) 0.5% in 20% (w/v) in TCA] was added followed by heating at 100ºC. Reaction mixture was centrifuged at 7500 × *g* for 5 min. Absorbance was noted at 532 and 600 nm. MDA was calculated using extinction coefficient of 155 mmol mL^{-1} as: MDA equivalents (nmol mL^{-1}) = [(A532-A600) / 155000] × 10^6.

***Determination of enzymatic antioxidants*:** For enzyme extraction, fresh plant material (0.1 g) was macerated in 0.9 mL of 50 mM cooled phosphate buffer (pH 7.8), and centrifuged at 15000 × *g* at 4°C and supernatant was collected and stored for enzyme assay.

Prior to the activities of the enzymatic antioxidants, total protein in the supernatant was measured with the dye-binding method of Bradford (1976). Activity of SOD was assayed by following the method of Giannopolitis and Ries (1977). Enzyme extract (50 µL) from the above was taken and mixed with 50 mM phosphate buffer (pH 7.8). To this 50 µM NBT was added followed by addition of 1.3 µM riboflavin and 13 mM methionine and 75 mM EDTA was added and this reaction mixture was kept in dark chamber coated with aluminum foil. Then it was illuminated under fluorescent lamps of 30 W for 5 min. The SOD activity was estimated by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm. Blank was used for comparison.

The activities of CAT and POD were estimated by following the method of Chance and Maehly (1955) with some modification. Enzyme extract (0.1 mL) was taken and mixed with 50 mM phosphate buffer (pH 7.8) and 5.9 mM H_2O_2 and diluted up to 3 mL. The absorbance was noted at 240 nm after 20 sec. CAT activity was expressed in units as µM of H_2O_2 decomposed per min. One unit was defined as an absorbance change of 0.01 units per min. For POD activity, reaction solution containing 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM H_2O_2 was taken. Enzyme extract (0.1 mL) was added and change in the absorbance was noted at 470 nm after each 20 sec. One unit POD

activity was defined as the change of 0.01 absorbance unit per min per mg of protein.

***Statistical analysis*:** The data presented here is the average of both the years 2010-2011. The differences between factors and their interactions were ascertained with analysis of variance computer software Statistix 8.1. Pearson's correlation coefficients were derived among different attributes using MS-Excel. Data were presented graphically by using MS-Excel.

RESULTS

***Oxidative stress parameters*:** Data on the H_2O_2 contents of leaves indicated significant ($P<0.01$) difference in the leaves and months with significant ($P<0.01$) interaction of these factors for H_2O_2 concentrations. In penultimate leaf H_2O_2 remained fairly constant during spring (February-April) season. It increased slightly in summer (May-July) but declined markedly in autumn (September-October) seasons. H_2O_2 increased again in winter (November-January) months. In middle leaf, with the onset of spring season, H_2O_2 consistently declined but then attained the greatest value in summer (in July) and a decline was observed in autumn, and then a rise in winter. In bottom leaf, except for late winter months, the trend of H_2O_2 accumulation was similar to penultimate and bottom leaves. The highest H_2O_2 level was observed in bottom, moderate in middle and lowest in penultimate leaf (Fig. 2).

The MDA content in leaves of lemongrass over months varied significantly ($P<0.01$) with a significant ($P<0.01$) interaction of these factors. Both penultimate and middle leaves exhibited similar contents and trend of MDA accumulation. In both these leaves, MDA contents were lower in spring season, which indicated a sharp increase at the onset of summer and the maximum values were noted in June−July. The MDA contents began to decrease sharply at the start of autumn but again increased in winter season. Bottom leaf showed increased contents of MDA contents from May and rack up the apical value in July, while a drop was detected in August, which continued till October. However, a steady-state rise was observed from October to January (winter season). In all leaves, the value of MDA increased during summer and winter seasons while minimum value was recorded in spring and autumn seasons (Fig. 3).

