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Research Article

# In Vitro Evaluation of Salt Stress on Seed Germination, Seedling Growth and Biochemical Parameters in Chilli (*Capsicum annuum* L.)

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**Abstract**

Salinity is an ever-increasing abiotic threat limiting crop produce. The effect of in vitro salt stress on seed germination, seedling growth and biochemical parameters was studied in chilli (Capsicum annuum L.) an important spice crop susceptible to salinity. Seeds of two commercial genotypes (Pusa Jwala and Sitara) were germinated on Murashige and Skoog's basal medium supplemented with graded series of salt stress (0, 60, 80, 100 and 120 mM NaCl). The seed germination percentage, shoot length, root length and fresh weight showed a declining trend with increasing levels of salt stress. The root to shoot ratio reduced indicating that the root growth was more susceptible compared to shoot growth. Sitara maintained higher seedling vigor index, germination stress tolerance index, shoot and root length stress tolerance index as compared to Pusa Jwala. The chlorophyll content declined while there was increment in proline under salt stress. The significance of this study is in rapid screening of commercial chilli genotypes for salinity stress using in vitro techniques and selection of tolerant genotypes for cultivation in salt affected areas.

**Keywords:** *chilli, salinity, germination, in vitro, chlorophyll, proline, abiotic stress.*

## Introduction

India is known as the "Spice Bowl" of the world due its rich diversity of spices which are relished around the globe. Chilli or Hot Pepper (*Capsicum annuum* L.), a member of Solanaceae family, is a popular spice cultivated over more than 7 lakh hectares in India. In 2022, the production of dry chillies ranged at 1.8 million tons in India which is highest in the world followed by Bangladesh, Ethiopia, Thailand and China (FAOSTAT). The ripe dried fruits of chilli are cherished for their taste, pungency, flavor and colour (Geetha and Selvarani, 2017). Capsaicin an alkaloid present in the placenta of the fruit imparts pungency (Chakrabarty, *et al.*, 2017) while capsanthin a carotenoid gives colour to the ripe fruits (Berry, *et al.*, 2021). Chillies are good source of vitamin A, C, E, act as powerful stimulant, carminative and also aid in the treatment of arthritis, rheumatism, herpes zoster etc. (Kochhar, 2009).

Salinity is an ever-increasing abiotic threat limiting crop produce. There are several reasons for increment in salt affected land. Some of the reasons are irrigation with low-quality water and unsustainable irrigation practices, deforestation and loss of deep-rooted vegetation, excessive use of fertilizers, over exploitation of coastal aquifers, inadequate drainage, and seawater intrusion into coastal areas (FAO). Nearly 2% of total agrarian land in India is affected by salinity (Mukhopadhyay, *et al.*, 2020). Salt-affected soils impair crop water uptake due to reduced water potential in the root zone, create nutritional imbalance because of lowered absorption and/or shoot transport of micronutrients. They also concentrate $Na^+$ and $Cl^-$ ions that are toxic to plants and may destroy soil structure making it unfit for cultivation (Parida and Das, 2005, Muhammad, *et al.*, 2024).

Chilli is moderately sensitive to salt stress therefore saline soils have negative impact on its production (Butt, *et al.*, 2016, Bhutia, *et al.*, 2018, Zamljen, *et al.*, 2022). Upon exposure to

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salt stress there is reduction in germination percentage, poor stand establishment, impaired growth and biochemical changes in chilli (Khan, *et al.,* 2009; Bhutia, *et al.,* 2018). Seed germination is the most sensitive stage towards salinity stress (Wu, *et al.,* 2019) thus, screening chilli during seed germination and early seedling stage for salinity tolerance is important for its successful establishment in salt affected soils. Direct screening for salinity stress under field conditions is not possible because salinity levels vary depending upon the location and climatic factors. In vitro systems provide a uniform and controlled environment to study the impact of salt stress in plants, are rapid and less labour intensive compared to field screening (Rai, *et al.,* 2011, Ozdemir, *et al.,* 2016, Sahu, *et al.,* 2023). Therefore, the present investigation was carried out to examine the effect of salt stress on seed germination, seedling growth, chlorophyll and proline content in chilli using tissue culture technique.

