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Moisture stress induced changes in root anatomy and antioxidants level in maize (*Zea mays* L.)

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Abstract
Maize is a drought sensitive crop whose growth and yield is seriously affected by this stress. Development of drought tolerant genotypes is the need of the hour and the only alternative to increase maize yield. In the present research, some biochemical and anatomical indices have been identified which can be used to screen the tolerant genotypes from a large population pool. The results obtained from the present study revealed that tolerant genotypes (LM 16, CM 140) as well as check (PMH 2) showed higher carotenoids, chlorophylls, proline and activities of SOD and POX enzymes, whereas, accumulation of H_2O_2 was lower than sensitive genotypes (LM 13, LM 20). Some morphological and anatomical changes were also induced in roots due to moisture stress. The number of metaxylem elements increased in both sensitive and tolerant genotypes. Diameter of metaxylem was reduced in tolerant genotype (CM 140) while number of root hairs increased in tolerant genotype (LM 16) with drought stress. Consequently, all these modifications in the root anatomy, enzyme activities and biochemical parameters could provide a useful tool to breeders for identification of moisture tolerant genotypes.

Keywords: Antioxidant enzymes, drought, maize, metaxylem and osmolyte

Introduction
Maize is the third most important cereal crop after wheat and rice and is known as corn which literarily means "that which sustains life" (Akinyele and Adigun, 2006) [1]. In Punjab, it was cultivated in 114 thousand hectares area, with production of 423 thousand tones and average yield of 37.08 quintal per hectare (Anonymous 2018) [2]. Abiotic stresses are the major causes for reduction of maize yield, globally. Among all the abiotic stresses, drought stress is the major limiting factor that affects global maize production. Drought causes root shrinkage, change in root anatomy, reduce photosynthetic pigments, loss of membrane integrity which leads to reduce yield (Praba *et al*., 2009) [3]. Continuous stress conditions lead to disturbances in plant metabolism and cause oxidative injuries leading to the production of reactive oxygen species (ROS) (Zhang *et al*., 2018) [4]. ROS are highly reactive and cause serious plant damage by affecting many cellular reactions like increasing lipid peroxidation, protein degradation, DNA fragmentation, membrane instability and enzyme inactivation (Zlatev and Lidon, 2012) [5]. Whether, ROS will act as damaging, protective or signaling factors, depends on the equilibrium between ROS production and their scavenging at the proper site and time (Mittler and Blumwald, 2010) [6]. Reactive oxygen radicals are scavenged by the antioxidant molecules or lipid soluble or water soluble scavenging molecules. Plants have many enzymatic antioxidants *viz.*, superoxide dismutase (SOD), Peroxidase (POX), Catalase (CAT) and non-enzymatic antioxidants such as α-tocopherol, carotenoids and flavonoids to avoid drought stress (Gill *et al*., 2011) [7]. Carotenoids have been reported to act as non-enzymatic scavengers of ROS under stress conditions (Jung *et al*., 2001) [8]. Non-enzymatic antioxidants help to maintain the integrity of the photosynthetic membranes under oxidative stress. Enzymatic antioxidants showed higher activity in drought tolerant genotypes of maize (Chugh *et al*., 2013) [9]. Plants also try to overcome drought stress by accumulating different types of organic solutes such as glycine betaine, proline, glutamate, sorbitol, mannitol, oligosaccharides, fructans, trehalose, sucrose, carnitine and inorganic ions like K^+ ions (Ashraf *et al*., 2011) [10]. These solutes lead to osmotic stability and protect the membrane as well as macromolecules.
As maize is a drought susceptible crop, it requires more amount of water after rice and sugarcane (Byakod, 2013) [11]. However, water stress is already a critical problem in many parts of the world and is expected to become more severe in the future. Thus, research efforts are required to develop tolerant varieties and there is a need to select drought tolerant genotypes. Antioxidant levels and changes in anatomy of rootsunder drought stress can be taken as indices for selection of tolerant genotypes.

These indices may help the breeders to find out tolerant genotypes from a large pool of population. Therefore, present research was planned to find out the antioxidants and anatomical changes in available tolerant and sensitive genotypes of maize.