***Antioxidants induction*:** For the activity of SOD, data showed significant ($P<0.01$) differences in the leaves of various ages and sampling months with a significant ($P<0.01$) interaction of leaves and sampling months. Seasonal changes produced a great impact on the SOD activity of lemongrass leaves. The SOD activity of the penultimate leaf was the lowest SOD in spring season, which increased greatly in summer season, attained the highest value in June (21.66 U/g protein/min), while a decline was observed in July and onward which continued till October. However, a gradual increase was observed from November to December. SOD activity of middle leaf was more or less similar to the penultimate leaf. However, bottom leaf indicated quite reduced SOD activity in most of the months except a rise was noted in May (Fig. 4).

The CAT activity of lemongrass leaves of three ages varied significantly ($P<0.01$) in different seasons with a significant

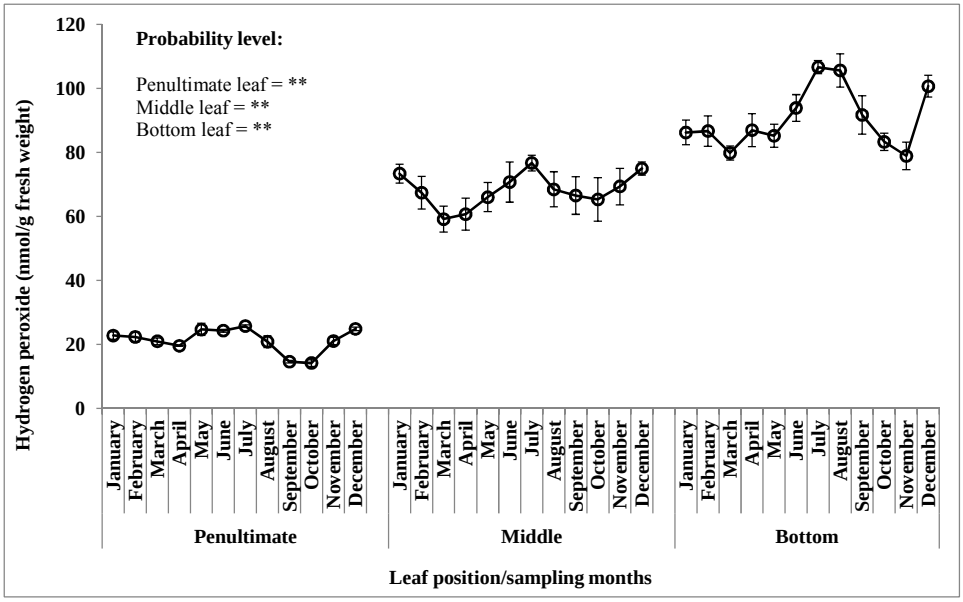

Figure 2. Effect of seasonal variation on hydrogen peroxide accumulation in the penultimate, middle and bottom leaves of lemongrass