## Materials and Methods

### In vitro seed germination

The seeds of commercial chilli (*Capsicum annuum* L.) genotypes Pusa Jwala and Sitara were procured from certified seed agencies (Pune, Maharashtra, India). The seeds were surface sterilized with 0.1% $HgCl_2$ for 5 min., rinsed thrice with sterile distilled water. They were briefly blotted and placed on Murashige and Skoog's basal medium (Murashige and Skoog, 1962) supplemented with graded series of NaCl (0, 60, 80, 100 and120 mM), sucrose (3%, w/v) and agar (0.8%, w/v) in culture tubes (25x100 mm). Non saline medium served as control. The pH of the medium was adjusted to 5.8±0.1 before autoclaving at 121$^{o}$C and 1.1 Kg cm$^{-2}$ for 20 min. The cultures were kept in complete darkness for germination. The germinated seedlings were maintained under 16-h light and 8-h dark photoperiod maintained by cool white fluorescent tube light of 32 µmol m$^{-2}$ s$^{-1}$ intensity and 25±4 $^{0}$C temperature. The seeds in which radicle emerged were considered germinated. Germination response was monitored daily for two weeks. Germination percentage was calculated as follows:

$$\text{Germination \%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds kept for germination}} \times 100$$

### Seedling Growth Parameters

The in vitro grown seedlings were harvested after six weeks of culture to study the growth parameters. The shoot length and root length of seedlings was measured using graduated ruler (cm). The fresh weight was determined using a digital balance (g).

a) Seedling Vigor Index was calculated by the formula (Korekar, *et al.,* 2013):

$$\text{SVI} = \frac{\text{Germination (\%) x Seedling length (cm)}}{100}$$

b) Germination Stress Tolerance Index was calculated by the formula (Tarchoun, *et al.,* 2022):

$$\text{GSTI} = \frac{\text{Germination percentage in salt stress}}{\text{Germination percentage in control}} \times 100$$

c) Shoot Length Stress Tolerance Index was calculated by the formula (Tarchoun, *et al.,* 2022):

$$\text{SLSTI} = \frac{\text{Shoot length in salt stress}}{\text{Shoot length in control}} \times 100$$

d) Root Length Stress Tolerance Index was calculated by the formula (Tarchoun, *et al.,* 2022):

$$\text{RLSTI} = \frac{\text{Root length in salt stress}}{\text{Root length in control}} \times 100$$

**Biochemical Estimations**

To estimate the amount of chlorophyll and proline, six weeks old in vitro grown chilli seedlings under control and different concentrations of NaCl were freshly harvested from five independent samples of same age. For chlorophyll estimation, the seedlings (0.5g; shoots) were homogenized in 10ml of 80% acetone using mortar and pestle. The homogenate was centrifuged at 5000 rpm for 5min. and the supernatant was transferred to a 100ml volumetric flask. The extraction was repeated till the residue became colourless. The final volume was made to 50ml with 80% acetone and the absorbance was measured at 645nm and 663nm using spectrophotometer (Sysytronics 105, Ahmedabad) against 80% acetone as blank (Witham, *et al.,* 1971). Total chlorophyll was calculated using the following formula:

$$\text{Total chlorophyll (mg gfwt}^{-1}) = 20.2\ (A_{645}) + 8.02\ (A_{663}) \times V/1000 \times W$$

Where A= absorbance at specific wavelength, V=final volume of chlorophyll extract in 80% acetone (ml), W=fresh weight of the seedlings (g)

For proline estimation, seedlings (0.5g; shoots) were homogenized in 10ml of 3% aqueous sulfosalicylic acid. The homogenate was filtered through Whatman (No. 2) filter paper. Two ml of the filtrate was taken to which 2ml acid ninhydrin and 2ml glacial acetic acid were added and the mixture was kept in water bath at 100°C for 1hour. The reaction was terminated in an ice bath. Four ml of toluene was added to the reaction mixture and vortexed. The toluene layer was separated and the absorbance was read in a spectrophotometer at 520nm (Sysytronics 105, Ahmedabad) using toluene as blank (Bates, *et al.,* 1973). Proline concentration was determined from L-proline standard curve and calculated as µg proline gfwt$^{-1}$.

**Data Analysis**

The experimental layout was completely randomized design. Each experiment comprised of five treatments containing five replicates and the experiments were repeated twice. The data is presented as means of five replicates per treatment and the standard error. Statistical analysis was performed using two-factor analysis of variance with replicates ($p$<0.05).