Materials & methods

Maize genotypes, two drought tolerant (CM 140 and LM 16), two sensitive (LM 13 and LM 20) and one check (PMH2) were procured from the Department of Plant Breeding and Genetics, PAU, Ludhiana. Maize seeds were sowed in polybags filled with 3kg soil. Polybags were arranged in completely randomized design and were replicated three times. Seeds were allowed to germinate for seven days and thereafter drought treatments were given at three days interval. Four treatments were control plants with normal moisture (100% of field capacity), withholding 60% irrigation level, 50% irrigation level and 40% irrigation level. Polybags were kept in rain shelter with day length of 10-12h. After 3 days of stress, root and stem samples were collected for biochemical and anatomical studies (roots).

Determination of Photosynthetic Contents

Photosynthetic contents *viz*., chlorophyll (Chl a, Chl b and total Chl) and carotenoids were determined by Arnon (1949)[12] method. 0.1g of leaves pelleted in 5ml of 80% acetone and centrifuged at 3000 rpm for 5 minutes and absorbance of the supernatant was recorded at 645, 665 and 480 nm for determination of photosynthetic contents. Acetone (80%) was used as blank. Photosynthetic contents were expressed in mg g^{-1} FW.

Proline content determination

0.1 g of root/shoot samples were homogenized in Methanol: Chloroform: water (12:5:1) reagent, followed by centrifugation and collected the upper layer. Proline content was determined by Bates (1973) [13] method. Ninhydrin reagent was added to extracted solution followed by benzene addition. Absorbance of upper layer obtained after benzene addition was recorded at 515nm.

Determination of Enzymatic Antioxidants

Fresh shoot/root samples were used to determine the antioxidants activity. Fresh 0.1 g shoot/root samples were homogenized in 50mM sodium phosphate buffer (pH 7.5) with pre-chilled pestle and mortar. The mixture was centrifuged and supernatant was used to determine the activity of peroxidase (POD; EC 1.11.1.7) and catalase (CAT; EC 1.11.1.6) enzymes. To determine the activity of superoxide dismutase (SOD; EC1.15.1.1) enzyme, the shoot/root samples were homogenized with 0.1M potassium phosphate buffer (pH 7.5) followed by centrifugation and collected supernatant. Peroxidase activity was determined following the method of Chance and Maehly (1954)[14]. Change in absorbance of mixture contained 0.05 M guaicol, 0.1 M sodium phosphate buffer (pH 6.5), 0.1 ml of enzyme extract and 0.8M H_2O_2 was recorded at 470nm for determination of POD activity. Activity of CAT was estimated by following the method Dhindsa and Matowe (1981) [15] method. 0.2 ml of enzyme extract was mixed with 50 mM sodium phosphate buffer (pH 7.5) and 30mM H2O2 and absorbance at 240 nm was measured. Marklund and Marklund (1974) [16] method was used to estimate the activity of SOD enzyme. 0.1 ml of enzyme extract was mixed with 0.1 M Tris HCl buffer (pH 8.2), 6 mM EDTA and 6 mM pyrogallol solution and change in absorbance was recorded at 420 nm. The change in activity of enzymes min^{-1} g^{-1} FW was noted.

Hydrogen Peroxide content determination

Hydrogen Peroxide (H_2O_2) content was determined by the method of Velikova *et al.* (2000) [17]. 0.1 g of fresh shoot/root samples were homogenized with 2ml TCA (0.1%) and centrifuged at 12000 rpm for 15 mins. Absorbance of mixture contained 0.5 ml of supernatant, 10mM phosphate buffer (pH 7.0) and 1M potassium iodide was observed at 390nm. H_2O_2 content was expressed in µmoles g^{-1} FW.

Anatomical changes in root

Fresh root samples after 3 days of stress were collected from all treatments. Transverse sections of root were hand cut with the help of blade for microscopic observations. Each section was stained with dilute safranin dye, placed on different slide in a drop of glycerin, covered with a cover slip. Three slides were prepared for each treatment and observed under a Leica Bright Field Research microscope coupled with digital camera and computer imaging systems using software NIS Elements F 3.0 at 4X objective. Change in root hairs, number and size of vascular tissues were observed in the root sections.

Statistically analysis: The data was statistically analysed using CPCS1 soft-ware to calculate CD at 5% level of significance.

