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# EFFECT OF SALINITY STRESS ON THE PHYSIOLOGY OF CHICKPEA (CICER ARIETINUM)

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## ABSTRACT

Chickpea is an extremely important crop plant. Climate change is causing water scarcity, increased salinity in soil and elevated carbon dioxide levels. These factors affect agriculture in a big way. The nutritional content of the crops is also getting threatened due to these alterations in the environment. The present study was undertaken to investigate the effect of increased concentration of salinity on germination of Cicer seeds and associated effects on the content of proline, total soluble protein, concentration of $H_2O_2$ and the antioxidant enzyme catalase in the desi variety of chickpea. The study clearly showed the negative impact of saline conditions on the germination of Cicer seeds. High salt concentrations while declining the percentage of germination also delayed the process of onset of seed germination. Further, the levels of $H_2O_2$ and the antioxidant enzyme catalase were found to decline with the increasing salt concentration. However, the study showed increased levels of total extractable proteins and proline.

**KEYWORDS:** Chickpea (Cicer arietinum L.), Germination, Salinity Stress, Proline, $H_2O_2$, Catalase.

## INTRODUCTION

In the present-day scenario, the abiotic stresses like salinity, elevated temperature, and drought are responsible for most of the reduction in crop yield. Climate change due to several anthropogenic factors is responsible for the decline in the crop productivity and the nutritional quality of the crops. Climate change is affecting agriculture due to change in the biotic and the abiotic component of the environment. The most common abiotic stress factor is the presence of NaCl in the soil. Climate change has impacted the entire globe and as a major consequence a large area of cultivable land is turning into highly unfertile barren land masses. Droughts and elevated temperatures are compounding the problem resulting in high salinity. Soils with high salt concentrations are responsible for several physiological or metabolic disorders in the plants due to ionic imbalance, decreased stomatal conductance and consequent reduced photosynthetic efficiency. High NaCl concentrations have been shown to inhibit several enzymes in crop plants. Chickpea is one of the most important legume crops for human nutrition grown in arid and semi-arid regions and is considered to be a salt sensitive species.[1] Cicer arietinum is a crop which is most universally utilized as a rich source of proteins. It is also used as a source of nutritious fodder for animals. Cicer being a leguminous crop has a crucial role to play in contributing to the fertility of soil.[2] It enriches soil nitrogen through its symbiotic association with Rhizobium. However, symbiotic nitrogen fixation by legumes is sensitive to environmental stresses, particularly to salinity.[3] High salt concentrations have shown to affect not only the overall plant growth but also cause ionic imbalance in the cell milieu. This stress caused due to ionic imbalance affects the symbiotic relationship between the legume and the Rhizobia.[4] Reduced plant productivity in legumes is attributed to diminished photosynthetic efficiency, nitrogen fixation and carbon metabolism.[5] Cicer crop exhibits adverse effects on cell metabolism under conditions of salinity. The effects are manifested more during the reproductive stage of the growth and development[6] In a comparative study it was observed that the form of chickpea kabuli was more vulnerable to increased soil salinity when compared with the desi variety.[7] In Chickpea study has shown that the percent germination of seeds is low as well as the onset of germination is also delayed.[8] Vegetative plant growth has also been reported to be suppressed under high salt concentrations.[9,10] It has been suggested that tolerance of chickpea seeds to increased salt stress is dependent upon the plant's genotype.[11] It therefore must be kept in mind that different species may exhibit different levels of stress tolerance. Further, it has also been shown that some plants exhibit salt tolerance at moderately high concentrations of NaCl. It is suggested that stress tolerance is attributed to pathways which help in the retention of water under high salt concentration, prevent chloroplast malfunctioning and despite high

salinity are able to maintain ionic balance in the plant. Increased uptake of salts continues till the plant has the ability to maintain endogenous osmotic adjustments. Once that capacity is exhausted extra salt starts moving towards the intercellular spaces. This leads to loss of water due to exosmosis.[12] Severe and prolonged salt stress can thus lead to dehydration and the plant may eventually perish. It is extremely essential to understand the mechanism adapted by the salt tolerant species so that attempts can be made to genetically engineer the crop plants to be salinity resistant. Research in this direction will not only increase crop productivity but will also help in utilizing and expanding agriculture to salt rich barren lands. In a biochemical study it has been demonstrated that salinity stress leads to oxidative burst, which generates reactive oxygen species (ROS) like $H_2O_2$, hydroxyl radicals, peroxy radicals, singlet oxygen etc. Production of these free radicals create a secondary stress in the cell in response to the primary salinity stress. The effect can therefore be countered only if there is increased synthesis of antioxidant enzymes or synthesis of some osmotically active molecules or chaperons which will help in maintaining ion balance and countering the stress of ROS thus produced.[13]