**Results and Discussion**

**Germination Percentage**

In Pusa Jwala the germination percentage was 95.25%, 74.88%; 55.85%, 42.58%, 22.54% on control, 60, 80, 100 and 120 mM salt stress respectively. The genotype Sitara recorded a germination percentage of 100% on control, 92.4% on 60 mM, 83.6% on 80 mM, 71.4% on 100 mM and 52.8% on 120 mM salt stress. The germination percentage decreased to 22.5% in Pusa Jwala and 52.8% in Sitara under 120 mM NaCl stress. With increasing concentration of salt stress, the germination percentage declined, however, Sitara maintained a higher germination percentage than Pusa Jwala at all levels of salt stress (Fig. 1A). The detrimental effect of salinity stress on germination was reported earlier in chilli (Howlader, *et al.,* 2018, Ai, *et al.,* 2021, Wahocho, *et al.,* 2021), bell-pepper (Tehseen, *et al.,* 2016) and pepper (Ozdemier, *et al.,* 2016). Since the osmotic pressure of the germinating medium is high due to the presence of salts, the seeds are unable to imbibe sufficient water. Further, salinity increases the ionic stress within the seeds due to accumulation of $Na^+$ and $Cl^-$ ions that are injurious to the young embryo. Salt stress also disturbs the hormonal balance in the germinating seeds by increasing ABA and lowering the GA levels. Reduction in GA levels causes a decline in α-amylase activity hindering the breakdown of starch to sugars and its mobilization to the developing embryo. The embryo, thus becomes deprived of reserved food material required during germination. The osmotic and ionic stress generate reactive oxygen species (ROS) which further damages the cellular machinery (Ucarli, 2022, Tehseen, *et al.,* 2016) lowering the seed germination percentage in chilli genotypes.

**Shoot Length and Root Length**

On control the shoot length of seedlings of genotype Pusa Jwala was 8.78 cm and Sitara 9.57 cm respectively. At 120 mM salt stress the shoot length of Pusa Jwala seedlings was 2.13 cm while it was 4.85 cm in Sitara. Pusa Jwala showed a decline of 76% while Sitara registered 49% decline in shoot length on 120 mM NaCl stress. The root length was 6.37 cm in Pusa Jwala and 8.04 cm in Sitara on control. In presence of 120 mM salinity stress the root length was 0.90 cm in Pusa Jwala and 2.74 cm in Sitara showing a reduction of 86% and 66% respectively compared to control. (Fig. 1B). The seedling length in Pusa Jwala was 15.15 cm while it was 17.61 cm in Sitara on control which declined to 3.04 cm and 7.59 cm at 120 mM salt stress respectively. Pusa Jwala registered a decline of 80% in seedling length while Sitara 57% at 120 mM NaCl. There was substantial decline in shoot length and root length resulting in reduction of seedling length with increment in salt stress (Fig. 1B). The reduction in shoot length and root length of seedlings in saline conditions is probably due to osmotic stress, ion toxicity and nutritional imbalance (Grozeva, *et al.,* 2023). Ion toxicity results in impaired functioning of plant growth regulators and enzymes thus reducing the shoot and root growth (Tehseen, *et al.,* 2016). Similar response was obtained in chilli pepper (Khaldi, *et al.,* 2021, Hand, *et al.,* 2017, Haseen, *et al.,* 2014, Yildirim and Guvenc, 2006), pea (Khan, *et al,* 2022) and tomato (Seth and Kendurkar, 2015). The root to shoot length ratio was 0.72 in Pusa Jwala and 0.84 in Sitara on control which declined to 0.43 in Pusa Jwala and 0.57 in Sitara at 120 mM salt stress. Root to shoot ratio declined by 42% in Pusa Jwala and 33% in Sitara at 120 mM salt stress. (Fig.1C). In the present study root to shoot ratio declined in presence of salinity stress indicating that the reduction in root length was more pronounced compared to shoot length. As the roots are directly exposed to salt stress and remain in contact with sodium ions, they suffer from osmotic stress resulting in reduced root turgor pressure which decreases the cell division and elongation of roots (Zhang, *et al.,* 2023, Nakamura, *et al.,* 2021). Similar response was observed in pepper (Yildirim and Guvenc, 2006), tunishian squash (Tarchoun, *et al.,* 2022) and rice (Kumari *et al.,* 2016).