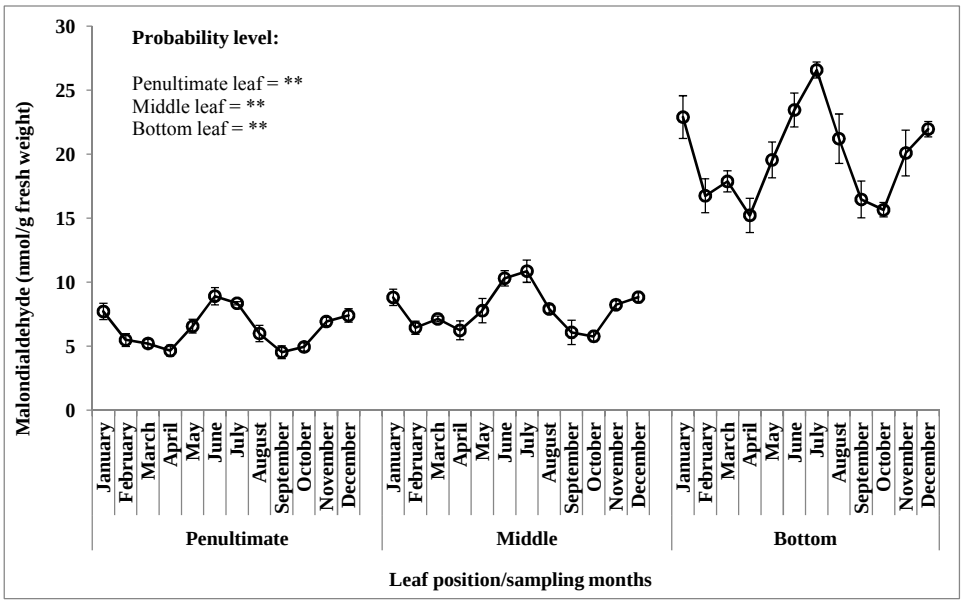

Figure 3. Effect of seasonal variation on MDA concentration in the penultimate, middle and bottom leaf of lemongrass

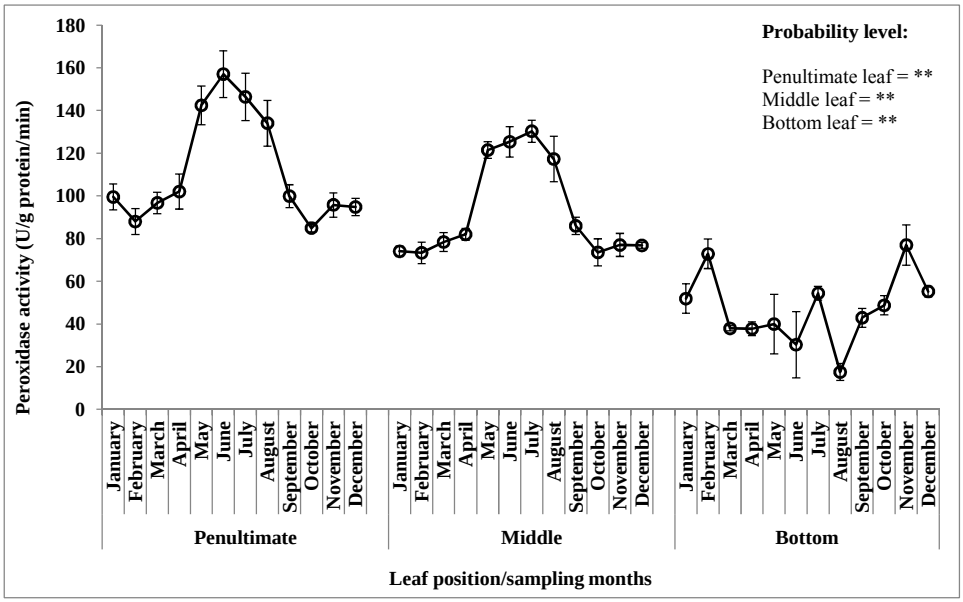

Figure 4. Effect of seasonal variation on SOD activity in the penultimate, middle and bottom leaves of lemongrass

($P<0.01$) interaction of leaf ages and sampling months. All leaves indicated individualistic behavior of CAT activity. The penultimate leaf showed the highest CAT activity in July (113.14 U/g protein/min). However, a reduction in CAT activity was noticed from August, which attained a steady-state level up to December. In the middle leaf, CAT in activity increased from January to March (winter to spring seasons) and attained the maximum value in July (87.84 U/g protein/min), while a decline was observed from August to December. On the other hand, bottom leaf over the seasons (sampling months) indicated lower CAT activity in winter months, declined in summer months, again increased in autumn. Overall, penultimate leaf showed the highest CAT activity followed by middle leaf (Fig. 5).