Results

Photosynthetic pigments

Moisture stress reduced the chlorophyll content in all maize genotypes including check (PMH 2) (Table 1). Reduction in Chlorophyll content was more in sensitive genotypes (LM 20and LM 13) than tolerant genotypes. LM 20 showed 55.61 % reduction than CM 140 at 60% stress level. Under control and stress conditions maximum chlorophyll degradation was observed in LM 20 than all other maize genotypes. However, maximum chlorophyll was observed in leaves of genotype CM 140 followed by LM 16, LM 13 and LM 20 at 60% level of stress. Chlorophyll b content was observed to be maximum in genotype CM 140 which was at par with PMH 2 and LM 20 had minimum chlorophyll b content. The drought tolerant genotype CM140 showed maximum total chlorophyll content (1.87 mg g^{-1} FW), while the

Table 1: Effect of moisture stress on total chlorophyll content (mg g^{-1} FW) in maize genotypes

Maize lines	Control			Different levels of stress									Mean		
				40%			50%			60%					
	Chl a	Chl b	Total chl	Chl a	Chl b	Total chl	Chl a	Chl b	Total chl	Chl a	Chl b	Total chl	Chl a	Chl b	Total chl
CM140	1.90	0.97	2.90	1.81	0.84	2.67	1.68	0.77	2.50	1.50	0.36	1.87	1.72	0.74	2.49
LM 16	1.87	0.92	2.81	1.61	0.79	2.42	1.54	0.70	2.27	1.34	0.30	1.65	1.59	0.68	2.29
LM 13	1.07	0.66	1.75	0.78	0.61	1.39	0.71	0.48	1.19	0.64	0.27	0.92	0.80	0.51	1.31
LM 20	0.97	0.68	1.67	0.76	0.55	1.31	0.66	0.46	1.12	0.58	0.25	0.83	0.74	0.49	1.23
PMH2	1.82	0.75	2.59	1.70	0.64	2.35	1.54	0.51	2.05	1.14	0.36	1.54	1.55	0.57	2.13

Mean	1.53	0.80	2.34	1.33	0.69	2.03	1.23	0.58	1.83	1.04	0.31	1.36			
CD (p=0.05)	Chl a = A= 0.014, B = 0.016, AB = 0.031 Chl b = A= 0.010, B = 0.011, AB = 0.022 Total Chl = A= 0.013, B = 0.014, AB = 0.028 A= Treatments, B = Genotypes, AB = (Treatments x Genotypes)														

drought sensitive genotype LM 20 was recorded with minimum total chlorophyll content(0.83 mg g^{-1} FW) at 60% stress level.

Carotenoid content showed increasing trend with increase in moisture stress level in all the genotypes (Table 2). At 60% stress level, maximum carotenoid content was recorded in LM 16 and it was 11.76 % and 7.84% more as compared to check PMH2 and CM 140, respectively. Under control conditions, LM 16 had more carotenoid content followed by PMH 2, CM 140, LM 20 and LM 13.Least mean carotenoid content was noticed in LM 13 genotype and maximum in LM 16 followed by CM 140 which was at par with check PMH 2, LM 20 and LM 13.

Table 2: Effect of moisture stress on carotenoid content (mg g^{-1}FW) in maize genotypes

Maize lines	Control	Different levels of stress			Mean
		40%	50%	60%	
CM140	0.40	0.45	0.48	0.55	0.47
LM 16	0.43	0.47	0.50	0.57	0.49
LM 13	0.26	0.31	0.32	0.35	0.31
LM 20	0.30	0.35	0.37	0.38	0.35
PMH 2	0.42	0.47	0.49	0.51	0.47
Mean	0.36	0.41	0.43	0.47	
CD (p = 0.05)	A = 0.012, B = 0.013, AB = 0.027 A= Treatments, B = Genotypes, AB = (Treatments x Genotypes)				

Proline content

Proline content increased continuously in maize root and shoot due to moisture stress (Fig. 1 and 2). Under control conditions, check PMH 2 had highest proline content in roots and minimum in LM 13. However, at 60% stress level, maximum proline content was recorded in roots of CM 140and it was 42.85 % more as compared to PMH 2. Minimum proline content was observed in roots of LM 20 (drought sensitive genotypes).In shoots, under control conditions maximum proline content was recorded in LM 16 followed by PMH 2, CM 140 and least proline content value in LM 13. While, check PMH 2 had more proline content and decrease in proline content was 21.70 % and 41.86 % as observed in shoots of CM 140 and LM 16 genotypes, respectively. Minimum proline content was observed in LM 13 at 60% level of stress.

