academicJournals

Vol. 13(3), pp. 434-440, 15 January, 2014
DOI: 10.5897/AJB2013.13196
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of fenugreek (*Trigonella foenum-graecum* L.) seedlings under moisture and heavy metal stress with special reference to antioxidant system

Nilima Karmakar[1]*, Arunabha Chakravarty[2], Pranab Kumar Bandopadhyay[2] and Prakash Kanti Das[3]

[1]Department of Soil Science and Agricultural Chemistry, N.M. College of Agriculture, Navsari Agricultural University, Navsari, Gujarat-396450, India.
[2]Faculty of Agriculture. Bidhan Chandra Krishi Viswavidyalaya Krishi Viswavidyalaya - 741252, Mohanpur, Nadia, West Bengal, India.
[3]Department of Genetics and Plant breeding. Institute of Agricultural Science. Calcutta University, 35-Ballygunj Road, Kolkata-19, West Bengal, India.

Accepted 23 December, 2013

In the present investigation, the impact of drought and heavy metal in fenugreek was critically monitored. Fenugreek seedlings were exposed to 1- bar polyethylene glycol (PEG) solution (osmotic stress) and 10 ppm solution of $HgCl_2$ (heavy metal). Within 3 days of seedling growth, mercury exposure induced relatively high level of adverse impact of root growth and biomass production where cell division was significantly reduced measured by mitotic index and development of root vascular bundle was affected indicating negative impact on nutrient transport chain. Mercury stress showed a five-fold increase in malonaldehyde (MAD) production along with antioxidant enzyme peroxidase (POD) activity and mercury stress showed simultaneous and lower antioxidant activity compared to osmotic stress increased activity in super oxide dismutase (SOD) and catalase (CAT) indicates that mercury pollution leads to serious damage in cell membrane through accumulation of lipid peroxides and POD activity to quench it whereas osmotic stress is not so serious and adopt the mechanism to quench the superoxides by SOD and followed by the detoxification of H_2O_2 by catalase and formation of lower level of MAD.

Key words: Fenugreek, antioxidant, heavy metal, osmotic stress.

INTRODUCTION

The fenugreek is a high value but low volume crop with multipurpose applications. It is popularly used as spice and its medicinal value is also highly appreciated for diabetes and heart ailments (Suresh Kumar et al., 2005).

In the present work, seeds of fenugreek (*Trigonella foenum-graecum* L.) were exposed to drought induced by polyethylene glycol (PEG). Simultaneously they were also allowed to grow in presence of mercuric chloride ($HgCl_2$) commonly usable form of heavy metal mercury and thereby pollution due to heavy metal was being simulated. These two different kinds of abiotic stresses were induced for fenugreek seedlings at the early stage

*Corresponding author. E-mail: nilimanau13@nau.in.

Abbreviations: PEG, Polyethylene glycol; **ROS,** reactive oxygen species; **BIS,** Bereau of Indian Standards; **MAD,** malonaldehyde; **CAT,** catalase; **POD,** peroxidase; **SOD,** superoxide dismutase; **DPPH,** 1,1-diphenyl-2-picrylhydrzyl; **RSA,** radicals scavenging activity.

of growth. The environmental stresses like drought (Hamidi and Safarnejad, 2010) and heavy metals (Adams et al., 2013; Smiri et al., 2013) are known to cause oxidative damage to plants either directly or indirectly by triggering an increased level of production of reactive oxygen species (ROS) which include superoxide radical ($O^{\cdot-}$), hydroxyl radical ($OH^{\cdot-}$) and hydrogen peroxide (H_2O_2). They cause damage to the biomolecules such as membrane lipid, proteins, enzymes, nucleic acid among others (Mishra and Singhal, 1992). According to the Bereau of Indian Standards (BIS), permissible level of Hg in drinking water and soil is 0.001 mg/lit and 0.01 mg/kg, respectively. But most of the industrial places of India namely Vatva, Vapi and Ankleswar of Gujarat, Panipat of Haryana and patancheru of Andhra Pradesh are suffering for Hg contamination by the effluents of different chemical industry. Among the three Gujarat is a major fenugreek producing state in India. Hence in the present investigation, the impact of drought and heavy metal pollution in fenugreek has been critically monitored and compared in terms of morphophysiological and cytological and also for biochemical responses.