**Fresh Weight**

On control the fresh weight was 3.00 gm in Pusa Jwala which declined to 0.83 gm at 120mM salt stress showing a reduction of 72.42% compared to control. In Sitara the fresh weight was 3.30 gm on control which lowered to 1.13 gm at 120 mM salt stress exhibiting decline of 65.88% compared to control. There was significant reduction in fresh weight with increasing levels of salt stress (Fig.2A). Decline in the fresh weight of the seedlings with increment in salt stress has been reported earlier in chilli (Wahocho, *et al.,* 2021), brinjal (Jameel, *et al.,* 2024), tunisian squash (Tarchoun, *et al.,* 2022) and

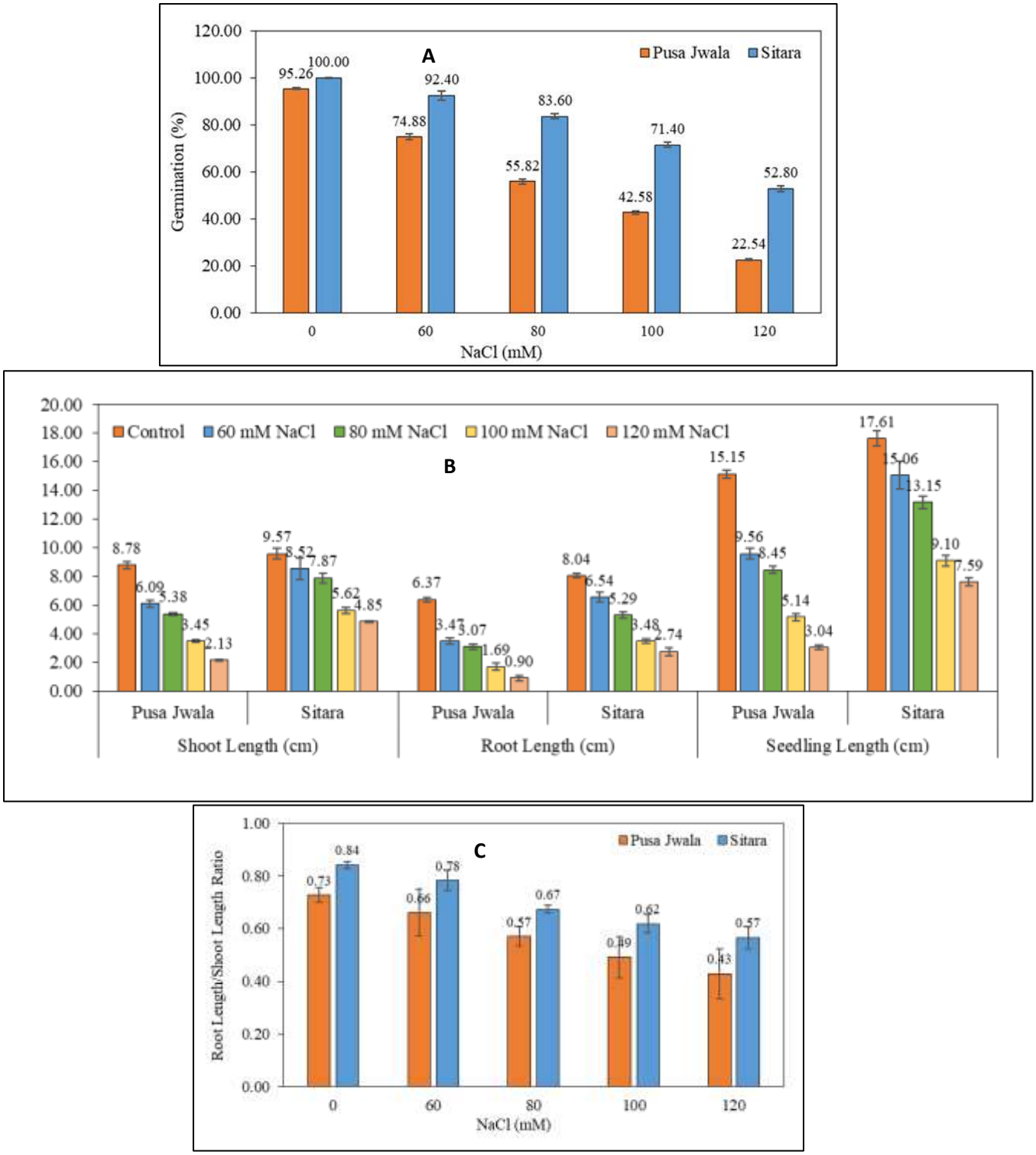



**Figure 1:** In vitro effect of graded levels of salt stress on seed germination (A); shoot length, root length, seedling length (B) and root length/shoot length ratio (C) in chilli genotypes. Each value represents mean (n=5) and vertical bars indicate SE of means.

