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Studies on the production of reactive oxygen species and their scavenging mechanism in lentil (*Lens culinaris* Medic.) seedlings

Aditya Pratap Singh, Siddhartha Singh and Pramod Kumar Pandey

Abstract
Among all the stresses crop plants experience during their lifecycle, drought has a significant importance particularly in areas where precipitation is scanty and rainfed irrigation is followed. Lentil is an important pulse in India and it is sensitive towards water deficit conditions. Water deficit triggers an array of biochemical responses in Lentil, ROS production being the major one. In the present study, we evaluated four lentil genotypes for their ROS production level under water deficit. H_2O_2, HO^-, lipid peroxidation, SOD, Catalase and Guaiacol peroxidise levels were determined. L_3 exhibited highest (48.17%) increase in H_2O_2 and L_4 showed highest OH- levels. Lipid peroxidation was found to be lowest in L_2 whereas minimum SOD increase was observed in L_3. The genotype L_3 showed maximum catalase activity. However, maximum increase in Guaiacol peroxidase activity was observed in L_3.

Keywords: Catalase, H_2O_2, Lentil, ROS, water deficit

1. Introduction
Plants constantly face a multitude of physical or chemical stress factors, which pose severe threats to agriculture and the ecosystem. Among abiotic stresses, drought is one of the most important environmental factors for a wide range of major crops. Generally, plants are considered to be under stress when they experience a relatively severe shortage of an essential constituent or an excess of potentially toxic or damaging substance, and the plants usually face both the situations simultaneously.
Athar and Ashraf (2009) [5] cite extensive agricultural production losses as a result of abiotic stresses. There are a wide range of abiotic stresses in modern agriculture, including cold, heat, droughts, floods, salinity, excessive light, metals, pollutants, mechanical injury, etc. The yield reduction of major crop plants is estimated to be over 50% due to these stresses. According to Monnéveux *et al*. (2006), Lafitte *et al*. (2007), Ashraf *et al*. (2008) [33, 26, 2], severity of losses depends on the development stage and intensity of the stress. Up to 26 percent of arable land is affected by drought, and over 20 percent of irrigated land is salt-affected (Rehman *et al*. 2005) [37]. Consequently, new crop varieties that are more resistant to climatic conditions are needed. Using cross-bred lines or genetic engineering, it is possible to produce crops that are stress tolerant. Nevertheless, to reach that goal, we need to better understand the mechanisms in plants that respond to stress. Modern breeding strategy must be adapted to incorporate modern knowledge for the development of new varieties that are better adapted to non-optimal environmental conditions. Stress-responsive proteins in plants are altered by changes in their relative abundance, which result in changes to their whole proteome, transcriptome, and metabolome (Folgado *et al*. 2013) [15].

1.1 Stress due to water deficit
It is believed that the condition of drought, which results in the lack of water in the soil, is the most devastating environmental stress for crops (Lambers *et al*., 2008) [28]. Drought severely reduces crop growth and biomass accumulation by substantially reducing plant growth and development. Drought results in a reduced rate of cell division and expansion, reduced leaf size, elongated stems and roots, and reduced root formation. As a result, stomatal oscillations become disturbed, plants' water and nutrient relations are disrupted, and crop productivity and water efficiency are decreased (Li *et al*. 2009, Farooq *et al*. 2009a) [29, 12].
Additionally, carbon fixation relies on photosynthetic pigments, such as chlorophyll, carotenoids and xanthophores, which absorb solar radiation to drive photosynthetic reactions.

Marigold chlorophyll a and chlorophyll b contents were relentlessly decreased due to drought stress (Asrar and Elhindi 2011) [4]. As a consequence of water stress, the photosynthetic rate of primary leaves of kidney beans declines with a declining photochemical efficiency (Fv/Fm). In drought-sensitive tomato seedlings, the antioxidant enzymes catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were found to decrease H_2O_2 and malondialdehyde (MDA) levels following the application of brassinolides (BRs) (Yuan *et al*., 2010) [44]. Cakmak and Marschner (1993) [8] found the ascorbate–glutathione cycle to be more effective than CAT and peroxidase (POX) in the breakdown of H_2O_2 in sorghum and sunflower. However, monodehydroascorbate reductase (MDHAR) and POX cytosolic activities were decreased by drought, while dehydroascorbate reductase (DHAR) cytosolic activities were increased (Zhang, 1992) [45].

