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Impact of ascorbic acid on seed germination, seedling growth and metabolism of salt-stressed *Vigna radiata*

• **Smriti Nandan** • **Anamika Kumari** • **Ranjana Kumari**
• **Pinky Prasad**

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Corresponding Author : Pinky Prasad

Abstract : *In the present study, effect of salinity (50 mM and 100 mM NaCl), and ascorbic acid (0.05 mg/ml and 0.1mg/ml concentrations) and a combination of two was monitored on seed germination, seedling growth and metabolism of Vigna radiata (mungbean). The treatment caused a decrease in germination percentage and germination rate, shoot length, root length, leaf area, fresh weight, dry weight, photosynthetic pigments and an increase in protein, carbohydrate, proline content and acid phoshphatase activity. Ascorbic acid treatment to the salt stress reduced the inhibitory effect of salt stress on germination, seedling growth and photosynthetic pigments and also reduced the protein, carbohydrate, proline content and acid phoshphatase activity. Significant effect was not observed in ascorbic acid treated seedlings, it showed attributes same as that of the control.*

Key words: *Salt stress, Germination, Growth and Metabolism*

Smriti Nandan
B.Sc. III year, Botany (Hons.),
Session : 2014-2017, Patna Women's College,
Patna University, Patna, Bihar, India

Anamika Kumari
B.Sc. III year, Botany (Hons.),
Session : 2014-2017, Patna Women's College,
Patna University, Patna, Bihar, India

Ranjana Kumari
B.Sc. III year, Botany (Hons.),
Session : 2014-2017, Patna Women's College,
Patna University, Patna, Bihar, India

Pinky Prasad
Head, Deptt. of Botany,
Patna Women's College, Bailey Road,
Patna – 800 001, Bihar, India.
E-mail : dr.pinky.prasad@gmail.com

Introduction :

The beginning of 21st century is marked by global scarcity of water resources, environmental pollution and increased salinization of soil and water. Various environmental stresses *viz*. high winds, extreme temperatures, soil salinity, drought and flood have affected the production and cultivation of agricultural crops, among these, soil salinity is one of the most important environmental stresses, which causes major reductions in cultivated land area, crop productivity and quality (Shahbaz and Ashraf, 2013). A saline soil is generally defined as the one in which the electrical conductivity of the saturation extract in the root zone exceeds approximately 40 mM NaCl at 25 °C.

Vigna radiata (mungbean) is an important short duration (65-90 days) legume crop of high nutritive value and nitrogen fixing ability. India is the largest producer and consumer of mungbean. Salinity stress is a major constraint in the production of this crop, where 50 mM NaCl can cause yield losses up to 70% (Saha et.al, 2010). Salt stress similar to many abiotic stress factors, known to induce oxidative damage to plant cells from reactive oxygen species that affect the physiology and biochemistry of plants and that can lead to reduction in yield (Azevedo Neto et.al, 2006). Ascorbic acid is an organic acid with antioxidant properties. Many oxidants, typically reactive oxygen species such as the hydroxyl radical (formed from hydrogen peroxide), contain an unpaired electron and thus are highly reactive and damage plant cells at molecular level. This is due to their interaction with nucleic acid, proteins, and lipids.

The purpose of this study was to observe the influence of ascorbic acid in reducing the inhibitory effect of salt stress on the seed germination, seedling growth and metabolic activity in *Vigna radiata.*

Materials and Methods :

Vigna radiata cv Samrat PD-139 seeds were used in the present study. The seeds were obtained from local market of Patna.

Seeds of uniform size and shape were selected. The selected seeds were surface sterilized by immersion in 0.1% mercuric chloride ($HgCl_2$).

The seeds were soaked in freshly prepared aqueous solution of ascorbic acid of two different concentrations, 0.1mg/ml and 0.05mg/ml for 4 hr at room temperature.