MATERIALS AND METHODS

Seed treatments

Disease free healthy seeds of fenugreek variety Rajasthan Methi (*T. foenum-graecum* L. Rmt-1) were obtained from Horticultural teaching Farm Jaguli, Bidhan Chandra Krishi Viswavidyalaya, Kalyani, West Bengal, India. Seeds (20 for each replication) arranged in a concentric manner in the petri plate (20 cm diameter) were imbibed with distilled water for 3 h and then treated with 1-bar solution of PEG and 10 ppm of $HgCl_2$. Being continuously exposed to the treatments, the germinated seeds were allowed to grow for 144 h. Simultaneously, seeds were germinated only in distilled water and grew for 144 h as controls. The whole set of experiment was conducted in biological oxygen demand (BOD) incubator at 20±1°C, 80% RH and repeated four times. During the entire period the chemical induced stress was maintained by intermittent application of chemicals with 10 ppm solution of $HgCl_2$ and 1- bar solution of PEG, respectively.

Seed germination and seedling growth

Percent germination (time) was recorded and seedling growth (at 144 h) were noted in terms of length (shoot and root) and weight (fresh and dry) following the method of Idrees et al. (2010).

Morphology and anatomy

Cross sections of hypocotyls were performed at 72 h of growth to pursue the deformation and root abnormalities at that point was also compared between treated and control plants.

Mitotic index

To study mitotic index (MI, %), slides were prepared from root tip tissue after 72 h of growth following the method of Sharma and Sharma (1994). Slides were observed under oil emersion lens of a Carl Zeiss Microscope (Germany). Mitotic index was recorded for control and treated to have a comparative account of induced moisture stress and heavy metal mercury exposure.

Lipid peroxidation assay

Following the extraction of plant sample in 20% trichloro acetic acid, malonaldehyde (MAD) concentration was estimated according to the method of Ohkawa et al. (1979) which would indicate the extent of lipid peroxidation under stress. MAD concentration was expressed as nmol gm^{-1} DW (Dry weight) of the seedling sample. MAD concentration was calculated using molar extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$ and was performed after 144 h of growth.

Antioxidative enzyme assay

Antioxidative enzymes namely, catalase (CAT), peroxidise (POD) and superoxide dismutase (SOD) were studied and was performed after 144 h of growth. CAT activity was assayed by the method of Beers and Sizer (1952). POD activity was determined by the method of Shannon (1966). SOD activity was assayed following the method of Beauchamp and Fridovich (1971). SOD activity was expressed as the amount of enzyme required to cause 50% inhibition under the experimental condition.

Total antioxidant activity

Total antioxidant activity of fenugreek seeds exposed to PEG and $HgCl_2$ was estimated and compared with the control on the basis of scavenging activity of the 1,1-diphenyl-2-picrylhydrzyl (DPPH) following the study of Braca et al. (2001) and the sample size that lowered the initial absorbance of DPPH solution by 50% was chosen for measuring the antioxidant activity. The percentage of the remaining DPPH radical (*%DPPHrem*) and antioxidant activity (*%AA*) were calculated for each concentration of the sample extract from the obtained absorbance for the sample (*As*) and the blank (*A0*) using the following equations:

$$\%DPPHrem = (As / A0) \cdot 100$$

$$\%AA = 100 - \%DPPHrem$$

The values of the calculated IC_{50} were expressed as µg of fenugreek sample in 1 ml extract that is needed for the scavenging of 50% of the DPPH radical present in the solution. The IC_{50} value is the concentration of antioxidant, required for 50% scavenging of DPPH radical in the specified time period.