1.2 Reactive Oxygen Species (ROS)

ROS (reactive oxygen species) generation are a primary consequence of stress on plants. In addition to ROS, hydrogen peroxide (H_2O_2), superoxide (O2.-), and hydroxyl radicals (OH.) can further react with a variety of other molecules and metabolites, such as lipids, proteins, DNA, pigments, and other essential cellular molecules, leading to a number of destructive processes (Lamb and Dixon 1997, Ashraf 2009) [27, 3]. Plants produce ROS scavangers in response to ROS production. Cells utilize ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics as nonenzymatic antioxidants. A number of enzyme antioxidants have been identified, such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), the enzymes of the ascorbate-glutathione (AsA-GSH) cycle, such as monodehydroascorbate reductase (MDHAR), the dehydroascorbate reductase (DHAR), and glutathione reducuctase. In addition, organs and tissues have different metabolic rates and oxygen requirements, and their antioxidant levels are different (Ashraf 2009) [5]. Under conditions of oxidative stress, cellular antioxidant defense systems perform poorly when ROS production is increased (Sharma *et al*. 2012) [39].

Lentil, an important pulse crop adapted to drought, is greatly impaired during water-deficit conditions, especially during germination, flowering, and pod filling stages, and the effects are exacerbated by a combination of different abiotic stresses arising in lentil cultivation zones, such as high temperatures. When lentil plants are osmotically stressed, their metabolism is affected and a large pool of compounds is produced that are involved in carbohydrate and amino acid metabolism, biosynthesis of secondary metabolites, glycolysis, and the TCA cycle.

2. Materials and Methods

2.1 Plant material and treatment levels

Experimental studies were conducted at the Department of Basic Sciences and Humanities, Central Agricultural University, Pasighat, Arunachal Pradesh. In pots containing purified quartz sea sand soaked in a Hoagland nutrient solution, four genotypes of lentil (L_1- L-303, L_2- IPL-406, L_3- DPL 15 and L_4- TRC L-11-2) were collected and grown. Water stress was induced by adding 0% (Control), 15%, 30% and 45% polyethylene glycol to Hoagland nutrient solution. Plants were uprooted after 72 hours and were then used in analysis.

2.2 Hydrogen Peroxide Quantification

A spectrophotometric method was used to measure H_2O_2 production using titanium sulphate following the method of Jana and Choudhuri (1981) [23]. 3 ml of 50 mM sodium phosphate buffer (pH 6.5) was used to extract about 150 mg of fresh shoot samples. The homogenate was centrifuged for 15 minutes at 6,000 x g. A mixture of 3 ml of extracted solution and 1 ml of titanium sulfate in 20 percent (w/v) H2SO4 was centrifuged for 15 minutes at 6,000 x g to determine H_2O_2 levels. We measured the yellow color intensity of the supernatant at 410 nm. H_2O_2levels were calculated using a molar extinction coefficient of 0.28 µM-1 cm-1 and expressed as nmol g-1 tissue fresh weight.

2.3 Determination of OH^- production

Deoxyribose assays were used to determine the amount of OH^- produced, according to Halliwell and Gutteridge (1981) [18]. Two mL of 50 mM sodium phosphate buffer (pH 7.0) containing 100 mg of fresh roots and shoots were homogenized and centrifuged for 10 minutes at 4 C. A mL of the supernatant was reacted for an hour in the dark with 0.8 mL of 2.5 mM 2-deoxyribose and 200 µL of 2 mM FeSO4. Following that, 0.25% thiobarbituric acid was prepared in 10% TCA and then added to the solution. Immediately following the boiling process, the contents were cooled for 10 minutes. Using a spectrophotometer, the absorbance at 532 nm was measured after centrifugation for 10 minutes at 3,000 g for 10 minutes.

2.4 Lipid peroxidation levels

The thiobarbituric acid reactive substances (TBARS) were used to determine lipid peroxidation products using Heath and Packer's (1968) [19] method. The tissue weighed 200 mg and was ground, soaked in a mixture of trichloroacetic acid and 0.25 percent 2-thiobarbituric acid and heated at 95oC for 25 minutes. An ice bath was used to cool the mixture and then a 10,000 g centrifuge was used to separate it for 10 minutes. To calculate the turbidity, the same sample's absorbance was measured at 532 nm and the absorbance at 600 nm was subtracted. TBA was diluted with 10 percent TCA in the blank. For the determination of the concentration of TBARS per mole of fresh tissue weight, we used an extinction coefficient of 155 mM-1cm-1 as the extinction coefficient for the measurement of lipid peroxides and oxidatively modified proteins.