Tomato (Seth and Kendurkar, 2015, Amini and Ehsanpour, 2006). The seedlings suffer from salt induced osmotic stress which results in lowered water and nutrient uptake thus reducing the seedling fresh weight (Shahid, *et al.*, 2020).

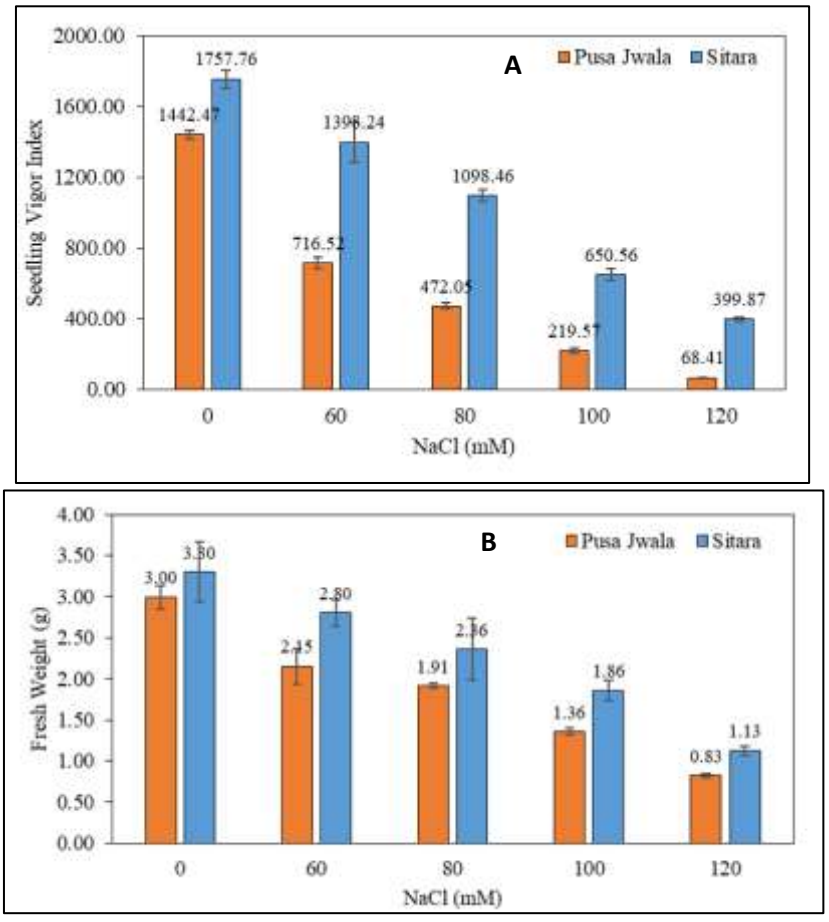
**Seedling Vigor Index**

The seedling vigor index was 1442.47 in Pusa Jwala on control and 68.41 at 120 mM salt stress showing a decrease of 95.26%. In Sitara the seedling vigor index was 1757.76 on

control and 399.87 at 120 mM salinity stress presenting a reduction of 77.25% (Fig.2B). Lowering of seedling vigor index with rising concentrations of salt stress was observed in pumpkin (Irik and Bikmaz, 2024), mungbean (Shaddam, *et al.*, 2024), pea (Khan, *et al.*, 2022), maize (Hoque *et al.*, 2014) and rice (Djanaguiraman *et al.*, 2003). Excess salts in the medium not only hamper water absorption but also interfere in the assimilation of important macronutrients such as phosphorous and potassium reducing seedling vigor index (Khan, *et al.*, 2022).

