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Influence of salt stress on the morphological physiological activity and anatomy of Cow pea plant (*Vigna unguiculata*)

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Abstract
In this study 21 days old seedlings of Cow pea plant (*Vigna unguiculata* L) were subject to different salt stress levels (0g, 0.1g and 0.2g NaCl) at germination and early seedling growth stage of plant development. Data were analyzed for growth parameters such as plant height, fresh and dry weight, leaf water content (LWC), and length of radicle and plumule during germination period, and biochemical parameters such as proline content, membrane stability index (MSI), malondealdehyde (MDA) content, chlorophyll content, and antioxidant enzyme activity Catalase (CAT) and Peroxidase (POD). In this study it was seen that the effect of salt stress reduced plant height, fresh and dry weight, LWC, radical and plumule length. Salt stress reduced the biochemical activities and also chlorophyll a, chlorophyll b and total pigment content. The decrease was 1.83, 0.82 and 2.65 respectively.
The result showed an increase in the activity of CAT enzyme in leaves and root with increasing salt concentration. An increase CAT activity were found with 0.1g, 0.2g NaCl treatment which represented values of relative increasing of 14.85g/L and 15.88 in leaves and 8.5g/L and 9.48g/L in root respectively. There was increase in the activity of POD enzyme in leaves and root with increasing salt concentration. The level of POD activity was found with 0.1g,0.2g NaCl treatment, which represented relative reduction 95.98g/L and 102.59g/L in leaves.

Keywords: Proline content, chlorophyll content, membrane stability index, malondealdehyde, catalase activity, peroxidase activity, anatomy of stem, salt stress

Introduction
Abiotic stress management is one of the most important challenges facing the agricultural sector of India. Plants are constantly confronted to abiotic stresses that affect their productivity. Abiotic stresses include salinity, drought, high or low temperature, pH, and heavy metal toxicities. The extremes of these stresses limit the crop productivity worldwide. Salinity is one of the most important abiotic stresses, particularly relevant these days because of decreasing fresh ground water. Salinity is defined as the presence of excessive amount of soluble salts that hinder the normal functions of plant growth. One of the most common forms of land degradation results from soil salinization. All over the world, and particularly in arid and semiarid regions, salinity is the one of the important problem and is increasing day by day. Increased salinity induces osmotic as well as toxic effects and hence affects growth and other Physico-biochemical process including photosynthesis.
Exposure to high salt concentration triggers production of toxic reactive oxygen species (ROS). It results in oxidative stress. Salinity cause a range of injurious effect such as inhibition of photosynthetic rate by decreasing chlorophyll content, damage to plasma membrane stability and other metabolic commotion, (Karimi *et al*., 2005 Ashraf and Praveen, 2002) [33, 11]. Salinity stress tolerance seems to be correlated with spur of antioxidant enzymes and enhanced ability to remove R0S and a higher concentration of CAT (catalase) is noted, (Santos *et al*., 2001; Bettaieb *et al*., 2007) [44, 16].
Catalase (CAT) and Peroxidase (POD) are principle enzymes which scavenge reactive oxygen species and reduce the protein degradation, (Foyeret *et al*., 1994; Smirnoff, 1993) [46]. These enzymes acts as the main defense against ROS produced various part of plant cells. CAT is a titrameric heme containing enzyme that is found in all aerobic organisms and serve to rapidly degrade water at an extreme rapid rate. The expression of catalase gene is not only

influenced by genetic and developmental signals both, physical and chemical signals, (Sneha *et al*., 2013). POD is a heme binding enzyme, which play a key role in detoxification of H_2O_2 in chloroplast. In plants, it is found in cytosol and chloroplast. The activity of POD increases with increase in activity of CAT.

Salinity influences the activity of both catalase and peroxidase. Mainly salinity cause oxidative stress which affect morphological, biochemical and enzymatic components in the plant cell under the salt stress condition.

The main objective of this study was to observe the effects of salt stress on growth parameters, chlorophyll contents, membrane stability index, malonaldehyde, proline content and antioxidant enzyme activity such as that of CAT and POD as well as to see whether salt stress affect the anatomy of the plant.

Methods and materials

The present study is done to analyze the effect of one of the abiotic environmental stress (salinity) response on plants. The plant material selected for the study is Cow pea plant (*Vigna unguilata* L) (Plates 1, 2 and 3), considering the ease in the growth of the plant.