Seeds were divided into six groups, comprising of 50 seeds each. One group of seeds was soaked in distilled water and was treated as Control; one group was soaked only in ascorbic acid solution of concentration 0.1mg/ml. Two groups of seeds were soaked separately in 50 mM and 100 mM concentrations of NaCl respectively and were treated as salt stressed seeds; and the rest two groups of seeds were pre-treated separately with 0.05 mg/ml and 0.1 mg/ml concentrations of ascorbic acid and subjected to 50mM and 100mM concentrations of NaCl, respectively. Seeds were left in dark for 18 hr at room temperature (±23°C).

The soaked seeds were transferred to petriplate containing double layer of filter paper moistened with their respective solutions. After 60 hr the germinated seeds were transferred to the pro-tray containing vermiculite treated with the respective solutions as used during soaking. Vermiculite as a growing medium was chosen because it is rich in minerals and nutrients. Photoperiod of 6 hr light and 10 hr darkness was provided to the seedlings in natural environment.

At a regular interval of 3 days, pro-trays containing seedlings were irrigated with their respective solutions. The 15 days old seedlings were used for the analysis.

Growth measurements in terms of shoot length, root length, leaf area, fresh weight and dry weight was taken.

RWC% was calculated using the following formula;

$$\mathbf{RWC\% = \frac{Fresh\ wt. - Dry\ wt.}{Turgid\ Wt. - Dry\ wt} \times 100}$$

Protein estimation was done by following the method of Lowry et al., (1951). Absorbance was taken at 600 nm. For carbohydrate, method of Dubois et al (1956) was followed and absorbance was taken at 490 nm.

Photosynthetic pigments were estimated as described by Arnon (1949). The optical density of the filterate was measured at wavelengths 663, 645 and 440.5 nm to estimate chlorophyll 'a', 'b' and carotenoids, respectively. The amount of pigment

present in each sample was calculated according to the following equations:

Mg chlorophyll 'a'/g-tissue = 12.7 (O.D.) 663-2.69(O.D.) 645×v/w

Mg chlorophyll 'b'/g-tissue = 22.9 (O.D.) 645-4.68(O.D.)'663×v/w

Mg total chlorophyll/g tissue = 20.2 (O.D.) 645+8.02(O.D.) 663×v/w

Mg carotenoids/g-tissue = 46.95 (O.D.440.5-0.268×chlorophyll'a'+'b')

Where W= the fresh weight by grams for extracted tissue; V= the final size of the extract in 80% acetone; O.D. = optical density at specific wave length.

0.5 g of leaf tissue was homogenized with 10 ml of 3% aqueous sulphosalicylic acid. It was filtered using muslin cloth. The filtrate was centrifuged at 2500 rpm for 10 min. The supernatant was separated. 2 ml of supernatant from each sample was taken in test tubes, 2 ml of glacial acetic acid and 2 ml of acid ninhydrin were added to each of the test tubes. Then, these test tubes were kept in the water bath at 90°C for 1 hr followed by terminating the reaction by placing tubes in ice bath. 4 ml toluene was added to each test tube and stirred for 20-30 sec. Toluene layer was separated and warmed to room temperature. The absorbance was taken at 520 nm.

250 mg of tissue from each treatment and control were extracted in pre-chilled 0.1M sodium acetate buffer (pH- 4.5). The homogenate was centrifuged at 2500 rpm for 15 min and supernatant was collected for enzyme assays. Acid phosphatase activity was assayed by measuring the amount of p-nitrophenol produced.

The assay mixture contained 1 ml 6mM p-nitrophenol phosphate, 2 ml acetate buffer and 1 ml extract in a total volume of 4 ml. This mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of 1 ml of 0.1 N NaOH. The amount of p-nitrophenol liberated was measured by recording absorbance at 400 nm in a spectrophotometer.

Results and Discussion :

The seeds of *Vigna radiata* were soaked in different solutions as mentioned above and grown in petriplates containing double layer of filter papers soaked in the respective solutions as shown in Fig. 1.