**Stress Tolerance Index**

The germination stress tolerance index was 78.62 in Pusa Jwala and 92.59 in Sitara at 60 mM sat stress which gradually declined to 23.66 and 52.91 at 120 mM salt stress. Pusa Jwala showed a decline of 70% while Sitara exhibited a reduction of 42.86% in germination stress tolerance index at 120 mM NaCl stress. The shoot length stress tolerance index was 69.45 in Pusa Jwala and 86.47 in Sitara at 60mM salt stress which reduced to 24.44 and 49.83 at 120 mM NaCl stress displaying a decrease of 65% and 43% respectively. The root length stress tolerance index in Pusa Jwala was 54.72 at 60 mM stress which lowered to 14.35 at 120 mM salt stress showing 77% reduction. However, Sitara exhibited root length stress tolerance index of 81.27 at 60 mM and 34.13 at 120mM salinity stress showing a 58% reduction. The germination stress tolerance index, shoot length stress tolerance index and root length stress



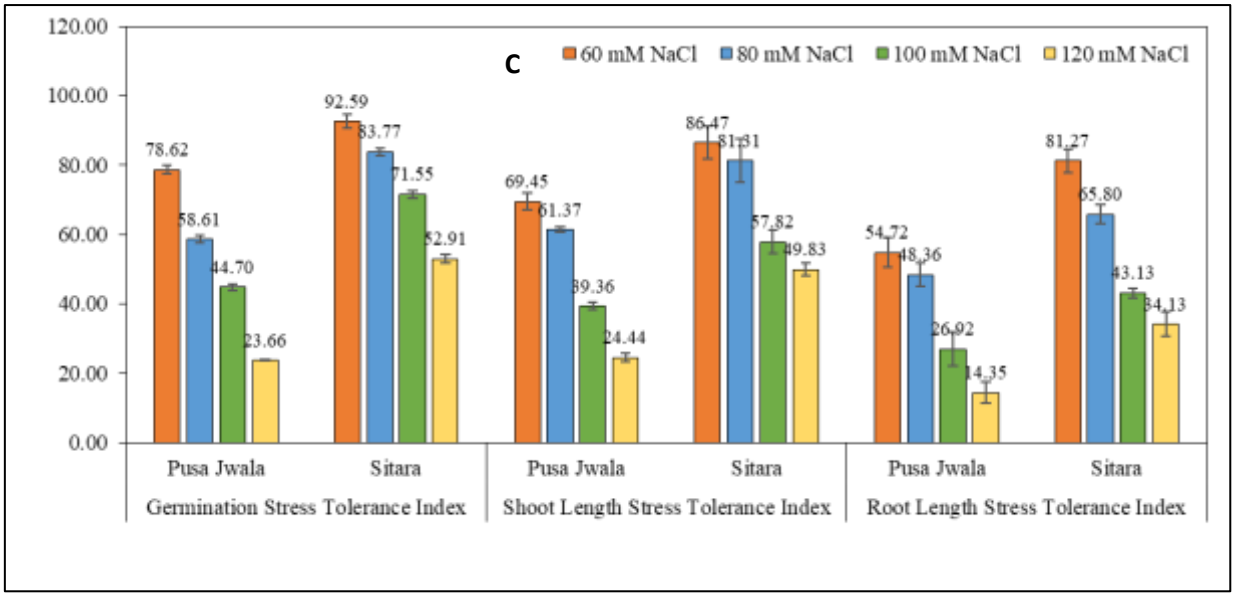



**Figure 2**: In vitro effect of graded levels of salt stress on fresh weight (A); seedling vigor index (B); and salt stress tolerance index (C) in chilli genotypes. Each value represents mean (n=5) and vertical bars indicate SE of means.

tolerance index showed inverse relationship with increasing salinity (Fig. 2C). Similar response on stress tolerance index was reported in mungbean (Kobir *et al.,* 2022). Two- factor analysis of variance validated these results and confirmed that the graded series of NaCl had significant impact on growth parameters in both the genotypes (Table 1). All the growth paraments showed interaction between salt stress and genotype except fresh weight, shoot length and shoot length stress tolerance index.