Statistical analysis

All biochemical estimates were repeated four times and their mean values (±SE) were computed. Statistical analysis was performed using analysis of variance (ANOVA). Significant differences between values of enzymatic activity in control and stress plants were determined at $P \leq 0.05$ according to Duncun's multiple range test (Duncan, 1955).

Morphological data (root and shoot length and their fresh and dry weight) for each replication were recorded from ten seedlings. Mean values (±SE) based on four replications were computed. The statistical analysis was done as above.

Table 1. Morphological and Cytological parameter recorded after 144 h seedling growths in different treatments.

Treatment	Length (RL)	Shoot length (SL)	Fresh weight (FW)	Dry weight (DW)	Mitotic index (MI %)
Control	6.0500±0.17559[a]	7.2850± 0.16520[a]	0.2243±0.00947[a]	0.0128±0.00085[a]	46.8175±0.57946[a]
PEG	3.7250±0.11087[b]	4.4750±0.17970[b]	0.1100±0.00481[b]	0.0085±0.00029[b]	33.7600±0.78722[b]
Mercury	2.4750±0.15478[c]	2.9000±0.09129[c]	0.0658±0.00464[c]	0.0043±0.00025[c]	22.1025±0.35945[c]

Data represent mean of four replicates±SE. In each column mean values followed by different letters are significantly different at 0.05 level (DMRT).

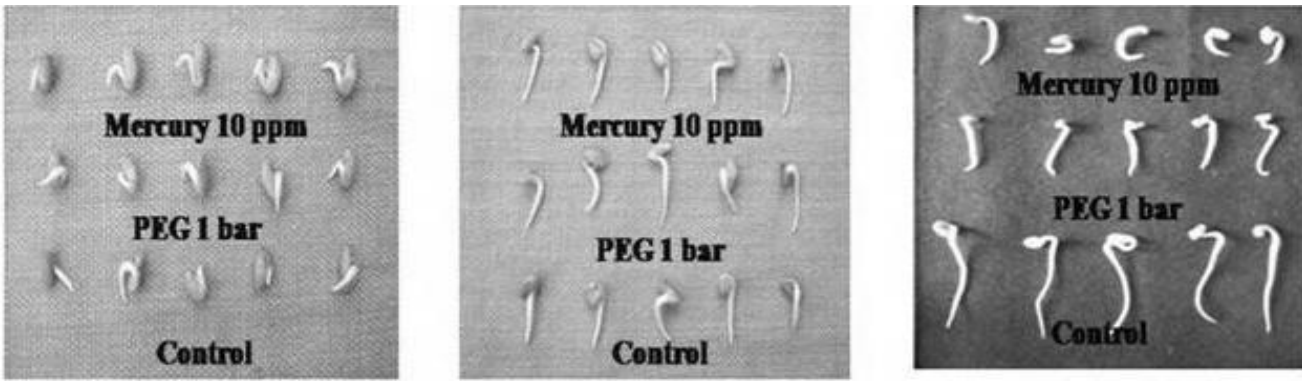

Figure 1. Fenugreek seeds under germination: A, Germination at 24 h, normal; B, germination at 48 h, normal; C, germination at 72 h, root abnormality under mercury and PEG treatment; root growth severely affected under mercury.

RESULTS

Seed germination and seedling growth

Following 4 h of imbibitions in water, seeds became metabolically active which triggered the process of germination and after 24 h, germination was close to 100% both in control and treated. The root, shoot length and the biomass produced in terms of fresh weight and dry weight of roots and shoots under PEG and $HgCl_2$ treatment along with the control are given in Table 1. At 144 h of exposure root and shoot length decreased both under PEG and mercury treatment. The effect of the heavy metal mercury on inhibition of root-shoot growth was found to be more pronounced. Consequently, fresh weight and dry weight of root and shoot was significantly depleted under mercury treatment. Thus biomass produced became almost half to that of PEG induced reduction (Table 1).