2.5 Superoxide dismutase activity

In the presence of oxygen, p-nitroblue tetrazolium chloride (NBT) was inhibited during reduction. SOD activity was determined using Beauchamp and Fridovich's (1971) [6] method. We homogenized 200 mg of roots/shoots of freshly uprooted plants in 5 mL of 100 mM K-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100, and 2% (w/v) polyvinylpyrrolidone (PVP). The supernatants of the cells were centrifuged at 22,000 xg at 4 C for 10 minutes against the extraction buffer for 6 h. Dialyses were then performed against 3-4 changes of the extraction buffer. SOD activity in the supernatant was determined. An enzyme activity of one unit of SOD equals a 50% inhibition of reduction of NBT at 560 nm.

2.6 Catalase activity

An assay for catalase activity was conducted according to Beers and Sizer (1952) [7]. Fresh tissue (±200 mg) was homogenized using chilled mortar and pestle in 5 ml of 50 mM Tris-HCl buffer (pH 8.0). We used EDTA as a buffer solution of 0.5 mM, Triton X-100 as a 5% solution, and polyvinylpyrrolidone as a 2% (w/v) solution. At 4°C, homogenates were centrifuged for 10 minutes at 22,000 xg. A UV-VIS spectrophotometer (Perkin Elmer, LAMBDA EZ 201, USA) was used to measure the decrease in absorbance of H_2O_2 at 240 nm after dialysis in cellophane membrane tubings for CAT assay. By measuring the amount of H_2O_2 oxidized per unit of protein per minute, the enzyme's specific activity was determined.

2.7 Guaiacol peroxidase activity

The activity of Guaiacol Peroxydase was measured according to Egley *et al*. (1983) [11]. 200 mg root/shoot samples were homogenized in 3 ml of chilled 50 mM Na-phosphate buffer (pH 7.0). Dialysis was performed on the supernatant after homogenization at 22,000 x g at 4 °C for 10 minutes. By using a spectrophotometer (Bausch and Lomb, Spectronic 20, USA) to measure absorbance changes at 420 nm, tetraguaiacohinone is formed (extinction coefficient is 26.6 mM-1cm-1) at intervals of 30 seconds to 2 minutes. Specific enzyme activity is measured in millimoles of H_2O_2 reduced per gram of protein per minute.

3. Results

3.1 Water deficit effects on H_2O_2 production

When experiments on the effects of water deficit on H_2O_2 level in lentil seedlings were conducted, it was observed that in shoots of the seedlings, H_2O_2 levels were elevated (Fig 1). The variety L_1 exhibited an increase of 11.61%, L_2 exhibited an increase of 10.91%, L_3 exhibited an increase of 48.17% and L_4 exhibited an increase of 13.01% as compared to control.