**Biochemical Parameters**

**Chlorophyll Content**

In Pusa Jwala the chlorophyll content was 0.436 mg gfwt$^{-1}$ on control which declined to 0.118 mg gfwt$^{-1}$ on 120 mM salt stress showing a reduction of 72.94%. The chlorophyll content was 0.786 mg gfwt$^{-1}$ in Sitara on control which reduced to 0.285 mg gfwt$^{-1}$ on 120 mM salinity stress registering a decline of 63.64 %. There was substantial decline in chlorophyll content with increasing levels of salt stress (Fig 3A). The reduction of chlorophyll in presence of salt stress is due to excessive accumulation of $Na^+$ and $Cl^-$ in the leaf tissues which disrupts the uptake of potassium and magnesium required for chlorophyll biosynthesis (Fu and Yong, 2023). Salinity increases the production of reactive oxygen species that triggers the formation of photolytic enzyme chlorophyllase causing degradation of chlorophyll (Taibi, *et al.,* 2016). Salt stress also damages the function of chloroplast by altering the permeability of chloroplast membranes and affecting the electron transport chain in the thylakoid membrane thus reducing the rate of photosynthesis (Sadeghi *et al.,* 2024). These results are in agreement with previous studies in chilli (Sharma, *et al.,* 2012, Gammoudi, *et al.,* 2016, Ozdemir, *et al.,* 2017, Hand, *et al.,* 2017), tunisian chilli pepper (Zhani, *et al.,* 2012), white pepper (Nouck, *et al.,* 2021), *Phaseolus* (Taibi, *et al.,* 2016) and Maize (Chattha, *et al.,* 2023) where chlorophyll content showed a declining trend in presence of salinity stress.