Plate 1, 2 and 3: Study material showing induction of salt stress

Sample Collection

Cow pea plant (*Vigna unguiculata* L) seeds were collected from Kerala Agricultural College, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala, India.

Morphological Parameters

Determination of plant height:

The plant height (centimeters) was measured with the help of scale at the time of harvest. The length was measured from the point where the root and shoot joins to the end of root for root length and to the top of shoot for shoot length. These lengths were then added to get the total plant height.

Determination of fresh and dry weight of shoot and root

After harvesting the seeding, the shoot was cut from root at the point where they joined together. The fresh weight was recorded for each part separately. And the sample was dried in an oven at 70 °C up to constant dry weight for the detection of moisture content.

Determination of length of radicle and plumule

The radicle and plumule length (centimeter) were measured with the help of a centimeter scale at time of germination. The length was measured from the point where the root tip and shoot joints to the end of root.

Determination of leaf water content

Leaf water content was estimated according to the method described by Smart and Bingham (1974) [45]. Leaf discs were punched from each treated plant and the fresh weight was determined. The same leaf discs were kept on water for 4 hrs and turgid weight recorded. The leaf sample was dried in oven at 85 °C for dry weight.

Determination of pigment content

The chlorophyll content was determined according to Dere *et al*., (1998). Leaf fresh materials (1g) were ground properly in 50ml of 100% acetone then centrifuged for 10min at 2500 x g, absorbance was read using a spectrophotometer (UV-1800 Shimadzu) at 662,645 and 470 nm. Pigment content was estimated following formula;

Chl a = (0.0127xODx663) - (0.00269xODx645)

Chl b = (0.0229xODx645) - (0.00468xODx663)

Total Chl = (0.0202xODx645) + (0.00802xODx663)

Biochemical Parameters

The study materials were analyzed for the following biochemical parameters.

Enzymatic Antioxidants

Determination of Catalase (CAT): The enzyme extract of V. *unguiculata* was prepared in phosphate buffer. The homogenate was centrifuged and supernatant was used for enzyme assay. H_2O_2 - phosphate buffer was taken in an experimental cuvette, followed by the rapid addition of 0.1ml of enzyme extract and thorough mixing. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer (UV-1800 Shimadzu).The enzyme solution containing H_2O_2 - free phosphate buffer was kept as control. One enzyme unit was

calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05units.

Determination of Peroxidase (POD): Enzyme extract A (20% homogenate) was prepared in 0.1M Phosphate buffer (pH 6.5). For this, plant tissue was taken from the various parts of the plant. This was clarified by centrifugation and supernatant was used for the assay. To 3.0ml of pyrogallol solution, 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to zero reading at 430nm.To the test cuvette, 0.5ml of H_2O_2 was added and was mixed. The change in absorbance was recorded every 30 seconds up to 3minute minutes in a spectrophotometer (UV-1800 Shimadzu). One unit of peroxidase is defined as the change in absorbance/minute at 430nm.

Non-enzymatic Antioxidants

Determination of proline contents: Dry weight (0.5g) was extracted by homogenization in 3% (w/v) aqueous sulphosalicylic acid. After the 20 min of centrifugation at 3000 x g, supernatant was collected and was mixed with acetic acid and ninhydrin. The mixture was boiled for 1 hour and then absorbance was read using a spectrophotometer (UV-1800 Shimadzu) at 520 nm using toluene as blank.

Determination of membrane stability index

Fresh leaf samples (0.1 g) were taken in test tubes in two sets containing 10 ml of double distilled water. One set was kept in water bath for half an hour at 40 °C and the electric conductivity was recorded (C1). Another set was kept in water bath at boiling temperature (100 °C) and EC was recorded (C2). MSI was calculated as per the formula:

$$(MSI) = [1 - (C1/C2)] \times 100$$

Determination of malonaldehyde content

Fresh leaves were ground in 1% (10 ml/g fresh weight) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 5 minutes. Reaction mixture containing 1.0 ml of supernatant and 4.0 ml of 0.5% (w/v) thiobarbituric acid (TBA) was heated at 95 °C for 30 min, cooled on an ice bath and centrifuged at 5000 rpm for 5 min for clarification. Absorbance of the supernatant was taken at 532 and 600 nm.

