IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 3, Issue 1 (Jan. – Feb.2017), PP 89-97
www.iosrjournals.org

Evaluation of Abiotic Stress Induced Physiological and Biochemical Changes in *Trigonella Foenum-Graecum*

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Abstract: *Plants species are often being exposed to unfavourable environmental conditions such as various abiotic stress factors. These factors play an important role in determining crop productivity and yield. Some examples of abiotic stresses include decreased water availability, extreme temperature, and decreased availability of soil nutrients, excess of toxic ions, excess of light, exposure to heavy metals. The ability of plants to acclimatise to different environments depends mainly on photosynthesis and reproduction. It has been understood after exploring and understanding plant abiotic responses at the whole-plant, physiological, biochemical, cellular and molecular levels that abiotic stresses elicit complex cellular responses .The aim of our study is to determine and understand the effects of salinity, osmotic stress and heavy metal stress on Trigonella foenum-graecum. The results suggest that these abiotic stress factors cause significant reduction in seed germination and also cause subsequent decrease in biochemical constituents like proteins, β-amylase, total soluble sugars and starch. Different concentrations of the stress also altered the relative water content. The results also indicated that certain parameters like total phenolics, free proline content, glycine betaine activity and catalase enzyme activity increased significantly with increase in the stress levels.*

Keywords: *Abiotic stress, catalase, reactive oxygen species, seed germination, Trigonella foenum-graecum.*

I. Introduction

There is an alarming increase in the world population and it is projected to rise by more than one billion by 2030 and over 2.4 billion by 2050.Therefore, it has been estimated that to meet the demands of the increasing population, food production by agricultural practices must be increased by 70 percent by 2050. The currently existing and projected changes in abiotic stresses such as drought, salinity, cold, heat and heavy metal will not only affect the plant growth , it will significantly limit the productivity and it is the leading cause of crop losses worldwide and thus affect our ability to feed the population.[1,2]

Salinity is a major abiotic stress. Depending on severity and duration of the stress its causes changes in various metabolic and physiological processes and ultimately inhibits crop production [4–7]. Soil salinity initially represses plant growth in the form of osmotic stress which is then followed by ion toxicity [4, 5]. Osmotic stress in the initial stage of salinity stress causes various physiological changes, such as interruption of membranes, nutrient imbalance, impairs the ability to detoxify reactive oxygen species (ROS), differences in the antioxidant enzymes and decreased photosynthetic activity [3, 5].

Salinity stress is also considered as a hyper ionic stress which causes the accumulation of Na^+ and Cl^- ions in plant tissues exposed to soils with high NaCl concentrations. Entry of both Na^+ and Cl^- into the cells causes severe ion imbalance and excess uptake might cause significant physiological disorder(s). High Na^+ concentration inhibits uptake of K^+ ions which is an essential element for growth and development that results into lower productivity and may even lead to death [4]. In response to salinity stress, the production of ROS, such as singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide, is enhanced [8,9]. Salinity-induced ROS formation can lead to oxidative damages in various cellular components such as proteins, lipids, and DNA, interrupting vital cellular functions of plants.

Osmotic stress is reported to reduce plant growth and productivity. Osmotic stress is said to cause the accumulation of the phytohormone abscissic acid (ABA), which induces several responses to osmotic stress. Osmotic stress signalling consists of an ABA-dependent and an ABA-independent pathway. Osmotic stress also caused increased ROS generation, which in turn elicits various cellular signalling networks resulting into physiological damage to plant cell. [10]. Heavy metal pollution is being considered as one of the important environmental problems worldwide. The remediation of metal pollutants from the soil, water and air is difficult because, unlike organic pollutants which degrade to harmless small molecules, toxic elements, such as lead, mercury, cadmium, copper and zinc, are immutable by biochemical reactions. [11]

