Physiological and Biochemical Responses of Chrysanthemum × morifolium to Salinity Stress under In-Vitro Conditions

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Research Article

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Abstract

Salinity is a critical abiotic stress that significantly limits plant growth and productivity by disrupting physiological and biochemical processes. This study investigated the effects of NaCl-induced salinity on the in vitro culture of *Chrysanthemum × morifolium* Ramat. Explants were cultured under varying concentrations of NaCl (0–300 mmol/L) to assess its impact on callus formation, shoot regeneration, physiological attributes, and biochemical responses. Results showed that increasing NaCl concentrations reduced callus formation percentages, shoot regeneration frequency, and shoot length, with the highest reduction observed at 300 mmol/L NaCl. The number of shoots per explant decreased from 13.03 under non-saline conditions to 0.90 at 300 mmol/L. Chlorophyll content, carotenoids, and protein levels declined significantly with increasing salinity, whereas the proline content increased, indicating its role in osmotic adjustment and stress tolerance. Antioxidant enzyme activities, including catalase and superoxide dismutase, were enhanced under salt stress, with maximum activity recorded at 300 mmol/L NaCl, suggesting their involvement in mitigating oxidative damage. Lipid peroxidation and protein oxidative damage also increased, further indicating the detrimental effects of salinity. During the hardening phase, optimal survival was achieved using a potting mixture of coco peat, sand, vermicompost, and garden soil (2:1:1:1 ratio). Plants grown under in vivo saline conditions exhibited reduced biomass, root growth, and shoot development, with severe effects at 400 mmol/L NaCl. These findings provide insights into the physiological and biochemical responses of *C. morifolium* under salt stress, contributing to the development of salt-tolerant varieties and improving in vitro propagation techniques.

1. Introduction

The global floriculture industry, one of the fastest growing agricultural sector, plays a significant economic role, with countries like the Netherlands, the United States, Japan and Germany being leaders in the production and export of cut flowers (Getu 2009). *Chrysanthamum x morifolium* species predominantly originate from East Asia, with China, Japan, Thailand, and India being major producers. With its diverse agro-climatic zones, India is well-suited for floriculture, with states like Tamil Nadu, Karnataka, Madhya Pradesh, and West Bengal leading in cultivation (Kumar et al. 2023). As India's floriculture industry expands, the area under *Chrysanthemum* cultivation has grown by 225.33%, with production increased by 391.77% from 2014 to 2024 (Kumar et al. 2024).

Chrysanthemum (*Chrysanthemum × morifolium*), commonly called as the "Queen of the East", belongs to the family Asteraceae. It is a highly attractive, short-day plant, often treated as a seasonal bloomer in late autumn despite being perennial. Chrysanthemum cut flowers are valued for floral arrangements due to their extended vase life and are cultivated for various decorative uses, including garlands, bouquets and floral decorations for social and religious events (Bohra and Kumar 2014, Patil et al. 2017). Apart from its economic value, Chrysanthemum also holds therapeutic importance in traditional medicine, used for treating inflammation, bruises, snake bites, and even as a remedy for fever (Ryu et al. 2019). Additionally, the petals are used in Chinese cuisine and for making herbal teas (Collins et al. 1997).

Abiotic stress factors such as salinity, drought, and extreme temperatures significantly affect *C. morifolium*, reducing its growth, flower longevity, and aesthetic value (Vinocur and Altman 2005). By 2050, salinity is predicted to affect about 33% of the world's irrigated agricultural land (Shao *et al.*, 2008). High salinity impacts various physiological processes in plants, inhibiting growth and damaging key metabolic activities like photosynthesis and protein synthesis (Munns and Tester 2008, Acosta-Motos et al. 2015). Salinity stress induces the accumulation of toxic products, such as reactive oxygen species (ROS), in plants, disrupting their metabolic balance and causing oxidative damage (Ahanger et al. 2017). In response to salt stress, plants undergo a series of physiological changes, including increased activity of antioxidant enzymes, and elevated proline content (AbdElgawad et al. 2016, Sarabi et al. 2017). These changes reflect the plant's response to salt stress at various levels, with some mechanisms protecting against oxidative damage and enhancing salt stress tolerance.

Analysis of stress responses in plants grown through traditional propagation methods is slow and labour-intensive. Plant tissue culture and micro-propagation techniques offer faster, more efficient alternatives for studying plant responses to stress conditions. This method enables the rapid production of large numbers of plants or herbal products from plant cells or tissues (explants) cultured on artificial nutrient media (Oseni, Pande and Nailwal 2018, Dogan, Karatas and Aasim 2018).

Salt stress can be simulated in plant tissue culture by adding sodium chloride (NaCl) to the media, offering a controlled environment to study plant responses at the cellular level (Shibli et al. 2007). This study was aimed to optimize NaCl concentrations for tissue-cultured Chrysanthemums under *in-vitro* conditions and subsequent application of these optimized concentrations to in vitro-developed plants under in vivo conditions within a polyhouse. The experiment involved the treating plants with varying NaCl concentrations to evaluate their impact on plant growth, stress tolerance, and to monitor associated physiological and biochemical changes during development.

2. Materials and Methods

The experiment was conducted in Plant Tissue Culture Laboratory, College of Forestry at Banda University of Agriculture and Technology, Banda, Uttar Pradesh during the years 2022-2024. The climate in Banda is semi-arid/tropical, with dry, hot summers (reaching 49°C) and cold winters (down to 2°C). Annual rainfall averages between 800 and 910 mm and mostly occurring from mid-June to September.

2.1. Explant Preparation: Nodal segments from healthy mother plants were treated with biocides (Hydroxyquinoline), fungicides (Carbendazim, Mancozeb), and a bleaching solution (sodium hypochlorite) before being sterilized with $HgCl_2$ for in-vitro culturing (Table S1).

2.2. Culture Media: The Murashige and Skoog (MS) medium(Murashige and Skoog 1962) was used which has composition as sucrose (30 g/L), vitamins, growth regulators such as kinetin (Kin), 6-benzylaminopurine (BAP), and thidiazuron (TDZ) adjusting pH to 5.7–5.8, and adding agar powder (7 g/L). Sterilization of the media and other necessary glassware, equipment was done by autoclaving at 121°C for 20 minutes at 15 psi pressure.