Seed germination percentage was reduced by salt stress as compared to the control. At 50 mM NaCl and 100 mM NaCl concentration, germination percentage was reduced by 8% and 28%, respectively. Germination rate was very slow at 100 mM NaCl concentration (Fig. 2).

Ascorbic acid treatment to non-salinized seeds, showed no effects on germination percentage but germination rate was little faster as compared to the control.

At the same time, seeds treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) subjected to 50 mM and 100mM NaCl concentrations, respectively, counteracted the inhibitory effects of salt stress conditions and showed germination similar to the control. A comparative result of the above observations is shown in Figure 2.

After 60 h, the seed sprouts were transferred to the portray containing vermiculite treated with respective solutions as used during soaking. The growth of eight days old seedlings of *Vigna radiata* is shown in Figure 3.

Salt stress resulted in decreased shoot length by 24.42% and 43.01%; root length by 10.91% and 42.55%; leaf area by 40% and 50%; fresh weight by 2.85% and 4.76%; and dry weight by 4.77% and 14.29% in 50 mM NaCl and 100 mM concentrations of NaCl, respectively.

Ascorbic acid treatment to non-salinized condition, showed positive effects on growth of the seedlings. Shoot length increased by 17.58%, root

length by 1.81%, leaf area by 36%, fresh weight by 13.33% and dry weight by 0.952% (Fig. 3).

The seeds treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, respectively, resulted in increased shoot length by 12.31% and 32.82%; decreased root length by 3.64% and 5.46%; and leaf area by 10% and 15%, respectively. Fresh weight decreased by 0.96% in 50 mM and 0.05mg/ml ascorbic acid and increased by 2.85% in 100 mM and 0.1 mg/ml ascorbic acid. The data are tabulated in Table 1.

Protein content increased in 50 mM NaCl and 100 mM NaCl treated seedlings by 9.42% and 13.91%, respectively. Protein value in control was found to be 56.83 mg/gm-dry tissue while in 50 mM NaCl and 100 mM NaCl treated seedlings, it was 60 mg/gm-dry tissue and 62.46 mg/gm-dry tissue, respectively.

Ascorbic acid treatment to non-salinized seedlings resulted in decreased protein value by 2.73%. 53.33 mg/gm-dry tissue protein was found.

The seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, respectively, resulted in decreased protein content (54.62 and 62.46 mg/gm-dry tissue) by 0.38% and seeds treated with 0.1 mg/ml and subjected to that of 100 mM NaCl concentrations, it increased by 9.66% (Fig. 4).

Carbohydrate content increased in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations by 7.15% and 6.22%, respectively. Carbohydrate value in control was found to be 75.61 mg/gm- fresh tissue and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 81.02 mg/gm- fresh tissue and 80.32 mg/gm- fresh tissue, respectively.

Ascorbic acid treatment to non-salinized seedlings resulted in decreased carbohydrate content (60 mg/gm- fresh tissue) by 20.65%.

In seedlings treated with 50 mM NaCl and 0.05 mg/ml ascorbic acid, an increase in carbohydrate content (79.28 mg/gm- fresh tissue) by 4.85% and in 100 mM NaCl and 0.1 mg/ml ascorbic acid treated seedlings an increase in carbohydrate content (78.56 mg/gm- fresh tissue) by 3.91% was observed (Fig. 5).

Under salt stress conditions, the amount of chlorophyll 'a' pigment was considerably reduced. At 50 mM and 100 mM NaCl concentrations, the amount was reduced by 77.28% and 79.27%, respectively. chlorophyll 'a' value in control was found to be 0.352 mg/gm-leaf tissue and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 0.08 mg/gm- leaf tissue and 0.073 mg/gm-leaf tissue, respectively.

Ascorbic acid treatment to non-salinized condition resulted in increased amount of chlorophyll 'a' (0.53 mg/gm-leaf tissue) by 50.56%.