Anatomy of stem

Preparation

Two to three centimeter long pieces of the material were taken for taking sections for seeing anatomy. Thin sections were taken using a razor. The thinnest section of the material was taken with the help of delicate brush. For staining, the sections were left for 3 – 5 minutes in a watch glass with stain. The sections were left in stain for 3-5 minutes. The stained sections were mounded on a watch galss and were viewed under a compound microscope with a camera attached.

Results and Discussion

Salinity stress is one of the major abiotic stresses that severely affect the crop production. In this study the 14 days old seeding of cowpea plant were subjected to short term salt stress in order to observe its effect on chlorophyll content, membrane stability index (MSI), malondealdehyde (M) and antioxidants enzyme activity (CAT and POD) response. The aim of the experiment was to evaluate the change in the content of above parameters upon stress in plants. The 14 days old seedling were subject to salt stress by supplementing Hoagland's solution with different concentration of NaCl (0.1 and 0.2g/L) salinity and the results were obtained as follows:

Morphological Parameters

The effect of salt stress on plant growth parameters is shown in Table1. Exposure of cow pea plant to various salinity conditions reduced fresh and dry weight of plant, plant height, leaf water content, length of plumule and radical during germination period with increasing NaCl Concentration treatment. Salinity induced reduction in weight was reported to be 1.23g (0.1g NaCl), 1.21g (0.2gNaCl), 0.98g (0.1g NaCl) and 0.87g (0.2g NaCl) in fresh and dry weight of plant against 2.12 g of fresh weight of control plant and 1.88g of dry weight of control plant. The length of the plumule was measured to be 13.21 cm (0.1g NaCl), 11.46 cm (0.2g NaCl) against 15.63 cm (control) and the length of the radicle were 15.5 cm (0.1gNaCl) and 9.99 cm (0.2g NaCl), against18.5cm in control plant, respectively at various treatments. Leaf water content (LWC) is 89.55(0.1g NaCl), 74.36(0.2g NaCl) against 92.11 in the control plant.

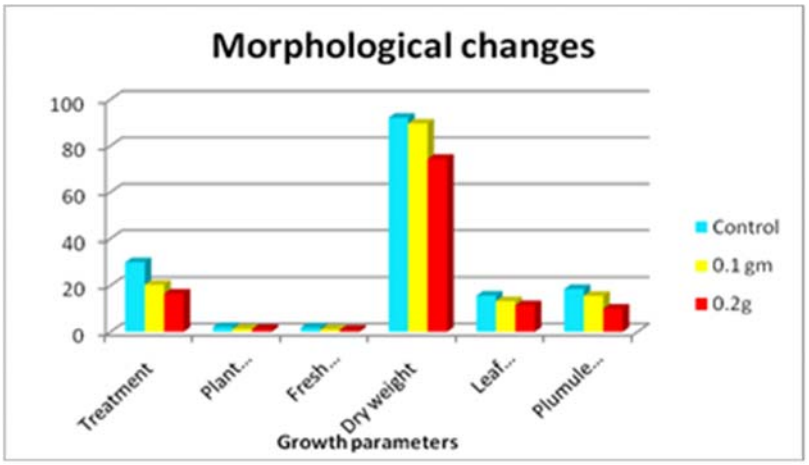
All the morphological parameters measured showed a reduction when compared to the control plant. The reduction in the parameters increased as the salinity given increased.

Reduction in morphological parameters may be due to the inhibition of cell division and reduced rate of cell elongation exerted by the high salinity levels as reported by Arco *et al*., (2013) [7] and Ahanger *et al*., (2014) [1]. Reduction in leaf relative water content is in concurrence with the results of Arulbalachandran *et al*., (2009) [9]; Alqarawi *et al*., (2014) [5] and Hasem *et al*., (2014). As the water content decreases with increasing salinity, it resulted in gradual decrease in the growth of the plant.