Lead (Pb) is considered as one of the most hazardous pollutants of the environment and Pb pollution is a growing ecological concern due to its impact on human health and environment. The main sources of Pb pollution in the environment are mining and smelting of Pb ore, industrial effluents, fertilizers, pesticides, and municipal sewage sludge .Lead toxicity leads to decreases in the percentage of seed germination, as well as

growth, dry biomass of roots and shoots, disruption of mineral nutrition, reduction in cell division and inhibition of photosynthesis. [12]. Lead is reported to produce reactive oxygen species (ROS) and enhance antioxidant enzyme activity in plants. Oxidative stress gives rise to ROS production which intern causes many harmful effects in plant cells, such as inhibition of photosynthetic activity, inhibition of ATP production, lipid peroxidation, and DNA damage. ROS damages cell membranes, nucleic acids and chloroplast pigments. [13]. The present study focuses on the evaluation of the physiological and biochemical changes induced by salinity, osmotic and heavy metal (Lead) stress on *Trigonella foenum-graecum*.

II. Materials And Methods

Salinity stress

Collection of Plant material and Germination

Seeds of *Trigonella foenum-graecum* were collected locally from south Bangalore and were used for the germination test. Fenugreek seeds were sterilized with 0.1% mercuric chloride for 5 min and washed thoroughly with distilled water. They were then placed to germinate in Petri dishes, distilled water (control) and different concentration of NaCl solutions (10, 30, 50 and 100 mM NaCl) at room temperature (25°C) in light. In each treatment, 5 Petri plates (each containing 25 seeds) were used. The seeds were considered germinated when the radical length reached 2 mm and the cumulative germination percentage was determined. [14]

Seedling analysis

Germinated seeds in distilled water and in saline solutions were sampled after 7 days from the beginning of incubation and then germination process was stopped. The tissues were weighed to obtain the fresh weight. The dry weight was obtained after drying the tissue at 75°C for 48 h and then the tissue water content was calculated as based on the (FW-DW/DW) ratio. [14]

Determination of starch content

Batches of 500mg of cotyledon pairs or axes were homogenized in an ice-cold mortar and pestle in a volume of 16 ml 80% (v/v) ethanol. The homogenate were centrifuged (30000xg, 10 min at 2°C) and then perchloric acid ($HClO_4$; 6 ml, 30%, v/v) was added to solubilize starch from the pellet. The slurry was left at room temperature for 6 h, starch was detected with I_2-KI reagent prepared by diluting 0.1 ml stock solution (0.06 g I_2 and 0.60 g KI in 10 ml deionized water) with 0.05M HCl just prior to the assay. Samples of 0.5 ml starch solution were mixed with 0.5 ml I_2-KI reagent, 1 ml 30 % (v/v) perchloric acid and then were vortexed and left standing at room temperature. The absorbance (620 nm) of the samples was compared to that of the standard curve of 0 to 5 mg/ml which was obtained using soluble starch dissolved in 30% $HClO_4$ and detected with the same I_2-KI reagent. The assay was conducted in triplicate for each sample. [17]

Determination of Soluble sugars

Three replicates of cotyledons or axes samples of germinated seeds were suspended in test tubes with 3 ml of 80% ethanol, the extract was evaporated to dryness in hot air oven and the residue was dissolved in 20 ml of distilled water. Total soluble sugars were determined by the phenol sulphuric acid method using glucose as standard. [18]

Determination of □-Amylase activity

The seeds were homogenised with 4ml of ice cold 16mM sodium acetate buffer (pH 4.8) and centrifuged at 12000g for 15 mins. The supernatant is used as the enzyme for β-amylase activity. To 0.5ml of 1% potato starch and 0.5ml of enzyme in 16mM sodium acetate buffer starch was added vortexed and incubated in shaker for 5min at 37°C.To this 0.5ml of DNS reagent was added and incubated in boiling water bath for 10min. Maltose was taken as standard concentration. The absorbance was read spectrophotometrically at 540nm. [19]

Determination of Total Phenolics

Fenugreek seeds (500mg) was soaked in 2.5 ml of 95% ethanol and kept in the freezer for 48h. The sample was homogenized and centrifuged (13000 rpm, 10 min). One ml of the supernatant was transferred to a test tube, and 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent were added. After an incubation period of 5 min, 1 ml of 5% Na_2CO_3 was added, mixed well and kept in the dark for an hour. The samples were vortexed and absorbance was measured at 725 nm using colorimeter. Phenolic content was reported as mg/g fresh weight (FW) and measured as gallic acid equivalents. [20]