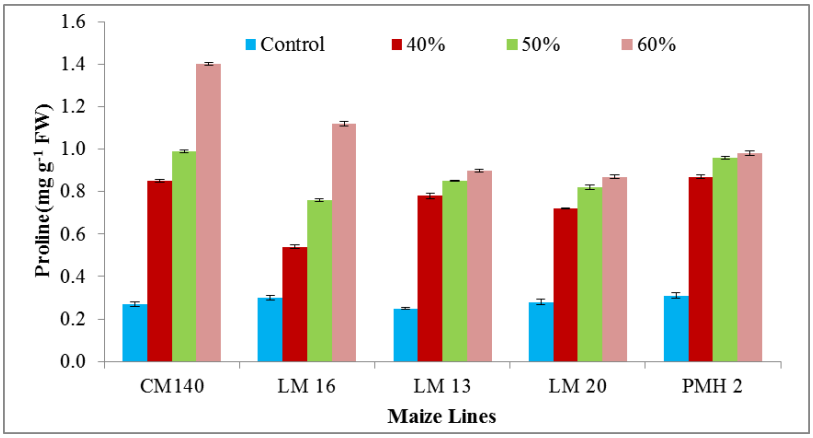

Fig 1: Effect of moisture stress on proline content in roots of maize genotypes

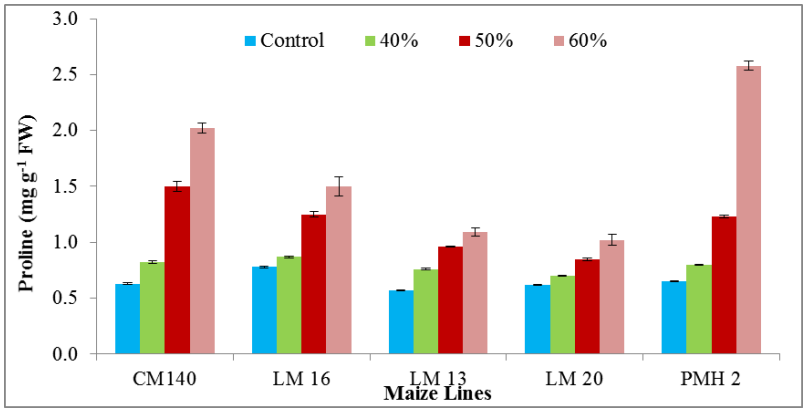

Fig 2: Effect of moisture stress on proline content in shoots of maize genotypes Enzymatic Antioxidants

Enzymatic Antioxidants

Water stress increased the activity of antioxidant enzyme with increase in stress level in both roots and shoots of all the maize genotypes (Fig. 3 and 4). Maximum activity of superoxide dismutase was observed in PMH 2 under control as well as stress conditions. While the least SOD activity was noticed in roots and shoots of genotype LM 20 at 60% stress level.

A clear difference in peroxidase activity was observed under control and at different levels of stress in maize genotypes (Fig. 5 and 6). Peroxidase activity increased under stress conditions. Under control conditions, maximum enzyme activity was found in the roots and shoots of CM 140. Both roots and shoots of CM 140 showed 36.58 % and 16.28 % increase in the enzyme activity than that of PMH 2 during 60% level of stress conditions. However, lowest peroxidase activity was recorded in roots and shoots of LM 20.

Effect of moisture stress on catalase activity varied between roots and shoots of maize genotypes (Fig. 7 and 8). Its activity decreased with increase in the stress levels in both roots and shoots of all the genotypes as well as check PMH 2. Under control conditions, the maximum activity of catalase was 16.75% and 2.12 % more in roots and of CM 140 and LM 16 as compared to check PMH 2. In shoots, enzyme activity was 17.49 % and 5.47% more in shoots of LM 16 and CM 140 as compare to PMH 2.

Hydrogen peroxidase content

Moisture stress enhanced the accumulation of H_2O_2 content in roots and shoots of maize genotypes subjected to different levels of moisture stress (Fig 9 and 10). Under non-stressed condition and at maximum stress level (60%), roots and shoots of sensitive genotypes had more H_2O_2 content as compared to tolerant genotypes. However, least H_2O_2 was recorded content in roots of genotypes LM 16 and in shoots of check PMH 2 at 60% level of stress.