Table 1: Influence of salt stress on growth of Cowpea plant

S. No	Treatment	Plant height (cm)	Fresh weight (g)	Dry weight (g)	Leaf water content (%)	Length of plumule (cm)	Length of radicle (cm)
01	Control	30±1.00	2.12±0.03	1.88±0.036	92.11±2.43	15.63±0.057	18.5±0.141
02	NaCl (0.1g)	20.3±0.702	1.23±0.01	0.98±0.01	89.55±1.23	13.21±0.032	15.5±0.028
03	NaCl (0.2g)	16.46±0.042	1.21±0.017	0.87±0.004	74.36±2.01	11.46±0.045	9.99±0.1007

The change in the growth parameters considered in control and in treatment can be better represented as a graph (Figure 1).



Chlorophyll content

The effect of salt stress on the production of plant pigment is presented in Table 2. It is seen that salinity reduced chlorophyll a, chlorophyll b and total pigment content in the experimental plant. The values obtained with respect to the salt stress given were as follows. Chlorophyll a showed values such as 1.83 (0.1g NaCl) and 1.02 (0.2g NaCl), where the corresponding values for the control plant was 2.14. The content of chlorophyll b was 0.82 (0.1g NaCl) and 0.53 (0.2g NaCl) as compared to 0.84 in control and total pigment content showed values 2.65 (0.1g NaCl) and 1.55 (0.2g NaCl), when the control plant showed a total chlorophyll content of 2.98 mg/g of fresh weight.

Reduction in chlorophyll contents due to NaCl treatment in this work is in confirmation with the finding of Rasool *et al*., (2013) [40]; Alqarawi *et al*., (2014a) [5]; Alqarawi *et al*., (2014b) [25]; and Hashem *et al*., (2014 b) [27] who have reported a considerable decline in chlorophyll contents. Rasool *et al*., 2013 [40]; Hameed *et al*., 2009 [23] and Wu *et al*., 2014) [49] reported that salinity cause reduction in synthesis of chlorophyll pigments.

Table 2: Influence of salt stress on pigment system of *Vigna unguiculata*

S. No	Treatment	Chlorophyll a (mg/g fresh weight)	Chlorophyll b (mg/g fresh weight)	Total chlorophyll content (mg/g fresh weight)
01	Control	2.14±0.16	0.84±0.06	2.98±0.11
02	NaCl(0.1g)	1.83±0.09	0.82±0.8	2.65±0.08
03	NaCl(0.2g)	1.02±0.07	0.53±0.4	1.55±0.02

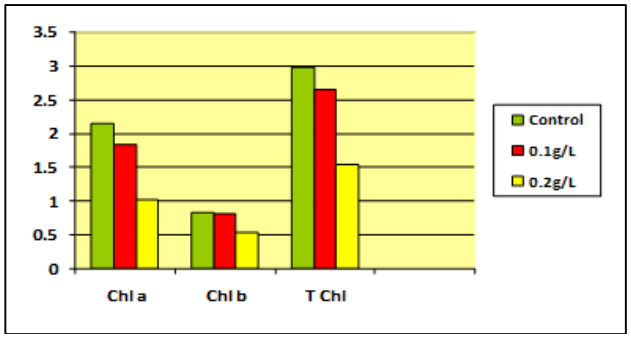


Fig 2: Effect of salt treatment on total chlorophyll content in Cow pea plant at different concentration

Table 3: Influence of salt stress on Membrane Stability Index (MSI %)

S. No:	Treatment	Membrane stability Index (MSI) (%)
01	Control	87.12±2.11
02	NaCl(0.1g)	71.06±3.14
03	NaCl (0.2g)	52.4±2.43

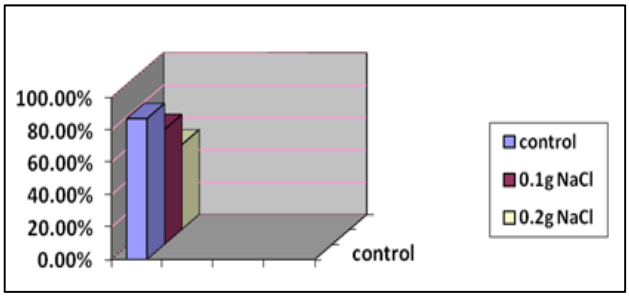


Fig 3: Effect of salt treatment on membrane stability index in cowpea plant

Biochemical parameters

Membrane Stability Index (MSI)

The results of MSI are given in table 3. It can be seen that both level of salinity stress induced reduced MSI in comparison to control. Salinity induced reduction is reported to be 71.06% (0.1g NaCl) and 52.4% (0.2gNaCl). Salinity stress reduced the membrane stability index depicted in our results corroborates with results of Ahmad, (2010) [2], who, in mustard demonstrated that exposure to salinity increase membrane leakage. Loss of membrane stability is also attributed to the increased peroxidation of membrane lipids on salinity stressed plants is in agreement with findings of Ahmad, (2010) [2] for mustard and Rasool *et al*., (2013) [40] for chick pea.