However, there are reports of some positive effects of moderate salt stress in certain vegetable crops. Moderate saline conditions have been shown to contribute towards the colour, firmness, aroma and taste of several fruits and vegetables. This has tremendous economic implications. Tomato plants have shown an improvement in its quality under conditions of moderate salt stress. Researchers have shown that exposing tomato plants to moderate stress helps to improve the nutritional quality of the tomato plant. Application of such positive stress/ moderate salinity can be utilized in obtaining desired results in plants of interest. In a study,[14] it was shown that there was increased production of phytochemicals like lycopene, vitamin C and beta carotenes in tomato plants grown in moderate salt concentrations. Therefore, it is important to understand the metabolic pathway adopted by these salt tolerant plants. They appear to be affecting carbon metabolism, nitrogen fixation and secondary metabolite production to name a few. Study on the effect of salinity on different crops and different species of the same genus may exhibit different results.

## MATERIAL AND METHODS

**Plant Material** – Seeds of Cicer arietinum were obtained from Indian Agriculture Research Institute, New Delhi. Seeds were used to check the effect of salinity on various parameters. After the process of surface sterilization, the seeds were used for germination and raising of seedlings under controlled culture conditions. They were grown in Murashige and Skoog (MS) medium containing different concentrations of sodium chloride for five days.

**Preparation of Culture Media** – Various concentrations of salt (0 mM – 200 mM) were prepared using MS medium. Five different culture media were thus prepared with 0 mM, 50 mM, 100 mM, 150 mM and 200 mM NaCl. Culture media were poured in different culture bottles. These bottles were autoclaved for 20 minutes at 100 kpa.

**Seed Germination** – Healthy and homogenous seeds of Cicer arietinum were used for experiments. Seeds were surface sterilized with 0.1% $HgCl_2$ for 3 minutes, washed with double distilled water and then about 10-12 seeds were inoculated in culture bottles containing culture medium with different salt concentrations inside the laminar air flow. All the inoculated bottles were incubated at 28 degrees, with a light-dark cycle of 16/8 hours in the culture room. Percent germination of seeds was calculated by recording the number of germinated seeds in all the sets every day. After 5 days of germination, seeds were taken out to determine the content of protein, hydrogen peroxide, proline and catalase.[15]

**Estimation of Proline** – After removing the seed coat from the germinated seeds, seedlings were weighed and homogenized in 3 percent sulphosalicylic acid, and then centrifuged at 15,000 rpm for 20 minutes at 22 degrees centigrade. One ml each of ninhydrin and glacial acetic acid were added to one ml of the supernatant obtained after centrifugation. This mixture was kept in a water bath at 100 degrees centigrade for one hour. The reaction was then terminated in an ice bath. To the terminated mixture 4ml toluene was added and then mixed in a cyclomixer. Toluene layer separated from the aqueous layers. Absorbance was measured at 520 nm against toluene as the blank. Proline concentration (micromole /g fresh weight) was calculated using the formula-

Proline content = OD x 33 x Dilution Factor x Tissue Extract x 100/ fr. wt.

**Estimation of Hydrogen Peroxide**

Seedlings were homogenized in 3ml of 5% trichloroacetic acid and then centrifuged at 10,000 rpm for 10 minutes at room temperature. To 1ml of this supernatant 4ml fox solution was added. After vortexing the contents were kept for 30 minutes at room temperature to allow the reaction to take place. Absorbance was measured against 1ml of 5% TCA and 4 ml of Fox solution as blank. Concentration of $H_2O_2$ was calculated using a standard curve prepared and expressed as micro mol/ G fresh wt.

$H_2O_2$ content = OD x 9.47x 1000 / fr wt.

**Estimation of Proteins**

Germinated seedlings were homogenized in an extraction buffer and centrifuged at 10,000 rpm for 30 minutes. The supernatant was used to estimate the total proteins by Bradford's method. The absorbance was measured at 595 nm. The protein content was calculated from a standard curve prepared and expressed as mg/ fresh wt.