2.3. Culture Media for Growth Stages: For shoot induction, MS media with BAP (0.5-2 mg/L) was used. For shoot proliferation of microshoots were transferred to MS media with varying concentrations of BAP, kinetin, or TDZ for shoot proliferation and rooting of elongated shoots were transferred to half-strength MS media with IBA and IAA for root induction. Sterilized explants were inoculated in culture media inside a laminar airflow chamber, and incubated at 25±1°C under a 16/8 hour light/dark photoperiod. Observations were recorded for shoot induction (%), number of shoots, shoot length (cm), root induction (%), number of roots per shoot and root length (cm).

2.4. Sub-culturing *In Vitro* plants on MS medium supplemented with NaCl

To subculture *in vitro* plants in MS media supplemented with NaCl (25 mM to 300 mM), first sterile NaCl stock solutions were prepared for each concentration. MS media was prepared according to the standard protocol, and NaCl stock solutions were added to the media. The pH of the NaCl-supplemented MS media was adjusted to 5.7–5.8, then autoclaved. After autoclaving, media allowed to cool. The plant material (explants) is surface sterilized and subcultured into the NaCl-enriched media. Plant growth was regularly monitored for signs of salt stress, including reduced growth rate, physiological changes like chlorophyll and carotenoid content, biochemical changes such as protein, proline levels, antioxidant enzyme activities, or altered root development, and these parameters were recorded to analyze the effects of NaCl stress on the plants.

2.5. Establishment of In-vitro propagated plants for hardening

Primary hardening of *Chrysanthemum morifolium* plants was carried out by transferring *in vitro* plants to a mixture of cocopeat, perlite and vermiculite (2:1:1) in trays under fiberglass house conditions for two weeks. For secondary hardening, plants were transferred to a mixture of cocopeat, sand and vermicompost (2:1:1) and maintained under shade net conditions for four weeks. During this period, physiological and biochemical changes were monitored under both *in vitro* stress and control conditions. These changes included alterations in growth rate, chlorophyll and carotenoid content, protein levels, proline accumulation, and antioxidant enzyme activity, providing insights into the plants' stress response mechanisms during hardening.

2.6. Physiological parameters

2.6.1.Chlorophyll and carotenoid contents of leaves

To analyse chlorophyll and carotenoid contents, fully matured open leaves were taken from both control and stressed conditions. The chlorophyll content (chlorophyll *a*, *b* and total chlorophyll) of the leaves was estimated using the method described by **Hiscox and Israelstam (Hiscox and Israelstam 1979). A** sample of 50 mg of leaves was placed in 10 ml of dimethyl sulfoxide (DMSO, Analytical grade) and incubated at 65°C for 4 hours. Post-incubation, the tubes were cooled to room temperature, and the absorbance was measured at 663, 645, and 470 nm using a UV-VIS spectrophotometer (5704 ECIL,

India) against pure DMSO as the blank. The chlorophyll and carotenoid contents were calculated using the following formulas:

Chlorophyll *a* (Chl-a) = 12.21 A663 − 2.81 A645

Chlorophyll *b* (Chl-b) = 20.13 A645 − 5.03 A663

Total Chlorophyll (mg g^{-1} DW) = (20.7 × OD645) + (8.02 × OD663) × volume × dilution / (1000 × weight of the sample)

Total Carotenoids = [1000 A470 - (3.27 Chl-a + 104 Chl-b)] / 229

All chlorophyll values were expressed in µg/g of fresh weight (fw). The ratio of chlorophyll *a* and *b* was calculated by dividing chlorophyll *a* by chlorophyll *b* to assess the balance between different chlorophyll types under stress and control conditions. The ratio of carotenoid to chlorophyll was calculated by dividing total carotenoids by total chlorophyll to analyze how stress conditions affected pigment composition.

2.6.2. Biochemical Parameters

2.6.2.1. Antioxidant Enzyme Activity:

The antioxidant enzyme activities were analyzed to assess oxidative stress in *C. morifolium* under in-vitro conditions. Leaf samples from each treatment were collected in an ice-box to prevent proteolytic degradation. One gram of leaf tissue was homogenized in a pre-chilled mortar and pestle with 5 ml of chilled phosphate buffer (50 mM, pH 7.0). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant was used for the following enzyme assays:

2.6.2.2. Superoxide Dismutase (SOD) Activity

SOD activity was determined according to **Fridovich (Fridovich 1975).** The reaction was based on the ability of SOD to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). Absorbance was recorded at 560 nm, and enzyme activity was expressed as unit/min/mg of protein.

2.6.2.3. Catalase (CAT) Activity

Catalase activity was measured using the method of **Luck (Lück 1965).** Residual hydrogen peroxide (H_2O_2) was quantified by titration with potassium permanganate. Results were expressed as µmol of H_2O_2 decomposed per minute per mg of protein.

2.6.3. H_2O_2 Content Estimation

H_2O_2 content was determined following the method of **Alexieva *et al.* (Alexieva et al. 2001).** Using 0.1% TCA for extraction, the H_2O_2 was reacted with potassium iodide (KI), and the absorbance was measured at 390 nm. Results were expressed as µmol g^{-1} of fresh weight.

2.6.4. Proline Content

Proline was quantified following the method of Bates et al. (Bates, Waldren and Teare 1973). The leaves were homogenized in 3% sulpho-salicylic acid, and the proline content was determined by the formation of a pink chromophore with acid ninhydrin, followed by toluene extraction. Absorbance was recorded at 520 nm, and proline content was expressed as µg/g of dry weight.

2.6.5. Protein Content

Protein content was estimated using Lowry's method (Lowry et al. 1951). The protein was precipitated using TCA, and absorbance of blue color developed after treatment with Folin-Ciocalteu reagent was measured at 660 nm. Protein content was calculated based on a standard curve prepared using bovine serum albumin (BSA).

2.6.6. Histochemical detection of superoxide and H_2O_2 by NBT and DAB staining

Superoxide and H_2O_2 were detected in leaf tissues using NBT and DAB staining techniques following Kumar et al. (Kumar et al. 2014). Theleaves were stained overnight, cleared with ethanol and visualized against a contrast background. Superoxide appeared as dark blue spots, while H_2O_2 as reddish-brown spots.