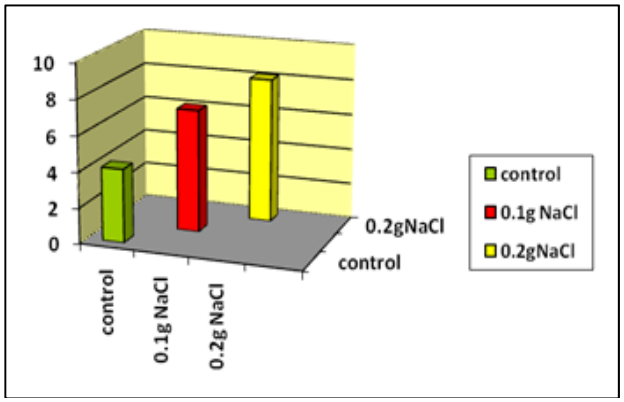
Malondealdehyde

In given table 4, Lipid peroxidation measured in terms of MDA content was reported to increase by 6.12 (0.1g NaCl) and 8.13(0.2g NaCl) treated cow pea plant. The control plant showed onle 4.12 g of MDA. The lipid peroxidation level, as indicated by MDA accumulation, increased significantly under the salt stress. This result is in agreement with the findings of Dionisiases and Tobia, (1998), who

reported an increase in lipid peroxidation in rice leaves during salt stress. However, Sudhakar *et al*., (2001) [47] reported that the level of lipid peroxidation, as indicate by MDA formation, was high in a salt-sensitive cultivar of mulberry (*Morus alba*)

Table 4: Influence of salt stress Malondealdehyde

S. No:	Treatment	Malondialdehyde (MDA) (g)
01	Control	4.12±0.42
02	NaCl(0.1g)	6.87±0.41
03	NaCl(0.2g)	8.13±0.089

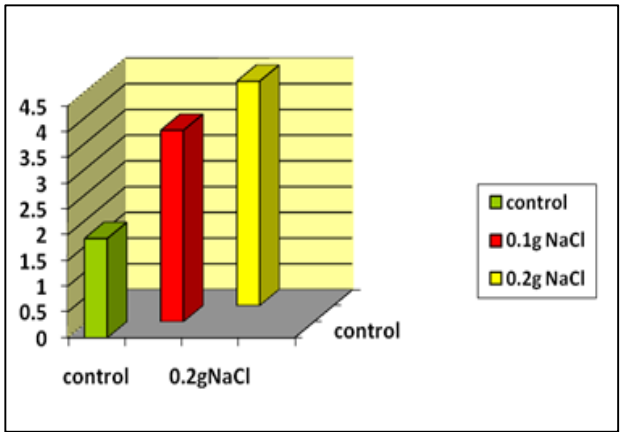


Proline Content

As seen in Table 5, increase in proline content was conspicuous in salt stressed plant. Salt stress 0.1g, 0.2g induced treatment showed a proline content of 3.72 g and 4.36 g respectively, where in the control plant showed 1.93 g only. The increase in proline content under present study is in concordance with Ahmad (2010) [2]; Alqarawi *et al*., (2014) [5] and Harinasut *et al*., (2014a). Harinasut *et al*., (2000) [24] reported manifold increase in proline accumulation in Marus alba plants subjected to the salt stress. Alteration in activities of proline synthesizing and proline degrading enzymes under salt stress has been demonstrated by Alqarawi *et al*., 2014a [25] and Hashem *et al*., 2015 [27].