Osmotic stress

Laboratory water stress was created by using Polyethylene Glycol 6000. [15] Five different water stress conditions (Control, -0.1, -0.3,-0.6 and -0.9MPa) were used in a completely randomized design. The osmotic

potential of the aqueous solutions of PEG 6000 was calculated according to the empirical equation. PEG 6000 was dissolved in water and placed in a shaker bed (25 ^{o}C) for 16 hr. [21]

Seed germination assay

Seed germination bioassay was carried out in sterilized Petri plates (9 cm inner diameter). The seeds were surface sterilized using 0.1% $HgCl_2$ for 5 min, followed by thorough rinsing with distilled water. About 25 healthy and uniform seeds were used for germination and 15 ml solution of respective concentration of PEG 6000 was added in each Petri plate. Seed germination bioassay was carried out at room temperature (25±3°C) and uniform light conditions. Seeds were considered to be germinated with radical emergence of 1mm. [14]

Seedling growth analysis

Seed germination and seedling growth was investigated and recorded. Total number of germinated seeds in each Petri dish on the seventh day was regarded as the final germination percentage (FGP). Five seedlings from each treatment were selected randomly and the average plumule and radicle length were measured. Then, they were oven-dried at 50 °C for 24 hrs to acquire their mean dry weight then the tissue water content was calculated as based on the (FW-DW/DW) ratio. [14]

Determination of Protein content and total sugars

Protein content of the seedlings was determined, were 500mg of seedlings were taken and homogenised with distilled water for all different concentration and centrifuged(10000g for 10 min) the supernatant was taken and protein estimation was carried out. [22]. Total sugars content was determined by the method described by Hedge and Hofreiter, 1962. [23]

Estimation of Free Proline content

Fresh seedlings were homogenized in 3% sulphosalicylic acid. The reaction mixture containing filtrate, GAA and ninhydrin was kept in boiling water bath for 1 hr, reaction terminated in ice bath. Reaction mixture was extracted with toluene, mixed and left at room temperature for 30 min until two phase separation was achieved. Upper layer was warmed to room temperature and optical density was measured. Proline content was determined from standard curve using D-Proline. The reaction was terminated by placing the tubes in ice bath and the red colour developed by addition of toluene was measured spectrophotometrically at 520 nm. [24]

Determination of Glycine betaine content

About 500mg of fresh seedlings were taken and were incubated in 1 N H_2SO_4, homogenised and centrifuged (12000 rpm for 10 min). The reaction mixture contained the supernatant, H_2SO_4 and cold I_2KI reagent. The tubes were then cooled to 4°C and centrifuged again. Ethylene dichloride was added and absorbance was recorded at 365 nm. [25]

Determination of Catalase Activity

The assay mixture contained 0.5 ml of H_2O_2, 1.0 ml of buffer and 0.4 ml of water. 0.2ml of the enzyme was added to initiate the reaction. To this 2 ml of the dichromate/acetic acid reagent was added after 0, 30, 60 90 seconds of incubation. To the control tube the enzyme was added after the addition of the acid reagent. The tubes were then heated for 10 minutes. And then the colour developed was read at 610nm. [26]

Heavy Metal stress

Seed material and Germination

Seeds were collected from local plants of fenugreek and were used for the germination test. Fenugreek seeds were sterilized with 0.1% mercuric chloride for 5 min and washed thoroughly with distilled water. They were then placed to germinate in Petri dishes, distilled water (control) and heavy metal of lead nitrate (0.01, 0.02 0.03mg) at room temperature (25°C) in light. In each treatment, 5 Petri dish (each one contained 25 seeds) was used. The seeds were considered germinated when the radicle reached 2 mm, cumulative germination percentage was determined. [14]

Seedling analysis

Germinated seeds in distilled water and in heavy metal solutions were sampled after 7 days from the beginning of incubation and then germination process was stopped. A part of these tissues were weighed to obtain the fresh weight. The dry weight was obtained after drying the tissue at 50°C for 24h; then the tissue water content was calculated as based on the (FWDW/DW) ratio. [14]