3. Results

The study investigated the sterilizing agent, the effects of basal media composition, phytohormones, and NaCl treatments on shoot induction, regeneration frequency, and shoot growth. Surface sterilization using an optimized combination of 2% NaOCl, 0.10% fungicide, 0.02% biocide, and 0.10% $HgCl_2$ resulted in the highest survival of *Chrysanthemum × morifolium* nodal explants, whereas higher concentrations significantly reduced viability (Table S1). Full-strength and half-strength MS media were supplemented with different concentrations of phytohormones, including BAP, TDZ, and kinetin, and the results demonstrated significant variations in shoot induction and growth. In full-strength MS medium, the addition of BAP at 2 mg/L yielded the highest shoot induction (69.67%) with an average of 3.57 shoots per explant and a mean shoot length of 5.43 cm. TDZ at 0.5 mg/L resulted in 59% shoot induction with 2.97 shoots and a shoot length of 2.77 cm. Among kinetin treatments, 1 mg/L produced the highest induction rate (50.33%), with an average of 3.23 shoots and a shoot length of 3.67 cm. However, higher concentrations of TDZ and kinetin reduced shoot induction and growth, indicating a concentration-dependent response. Half-strength MS medium supplemented with 2 mg/L BAP was the most effective for shoot induction and growth. It achieved the highest shoot regeneration frequency (89.67%), producing an average of 13.03 shoots per explant with a mean shoot length of 8.9 cm. BAP at 0.5 mg/L also showed high efficacy, with an 80.33% induction rate and 11.67 shoots per explant (Figure S1a&b and Table 1).

The addition of NaCl to half-strength MS medium supplemented with 2 mg/L BAP significantly reduced shoot regeneration frequency and growth. Under control conditions (T0, 0 mM NaCl), the regeneration frequency was 89.33%, with a mean shoot length of 7.83 cm. At 25 mM NaCl (T1), the regeneration frequency decreased to 79.87%, and shoot length reduced to 5.97 cm. With increasing NaCl concentrations, the inhibitory effect became more pronounced. At 100 mM NaCl (T4), regeneration frequency dropped to 54.73%, and shoot length decreased to 3.27 cm. At 300 mM NaCl (T8), the lowest regeneration frequency (5.33%) and shoot length (0.9 cm) were observed, reflecting the severe impact of salt stress on shoot development. The result showed that the BAP was the most effective phytohormone for promoting shoot induction and elongation under both normal and stress conditions. However, salt stress caused a substantial decline in regeneration frequency and shoot length, with the inhibitory effects increasing with higher NaCl concentrations. These findings highlight the importance of optimizing phytohormone concentrations and managing salt stress for successful in vitro shoot regeneration (**Figure 1 a & b; Table 1).**

3.1. Role of different NaCl concentrations on root regeneration

Root regeneration in *C. morifolium* was most effective with 0.2 mg/L IBA in half-strength MS medium, yielding the highest root induction (90.4%) and the maximum roots per shoot (11.66). The longest roots (12.30 cm) were observed at 0.4 mg/L IBA. IAA showed lower efficiency, with 70.43% root induction, 10 roots per shoot, and a maximum root length of 11.40 cm at optimal concentrations. Higher auxin levels (1.0 mg/L) significantly reduced root induction, number, and length. These results underscore the efficacy of low IBA concentrations for root regeneration **(Figure S2; Table S2).**

3.2. Physiological and biochemical changes in *C. morifolium* under in-vitro salt stress:

The study examined the physiological and biochemical responses of *C. morifolium* under varying salinity stress. Chlorophyll A and B content decreased with increasing salt levels, with the highest chlorophyll A (113.34 µg/g) and B (72.30 µg/g) observed at 25 mmol/L NaCl, and the lowest at 300 mmol/L NaCl. The chlorophyll A/B ratio increased with salinity, while total chlorophyll and carotenoid content also declined, with the highest total chlorophyll (207.49 µg/g) and carotenoid (38.13 µg/g) at 25 mmol/L NaCl (Figure 2a). Proline content, a key stress marker, significantly increased with salinity, peaking at 300 mmol/L NaCl (92.33 µg/g) **(Figure 2b**). Superoxide Dismutase (SOD) and Catalase (CAT) activities elevated under high salt stress, with the highest SOD activity (107.20 unit/min/mg protein) and CAT activity (137.86 µmoles of H_2O_2 decomposed min-1 mg-1 protein) recorded at 300 mmol/L NaCl. Hydrogen Peroxide (H_2O_2) levels also significantly increased under salinity, reaching a maximum of 133.52 µmoles g-1 fw in 300 mmol/L NaCl **(Figure: 2c-2e).** These findings highlight how *C. morifolium* responds to salinity stress by activating stress-responsive biochemical pathways, including protein degradation, proline accumulation, and enhanced antioxidant enzyme activity **(Figure 2 a-e).** In contrast, at 25 mmol/L NaCl (T1), ROS accumulation was much lower. These findings align with increased H_2O_2 and SOD levels, confirming that oxidative stress increases with salinity **(Figure 2).**

3.3. Histochemical detection of Superoxide and H_2O_2 by NBT and DAB staining of *Chrysanthemum* leaves under NaCl treatment

The histochemical analysis of *C. morifolium* leaves under NaCl-induced salinity showed significant accumulation of ROS, including hydrogen peroxide (H_2O_2) and superoxide anions. At 300 mmol/L NaCl (T8), DAB staining revealed intense brown pigmentation, indicating high H_2O_2 accumulation **(Figure 3a),** while NBT staining showed dark blue spots, indicating superoxide build up **(Figure 3b).**