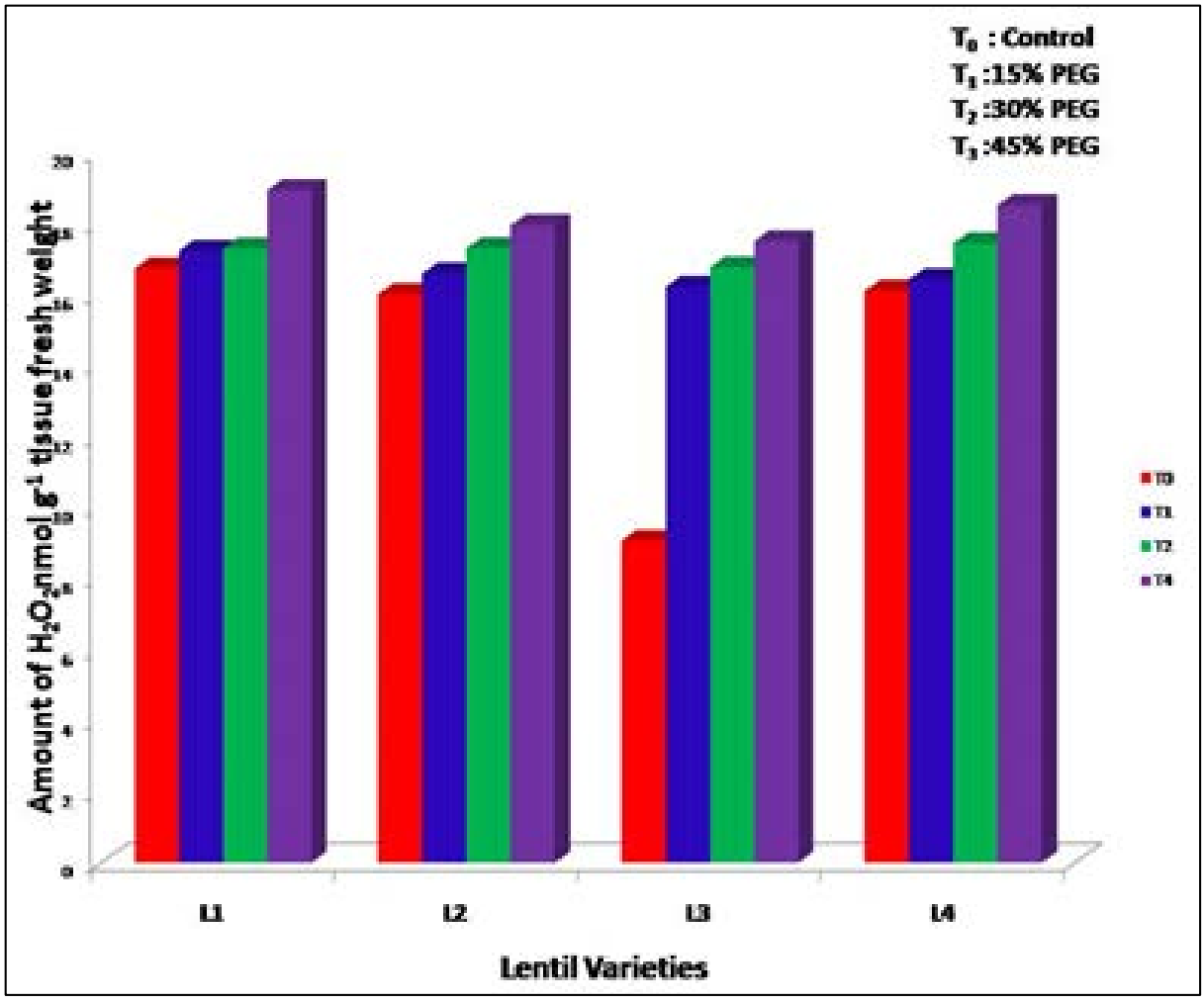

Fig 1: Effect of drought on hydrogen peroxide (H_2O_2) production in different varieties of Lentil

3.2 Water deficit effects on OH^- production

Following thiobarbituric acid (TBA) condensation of the degradation product malonaldehyde with 2-deoxyribose oxidative degradation, the formation of HO^- was quantified in the seedlings. Under drought conditions, all the lentil varieties showed an increase in hydroxyl free radicals as compared with the control (Fig 2). The maximum increase was observed in the variety L_4 (Fig 2.).