Morphology and anatomy

No physical deformities were found either at 24 or 48 h (Figure 1: Plate 1a and b). Significantly at 72 h, radical development was conspicuously different and suffered most in the mercury exposure (Figure 1: Plate 1c). However, after 144 h root abnormality became visibly pronounced. Roots were very thick and highly twisted and they were devoid of any secondary root initiation under both PEG and mercury (Figure 2: Plates 2a and b) where controls exhibited normal root growth (Figure 2: Plate 2c). It was also observed that highly thickened primary leaves remained closed in stress induced seedlings when seedlings without stress exhibited opened normal primary leaf.

The anatomy of hypocotyl region further revealed that the heavily bulged root hair showed either burst open tip or blunt club shaped tip under mercury exposer following 72 h (Figure 3a). In contrast the PEG exposed very lanky overgrown root hair (Figure 3b) when gradual root hair development was found under control (Figure 3c). A very conspicuous effect on vascular bundle development was recorded at 72 h stress exposer. Three rows of bundles observed more frequently under mercury stressed (Figure 4a), in case of PEG induced drought condition rows of vascular bundle were seen with reduced size (Figure 4b) instead of normal four rows of vascular bundles in control (Figure 4c).

Mitotic index

Mitotic index which indicates the frequency of the cells

Figure 2. Root and primary leaf after 144 h. A, Under mercury treatment severe root abnormality and primary leaf remained closed, no secondary root formation; B, under PEG swelling of root growth; C, under control normal root growth and primary leaves opened.

Table 2. Biochemical parameters recorded after 144 h seedling growths in different treatments.

Treatment	Lipid peroxidation (MAD) nmol gm^{-1} DW	Super oxide dismutase (SOD) A_{560} min^{-1} mg^{-1} protein	Catalase (CAT) µ mol H_2O_2 oxidised min^{-1} mg^{-1} protein	Peroxidase (POD) A_{430} min^{-1} mg^{-1} protein
Control	29.42 ± 2.70[c]	2.5075±0.065[c]	198.82 ± 7.19 [a]	2.66 ± 0.36[c]
PEG	86.44 ± 3.41[b]	4.4100±0.037[a]	104.43 ± 9.17 [b]	5.89 ± 0.76 [b]
Mercury	146.49±5.55[a]	3.6475±0.057[b]	51.56 ± 4.48[c]	10.43 ± 1.01 [a]

Data represent mean of four replicates±SE. In each column mean values followed by different letters are significantly different at 0.05 level (DMRT).

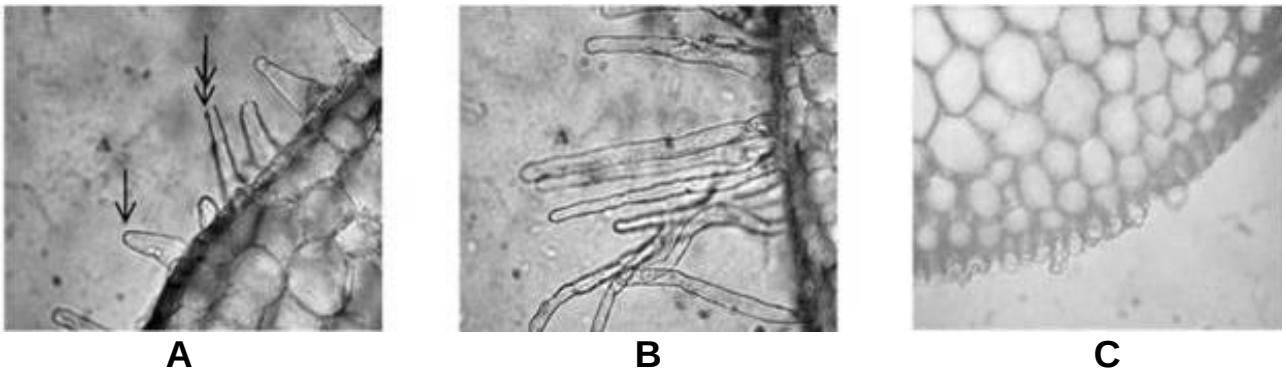

Figure 3. Root hairs under 72 h exposure. A, Under mercury club shaped root hair (→), and bursting root tip (↠); B, under PEG treatment root showing lanky over growth; C, under control normal root hair formation.

under division was computed and found to be significantly influenced by the two different kinds of stresses, confirmed by Duncun's multiple range test (DMRT) (Table 1). Mitotic index decreased and it was found minimum in mercury exposed seedlings which were reduced to more than half than that of control. In osmotic stress, a significant reduction of mitotic index has been found.