**Estimation of Catalase**

The activity of the enzyme was measured by taking 2ml of 50mM potassium phosphate buffer (pH 6.5), 20 microlitre of 50% $H_2O_2$ and 50 microlitre of enzyme and taking the absorbance at 240 nm against a blank of 2ml of 50mM potassium phosphate buffer pH 6.5 and 20 microlitre of $H_2O_2$. The absorbance was taken at an interval of 15 seconds over a time period of 3 minutes. The absorbance was followed by a decrease in absorbance. The activity of the enzyme was expressed as micro mol catalase oxidized / minute /mg protein.

The activity of catalase was calculated using the formula:

Y = OD x 30 x 1000 x 1000 / fr wt x Extinction Coefficient

Catalase oxidized /min/mg protein= Y/Protein Content

**RESULTS**

**Seed Germination**

Cicer seeds were germinated under the variable concentrations of NaCl (0-200mM). Germination of seeds started from the second day in bottles with salt concentration up to 100mM. However, there were no signs of germination in seeds, treated with NaCl concentration of 150 and 200 mM at the end of two days. After 5 days there was significant germination in seeds with salt treatment up to 100mM. Seeds started germinating after two days in sets with higher salt concentration.

At the end of five days percent germination was fairly high (91.67%) in untreated and 50mM salt treated seeds. Higher salt concentration resulted in a decline of percentage of germination. At 200 mM strength the decline in germination was to the effect of 54.54%. This suggested negative effect of salinity stress on the process of germination in Cicer seeds (Table: 1).

**Table 1: Percent germination of Cicer seeds as a function of NaCl concentration in the culture medium.**

| Strength of NaCl (mM) | No. of seeds /bottle | No. of seeds germinated/bottle | Germination % = Germinated seeds x 100 / Total no. of seeds |
|---|---|---|---|
| 0 | 12 | 11 | 91.67 |
| 50 | 12 | 11 | 91.67 |
| 100 | 12 | 9 | 75.00 |
| 150 | 11 | 8 | 72.73 |
| 200 | 11 | 6 | 54.54 |

**Proline Accumulation**

The results clearly depicted a tremendous rise in the level of proline at different concentrations of NaCl. Content of proline showed a sharp increase in seeds treated with higher salt concentration compared to low NaCl treated seeds. Its levels rose from 0.3414 to 341.4 micro mol/gm fresh weight at 200 mM NaCl concentration (Table: 2).

**Table 2: Effect of NaCl concentration on the Proline concentration in germinating Cicer seeds.**

| Strength of Nacl (mM) | Fresh Wt. (mg) | O.D. | Proline content (micro mol/g fr. wt.) |
|---|---|---|---|
| 0 | 1336.7 | 0.461 | 0.3414 |
| 50 | 1141.7 | 0.765 | 66.3353 |
| 100 | 1056.0 | 1.689 | 158.3438 |
| 150 | 846.8 | 2.298 | 265.6608 |
| 200 | 788.9 | 2.721 | 341.4615 |

**Concentration of Hydrogen Peroxide**

Unlike the content of Proline, the level of hydrogen peroxide under the increasing concentrations of salinity were found to drop significantly from 0.49 nmol/gm fr wt to 0.20 nmol/gm fr wt (Table:3)

**Table 3: Effect of NaCl concentration on the concentration of $H_2O_2$ in germinating Cicer seeds.**

| Strength of NaCl (mM) | Fresh Wt. (mg) | O.D. | $H_2O_2$ (n mol/g f.wt) |
|---|---|---|---|
| 0 | 1258.4 | 0.066 | 0.4967 |
| 50 | 1214.0 | 0.058 | 0.4524 |
| 100 | 1004.4 | 0.045 | 0.4243 |
| 150 | 853.7 | 0.019 | 0.2108 |
| 200 | 779.3 | 0.017 | 0.2066 |

**Total Soluble Proteins**

Amount of total soluble proteins showed a marked increase in the concentration of proteins from 0.11mg/fr wt to 0.42 mg/fr wt thereby depicting an overall increase of about 3.7 times (Table:4).