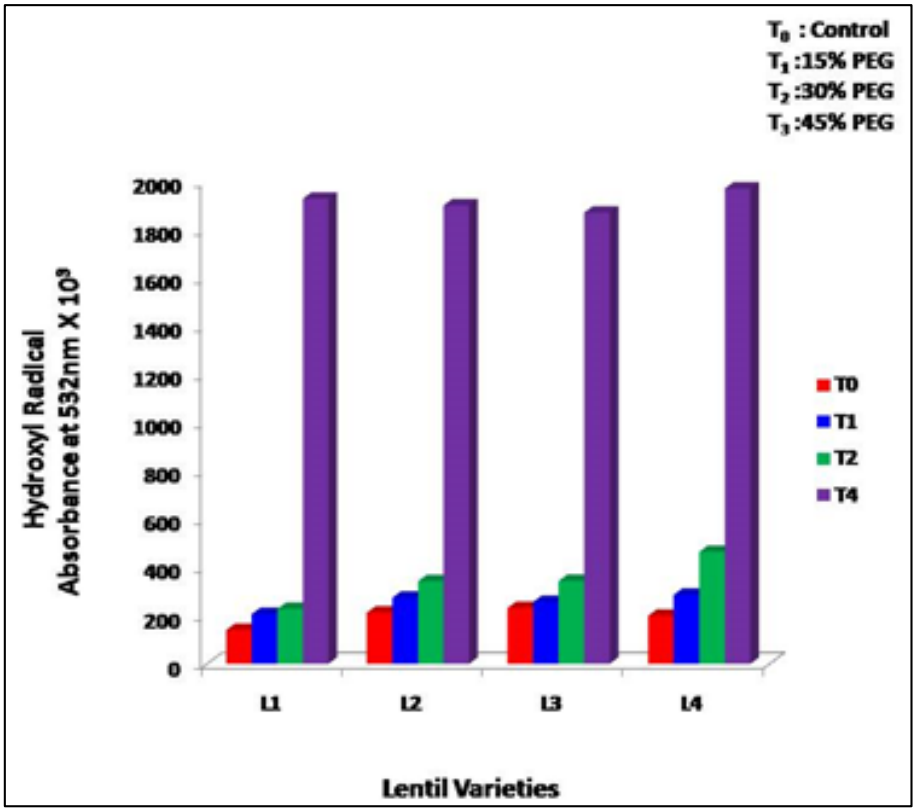

Fig 2: Effect of drought on production of hydroxyl free radical in different varieties of Lentil

3.3 Lipid peroxidation as affected by water deficit

When the level of lipid peroxidation products was measured in terms of TBARS, it was observed that water deficit treatments caused increase in the level of TBARS in all the varieties of lentil with highest increase in L_4 and lowest increase was observed in L_2 (Fig.3.). However, variety L_1 was found to at par with L_4.

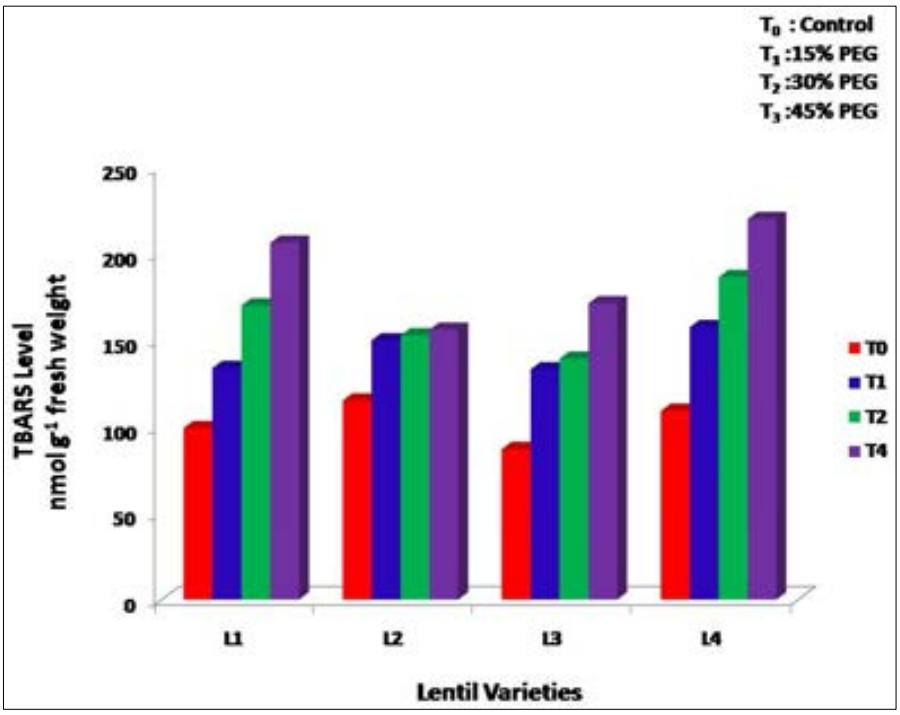

Fig 3: Effect of drought on peroxidation of lipids in different varieties of Lentil

3.4 Superoxide dismutase activity under water stress

Superoxide dismutase activity was assayed in the different varieties of lentil. All the varieties exhibited an increase in superoxide dismutase activity (Fig 4). The maximum increase was observed in L_1 (34.86%) followed by L_2 (33.92%), L_4 (28.49%) and L_3 (10.19%) (Fig4).