Seedlings treated with 50 mM NaCl and 0.05 mg/ml ascorbic acid concentrations overcame the negative effect of salt and resulted in increased amount of chlorophyll 'a' (0.503 mg/gm-leaf tissue) by 42.89% as compared to the control. However 100 mM NaCl and 0.1 mg/L ascorbic acid treatment though overcame the negative effect of salt to an extent but it was still less (0.22 mg/gm-leaf tissue) by 36.97% than the control (Fig. 6).

Under salt stress conditions, the amount of chlorophyll 'b' pigment was considerably reduced. At NaCl 50 mM and 100 mM concentrations, the amount was reduced by 58.88% and 67.75%, respectively. chlorophyll 'b' value in control was found to be 0.214 mg/gm-leaf tissue and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 0.088 mg/gm- leaf tissue and 0.0695 mg/gm-leaf tissue, respectively.

Ascorbic acid treatment to non-salinized condition resulted in increase in the amount of chlorophyll 'b' (0.330 mg/gm-leaf tissue) by 54.62%.

Seedlings treated with 50 mM NaCl and 0.05 mg/ml ascorbic acid concentrations overcame the

negative effect of salt and resulted in increased amount of chlorophyll 'b' (0.305 mg/gm-leaf tissue) by 42.94% as compared to the control. However, 100 mM NaCl and 0.1mg/L ascorbic acid treatment overcame the negative effect of salt to an extent but it was still less (0.157 mg/gm-leaf tissue) by 26.64% as compared to the control (Fig. 7).

Under salt stress conditions, the amount of carotenoids was considerably reduced. At 50 mM and 100 mM NaCl concentrations, the amount was reduced by 66.75% and 77.47%, respectively. Carotenoids value in control was found to be 1.829 mg/gm-leaf tissue and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 0.608 mg/gm- leaf tissue and 0.412 mg/gm-leaf tissue, respectively.

Ascorbic acid treatment to non-salinized condition resulted in increased amount of carotenoid (2.61 mg/gm-leaf tissue) by 42.70%.

Seedlings treated with 50 mM NaCl and 0.05 mg/ml ascorbic acid concentrations overcame the negative effect of salt and also resulted in increased amount of carotenoid (2.734 mg/gm-leaf tissue) by 49.48% as compared to the control. 100 mM NaCl and 0.1 mg/ml ascorbic acid treatment overcame the negative effect of salt to an extent but it was still less (1.386 mg/gm-leaf tissue) by 24.17% than the control (Fig. 8).

Under salt stress conditions, the content of proline was increased as compared to the control. The amount of proline was increased considerably by 65.57% in 50 mM NaCl and 4.91% in 100 mM NaCl treated seedlings, respectively. Proline value in control was found to be 30.5 µg/gm- ml and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 50.5 µg/gm-ml and 32 µg/gm-ml, respectively.

Ascorbic acid treatment to non-salinized condition resulted in decreased proline level (24 µg/gm- ml) by 21.32%.

The seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, resulted in low level of proline by 6.23% and 13.12%, respectively, as compared to the salt stressed values and these were close to control value. The seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, level of proline was found to be 28.6 µg/gm- ml and 26.5 µg/gm- ml, respectively (Fig. 9).

Acid phosphatase activity increased during salt-stress. At lower salt concentration i.e., 50 mM salt concentration, it increased by 131.57% and at 100 mM NaCl concentration, it was 54.88%. Acid phosphatase value in control was found to be 133 µmol/min.ml and in the seedlings treated with 50 mM NaCl and 100 mM NaCl concentrations, it was 308 µmol/min.ml and 206 µmol/min.ml, respectively.

Ascorbic acid treatment to non-salinized condition resulted in a little decrease in acid phosphatase activity (130 µmol/min.ml) by 2.26%.

Seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, showed increased acid phosphatase activity by 59.39% and 28.57%, respectively, as compared to the control. The seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, acid phosphatase activity was found to be 212 µmol/min.ml and 171 µmol/min.ml, respectively (Fig. 10).

Salt stressed seedlings resulted in reduced RWC % values as compared to the control. A reduction in RWC % by 1.51% and 3.14% at 50 mM and 100 mM NaCl, respectively was observed.