3.4. Principle component analysis

The Principal Component Analysis (PCA) biplot **(Figure S3)** provides a clear visualization of the impact of various treatments on plant growth and stress responses. The first principal component (F1), accounting for 98.78% of the variance, is primarily influenced by growth-promoting factors such as shoot regeneration frequency, total chlorophyll, chlorophyll A, chlorophyll B, carotenoids, protein content, and shoot length. Treatments T1, T2, T3, and T0, positioned on the right side of the biplot, show strong positive associations with these variables, indicating favourable conditions for growth, chlorophyll synthesis, protein accumulation, and shoot regeneration. These treatments likely provided optimal environmental or nutrient conditions, enabling the plants to allocate energy toward growth and development. In contrast, the second principal component (F2), which explains only 0.80% of the variance, is associated with stress-related variables like superoxide dismutase (SOD), hydrogen peroxide (H_2O_2), and proline. Treatments T4 and T5, positioned closer to these stress markers, indicate a moderate level of stress, activating antioxidant defense mechanisms to counteract oxidative and osmotic stress. Proline accumulation suggests osmotic stress responses. Conversely, treatments T8, T6, and T7, positioned on the far-left side of F1, are negatively correlated with growth-related variables, indicating detrimental effects on plant growth. The large separation between these treatments and growth parameters suggests that they inhibited shoot regeneration and reduced biomass accumulation, possibly due to poor environmental conditions, nutrient deficiencies, or other stress factors. (**Figure S3**). The biplot clearly shows the trade-off between growth and stress responses. Treatments such as T1, T2, T3, and T0 promoted growth, enhancing shoot regeneration and chlorophyll synthesis. In contrast, treatments like T4 and T5 activated stress-related pathways, redirecting resources toward antioxidant and osmotic adjustments, which limited biomass accumulation. This is reflected by the increased SOD, H_2O_2, and proline levels, indicating oxidative and osmotic stress. The PCA biplot effectively differentiates between treatments that favor growth and those that trigger stress responses. Treatments T1, T2, T3, and T0 provided optimal conditions for plant development, while treatments like T4 and T5 activated defense mechanisms. Treatments T8, T6, and T7 were the least effective, resulting in suppressed growth. The biplot emphasizes the need to balance growth and stress responses for successful regeneration and development under varying conditions.

3.5. Establishment of *In-vitro* propagated plants for hardening

The tissue culture-raised *C. morifolium* plants under salt stress were successfully acclimatized and hardened through a sequential process. In the primary hardening stage, the plants exhibited significant

growth in a medium comprising cocopeat, perlite, and vermiculite in a 2:1:1 ratio. During secondary hardening, the plants continued to grow well in a mix of cocopeat, sand, and vermicompost (2:1:1). Finally, the acclimatized plants were transplanted into a healthy potting medium of vermicompost, garden soil, sand, and cocopeat in a 2:1:1:1 ratio, where they thrived under controlled conditions in a polyhouse (Figure S4; Figure 4a and b).

The impact of varying NaCl concentrations on tissue-cultured *C. morifolium* plants under in-vivo conditions was evaluated at 15, 30, and 45 DAS. Significant effects were observed on parameters such as the number of leaves (Figure 4c), plant height (Figure 4d), secondary shoots (Figure 4e), and plant spread (Figure 4f), (Figure 4 c-f and Table 2). The highest number of leaves and plant height were recorded in treatments S2 and S3 (Figure 4c). Secondary shoots peaked at S3 (19.25 at 30 DAS) and S5 (14 at 15 DAS), while plant spread was greatest in S1 (18.45 cm at 30 DAS). Shoot diameters were maximized under S4 and S3, respectively (Table 2). The higher NaCl concentrations generally suppressed plant growth, with S1 and S3 performing best across most parameters. Negative controls consistently showed inferior results compared to positive controls. Root length measurements at the end of the experiment (45 DAS) revealed the longest roots under S1 salt treatment (15.22 cm), followed by S2. Negative controls produced shorter roots compared to positive controls (Figures 5a,b). Soil conductivity (E.C.) and pH were also influenced by NaCl levels. At 45 DAS, S5 had the highest E.C. (25.10), while S1 recorded the lowest (8.13). Soil pH was highest in S1 and S2 (7.69), and lowest in S5 (7.49). Negative controls showed consistently poorer performance compared to positive controls in both plant and soil parameters (Table S3).

3.6. Effect of NaCl on physiology and biochemical changes of tissue cultured raised *C. morifolium* under in vivo salt stress

This study investigated the impact of different NaCl concentrations on tissue-cultured *C. morifolium* under in-vivo conditions, highlighting significant physiological and biochemical changes. Chlorophyll A and B contents decreased progressively with increasing NaCl concentrations, with the highest levels recorded at S1 (105.71 µg/g for chlorophyll A and 61.57 µg/g for chlorophyll B), while the lowest were observed at S5 (39.27 µg/g for chlorophyll A and 18.74 µg/g for chlorophyll B) (Fig. 6a). The chlorophyll A/B ratio increased under higher salt stress, peaking at S5 (2.09) (Fig. 6a). Total chlorophyll content also showed a decreasing trend, with S1 exhibiting the highest (167.28 µg/g) and S5 the lowest (58.01 µg/g) carotenoid content similarly declined with increasing NaCl, reaching the highest in S1 (33.16 µg/g) and the lowest in S5 (6.49 µg/g), resulting in a carotenoid/chlorophyll ratio of 0.19 in S1 and 0.11 in S5 (Figure 6a). Proline content, an osmoprotectant, increased markedly under high salt stress, peaking at S5 with 98.63 µg/g of fw, compared to the lowest content in S1 (40.88 µg/g of fw) (Figure 6b). Antioxidant enzyme activities, including SOD and catalase, were significantly elevated in response to salt stress, with catalase activity peaking at 143.87 µmoles of H_2O_2 decomposed/min/mg of protein in S5 (Figure 6c), and SOD activity reaching a maximum of 113.19 unit/min/mg of protein in S5 (Figure 6d). H_2O_2 accumulation also increased under higher salt conditions, with the highest content recorded in S5 (139.14 µmoles/g of fw), compared to the lowest in S1 (74.37 µmoles/g of fw) (Figure 6e). Negative

controls consistently showed lower values compared to positive controls across all parameters, confirming the detrimental effects of higher NaCl concentrations on plant growth, chlorophyll synthesis, protein content, and antioxidant defense mechanisms.

3.7. Histochemical Detection of superoxide and H_2O_2 by NBT and DAB Staining of *In-vivo* raised tissue cultured plants

Histochemical analysis of *C. morifolium* leaves under different NaCl concentrations (0–400 mmol) revealed hydrogen peroxide (H_2O_2) and superoxide anion accumulation. In leaves collected 45 DAS, DAB staining showed brown spots indicating H_2O_2 accumulation, while NBT staining revealed blue pigments, signifying superoxide anion presence. Severe cell damage was observed at 400 mmol NaCl (S5), with intense brown and blue pigmentation, while fewer symptoms were seen at 25 mmol NaCl (S1). These findings align with biochemical analysis, where higher H_2O_2 and SOD activity were recorded in S5 compared to the control **(Figure S5).**