As regards POD activity, the data indicated significant ($P<0.01$) differences in the leaves and months while there was significant ($P<0.01$) interaction of these factors. Different leaves presented POD activity with value ranging

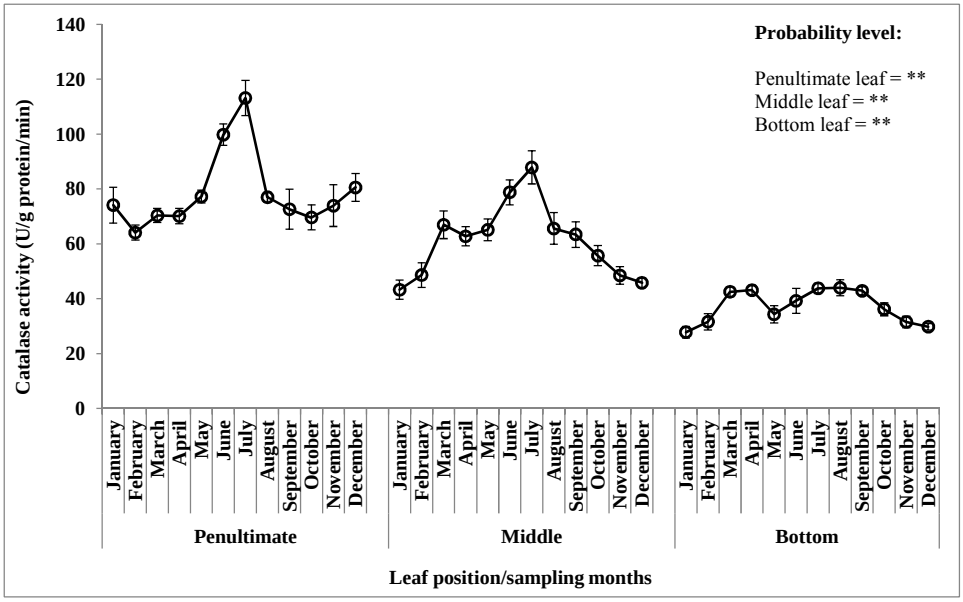

Figure 5. Effect of seasonal variation on CAT activity in the penultimate, middle and bottom leaves of lemongrass

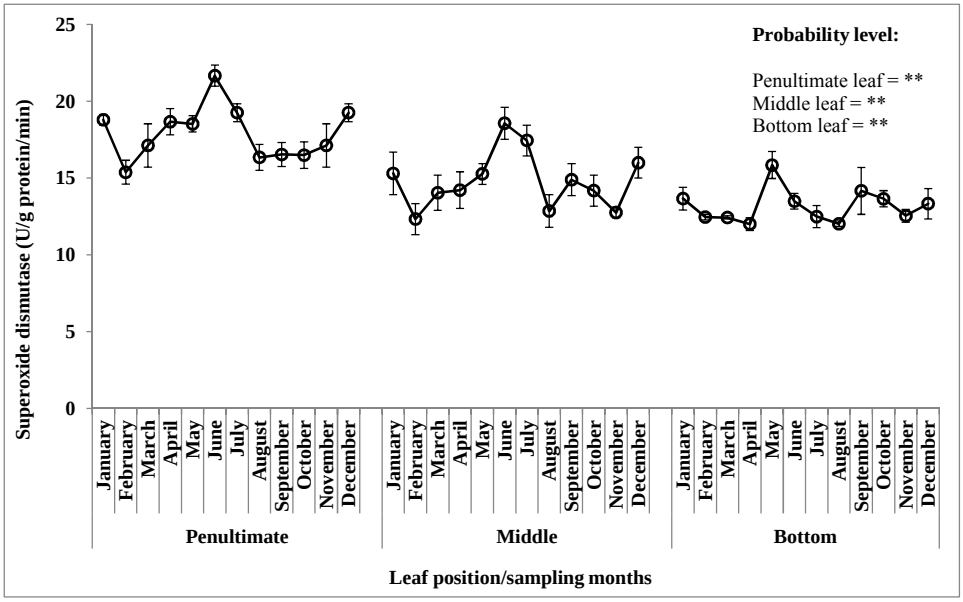

Figure 6. Effect of seasonal variation on POD activity in the penultimate, middle and bottom leaves of lemongrass

from as low as 17.46 U/g protein/min in bottom leaf to as high as 157.06 U/g protein/min in penultimate leaf. The POD activity of penultimate and middle leaves was lower in winter and autumn but higher in summer months. It declined from August and attained steady-state level thereafter. However, bottom leaf exhibited a differential behavior of POD activity; three distinct peaks were observed in February, July and November. Overall, penultimate leaf indicated the highest POD activity followed by middle leaf (Fig. 6).