Lipid peroxidation

Very often, MAD levels have been utilized as suitable marker for membrane lipid peroxidation (Masia, 2003). The study revealed significant increase in levels of MAD following PEG and mercury treatments (Table 2). The MAD content under PEG treatment and mercury treatment was 86.44 and 146.49 nmol gm^{-1} which was

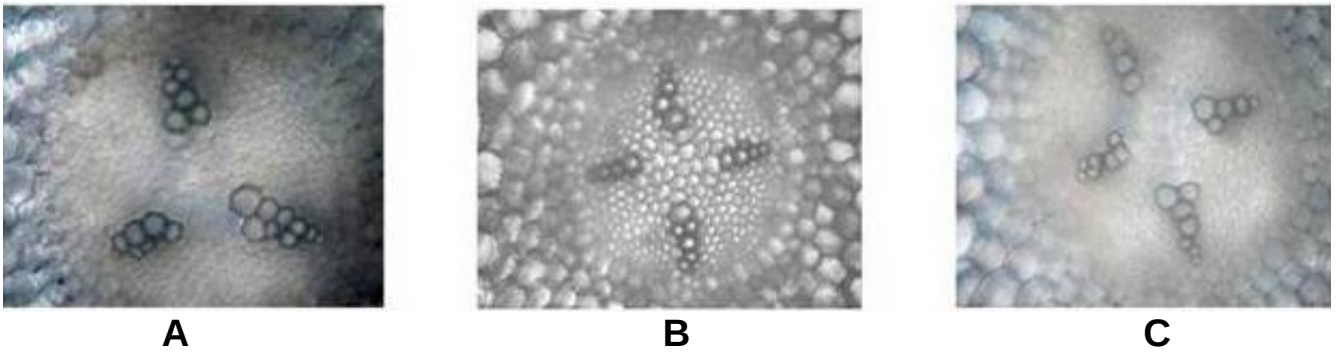

Figure 4. Root vascular bundle under 72 h. A, In mercury 3 rows of vascular bundles instead of 4; B, in PEG 4 rows of vascular bundle of reduced size; C, in control 4 rows of normal vascular bundles.

three and five fold higher than control plants, respectively. Consequently there was an enhancement in the free radical mediated lipid peroxidation under mercury toxicity.

Antioxidative enzyme

The present set of experiments in fenugreek demonstrates increased activity of the antioxidant enzymes like SOD and CAT activity is increased in PEG induced stress (Table 2). A drastic decrease in CAT activity and fivefold increase in POD activity in heavy metal induced stress were observed (Table 2). In both of the stresses, SOD and POD activity increased.

Total antioxidant activity

A significant decline of antioxidant activity was recorded under both PEG and Mercury treatments and activity was depressed significantly between the treatments confirmed by DMRT test. Under mercury treatment, fenugreek seedlings showed minimum level of antioxidant activity indicating a high degree of failure in free radicals scavenging activity (RSA) under mercury exposure. The IC_{50} value of DPPH radical scavenging in case of moisture stress is found to be 70 µg/ml, while it was above 100 µg/ml for the heavy metal mercury induced stress (Figure 5).