Determination of protein constituents

Protein content of the seedlings was determined, were 500mg of seedlings were taken and homogenised with distilled water for all different concentration and centrifuged(10000g for 10 min) the supernatant was taken and protein analysis was carried out. [22]

Estimation of Free proline content

Fresh seedlings were homogenized in 3% sulphosalicylic acid. The reaction mixture containing filtrate, GAA and ninhydrin was kept in boiling water bath for 1 hr, reaction terminated in ice bath. Reaction mixture is extracted with toluene mixed and left at room temperature for 30 min until two phase separation happens. Upper layer is warmed to room temperature and optical density measured. Proline content is determined from standard curve using D-Proline. The reaction was terminated by placing the tubes in ice bath and the red colour developed by addition of toulene was measured at 520 nm. [24]

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III. Results And Discussion

Salt stress

The influence of NaCl on the growth and water content of embryonic axes and cotyledons in germinated seeds was studied. In the cotyledons the FW (fresh weight) values showed significant differences between control and salt solution. However, in embryonic axes, the FW values decreased by 50 mM NaCl treatment. The DW (dry weight) displayed significant differences on one hand between control and 100 mM NaCl treatment. In embryonic axes, between control and 50 mM NaCl treatment on the other hand. Further, a significant decrease in water content of embryonic axes was observed from 40 mM NaCl. The lowest value of this parameter was observed at 50 and 100 mM NaCl. In cotyledons, the water content varied only slightly at this stage of germination (radicles length 2 mm) water content vary from 3.289 (0 mM NaCl) to 0.887 (100 mM NaCl).

Embryonic Axes	**Control**	**Concentration of NaCl (mM)**			
		10mM	**30mM**	**50mM**	**100mM**
FW (mg)	24.2± 3.340	22.467±1.234	22±1.562	18.533±2.541	13.933±0.521
DW(mg)	3.000±0.230	3.5333±0.581	3.666±0.405	3.8666±0.176	4.000±0.346
Relative water Content	7.332±1.802	5.637±0.9323	5.203±1.0392	3.793±0.6425	2.560±0.4418
Cotyledons	**10mM**	**30mM**	**50mM**	**100mM**	
FW(mg)	32.933±2.257	27.333±7.802	25.8±1.803	24.933±3.1205	23.266±3.5802
DW(mg)	8.8±1.800	9.333±1.034	9.8±0.416	10.6±1.858	12.266±0.466
Relative water content	3.289±1.344	1.828±0.484	1.646±0.236	1.459±0.459	0.887±0.258

Table 1: Effect of salinity stress on relative water content of fenugreek seeds

(Values are expressed as mean ± SD, n = 3)

Fenugreek seeds do not contain starch as carbohydrate reserve. Synthesis of starch is initiated after germination. As starch is determined in certain amount after germination. Figure 2 shows the influence of NaCl concentrations on starch level in cotyledons. During germination, the amount of starch in embryos increased clearly in control (seeds treated with distilled water) and cotyledons treated with NaCl contained lower levels of starch than that treated with distilled water. Gradual Decrease of starch amount was observed in cotyledons of seeds treated with different concentration of NaCl. A large decrease of starch was observed in cotyledon treated with control and 100 mM NaCl.

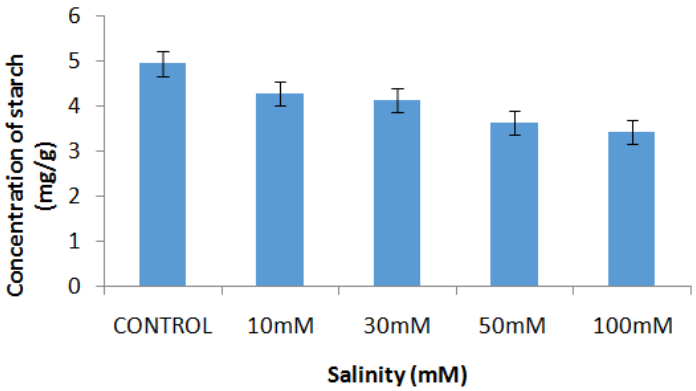

Figure 1: Changes in levels of starch in cotyledons of germinating fenugreek seeds treated with distilled water (control) and saline solutions

The activity of β- amylase detected in seeds germinated in the control treatment was higher compared to those detected in stressed seeds. Fig. 3 shows the influence of the presence of various concentrations of NaCl on the β-amylase activity in cotyledons of germinated seeds. The activity of this enzyme showed decreasing trend at 50mM and showed slight increase in 10mM and 30mM when compared to control but was negligible (about 0.02mg/ml). B-Amylase activity decreased particularly by 61.3% in 100mM concentration.