Ascorbic acid treatment to non-salinized seedlings also resulted in reduction of RWC% by 1.22%.

The seedlings treated with different concentrations of ascorbic acid (0.05 mg/ml and 0.1 mg/ml) and subjected to 50 mM and 100 mM NaCl concentrations, respectively, resulted in less

increment in RWC% as compared to the salt stressed conditions but it was still less than the control. For 0.05 mg/ml ascorbic acid and 50 mM NaCl concentration, it was 81.80 and for 0.1 mg/ml and 100 mM NaCl concentration it was 79.59 (Table 2).

It was observed that salt stress resulted in decreased germination percentage and germination rate. However, pre-treatment of seeds with ascorbic acid overcame the inhibitory effects of different concentrations of sodium chloride.

Growth of the seedlings in salt stress condition was reduced. Reduced shoot length, root length, leaf area, fresh and dry weight occurred in salt stress conditions. Addition of ascorbic acid counteracted the negative effects of salt stress on growth.

Biochemistry of the seedlings was affected by the salt stress conditions. Protein and Carbohydrate content had increased during salt stress which agrees with the result of Singh *et.al,* 1987 and Parida et.al, (2002). Photosynthetic pigments chlorophyll 'a', chlorophyll 'b' and carotenoids were affected negatively by salt stress. Salinity caused swelling of membranes in chloroplasts of sensitive plants which affects their chlorophyll content, or due to excess ion in leaves which induced loss of chlorophylls (Wahid et.al, 2004). Applications of ascorbic acid to the salt stressed seedlings showed biochemical contents like that of control.

Proline content increased during salt stress. Salt stress causes significant increases in proline contents, an important metabolite, with increasing salt concentration (Misra et.al, 1996). However, in the present investigation proline accumulation decreased with increasing salt concentration.

Addition of ascorbic acid to the salt stressed seedlings brought proline level close to that of the control.

Acid phosphatase enzyme activity was increased during salt-stress which is similar to the reportings of Olmos and Hellin, 1997. However, acid phosphatase enzyme activity was decreased on increasing salt concentration. Ascorbic acid treated seeds subjected to salt stress shown to have acid phoshphatase enzyme activity more than the control but less than the salt stressed seedlings.

Relative water content percentage was decreased in salt stressed seedlings. The same was a little increased in seedlings treated with ascorbic acid and subjected to salt stressed condition as compared with the salt stresed seedlings. This is similar to the result interpreted by Romero-Aranda et.al, (2001).

Dehghan et.al, (2011) reported that exogenously applied ascorbic acid counteracts the adverse effects of salt stress on growth of *Glycine max* seedlings which was cultivar specific. Salt stress is one of the most harmful environmental stresses which reduce the plant growth and development. Among the various abiotic stresses, osmotic stress (drought and salinity) causes a variety of biochemical, physiological and metabolic changes in plants, ultimately reducing the yield (Xiong and Zhu, 2002).

Conclusion :

In this study, it has been observed that salt stress negatively affects the seed germination, seedling growth and metabolic activities of the plant resulting in poor growth and development and hence reducing the productivity. It has also been observed that pre-treatments of seeds with ascorbic acid and then subjecting it to salt stress, overcame the inhibitory effects of salt stress and had growth and metabolic activities like normal seedlings. Therefore it may be concluded that, before sowing the seeds in saline soil, it should be pre-treated with ascorbic acid according to the concentration of the salt in the soil to have better productivity.