**Table 4: Effect of NaCl concentration on the content of total soluble protein in germinating Cicer seeds.**

| Strength of Nacl(mM) | Fresh Wt. (mg) | O.D. | Protein content (mg)/g f.wt |
|---|---|---|---|
| 0 | 1407.6 | 0.455 | 0.1164 |
| 50 | 1227.3 | 0.666 | 0.1954 |
| 100 | 951.6 | 0.921 | 0.3484 |
| 150 | 891.6 | 0.932 | 0.3763 |
| 200 | 792.7 | 0.945 | 0.4292 |

**Estimation of Catalase Activity**

High salt concentration had a tremendous effect on the activity of the enzyme catalase**.** The activity of the enzyme declined from 860 micromoles/ min/mg protein to 46.29 micromoles/ min/mg protein (Table:5).

**Table 5: Effect of NaCl concentration on the activity of the antioxidant enzyme catalase in germinating Cicer seeds.**

| Strength of NaCl (mM) | Fresh Wt. (mg) | O.D. | Y (micro mol /min/g fr.wt) | Activity (micro mol enzyme /min/mg protein) |
|---|---|---|---|---|
| 0 | 1407.6 | 0.188 | 100.1705 | 860.5713 |
| 50 | 1227.3 | 0.131 | 80.0538 | 409.6919 |
| 100 | 951.6 | 0.100 | 78.8146 | 226.2187 |
| 150 | 891.6 | 0.052 | 43.7416 | 116.2413 |
| 200 | 792.7 | 0.021 | 19.8688 | 46.2926 |

## DISCUSSION

In our study, experiments were designed to investigate the effect of salinity stress on various parameters like germination, protein concentration, proline concentration, levels of $H_2O_2$ and the antioxidant enzyme catalase in Cicer arietinum.

It was very apparent that there was germination of seeds under increasing concentrations of NaCl but the process was much slower under high salt concentration. The results suggest that high salt concentration retards the process of germination but does not completely inhibit it. The increased salt concentration was found to exhibit its impact also on the amount of free proline in the system. The role of accumulated proline is still not very clear. There are certain studies which indicate the osmo - protective role of proline under stress conditions. It has been demonstrated that it acts as a metal chelator besides being an antioxidative amino acid molecule.[16] It is also hypothesized that higher proline concentration is responsible for inhibition of growth.

Protein concentration was found to be higher under conditions of high salinity. It is speculated that the increase in protein content could be because of better extraction and not because of higher rates of synthesis of proteins.

The content of $H_2O_2$ was observed to decline as a result of increasing salt concentration. Release of $H_2O_2$ during conditions of abiotic stress is normally considered to be the earliest response of plant cells towards attempted invasion of phytopathogenic microbes. In our study we observed decline in the levels of $H_2O_2$ which indicates probably a signal towards protective mechanism.

Further study of the activity of the antioxidant enzyme catalase further exhibited decline in activity as a result of increasing salt concentration. The reduced activity of this enzyme and simultaneous observation of reduced $H_2O_2$ concentration under the conditions of high salinity indicate their role in overall metabolism of the plant during its germination period.

Cicer being a leguminous crop has its roots in association with Rhizobia. It has been proposed that the soil microbes exert a very positive influence on the growth of plants growing under conditions of abiotic stress. Rhizospheric bacteria are known to influence plant metabolism in a manner that they exhibit tolerance towards abiotic stresses like drought, metal toxicity, chilling and salinity.[17] They are thus a big factor towards helping in alleviation of salt stress. Future research in establishing association of microbes with plant roots can prove to be immensely beneficial in combating problems associated with salinity.

## CONCLUSION

Our study has shown that salinity has a profound effect on the physiology of Cicer seed germination and subsequent seedling growth. It was observed that seeds grown under saline conditions exhibited a tremendous drop in percent germination of the Cicer seeds. This abiotic stress therefore is responsible for reduction in crop yield. Salinity stress also resulted in the increased levels of proline. These molecules appear to act as signalling molecules under the conditions of salt stress.

Abiotic stresses, including salinity stress, are instrumental in bringing about positive or negative effects at various levels of cell growth and metabolism. Stress has also been shown to cause altered gene expression. However, there is still a long way to go in completely understanding the molecular mechanisms behind it. The need of the hour is to develop more and more salt resistant varieties so that the increased levels of salinity due to climate change do not pose problems in causing diminished crop productivity. At present It is essential to understand the mechanism to overcome vulnerability of legumes to saline conditions. These resistant varieties can be used to reclaim marginal lands. Their ability to enrich soil nitrogen through symbiotic association with nitrogen fixing bacteria will contribute in improving crop productivity under changing climatic conditions.

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Shruti Kandwal performed the review, data collection, experiments and writing of the manuscript.

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