DISCUSSION

Perusal of the above result seemed that the concentration used for the PEG and mercury was not significantly high enough to affect the germination because the germination of seeds were 100% in each case but severe abnormality was found in seedlings of different time intervals. The inhibition of root growth can be attributed to the inhibition of mitosis (reduced MI%) and the depletion in biomass production due to the abnormality of vascular bundle formation revealed from root anatomy concerned with transport chain. There is a good ground to suggest that the root growth of fenugreek seedlings is more sensitive to the mercury. Similar trend is also reported in lentil by Kiran and Sahin (2006) in lentil with the heavy metal cadmium. The club shaped root hair formation under mercury stress and the lanky overgrowth of root hairs under osmotic stress suggest different levels of harmful effects under different stresses. However, once stress is set, stress tolerance responses preserve cell organelles and tissue structures (Kramer, 1983). The decreased mitotic index is attributed by the formation of aberrant cells and the result is very much similar with Patel and Patel (2013) in the same crop fenugreek with cadmium stress.

The increased activity of antioxidant enzymes such as SOD and POD are found to occur during stress (Shah et al., 2001; Diaz et al., 2005), to resist oxidative damage leading to adaptation and survival of plants during periods of stress (Malecka et al., 2001). Contrary to this, SOD activity became significantly more under PEG (Patade et al., 2011) than that of mercury. Increase in SOD activity in response to stress appears to be due to de novo synthesis of the enzyme protein (Lozano et al., 1996). Such over expression of SOD would deliver an overall defense system of plants subjected to oxidative stress. At the same time, an enhancement of activity of POD indicates that this enzyme serves as an intrinsic defense too, to resist mercury and PEG induced oxidative damage. The increased POD activity in mercury stressed fenugreek seedlings might be possibly due to increased release of peroxides localized in the cell walls. Under metal toxicity condition, the level of the POD activity has been used as a potential biomarker for evaluating the intensity of stress (Shah et al., 2001). The phenomenal increase in the level of POD activity observed in the