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Table 1. Growth measurements of 15 days old seedlings of *Vigna radiata*

Growth Parameters	Control	Effect of Ascorbic acid	Effect of salinity		Effect of ascorbic acid treated salt stressed conditions	
			50 mM NaCl	100 mM NaCl	0.05 mg/ml Ascorbic acid and 50 nM NaCl	0.1 mg/ml Ascorbic acid and 100 mM NaCl
Shoot length	17.86 cm	21.00 cm	13.50 cm	10.18 cm	20.06 cm	12.00 cm
Root length	5.50 cm	5.6 cm	4.90 cm	3.16 cm	5.30 cm	5.20 cm
Leaf Area	2cm^2	2.27cm^2	1.20 cm^2	1.00cm^2	1.80cm^2	1.70 cm^2
Fresh Weight	1.05 gm	1.19 gm	1.02 gm	1.00 gm	1.04 gm	1.08 gm
Dry Weight	0.105 gm	0.106 gm	0.100 gm	0.090 gm	0.104 gm	0.100 gm

Table 2. Effects of respective treatments on Relative Water Content (%)

Treatments	Control	Effect of Ascorbic acid	Effect of salinity	Effect of ascorbic acid treated salt stressed conditions		
			50 mM NaCl	100 mM NaCl	0.05 mg/ml Ascorbic acid and 50 mM NaCl	0.1 mg/ml Ascorbic acid and 100 mM NaCl
Relative water Content (%)	82	80.78	80.49	78.86	81.80	79.59

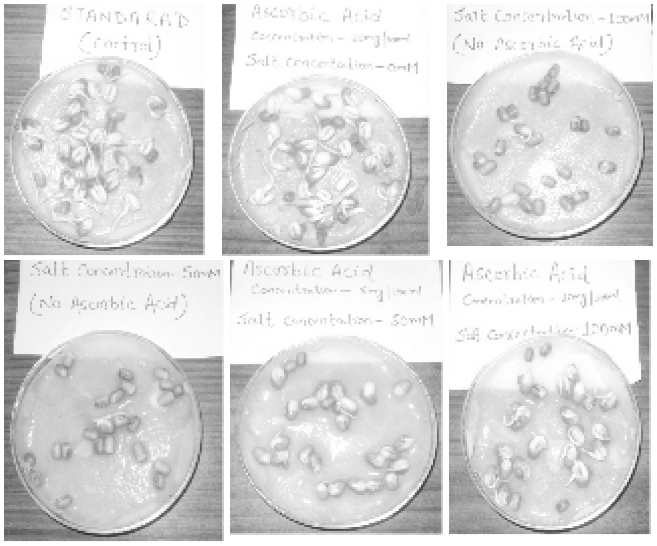

Fig. 1. Germinating seeds of *Vigna radiata* in different solutions

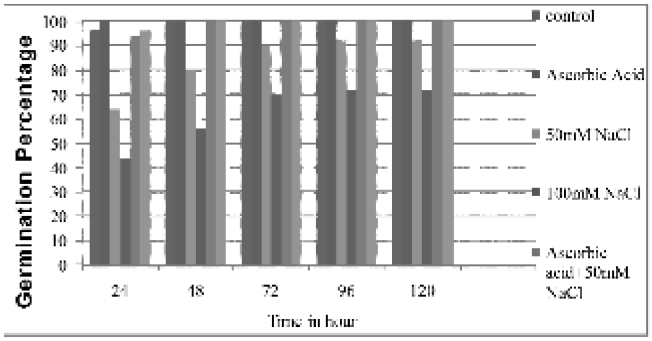

Fig. 2. Effect of respective treatments on seed germination in the seeds of *Vigna radiata*.