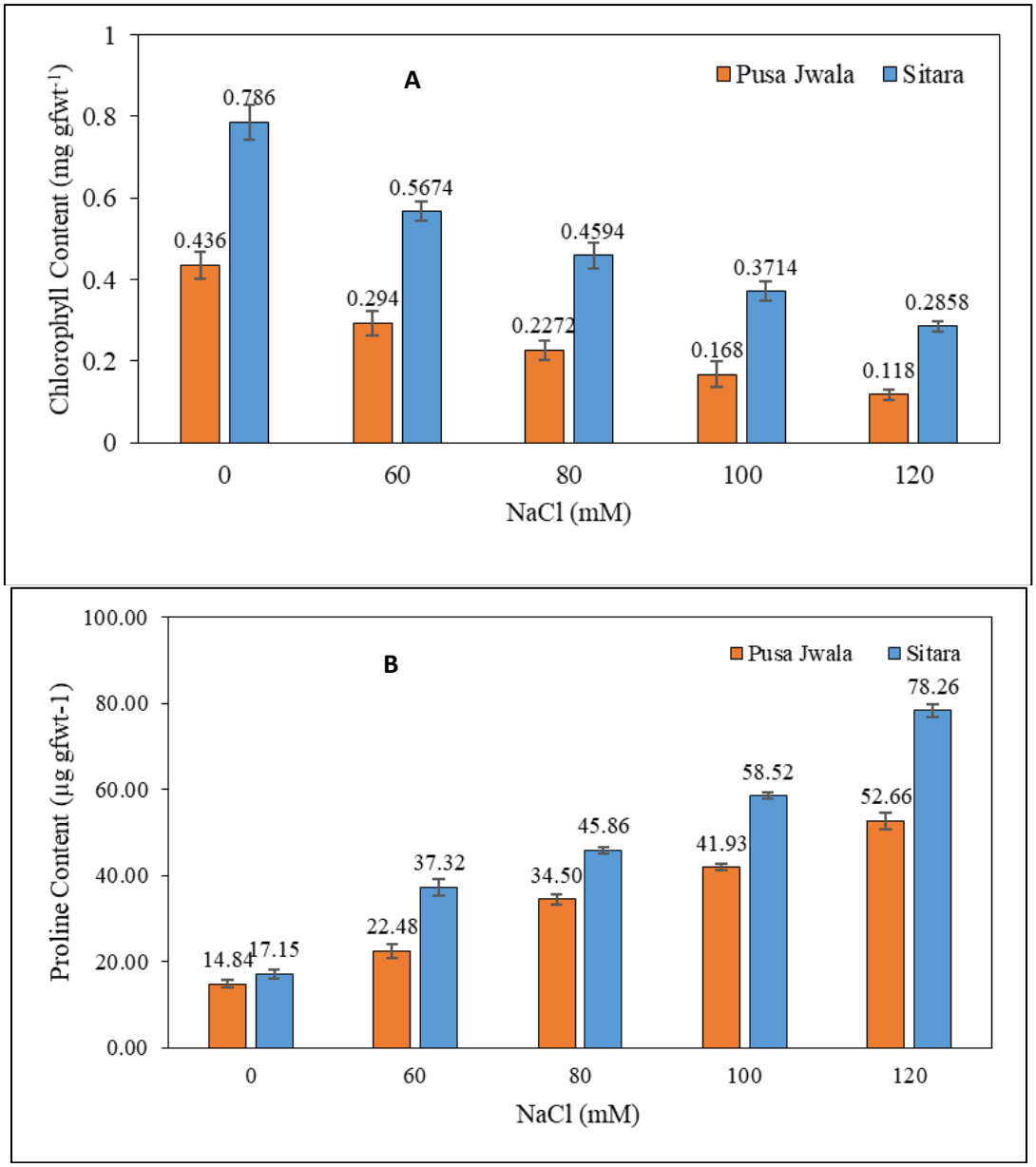



**Figure 3**: In vitro effect of graded levels of salt stress on chlorophyll content (A) and proline content (B) in chilli genotypes. Each value represents mean (n=5) and vertical bars indicate SE of means.

**Proline Content**

On control the proline content was 14.84 µg gfwt$^{-1}$in Pusa Jwala which increased to 52.66 µg gfwt$^{-1}$ at 120 mM NaCl stress showing an increment of 3.55 folds. The proline content was 17.15 µg gfwt$^{-1}$ in Sitara on control which increased to 78.26 µg gfwt$^{-1}$ on 120mM salt stress registering an increase of 4.56 folds (Fig.3B). Proline functions as an osmoprotectant by maintaining the water balance and turgidity within plant cells under saline conditions (Nguyen, *et al.,* 2021). It also stabilizes the subcellular structures like proteins, membranes and enzymes, scavenges reactive oxygen species and helps in maintaining the cellular redox potential (Shahid, *et al.,* 2020). Proline also serves as an important source of nitrogen and carbon during the recovery phase in several plants (Ghosh, *et al.,* 2021). Increment in proline content in response to salt stress was observed in earlier studies on chilli (Kaouther, *et al.,* 2012, Sharma, *et al.,* 2012, Lopez-Serrano, *et al.,* 2021, Perez-Gomez, *et al.,* 2024), white pepper (Nouck, *et al.,* 2021), tomato (Seth and Kendurkar, 2016), rice (Nguyen, *et al.,* 2021) and wheat (Masarmi, *et al.,* 2023). The two-factor analysis of variance showed significant impact of NaCl on biochemical parameters (chlorophyll and proline content) within the genotypes along with the interaction between salt stress and genotype (Table 1).

**Table-1:** Two-factor Analysis of Variance (Mean of squares) for seed germination, seedling growth and biochemical attributes in chilli under graded levels of salinity stress

| Source of Variation | DF | GP | SL | RL | FW | SVI | CL | PR | DF | GSTI | SLSTI | RLSTI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotype | 1 | 5931.57 | 56.163 | 55.9894 | 2.40331 | 2846192 | 0.75252 | 2223.78 | 1 | 5666.91 | 4080.97 | 3997.94 |
| Salt stress | 4 | 5385.96 | 50.9066 | 44.8533 | 6.88621 | 2893786 | 0.25197 | 3682.69 | 3 | 4040.52 | 3659.83 | 4002.24 |
| Genotype x Salt stress | 4 | 295.387 | 1.46341 | 0.81358 | 0.05487 | 71050.3 | 0.0123 | 174.281 | 3 | 114.54 | 33.4523 | 53.2004 |

DF: degree of freedom; GP: germination percentage; SL: shoot length; RL: root length; FW: fresh weight; SVI: seedling vigor index; CL: chlorophyll content; PR: proline content; GSTI: germination stress tolerance index; SLSTI: shoot length stress tolerance index; RLSTI: Root length stress tolerance index ($p<0.05$)

## Conclusion

The results from this investigation indicated that seed germination and seedling growth parameters (shoot length, root length and fresh weight) declined significantly with increasing levels of salt stress in Chilli under in vitro conditions. Higher levels of salt stress (100 and 120 mM) were inhibitory compared to lower stress levels. With increasing levels of stress there was substantial reduction in the chlorophyll content, however, there was considerable increment in proline content in both the genotypes. On the basis of seed germination, seedling growth and biochemical attributes genotype Sitara demonstrated better tolerance potential towards graded series of salt stress as compared to Pusa Jwala. The significance of this study is in rapid screening of commercial chilli genotypes for salt stress at an early growth phase (germination and seedling stage) using in vitro techniques. The tolerant chilli genotypes can be recommended for cultivation in salt affected fields and also incorporated into plant breeding programs.

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