Anatomical changes in roots

Differences in root anatomy were observed in the different maize genotypes under control and stress conditions (Plate 1). Firstly, more density of metaxylem under stress conditions as compared to control in all the maize genotypes as well as in check PMH 2 was noticed which helped to increase the water transport capacity. Secondly, under stress conditions, number of root hairs was increased only in LM 16 genotype and PMH 2 (check). Thirdly, in CM 140 the diameter of metaxylem elements was reduced under stress.

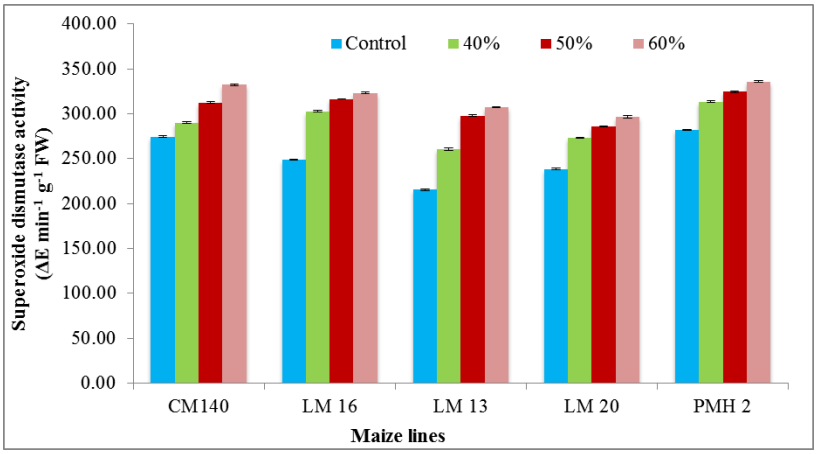

Fig 3: Effect of moisture stress on superoxide dismutase activity in root of maize genotypes

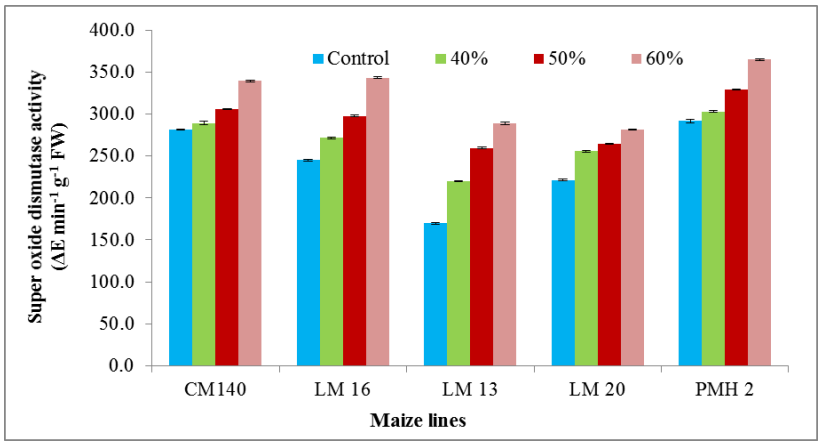

Fig 4: Effect of moisture stress on superoxide dismutase activity in shoots of maize genotypes

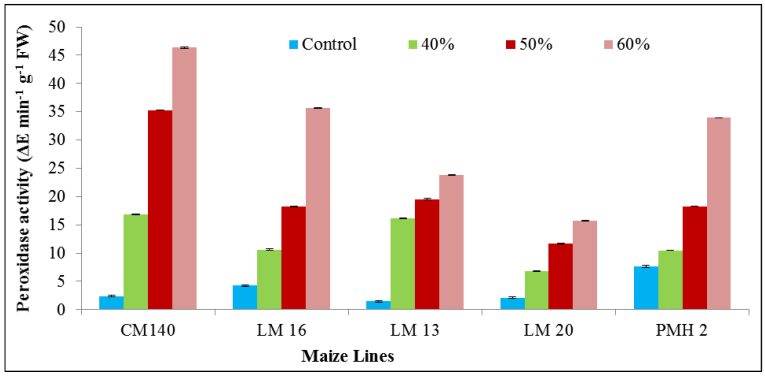

Fig 5: Effect of moisture stress on peroxidase activity in roots of maize genotypes