3.8. Principle component analysis:

The Principal Component Analysis (PCA) biplot **(Figure S6)** reveals the relationships between physiological and biochemical variables (active variables in red) and treatments (active observations in blue), explaining a total variance of 84.04% through two principal components, F1 and F2. The F1 accounts for 71.06% of the variance and is strongly influenced by stress-related variables like proline, hydrogen peroxide (H_2O_2), and superoxide dismutase (SOD), which are prominently associated with higher salinity treatments such as S5 (400 mM) and S4 (200 mM). These treatments show a positive correlation with oxidative stress and osmotic regulation responses, indicating that they induce significant stress in the plant. Conversely, F2, which explains 12.99% of the variance, is dominated by growth-related variables such as plant height at 30 and 45 days, root length, and the number of leaves at 15 days. These growth parameters are positively associated with lower salinity treatments like S1 (25 mM) and S2 (50 mM), demonstrating that mild salinity levels promote plant growth. The negative control (So) is centered near the origin, showing a moderate response across all variables, while the positive control (So+) clusters near chlorophyll content (Chl A and Chl B), highlighting optimal conditions for photosynthesis and plant health under non-stress conditions. The biplot distinctly separates growth-related responses, aligned with the positive side of F2, from stress-related responses, positioned on the positive side of F1. This separation emphasizes that higher salinity levels hinder growth while activating stress defense mechanisms. In summary, the PCA biplot effectively visualizes the differential impacts of salinity treatments on plant growth and stress, demonstrating that lower salinity fosters growth, whereas elevated salinity triggers biochemical stress responses.

4. Discussion

4.1. Regeneration and multiplication of *Chrysanthemum* under different phytohormones condition

This study focuses on identifying optimal NaCl concentrations that do not adversely affect *Chrysanthemum* growth in both *in-vitro* and *in-vivo* (pot culture) conditions. Field-grown explants face microbial contamination challenges in in-vitro cultures. Pre-treatments with 0.1% Carbendazim, 0.02% 8-HQC (30 min), and 0.1% $HgCl_2$ (3 min) resulted in 51.00% survival. Higher survival rates (75.56%) were reported with 0.2% Mancozeb, 0.2% Carbendazim, and 200 mg/L 8-HQC (Verma et al. 2012). Similarly, 0.1% $HgCl_2$ was most effective in *Stevia* (Sharuti Verma, Kuldeep Yadav and Narender Singh 2011) and yielded 65.08% survival in *Chrysanthemum* (Anjum et al. 2023). For shoot induction, half-strength of MS medium with 30 g/L sucrose, 2.0 mg/L BAP, and 7 g/L agar achieved 89.66% induction. Comparable rates were reported in *Chrysanthemum* (76.66%, 2.0 mg/L BAP; (Yesmin et al. 2014), 83.30% with 2.0 mg/L BAP + 0.5 mg/L IAA (Kashif Waseem et al. 2011) and 84.74% in *Xanthosoma sagittifolium* (Bansal et al. 2023). Jahan et al. (Jahan et al. 2021) recorded 100% regeneration with 2.0 mg/L BAP, while Boonkamjat et al. (Boonkamjat, Saetiew and Teerarak) reported 93.33% with 1 mg/L BA + 0.1 mg/L IAA in *Chrysanthemum*. Shoot proliferation was highest (13.03 shoots) in half-strength MS medium supplemented with 2.0 mg/L BAP. Similar findings were reported in *Chrysanthemum* (7.7 shoots) (Kashif Waseem et al. 2011) and *Xanthosoma sagittifolium* (13.44 shoots) (Bansal et al. 2023). Shoot length (8.90 cm) was optimal with 2.0 mg/L BAP, consistent with Ghosh et al.(Ghosh et al. 2021). Root induction was highest (90.40%) with half-strength MS medium + 0.2 mg/L IBA as supported by studies of researchers (Yesmin et al. 2014), (Rashid et al. 2009), (Kashif Waseem et al. 2011), and (Sushmarani, Venkatesha Murthy and Deeksha Raj 2021). Higher auxin concentrations inhibited rooting (Kaul et al. 1990) and longest roots (12.30 cm) were obtained with 0.4 mg/L IBA (Komalavalli and Rao 2000), while maximum roots (11.66 per shoot) were observed with 0.2 mg/L IBA, aligning with other researchers (Yesmin et al. 2014, Deltalab et al. 2024).

The development of salt-tolerant plants through in-vitro techniques, such as callus and shoot cultures is a non-transgenic route. Since, salt stress typically reduces plant growth (Queiros et al. 2007), as slower growth linked to improved stress survival. In our study, the highest shoot regeneration frequency (79.86%) was recorded at 25 mmol/L NaCl, with the lowest observed at 300 mmol/L. Salt stress reduced shoot regeneration frequency due to impaired cell division and photosynthesis, as reported in similar studies on Chrysanthemum (Thongpukdee et al. 2012) and *Bacopa monnieri* (Sable et al. 2018).

4.2. Physiological, biochemical and histological analysis of *in-vitro* regenerated plant under NaCl stress

Under salinity stress, *C. morifolium* displayed significant physiological and biochemical alterations compared to control conditions. Salinity treatment influenced photosynthetic pigments, including chlorophyll and carotenoids. The highest levels of chlorophyll A, chlorophyll B, total chlorophyll, and carotenoids were observed at 25 mmol/L NaCl (T1). However, the chlorophyll A/B ratio and carotenoid/chlorophyll ratio (0.231) at 200 mmol/L NaCl (T7). This decline in chlorophyll is attributed to pigment-protein complex instability under stress, as noted in previous studies (Dogan 2020, Rai et al. 2020). Moreover, the proline and protein levels varied significantly under salt stress. Maximum protein content was observed at 25 mmol/L NaCl, while proline accumulation peaked at 300 mmol/L, reflecting

the role of osmolites in stress mitigation. Antioxidative enzymes showed increased activity, with SOD, CAT, and H_2O_2 scavengers rising by 2.3-, 2.8-, and 3.1-fold, respectively, at 300 mmol/L NaCl, supporting previous findings in *Chrysanhemum* (Guan et al. 2012). NBT and DAB staining confirmed increased O_2^- and H_2O_2 accumulation under salt stress, leading to oxidative damage, reduced membrane integrity, and necrotic lesions at 300 mmol/L NaCl, consistent with studies on *Brassica napus* and *Chrysanthemum* (Banerjee and Roychoudhury 2017, Ahmad et al. 2019).