Table 5: Influence of salt stress on proline content of *Vigna unguiculata*

S. No	Treatment	Proline content
01	Control	1.93 ±0.25
02	NaCl(0.1g)	3.72± 0.21
03	NaCl(0.2g)	4.36±0.42



Enzymatic antioxidants (CAT and POD)

Salinity cause a wide range of responses in plants such as decreased growth, increased osmotic potential and most of important production of ROS due to oxidative stress in the cell. ROS are highly reactive species which readily oxidize protein, lipids, nucleic acid. As reported in many plants both enzymatic and non-enzymatic antioxidant plays an important role in scavenging the ROS. Result of antioxidant content in this study showed that CAT and POD activities increased with salinity stress. Increase in CAT and POD of salinity enhancement was 14.85 (0.1g NaCl) and 12.58 (0.2g NaCl) as compared to 12. 58 in control plant in leaf samples. The activity was recorded as 8.57 (0.1 g NaCl) and 9.48 (0.2 g NaCl) as compared to a 7.16 in control in root samples. From this study, it can be concluded that enzymatic antioxidants CAT and POD do play an important role in cowpea plant under salt stress. It has already been reported CAT and POD plays a major role in ROS scavenging mechanism under salt stress as reported in Barly, Onion, French bean, Wheat, Rice and Horse gram (Yildiz and Terzi, 2013; Kim *et al*., 2008; El-Baky *et al*., 2003 and Babu and Devaraj, 2008) [34, 19, 14].

Table 6: Catalase content in salinity stress in Cow pea plant (*Vigna unguiculata* L)

S. No:	Treatment	Catalase(CAT)	
		Leaf	Root
01	Control	12.58±0.106	7.16±0.029
02	NaCl(0.1g)	14.85±0.07	8.57±0.044
03	NaCl(0.2g)	15.88±0.051	9.48±0,042

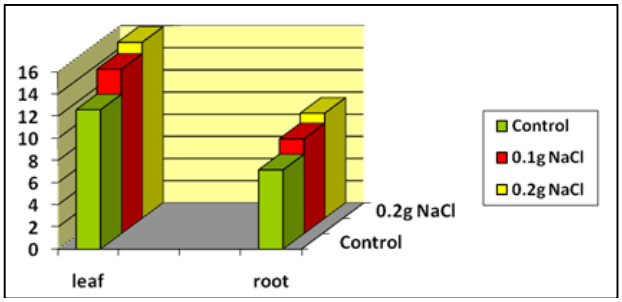


Fig 6: Catalase activities in stress induced study material

Table 7 shows the result of peroxidate enzyme activity in the study plants. High levels of peroxidase enzyme (POD) activity in leaves were found with 0.1g and 0.2g NaCl treatment (95.98 and 102.59 g H_2O_2 mg protein, respectively) with respect to 71.14 mg in leaf tissues of control plant. The values of peroxidase in root tissue in control plant was found to be 1549 mg and the stressed plant showed values 1649.43 mg and 1758 mg for 0.1 g NaCl and 0.2 g NaCl salt concentrations respectively. The results of the study showed an increase in the POD activity. Wang and Han, *et al*. (2009) also found a significant increase in POD activity compared to the control in a similar work. Similarly, Kant and Turan, (2011) [31] found an increase of POD activity in leaves of bean with increasing salinity. Hassanenin *et al*., (2009) observed that salts stress increased the activities of antioxidant enzymes in leves of Zea mays plants. Farag, (2009) [20] reported that in *Pisum sativum* (ev.puget), high concentration of NaCl enhanced the activity of antioxidant. Increased activity of POD is considered to be salt-tolerance mechanism in most plants (Ashraf, 2009; Hu *et al*., 2012) [10, 22].

Table 7: Peroxidase content in Salinity stress in Cowpea plant (*Vigna unguiculata* L)

S. No:	Treatment	Peroxidase	
		Leaf	Root
01	Control	71.14±0.056	1549.36±0.33
02	NaCl(0.1g)	95.98±0.07	1649.43±0.166
03	NaCl(0.2g)	102.59±0.010	1758.1±0.1