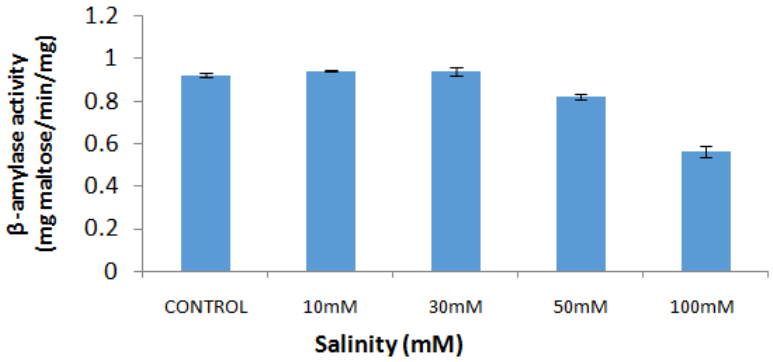

Figure 2: Influence of NaCl on the activity of amylase in cotyledons of germinating fenugreek seeds

The total phenolics content of germinated fenugreek seeds was different across NaCl treatments (Fig.3); within untreated fenugreek seeds contain 0.58mg/g. The amount of GAE mg/g increased with salinity, principally, seeds treated with 30 and 100 mM NaCl showed the greater accumulation of polyphenols which was around0.76mg/g and 0.778 mg/g.

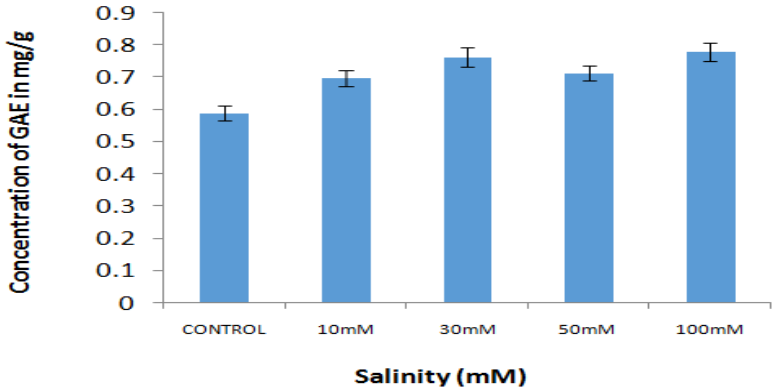

Figure 3: Total phenolics content in germinated fenugreek seeds

The influence of salinity levels on total soluble sugar content of germinated seeds is shown in fig. 4 compared with control, imposition of NaCl treatment resulted in a significant increase in total sugar content in cotyledons, The level of total soluble sugars increased to a smaller extent over stressed seeds in cotyledons, no significant differences were displayed under different salt conditions. Under the influence of high concentrations of NaCl (50 to 100mM) soluble sugars in cotyledons was around 63.85 mg/g and 67.22mg/g.