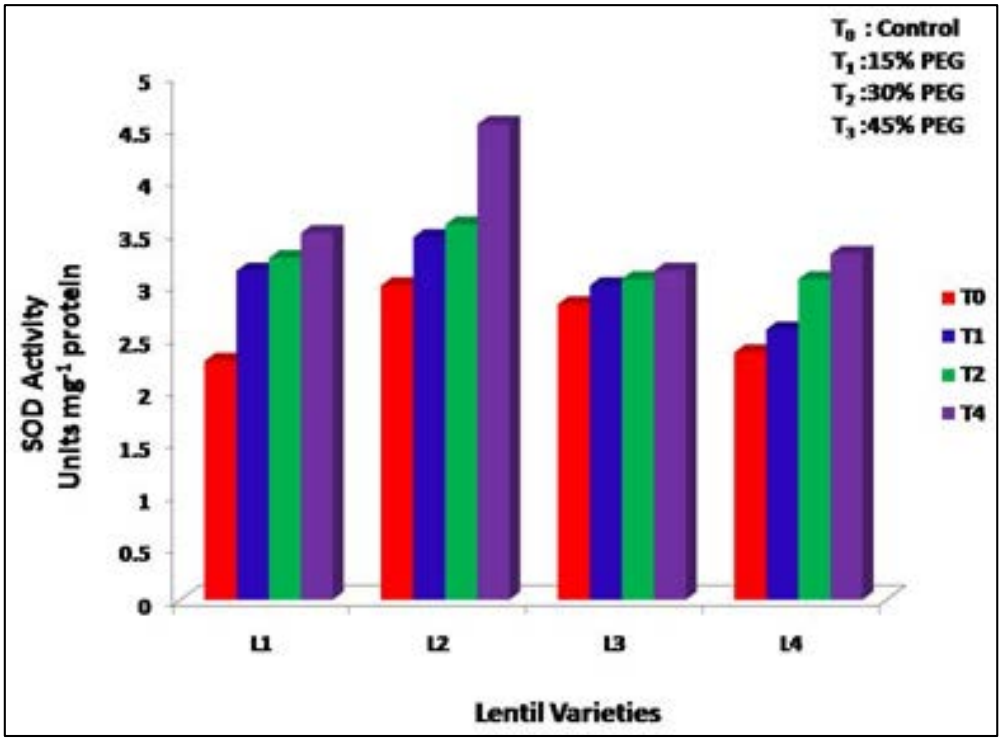

Fig 4: Effect of drought on superoxide dismutase activity in different varieties of Lentil

3.5 Catalase activity under water deficit

Catalase activity was assayed in the different varieties of lentil. All the varieties exhibited an increase in catalase activity (Fig 5). The maximum increase was observed in L_3 followed by L_4, L_1 and L_2 (Fig 5).