***Correlation*:** Parallels drawn of oxidative stress parameters with antioxidants activities revealed that H_2O_2 accumulation was positively related to MDA contents of all leaves. Among the antioxidants, SOD activity was positively correlated with H_2O_2 accumulation in penultimate and middle leaves. MDA contents indicated a positive relationship in case of penultimate and middle leaves with the activities of SOD and POD while only with penultimate leaf in case of CAT activity. Bottom leaf did not exhibit any association with the oxidative stress parameters and activities of antioxidants (Table 1).

Table 1. Correlations coefficient (r) of metrological attributes with the H_2O_2, MDA, SOD, CAT and POD of lemongrass leaves collected at penultimate, middle and bottom positions

X-Variable	Y-Variable	Penultimate	Middle	Bottom
a. Correlations of oxidative stress parameters with antioxidants				
H_2O_2	MDA	0.777**	0.761**	0.610*
	SOD	0.581*	0.594*	-0.193ns
	CAT	0.554ns	0.014ns	0.347ns
	POD	0.548ns	0.309ns	-0.355ns
MDA	SOD	0.753**	0.711**	-0.009ns
	CAT	0.774**	0.430ns	-0.083ns
	POD	0.610*	0.622*	-0.034ns
b. Correlations of seasonal factors with oxidative stress parameters and antioxidant				
Max. temperature	H_2O_2	-0.062ns	-0.188ns	0.205ns
	MDA	0.023ns	0.107ns	0.038ns
	SOD	0.173ns	0.371ns	0.021ns
	CAT	0.424ns	0.759**	0.632*
	POD	0.707*	0.759**	-0.474ns
Min. temperature	H_2O_2	-0.069ns	-0.100ns	0.346ns
	MDA	0.043ns	0.163ns	0.136ns
	SOD	0.120ns	0.367ns	0.043ns
	CAT	0.488ns	0.831**	0.729**
	POD	0.756**	0.823**	-0.470ns
Relative humidity	H_2O_2	-0.210ns	0.377ns	0.346ns
	MDA	-0.019ns	0.027ns	0.187ns
	SOD	-0.281ns	-0.091ns	0.331ns
	CAT	0.002ns	-0.145ns	0.273ns
	POD	-0.205ns	-0.145ns	0.273ns
Evapotranspiration	H_2O_2	0.158ns	-0.169ns	0.146ns
	MDA	0.169ns	0.203ns	0.101ns
	SOD	0.163ns	0.241ns	-0.178ns
	CAT	0.454ns	0.721**	0.474ns
	POD	0.758**	0.760**	-0.513ns
Average rainfall	H_2O_2	-0.136ns	0.218ns	0.616*
	MDA	0.050ns	0.206ns	0.246ns
	SOD	-0.031ns	0.265ns	0.315ns
	CAT	0.495ns	0.649*	0.671*
	POD	0.492ns	0.566ns	-0.253ns

Significant at: ns, non-significant; * and **, significant at P<0.05 and P<0.01 levels

As for the seasonal changes, POD activity of penultimate leaf was positively correlated with maximum and minimum temperatures, and evapotranspiration. CAT and POD activities of middle leaf were positively correlated with maximum and minimum temperatures, evapotranspiration and average rainfall. In case of bottom leaf, the CAT activity was correlated with maximum and minimum temperatures and average rain fall while H_2O_2 accumulation was related to average rainfall (Table 1).