4.3. The impact of varying NaCl concentrations on tissue-cultured *C. morifolium* plants under in-vivo conditions

Salinity stress significantly impacted plant biomass by altering water potential, increasing ion toxicity, and inhibiting cell wall expansion, leading to reduced root growth and plant height. A decline in growth parameters was observed with increasing NaCl concentrations, with the most significant biomass reduction at 45 DAS in S5 (400 mmol/L NaCl). Leaf number, plant height, and secondary shoot numbers varied across treatments, with S2, S3, and S1 showing the highest values at different stages. Plant spread was highest in S1, while primary stem diameter peaked in S4 and secondary shoot diameter in S3, S4, and S2 at different DAS intervals. These results align with previous studies on ornamental plants like *Tagetes erecta* (Sayyed et al. 2014), *Chrysanthemum* (Bañón et al. 2010), and marigold (Bahmanzadegan and Aboutalebi 2013), where salt stress-induced growth reduction was attributed to osmotic and ionic imbalances, inhibited cell division and elongation, and decreased cell wall extensibility. Prolonged salinity exposure led to severe symptoms such as leaf scorching, necrosis, and leaf drop, potentially resulting in plant death.

Salinity significantly affects root growth by limiting nutrient and water uptake, ultimately impacting plant health and yield. In our study, maximum root length was recorded under S1 (25 mmol/L NaCl), while the negative control exhibited the least growth. Similar reductions in root length under increasing salinity have been reported in *Chrysanthemum indicum* (Ranganayakulu, Veeranagamallaiah and Chinta Sudhakar 2013, Mazhar et al. 2012). Additionally, soil EC, a key indicator of soil health, increased with higher NaCl concentrations, peaking in S5 (400 mmol/L NaCl) at 45 DAS. In contrast, soil pH remained highest in S1 and S2 at different time points.

4.4. Effect of NaCl on physiology and biochemical changes of tissue cultured raised C. morifolium under In vivo salt stress

Salinity stress significantly impacts photosynthesis by inducing chlorosis, reducing pigment synthesis, and increasing oxidative stress(Shaw 1995, Parida and Das 2005). Carotenoids, which protect against ROS, decline under salt stress, leading to chlorophyll degradation (Rao and Rao 1981). In our study, the highest chlorophyll A (105.71 µg/g), chlorophyll B (61.57 µg/g), total chlorophyll (167.28 µg/g), and carotenoids (33.16 µg/g) were observed in S1 (25 mmol/L NaCl), while the highest chlorophyll A/B ratio (2.09 µg/g) was found in S5 (400 mmol/L NaCl), consistent with previous reports in corn (Molazem, Qurbanov and Dunyamaliyev 2010), cucumber (Malik et al. 2010), and purslane (Parvaneh, Shahrokh and Meysam 2012). Protein content, crucial for osmotic adjustment, was highest in S1 (47.87 µg/g),

indicating a balance between synthesis and degradation under moderate stress (Dogan 2020, Shatnawi et al. 2010). Proline, a key osmo-protectant, peaked in S5 (98.63 µg/g), supporting its role in salinity tolerance as reported by other researchers (Ashraf and Tufail 1995, Mansour et al. 2005).

ROS scavenging enzymes, such as SOD and CAT, play a vital role in mitigating oxidative stress (Yasemin et al. 2021, Parvin et al. 2019). The highest SOD (113.19 unit/min/mg), CAT (143.87 µmoles H_2O_2 decomposed min-1 mg-1), and H_2O_2 content (139.14 µmoles g-1 FW) were observed in S5, indicating increased ROS detoxification at higher salinity (Zandalinas et al. 2017, Laxa et al. 2019). High ROS levels, including superoxide anions (O_2^-) and H_2O_2, lead to oxidative damage and plasma membrane disruption, as observed in *C. morifolium* under severe salt stress (Banerjee and Roychoudhury 2017, Su et al. 2019). Similar trends have been reported in *Brassica napus* (Huang et al. 2022), where enhanced CAT and GR activity contributed to stress tolerance (Bor, Özdemir and Türkan 2003, Yaşar and Ellialtıoğlu 2013).

5. Conclusion

The present study established a standardization protocol that can be used to produce true-to-type plants, which are otherwise challenging to obtain for this species. This well-standardized protocol may facilitate large-scale multiplication of desired types, thereby meeting the demand for high-quality planting material of *C. morifolium*.

The second main focus of the present investigation was to analyze the morphological, physiological, and biochemical responses of *Chrysanthemum* to NaCl-induced salinity stress under *In-vitro* and *In-vivo* (pot culture) raised tissue-cultured plants. Based on the findings, it is concluded that salinity imposes both osmotic and ionic stress, impairing plant cell functions, damaging cell membranes, and affecting the photosynthetic apparatus. Salt stress reduces biomass, plant growth, chlorophyll content, carotenoid content, and restricts the accumulation of beneficial nutrients in plant tissues. Plants adapted to salt stress through mechanisms such as upregulation of antioxidant enzyme activities and accumulation of osmolytes like proline, which protect plant cells from the harmful effects of Na^+ and Cl^- ions. Plants that performed better under varying NaCl concentrations likely employed these mechanisms to maintain ionic homeostasis and protect against reactive oxygen species generated under salinity stress. These findings are commercially valuable for developing salt-tolerant varieties, ensuring better survival and productivity in saline environments.

Declarations

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Author contributions: SP, RK: conceived the idea and drafted the manuscript; RM: performed Plant tissue culture work; VC: analysis of physiological and biochemical work and editing work; SK edited the manuscript.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Supplementary Material

The Supplementary Tables are not available with this version.