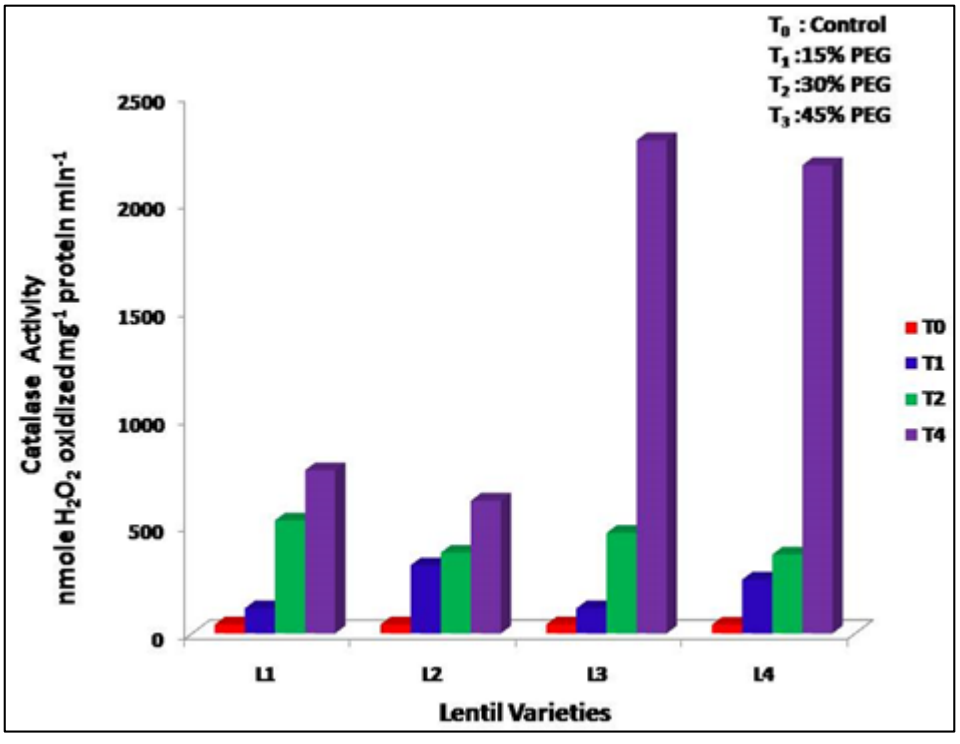

Fig 5: Effect of drought on Catalase activity in different varieties of Lentil

3.6 Guaiacol Peroxidase levels:

Guaiacol peroxidase activity was assayed in the different varieties of lentil. All the varieties exhibited an increase in Guaiacol peroxidase activity (Fig 6). The maximum increase was observed in L_3 followed by L_1, L_4 and L_2 (Fig 6).

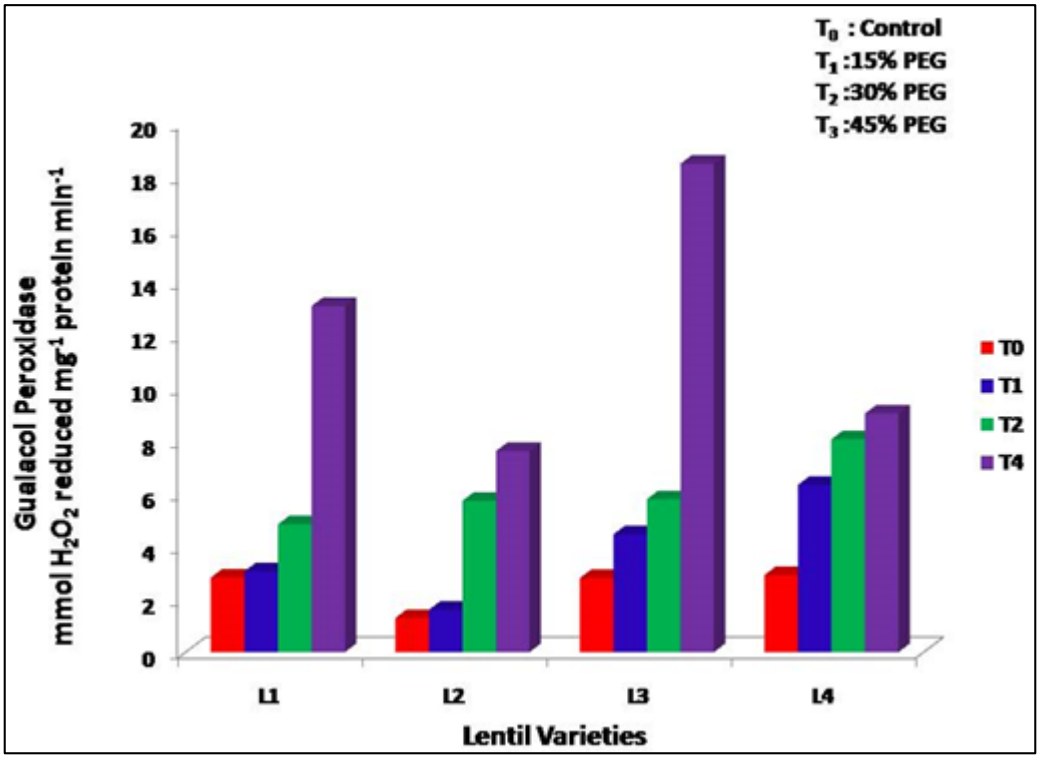

Fig 6: Effect of drought on Guaiacol Peroxidase activity in different varieties of Lentil

4. Discussion

Water deficits are one of the main abiotic stresses that limit worldwide crop production, especially in tropical regions. There are lots of evidences that point out oxidative stress as a major factor causing damage to plants under abiotic stress conditions such as metal toxicity and water deficit (Sgherri *et al.* 1996; Sharma *et al.* 2012) [38, 39]. Research has shown that drought-tolerant plants possess higher antioxidant capacities to protect themselves from oxidative damage caused by stressful conditions (Sharma *et al*., 2012; Mishra *et al*., 2013) [39, 30].