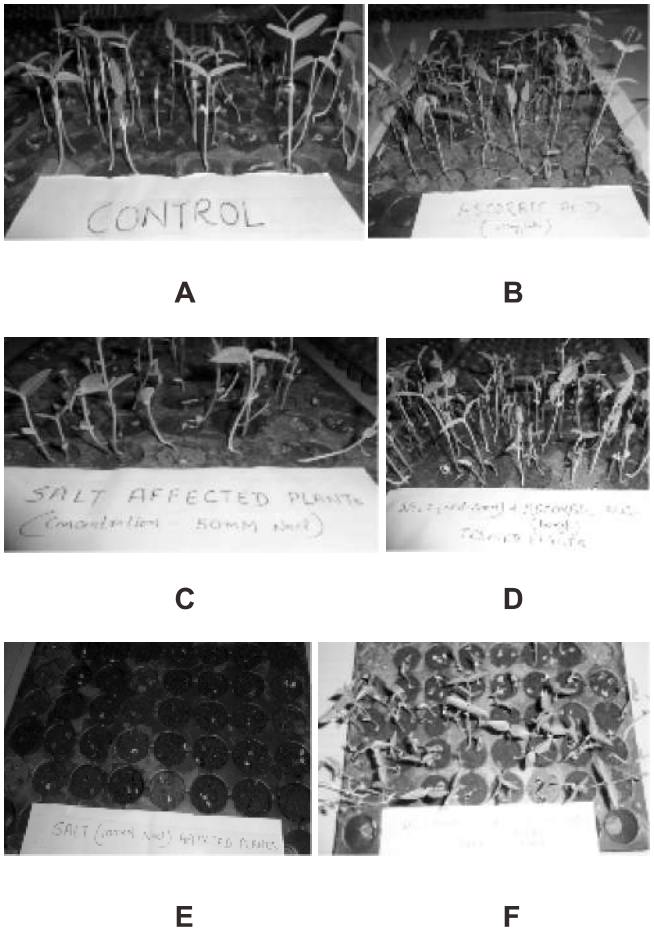

Fig. 3: Eight days old seedlings of *Vigna radiata* under different treatments (A): Control, (B): Ascorbic Acid (0.1 mg/ml) treated seedlings, (C): 50 mM NaCl treated seedlings, (D): 0.05 mg/ml and 50 mM NaCl treated seedlings, (E): 100 mM NaCl treated seedlings and (F): 0.1 mg/ml and 100 mM NaCl treated seedlings growing in portray containing vermiculite.