Figures

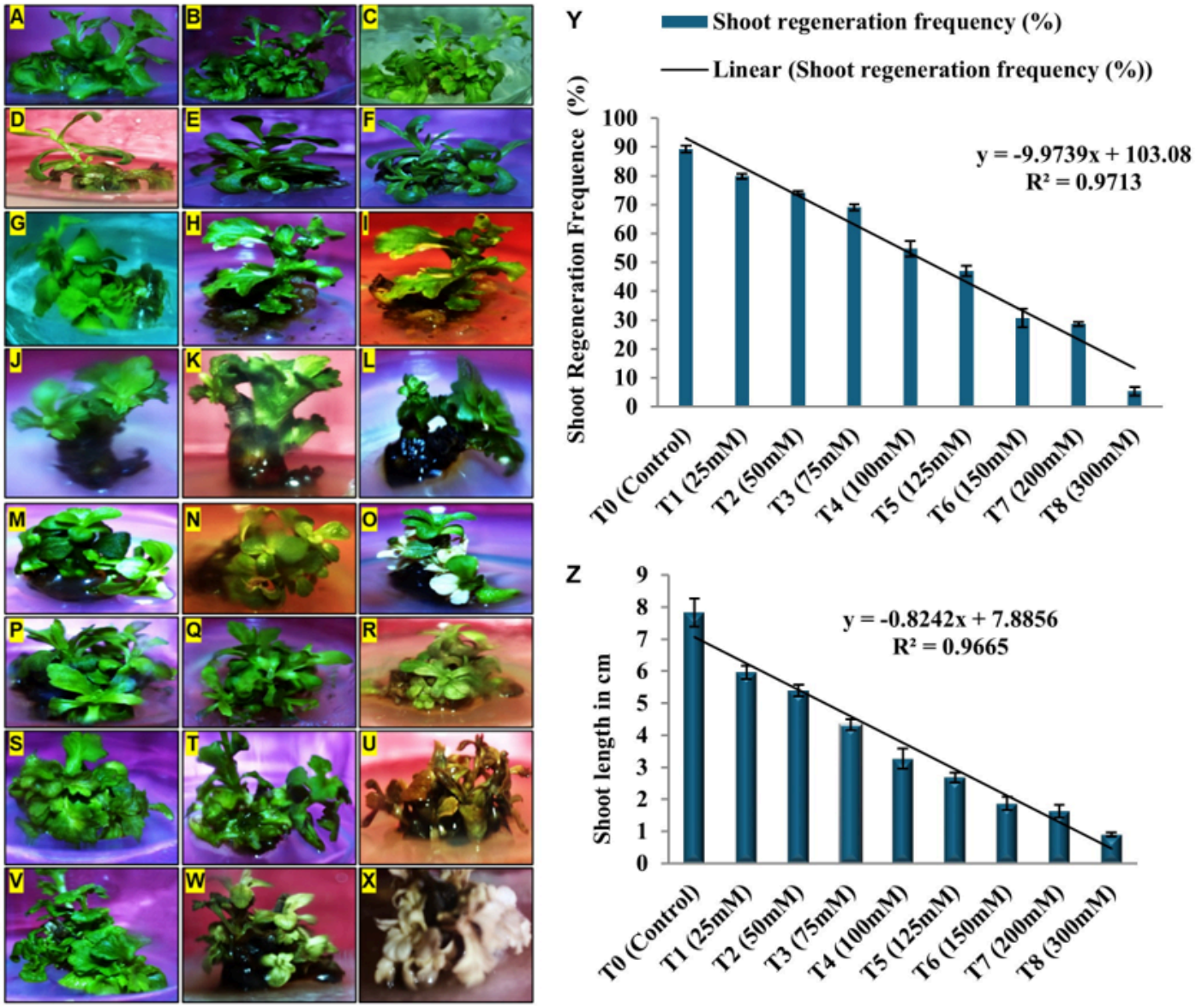

Figure 1

(**a)** Shoot multiplication in Murashige and Skoog (MS) media under different concentrations of NaCl salt stress: 25 mM (A - 10 days, B - 20 days, C - 30 days), 50 mM (D - 10 days, E - 20 days, F - 30 days), 75 mM (G - 10 days, H - 20 days, I - 30 days), 100 mM (J - 10 days, K - 20 days, L - 30 days), 125 mM (M - 10 days, N - 20 days, O - 30 days), 150 mM (P - 10 days, Q - 20 days, R - 30 days), 200 mM (S - 10 days, T - 20 days, U - 30 days), and 300 mM (V - 10 days, W - 20 days, X - 30 days). Figure demonstrates the effect of varying NaCl concentrations on shoot growth and multiplication over time. **b)** Shoot regeneration frequency at different concentration of NaCl (25mM, 50mM, 75mM,100mM,125mM,150mM, 200mM,200mM) in half MS media. **c)** Shoot length at different concentration of NaCl (25mM, 50mM, 75mM,100mM,125mM,150mM, 200mM,200mM) in half MS media.