Four varieties of lentils were studied in order to assess their ability to withstand water deficit as well as to identify the sensitive and tolerant varieties of lentils based on ROS production, lipid peroxidation, and antioxidant capacity. Water deficit was induced in the present experiments in sand cultures by using polyethylene glycol-6000 (PEG-6000) in the growth medium. PEG-6000 has the advantage of minimal penetration into the apoplastic and symplastic spaces because of its high molecular weight and size (Hohl and Shopfer 1991) [21], and consequently mimics plants' responses to water deficit under field conditions (Couper and Eley 1984). Most stressful environmental conditions trigger an overproduction of ROS, such as O_2^-, H_2O_2, and HO. in plant cells.

Peroxidation of membrane lipids, oxidation of proteins and fragmentation of nucleic acids can cause serious damage to vital organelles such as chloroplasts, mitochondria and plasma membranes (Gill and Tuteja 2010) [16]. Based on our results, we found higher levels of ROS, H_2O_2, and OH generation as well as coupled with high lipid peroxidation in the seedlings because of water deficit. Compared to control under water deficit, lentil seedlings developed higher levels of lipid peroxidation, which may result in increased electrolytic leakage and more oxidative damage to biomolecules in the plant, ultimately resulting in reduced growth. The highest increase in lipid peroxidation was observed in L_4 and lowest increase was observed in L_2 (Fig.3.). However, variety L_1 was found to at par with L_4.

An intricate system of antioxidative enzymes scavenges reactive oxygen species enzymatically (Apel and Hirt, 2004) [1]. The SOD enzyme family plays an important role in protecting cells from oxidative damage by dismutating O2.- to H_2O_2 and O2. SOD activity is used to monitor changes in O2 generation (Wang *et al.* 2005) [42]. Plant cells under stressed conditions have been shown to increase SOD activity including Cu/Zn SOD, Fe-SOD and Mn SOD. This may be due to the cells' response to oxidative stress in the cytosol, mitochondria, and chloroplasts (Ushimaru *et al.* 1995) [41]. The ROS scavenging system begins with this reaction. In all varieties of lentils, we observed an increase in the activity of superoxide dismutase (Fig. 4). This enzyme might be involved in scavenging increasingly produced oxygen in lentils. A maximum increase of 34.86% in L_1 was observed and a minimum increase of 10.19% in L_3. This indicated that L_1 was more resistant to ROS damage.

H_2O_2 accumulates in the tissues and therefore has to be eliminated through catalase and guaiacol peroxidase-mediated reactions. In our result all the varieties exhibited an increase in catalase activity (Fig 5). The maximum increase was observed in L_3 followed by L_4, L_1 and L_2 (Fig 5). It seems that lentil seedlings are more tolerant to oxidative stress because of increased catalase activity. As an enzyme that decomposes H_2O_2 into water and oxygen, catalase is considered to be an important player in the cellular defense strategy against oxidative stress. Due to the high turnover rate of its enzymes, catalase is crucial for oxidative stress tolerance. The light may be absorbed by the heme group or perhaps inactivated by H_2O_2 (Sharma *et al.* 2012) [39]. Plants subjected to salts and heavy metal toxicity showed increased catalase activity, preventing H_2O_2 formation (Hsu and Kao 2004, Kim *et al.* 2005) [22, 25].

In stressed plants, peroxidase activity increases, resulting

from oxidative reactions in cells caused by an increase in free radicals and peroxides (Radotic *et al*. 2000) [35]. Abiotic and biotic stress can be identified by the activity of peroxidases scavenging H_2O_2 in plants (Castillo *et al*. 1992) [9]. Several environmental stresses have been reported to increase guaiacol peroxidase activity, including drought, salinity, and metal toxicity (Mishra *et al*. 2011; Sharma *et al*. 2012) [31, 39]. All varieties in our experiment showed a significant increase in guaiacol peroxidase activity (Figure 6). L_3 showed the greatest increase, followed by L_1, L_4 and L_2 (Fig 6). In lentil plants exposed to water deficit conditions, GPX plays a crucial role in scavenging H_2O_2.

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