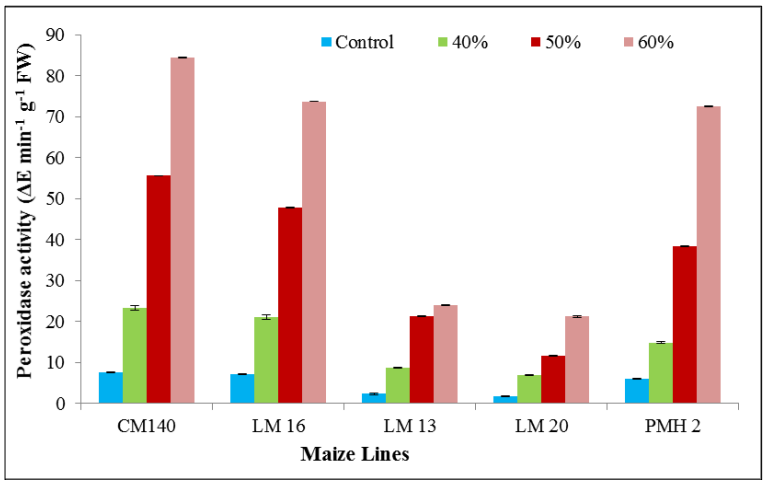

Fig 6: Effect of moisture stress on peroxidase activity in shoot of maize genotypes

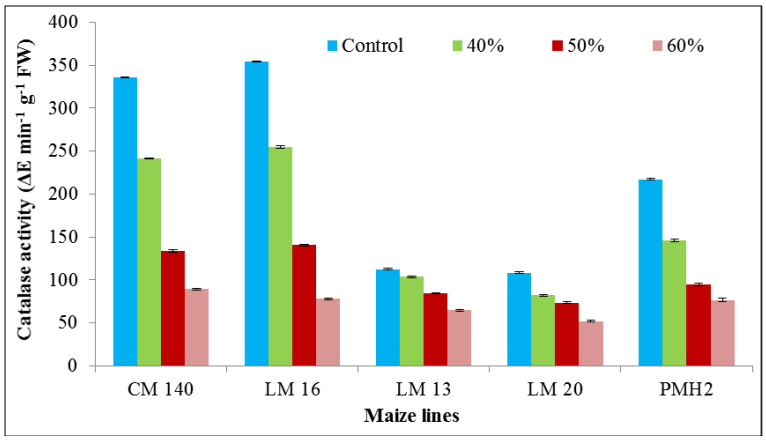

Fig 7: Effect of moisture stress on catalase activity in root of maize genotypes

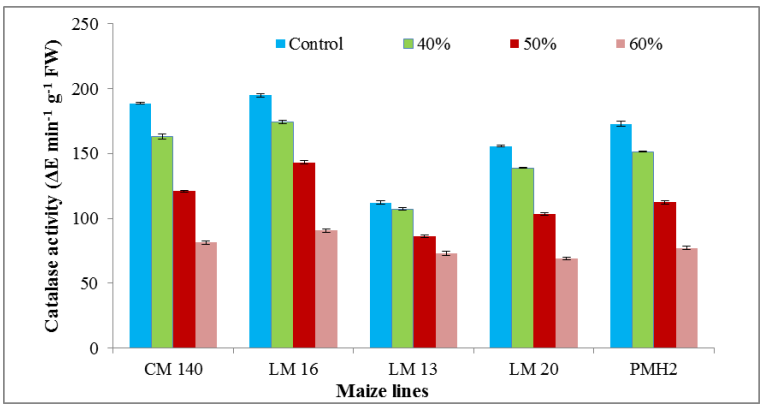

Fig 8: Effect of moisture stress on catalase activity in shoots of maize genotypes

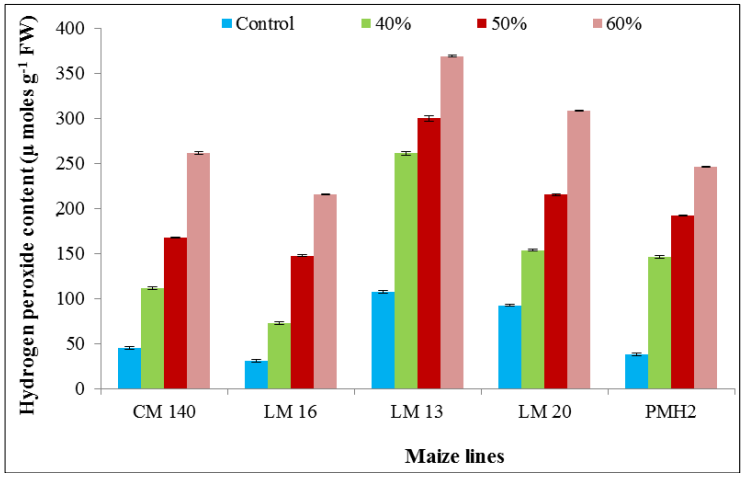

Fig 9: Effect of moisture stress on hydrogen peroxide content in root of maize genotypes