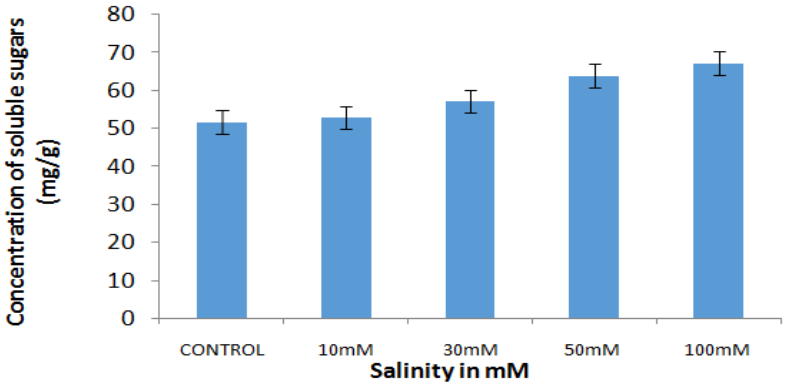

Figure 4: Total soluble sugar content (mg/g FW) in cotyledons of fenugreek seeds

Osmotic stress

Relative water content was highest in control, -0.1MPa and there was a significant decline in final relative water content with decrease in osmotic potential as shown in table 2. The fresh weight decreased significantly with increase in PEG concentration and a negligible increase in dry weights was observed. High concentration of PEG might hamper the process of water uptake by seeds and thereby inhibiting the process of seed germination.

	Control	-0.1MPa	-0.3MPa	-0.6MPa	-0.9MPa
Fresh weight (mg)	0.316±0.0738	0.331± 0.0241	0.315± 0.0432	0.259± 0.0111	0.258±0.0340
Dry weight (mg)	0.061±0.007	0.0623±0.011695	0.0683±0.0100	0.0683±0.0048	0.070±0.0104
Relative water content	4.050±0.310	4.542±0.419	3.717±0.591	2.804±0.056	2.685±0.064

Table 2: Effect of different levels of osmotic stress on relative water content of fenugreek seeds

PEG induced osmotic stress caused a significant reduction in protein content (Fig.5) of treated seedlings as compared to control. There was about 45.75% reduction in protein content at osmotic potential of -0.9MPa as compared to control.

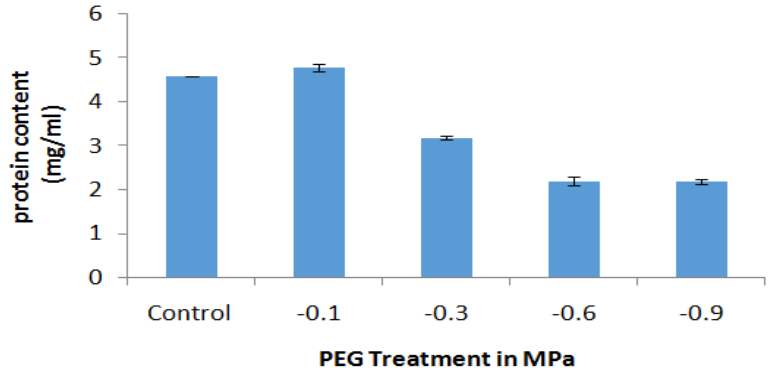

Figure 5: Effect of different levels of osmotic stress on total protein content of fenugreek seeds

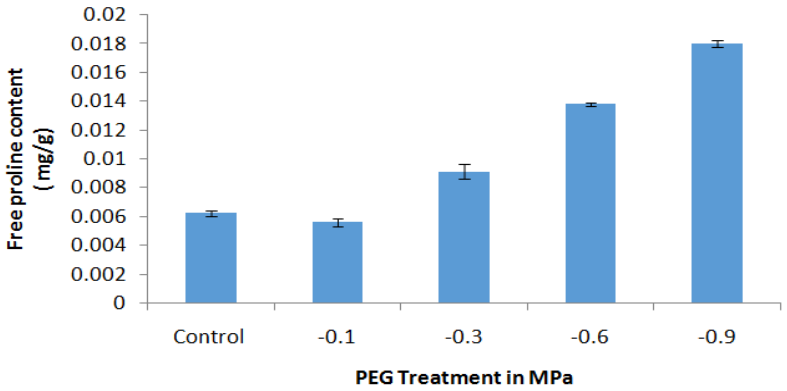

Figure 6: Effect of different levels of osmotic stress on free proline content of fenugreek seeds