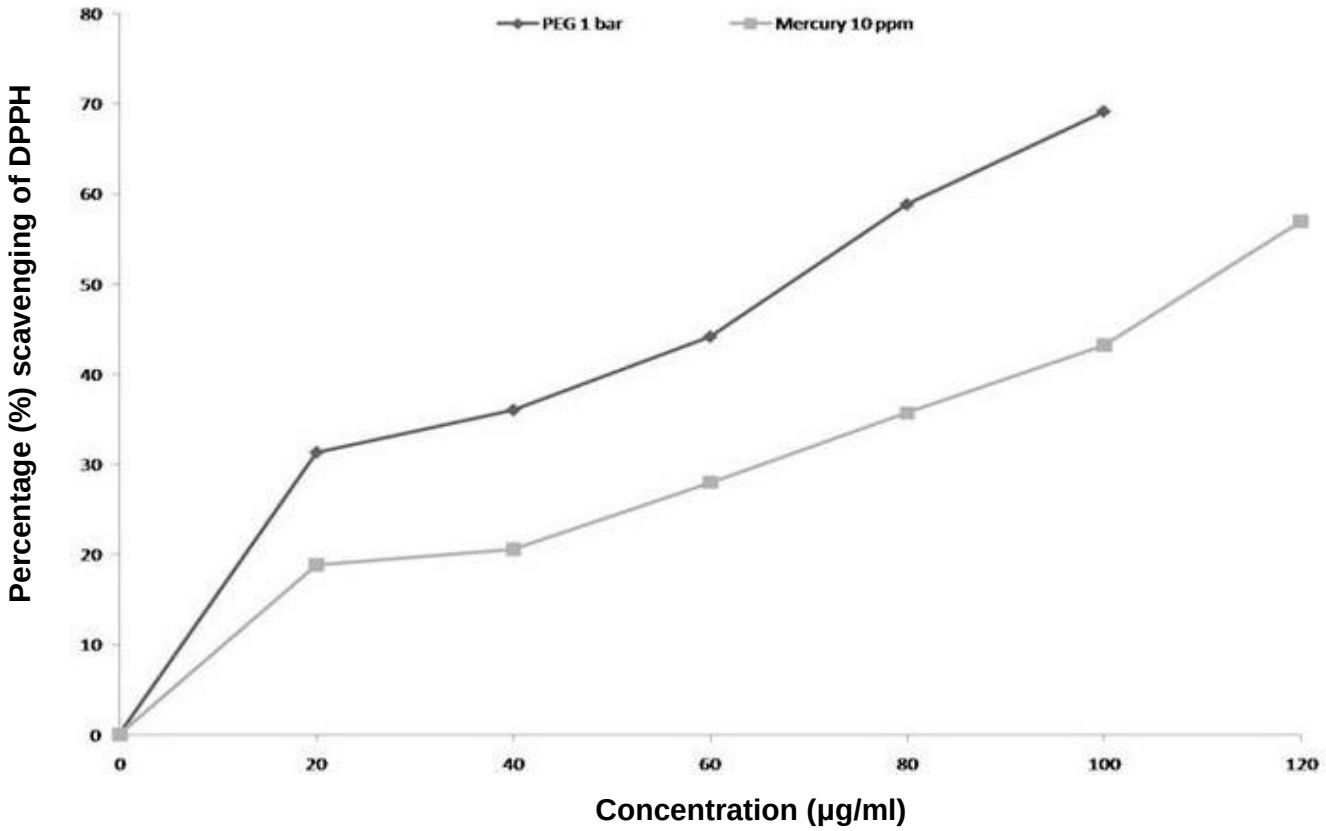

Figure 5. DPPH radical scavenging activity of different treatments of fenugreek seedlings after 144 h.

present experiment due to mercury proves greater intensity of stress induced by mercury than that of PEG. Declining catalase activity was reported in wheat under drought stress (Zhang and Kirkham, 1994) and in rice seedlings under Pb toxicity (Verma and Dubey, 2003). However, mercury causes a significant decline in catalase activity compared to PEG. Such marked depression in catalase activity under mercury toxicity observed would suggest a delay under mercury toxicity in removal of H_2O_2 and toxic peroxide mediated by catalase which is known to be a key enzyme involved in removal of toxic peroxides and decomposition of H_2O_2 (Lin and Kao, 2000).

Thus, mercury induced oxidative stress is much more intense than that of PEG and plant cell integrity is threatened seriously due to mercury toxicity causing the inactivation of membrane bound proteins and increase in membrane permeability. This is very much at par with the result found in *phaseolus* sp. at seedling stage due to mercury treatment (Shaw, 1994) and due to water deficit stress (Sofo et al., 2004). The huge increase of MAD concentration in heavy metal stress follows that the cell integrity is more seriously challenged and severely damaged though PEG also adversely influenced lipid peroxidation mechanism and due to the higher accumulation of ROS in heavy metal induced stress compared to osmotic stress the plant system has failed to quench the ROS which has been reflected in total antioxidant activity.

Mercury exposure conspicuously affected the quenching ability of the antioxidant to DPPH radical compared to osmotic stress. In the presence of mercury which is often detected as soil pollutant, fenugreek seedling would readily succumb due to its utter inability to prevent free radical damage. This indicates that mercury induces significantly higher level of lipid peroxidation as compared to the moisture stress and it can further be argued that under such situation, there would be the production of increased level of free radicals due to severe loss in cell membrane integrity in presence of heavy metal pollutant like mercury.

The higher level of lipid peroxide production adopted a mechanism which was balanced by the peroxidase enzyme in heavy metal stress whereas comparatively the lesser amount of ROS production in case of osmotic stress adopted the mechanism where the singlet oxygen was quenched by SOD and the byproduct H_2O_2 was detoxified followed by catalase. So, in different stress, the same plant species can adopt different mechanism for tolerance. On the other hand, if drought is being imposed,

fenugreek seedlings would not suffer to that extent.

REFERENCES

Adams E, Abdollahi P, Shin R (2013). Cesium Inhibits Plant Growth through Jasmonate Signaling in *Arabidopsis thaliana.* Int. J. Mol. Sci. 14:4545-4559.

Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44:276-287.

Beers RF, Sizer IW (1952). A Spectrophotometric Method Measuring the Breakdown of Hydrogen Peroxide by Catalase. J. Biol. Chem. 195:133-149.