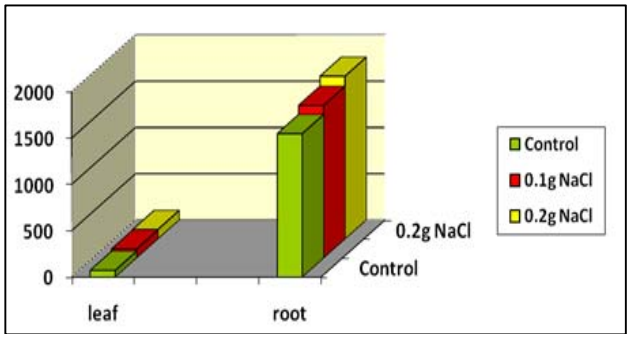


Fig 7: Peroxidase activity in stress induced study material

Anatomy of stem

The cross section (anatomy) of stem of *Vigna unguiculata* was analyzed to assess the effect of various salt concentrations and the anatomical adaptation of this plant to be acclimatized under salinity stress. There was obvious alteration in anatomical feature of stem of cow pea seedings imposed to various level of salinity. Transverse section of stem *V. unguiculata* showed decrease in thickness of upper epidermal layer at 0.1g NaCl and 0.2g NaCl compared to control. The thickness of cortex layers of stem was reduced by different concentration level (Plate 4). The thickness of hypodermal layer and pith area of stem were increased by 0.1g NaCl, 0.2g NaCl treated plants. The pith area got destructed in stressed plant lets of same age as compared to control. There was no significant change observed in the xylem vessels diameter of the stem of cow pea plant. The decrease in the stem cortical cell thickness is mainly due to the collapse to severe salinity (Alam *et al*., 2015) [3]. This may be beneficial to curtail growth under salinity conditions by conserving essential energy for survival. (Alam *et al*., 2015; Nawaz *et al*., 2016) [3, 38]. There was no significant changes in xylem vessel diameter in stem of v. unguiculata with increasing salinity. In contrast to our results, salinity induced decrease in xylem vessels diameter causing decrease in water and mineral conductivity have been reported in many plants. (Sandalio *et al*., 2001; Ortega *et al*., 2006; Rewald at al., 2012; Atabayeva *et al*., 2013) [43, 39, 41, 12].

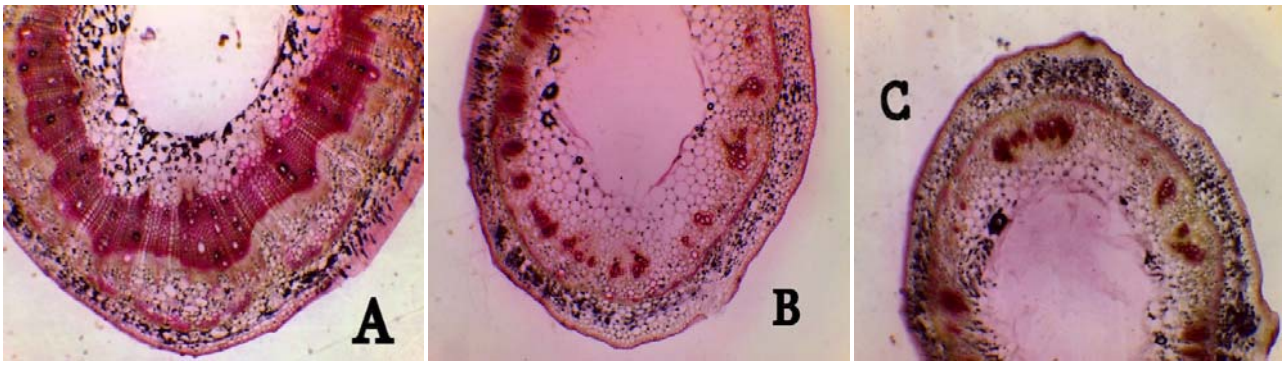


Plate 4: Figures show effects of salinity on stem anatomy (10x magnification) of *Vigna unguiculata* seedling treated with various concentration of salinity: figure (A) Control, figure (B) 0.1g/L NaCl and figure (C) 0.2g/L NaCl

Conclusion

Present study revealed that salinity stress caused a number of morphological and physiological and anatomical changes in the Cow pea plant (*Vigna unguiculata*). The study indicate decreased plant growth, fresh and dry weight of plant, and increase in the Proline content, Malodialdehyde (MDA) as well as activity of antioxidant enzymes (CAT and POD). The study concludes that environmental stress such as salt greatly influence the activity of both Catalase and Peroxidase. Salinity cause oxidative stress which affect the biochemical and enzymatic component in plant cell salt stress.

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