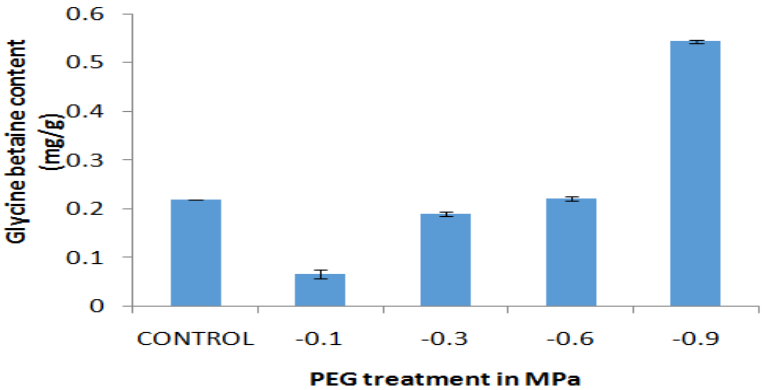

Figure 7: Effect of different levels of osmotic stress on glycine betaine content of fenugreek seeds.

Experimental findings on antioxidant system indicate that responded differently under normal and water stress conditions. Decrease in osmotic potential showed significant increase in antioxidant contents like proline and glycine betaine as compared to control. (Fig. 6 and 7). Proline and glycine betaine content was significantly increased with decreasing osmotic potential. During stress conditions, protein degrades and consequently the proline and glycine betaine increases. High level of proline enabled the plant to maintain low water potentials.

CAT activity increased sharply in relative to the control. This enzyme activity increased gradually upto the level of -0.9 PEG, and higher than the control. Furthermore, water stress induced a significant increase in CAT activity in all the treatments. Comparatively it showed higher CAT activity in control than that of -0.1 PEG concentrations. (Fig. 8)

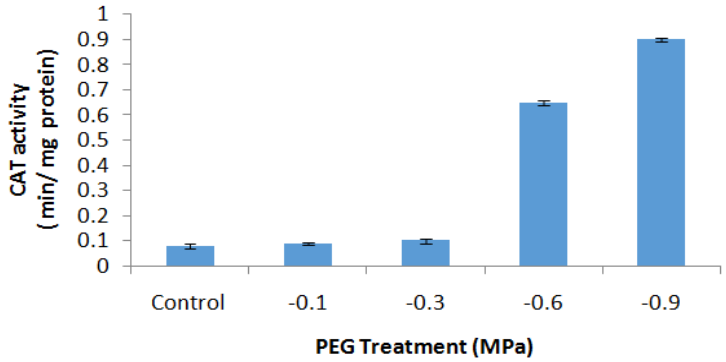

Figure 8: Effect of different levels of osmotic stress on catalase activity content of fenugreek seeds.

Heavy metal stress

The heavy metals treatment with Pb markedly decreased relative water content. seed germination was consistently reduced with increased concentration of lead (Pb) and maximum suppression of plant growth was recorded at the highest concentration of heavy metals Pb) at whose relative water content was reduced up to 49.4% as compared to control. (Table 3)

Radicle	**Control**	**Concentration of Pb (mg/mL)**		
		10mg/mL	**20mg/mL**	**30mg/mL**
Fresh weight (mg)	10.020±0.0075	8.500±0.0110	8.530±0.0075	6.320±0.003
Dry weight (mg)	7.50±0.0025	8.5000±0.0005	1.20±0.003	1.110±0.002
Relative water content	9.326±0.0077	7.352±0.0183	5.733±0.0183	4.454±0.0197
Cotyledons				
Fresh weight (mg)	26.900±0.0055	25.400±0.013	22.000±0.013	21.5±0.014
Dry weight (mg)	5.450±0.0015	4.7000±0.0085	4.5000±0.0019	3.5±0.0021
Relative water content	4.404±0.0106	3.962±0.0155	3.944±0.010	3.888±0.0043

Table 3: Effect of different levels of heavy metal stress on relative water content of fenugreek seeds
(Values are expressed as mean ± SD, n = 3)