Braca A, Tommasi ND, Bari LD, Pizza C, Politi M, Morelli I (2001). Natural anti- oxidants from plant material in phenolic compounds in food and their effects on health. J. Nat. Prod. 64:892-895.

Diaz P, Monza J, Marquez A (2005). Draught and saline stress, In: *Lotus japonicas* Handbook. pp. 39-50.

Duncan DB (1955). Multiple range and multiple F test. Biometrics 11:1-42.

Hamidi H, Safarnejad A (2010). Effect of Drought Stress on Alfalfa Cultivars (*Medicago sativa* L.) in Germination Stage**.** Am. Eurasian J. Agric. Environ. Sci. 8(6):705-709.

Idrees M, Khan MA, Aftab T, Naeem M, Hasmi N (2010). Salicylic acid induced physiological and biochemical changes in lemon grass varieties under water stress. J. Plant Int. 5:293-303.

Kiran Y, Sahin A (2006). The effect of cadmium and seed germination, root development and mitotic of root tip cells of lentil (*Lens culinaris* Medik). World J. Agric. Sci. 2(2):196-200.

Kramer P (1983). Water realations of plants. Academic Press. pp.489.

Lin CC, Kao CH (2000). Effects of NaCl stress on H_2O_2 metabolism in rice leaves. Plant Growth Regul. 30:151-155.

Lozano R, Azcon R, Palma JM (1996). SOD and drought stress in *Lactua sativa.* New Phytol. 136:329-331.

Malecka A, Jarmuszkiewicz W, Tomaszewska B (2001). Antioxidative defence to lead stress in subcellular compartmentsof pea root cells. Acta. Biochim. Polo. 48:687-698.

Mishra RK, Singhal GS (1992). Function of photosynthetic apparatus of intact wheat leaves under high light and heat stress and its relationship with peroxidation of thylakoid lipids. Plant Physiol. 98:1-6.

Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxidase in animal tissue by thiobarbituric acid reaction. Anal. Biochem. 95(2):351-358.

Patade VY, Bhargava S, Suprasanna P (2011). Salt and drought tolerance of sugarcane uner iso-osmotic salt and water stress:growth and osmolyte accumulation and antioxidant defense. J. Plant Int. 6:275-282.

Patel KP, Patel KM (2013). Cytological changes in *Trigonella foenum-graecum* (l.) under the cadmium stress. J. Life Sci. Technol. 1(1):10-13.

Shah K, Kumar RG, Verma S, Dubey RS (2001). Effect of cadmium on lipid peroxidation, super oxide anion generation and activities of antioxidant enzymes in growing rice seedlings. Plant Sci. 161:1135-1144.

Shannon J (1966). In: Methods of enzymatic analysis 2 (Ed Bergmcyer). Academic Press New York. pp. 685.

Sharma AK, Sharma A (1994). Chromosome Techniques: A Manual. Harwood academic publishers.

Shaw BP (1994). Effects of mercury and cadmium on the activities of antioxidative enzyme in the seedling of *Phaseolus aureus*. Biol. Plantarum. 37(4):587-596.

Smiri M, Jalali N, Ghoul EJ (2013). Cadmium affects the thioredoxin reductase/ thioredoxin system in germinating pea seeds. J. Plant Int. 8:125-133.

Sofo A, Bartolomeo D, Xyloyannis C, Massia A (2004). Lipoxigenase activity and proline accumulation in leaves and roots of olive trees in response to drought stress. Physiol. Plant 121:58-65.

Suresh Kumar G, Shetty AK, Sambaiah K, Salimath PV (2005). Antidiabetic property of fenugreek seed mucilage and spent turmeric in streptozotocin- induced diabetic rats. Nutr. Res. 25:1021-1028.

Verma S, Dubey RS (2003). Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing in rice plants. Plant Sci. 164:645-655.

Zhang J, Kirkham MB (1994). Drought-stress induced changes in activities of super oxide dismutase, catalase and peroxidase in wheat species. Plant Cell Physiol. 35:785-791.