DISCUSSION

Plants respond to the seasonal changes by showing adjustments in the metabolism. One of the most common consequences of heat stress is the generation of ROS leading to membrane damage and disruption of cellular phenomena (Wahid *et al.*, 2013). In this experiment, monitoring the leaf H_2O_2 contents indicated that its greater production took place all round the year in all leaves, although bottom leaf indicated the highest while penultimate the lowest amounts (Fig. 2). With the induction of oxidative stress, MDA is produced due to β-oxidation of membrane lipids (Sharma *et al*., 2012), which perturbs the plant phenomena including photosynthesis and respiration (Taylor *et al*., 2004). In the present study, there was substantial increased in the MDA contents of all the studied leaves in the harsh conditions of summer and winter, although leaf age had a great effect. The MDA contents indicated the pattern more or less similar to

H_2O_2 production (Fig. 2, 3). This was also evident from the positive correlation of H_2O_2 and MDA for all leaves (Table 1). Savicka and Skute (2010) observed that high temperature accelerates MDA production (up to 58%) at later growth stages as compared with early seedling stage (by 27%). Enhanced lipid peroxidation during hot months could be associated with the damage to cell membranes (Huang *et al.*, 2004) and in rice, it reduced antioxidant enzyme activities (Cao *et al*., 2009; Ahmed *et al*., 2012; Farhoudi *et al.,* 2012). It was important to note here that both seasonal changes and leaf age have great impacts on the production of MDA, whereas cooler months and younger (penultimate) leaf exhibited lower MDA production.

Both plant age and seasonal variation influence antioxidant enzyme activities of plants, which are important indices of plant responses (Sen and Mukherji, 2007). Temperature above (33°C) induced oxidative stress, which damage cell membrane due to degradation of protein also decreased enzyme activities in wheat (Bavita *et al*., 2012). To circumvent enhanced ROS produced by seasonal variation, plants cells activate the key antioxidant enzyme like SOD, CAT and POD (Mittler, 2002; Ozden *et al*., 2009). These antioxidants are the first line of defense against ROS, which catalyzes the dismutation of O^{2-} and dousing of other activated ions (Takahashi and Asada 1983; Scandalios, 1993; Wahid *et al.*, 2013). In the present study, the SOD, CAT and POD activities in lemongrass leaves were increased markedly in summer months (Figs. 4-6). However, leaves of different ages indicated differential response to the induction of antioxidant defense. Correlation data indicated that there was a close association of the MDA and H_2O_2 accumulation with the SOD and CAT activities in penultimate and middle leaves (Table 1). A conspicuous increase in ROS scavenging activity with the induction of antioxidant systems was of great advantage to these leaves. Under higher temperatures, plant respiratory rates are higher which enhanced antioxidant response due to subsequent higher ROS level in mitochondria. According to Dizengremel (2001), SOD formation increase with increase in NADH synthesis during higher respiration. Similarly, high temperature stress enhanced the respiration rate along with a significant increase in SOD-manganese activity in *Nicotiana plumbagifolia* (Bowler *et al*., 1992).

Among the environmental variables, changes in the ambient temperature and evapotranspiration were of greater significance. These variables cause osmotic strain on the leaves and induction of enzymatic antioxidants has been well reported under abiotic stresses including cold stress (Wu *et al*., 1999; McKersie *et al*., 1999), heat stress (Wahid *et al.*, 2007), high light intensity (Sen Gupta *et al*., 1993) and drought stress (Farooq *et al.*, 2009). In present study, CAT and POD activities increased in lemongrass leaves in summer period during month of June and July (Fig. 5-6), which has close association with high and low temperatures and evapotranspiration (Table 1). Reports show that increased CAT under adverse conditions is a prerequisite for plants against fatal H_2O_2 accumulation (Streb *et al*., 1997; Engel *et al*., 2006). This notion stands fast in the current study too because high and low temperatures and evapotranspiration are great stressing factor for plant growth, while POD protects the membrane from damage due to H_2O_2 (Farooq *et al.*, 2009).

***Conclusion*:** Seasonal factors have great impact on lemongrass antioxidant activity. The antioxidant enzymes are capable to minimize effect of oxidative damage by scavenging H_2O_2 and reducing MDA production in extreme environmental conditions, and thus enabling the lemongrass to display sustained growth in harsh seasonal conditions.

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