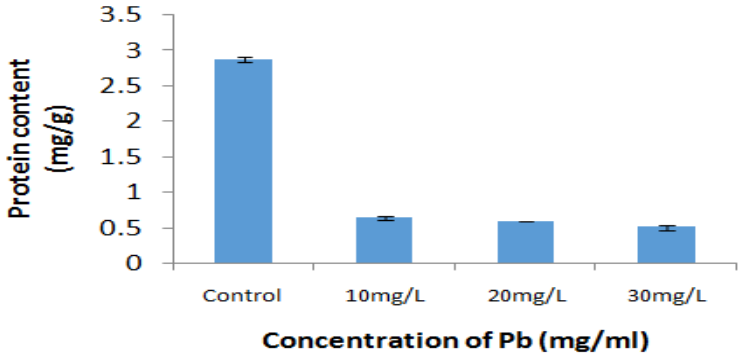

Figure 9: Effect of heavy metal on protein content of fenugreek seeds

The protein content under heavy metal stress found more pronounced decrease in the protein content with Pb treatment. The decrease in protein content may be caused by enhanced protein degradation process as a result of increased protease activity that is found to increase under stress conditions. It was about 82% decrease in the protein content in 30mg/l when compared with control as shown in fig. 9.

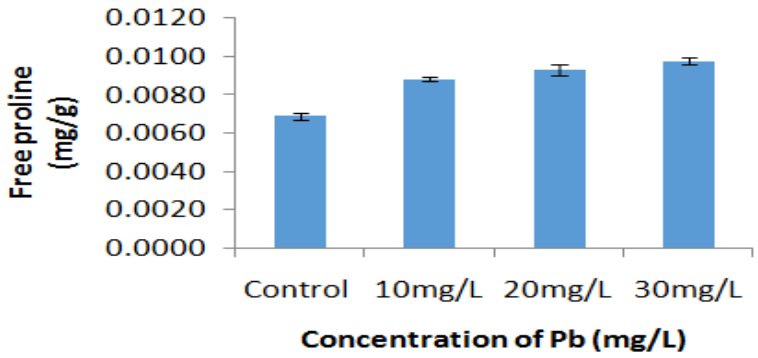

Figure 10: Effect of different levels of metal stress on free proline content of Fenugreek seeds.

Significant increase in contents of proline was noted in seedlings of fenugreek seeds treated with different concentrations of Pb as compared to control with maximum accumulation in 30mg/l Pb treated seedlings as shown in fig. 10.

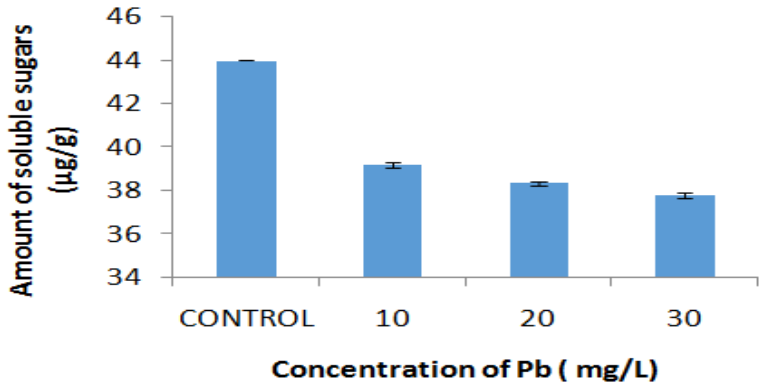

Figure 11: Effect of different levels of Metal Stress on total soluble sugar content of Fenugreek Seeds

The results related to the soluble sugar content are depicted in above Fig. 11, which revealed that lower concentration Pb (lead) increased the soluble sugar content; however, higher concentrations of 30 mg/l of Pb showed a decrease of 85.9% in soluble sugar content compared to control.

IV. Conclusion

The results from the present study indicate that with the increasing concentration of abiotic stress induced on the *Trigonella foenum-graecum* seeds, it resulted in reduced seed germination and it also caused subsequent metabolic changes. In the case of salt stress, we observed that as the salt concentration increased there was proportional increase in total phenolics and soluble sugars whereas there was a decrease in starch content and beta amylase activity. It had a negative impact on seed germination. It can also be observed that, water is a major limiting factor for seed germination and osmotic potential plays an important role, as it decreases protein content decreases but antioxidant contents increased. As the concentration of PEG 6000 increased the germination percentage of the seed decreased. Heavy metal stress in plants and seeds decreases the relative water content, soluble sugar content and protein contents due to increase in the protease activity consistently the seed germination reduced with increased in heavy metal stress which hinders the metabolic processes, growth of the plants and the crop yield.

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