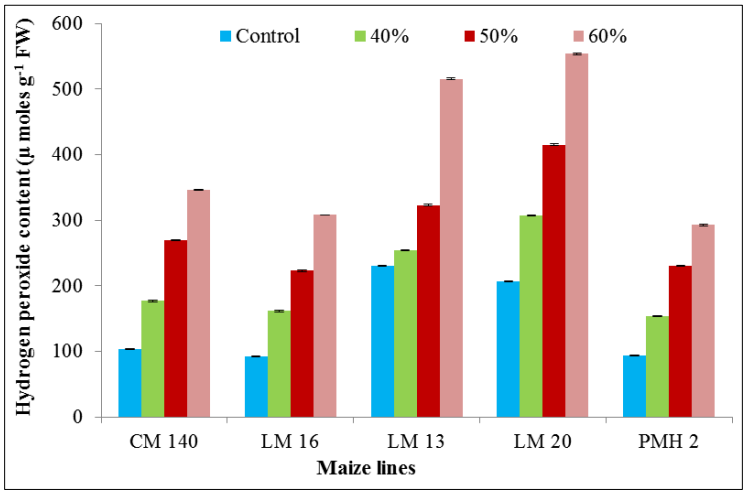

Fig 10: Effect of moisture stress on hydrogen peroxide content in shoots maize genotypes

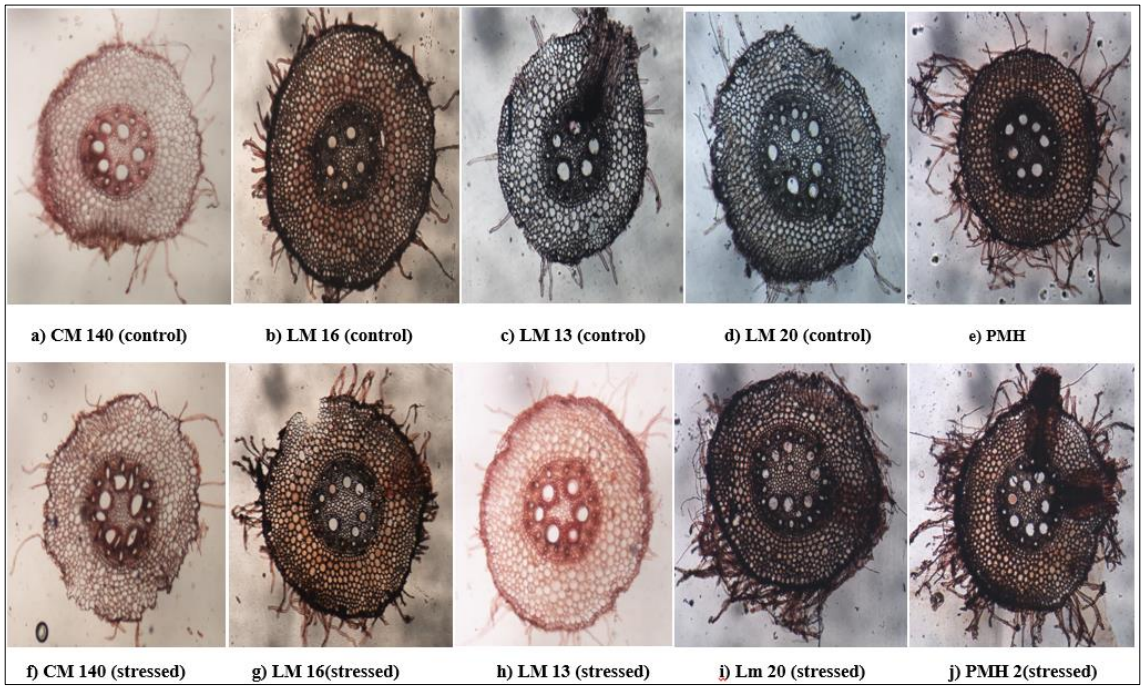

Plate 1: Variations in root anatomy of maize genotypes under control and stress conditions