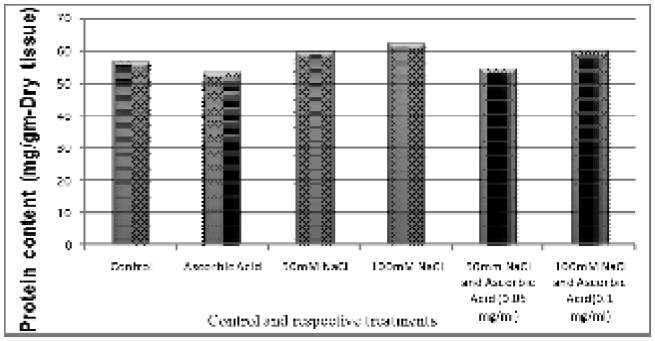

Fig. 4. Effect of respective treatments on Protein content

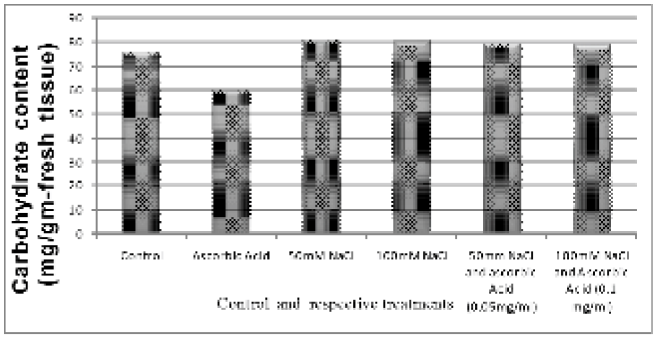

Fig. 5. Effect of respective treatments on Carbohydrate content

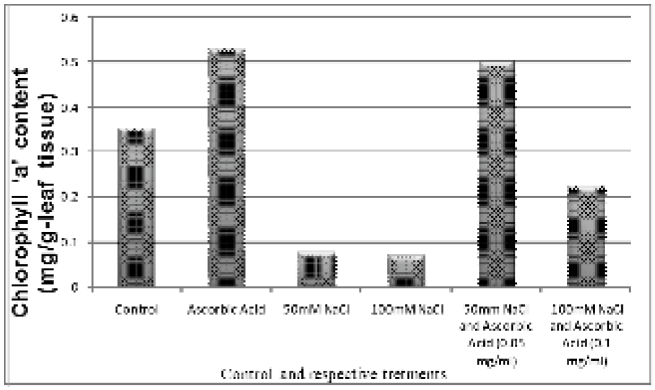

Fig. 6. Effect of respective treatments on Chlorophyll 'a' content

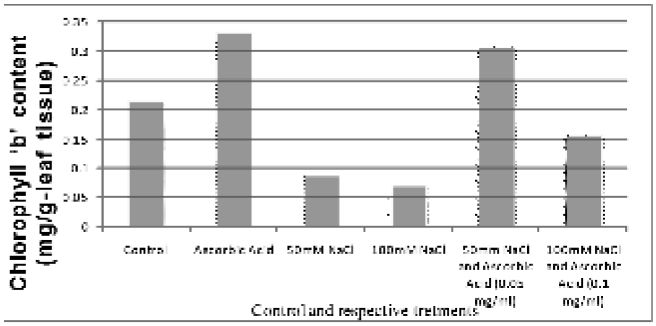

Fig. 7. Effect of respective treatments on Chlorophyll 'b' content

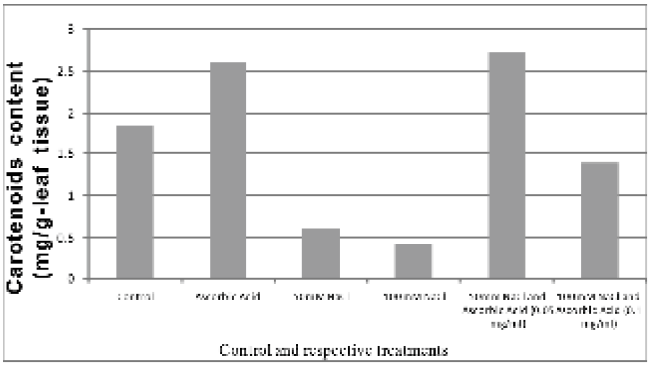

Fig. 8. Effect of respective treatments on Carotenoids content

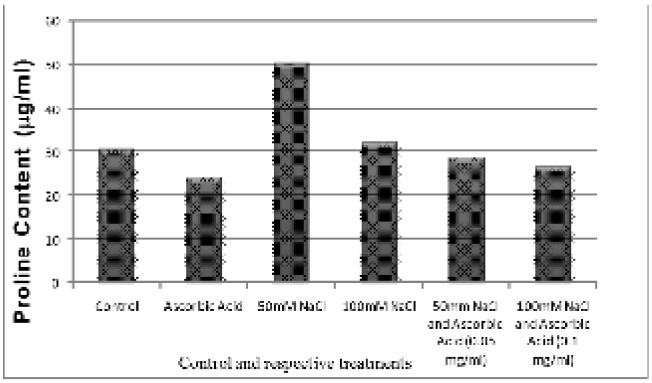

Fig. 9. Effect of respective treatments on Proline content

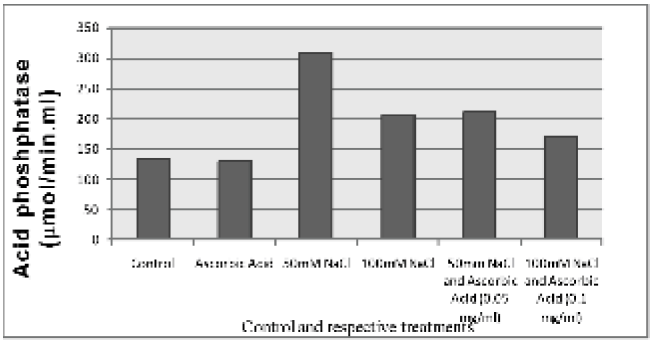

Fig. 10. Effect of respective treatments on acid phosphatase enzyme activity

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