Discussion

Our analysis of different biochemical parameters revealed a variation between maize genotypes and also among levels of stress. We found that different levels of stress had negative impact on the chlorophyll a, chl b and total chl content and it decreased with an increment in the levels of stress. Our results are concurrent with the findings of Morshedloo (Morshedloo *et al*., 2017) [18] in oregano. In addition to chlorophyll

pigments, plants have carotenoids which act as accessory pigments for photosynthesis. They also have a role in photo protection since they are very efficient physical and chemical quenchers of $^1O_2$ and potent scavengers of other free radicals (Cazzonelli, 2011) [19]. In our study, moisture stress led to an increase in the carotenoid content in leaves of maize as found by various researchers in sunflower (Ghobadi *et al*., 2013) [20] and eggplant (Mibei *et al*., 2017) [21].

To avoid water loss under stress, plants accumulate compatible solutes such as proline, sugars, glycine-betaine etc. These osmolytes stabilize and protect the structure of proteins and enzymes, maintain membrane integrity and scavenge ROS. We also observed rising level of osmolyte proline during drought stress in maize. Similar result was also obtained in *Foeniculum vulgare* under drought stress (Poudineh *et al*., 2018) [22].

In plants, H_2O_2 plays dual role, at low concentration it acts as signaling molecules and at high level, it acts as a toxic ROS molecule causing cell injuries or cell death (Niu and Liao, 2016) [23]. Increased accumulation of H_2O_2 content during water stress in our study is concurrent with the findings in wheat (Dong *et al*., 2018) [24] and chilli seedlings (Sahitya *et al*., 2018) [25].

Enzymatic antioxidants have a great role in ROS scavenging. Superoxide dismutase catalyzes the dismutation of O^{2-} to H_2O_2 and O_2. However, enhanced SOD enzyme activity may be due to high concentration of O_2 (Xie *et al*., 2018) [26] and it increased more in tolerant genotypes as compared to sensitive ones in sorghum (Guo *et al*., 2018) [27] and in chilli (Sahitya *et al*., 2018) [25]. Peroxidase further takes part in decomposition of H_2O_2 into water and oxygen. Changes in peroxidase activity have been frequently correlated to the response of resistance or susceptibility of plants to stresses (Zoz *et al*., 2013) [28]. Catalase also decomposes H_2O_2 into water and oxygen but at different cellular locations. Catalase is heterogeneous in nature under drought stress. It might be increased and remain unchanged or decreased on exposure to water stress. Our results are in agreement with the earlier findings (Anjum *et al*., 2017) [29], (Xie *et al*., 2018) [26] where decrease in CAT activity due to water stress was observed in maize hybrids.

Anatomical changes

Environment plays an important role in modifying the anatomical features. Various changes occur in the root anatomy due to moisture stress. Regarding root anatomy, increase in the number of metaxylem elements was observed in all the maize genotypes under stress conditions as compared to control (Plate 1). Greater amount of metaxylem for drought tolerant corn genotypes suggested that these characteristics may be related to higher hydraulic conductivity, which increases the water transport capacity (Souza *et al*., 2016) [30]. Decrease in the diameter of metaxylem elements under stress was also observed in CM 140 (Plate 1. a). It could support the retention of water in plants (Souza *et al*., 2016) [30]. Transverse Section of root in LM 16, PMH 2 showed an increase in number of root hairs under stress conditions [Plate 1 (g, j)]. Root hairs provide a mechanism by which the plant root contact with the soil can be maximized. Since root hairs are meant for absorption of water. So, increase in their number might have helped in extracting more water by the plants under stressful environment.

Conclusion

High carotenoid, proline content and activities of enzymes like SOD, POX and lesser accumulation of H_2O_2 content in tolerant maize genotypes helped to ameliorate the adverse effects of moisture stress. Changes in root anatomy like increase in number of metaxylem elements, reduced diameter of metaxylem (in CM 140) and increase in root hair density (in LM 16 and check PMH 2) are also important to provide stress tolerance. These biochemical and anatomical changes may act as stress indices and help the breeders to screen the tolerant lines from a large population.

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