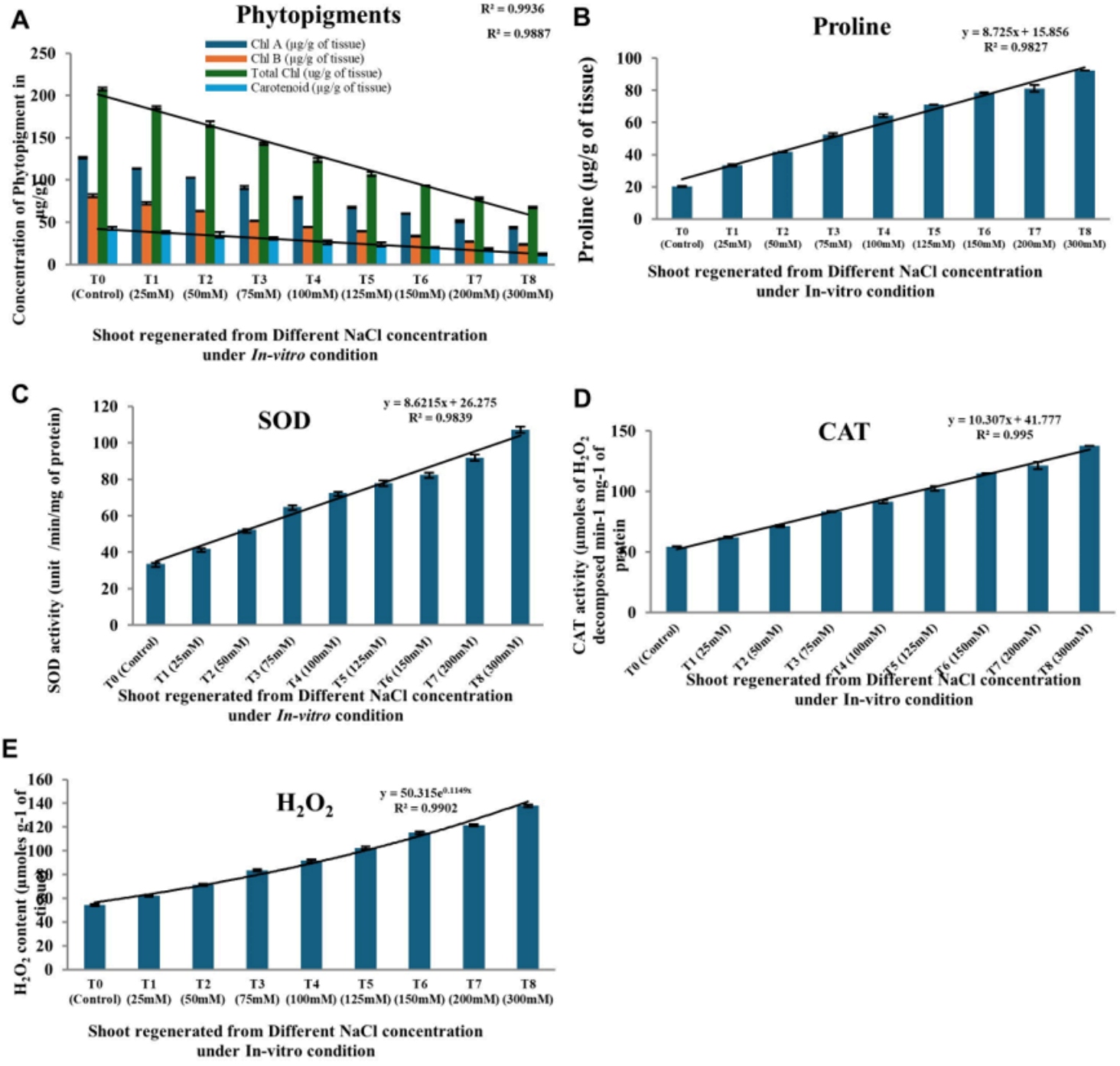

Figure 2

Physiological and Biochemical analysis of leaves collected from tissue culture raised plants at different concentration of NaCl (Control, 25mM, 50mM, 75mM,100mM,125mM,150mM, 200mMand300mM) in half MS media. (**A)** Chlorophyll and Carotenoid; (**B)** Protein; (**C)** Proline : (**D)** CAT; (**E)** H_2O_2; (**F)** SOD estimation by spectrophotometer methods.

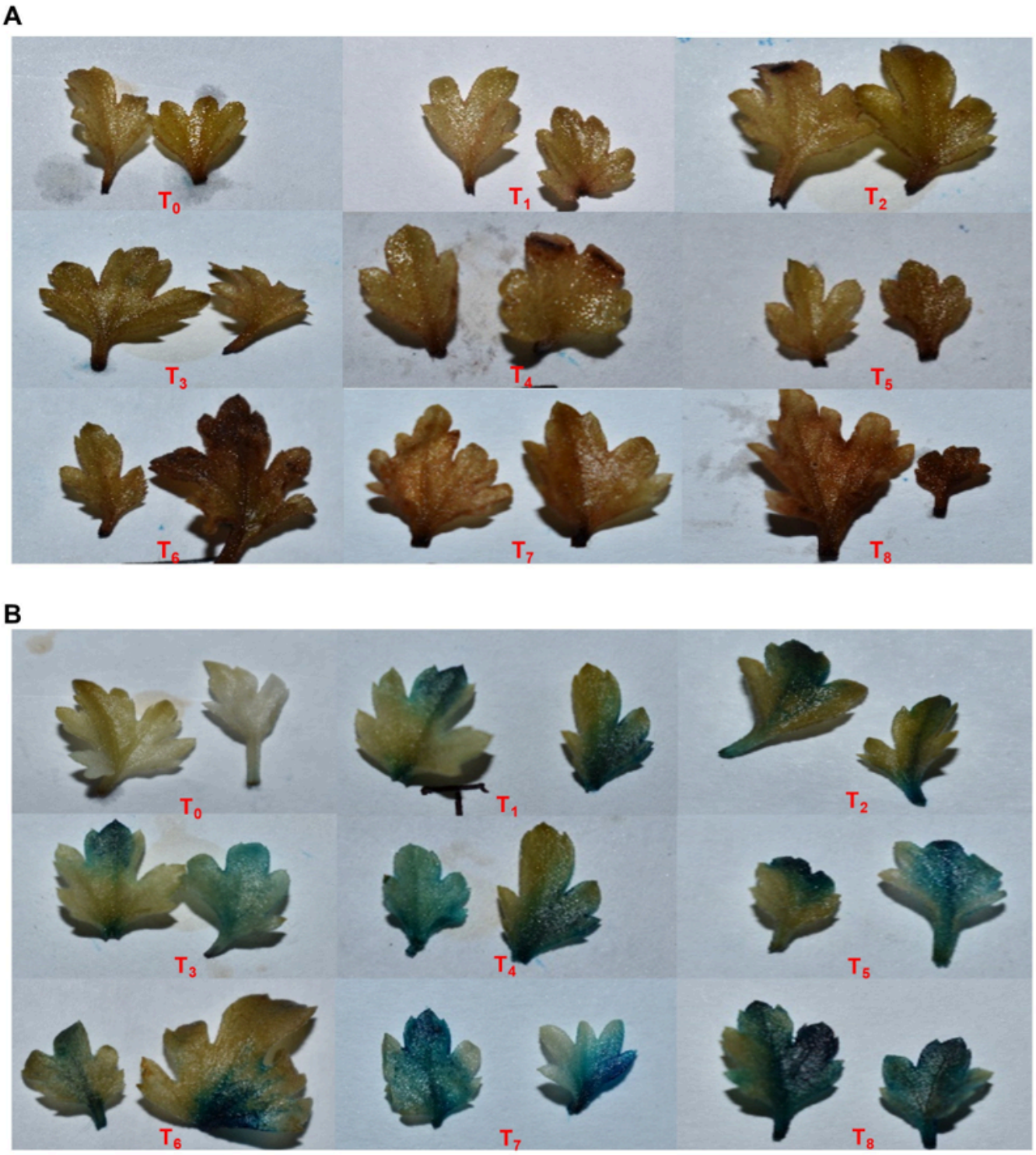

Figure 3

The histochemical analysis of *C. morifolium* leaves grown under tissue culture half MS media supplemented with different concentration of NaCl : Control (T_0) 25mM (T_1), 50mM (T_2)75mM(T_3),100mM(T_4),125mM(T_5),150mM(T_6), 200mM(T_7),300mM(T_8); **(a)** DAB Staining showed the accumulation of H2O2; (**b)** NBT Staining showed the accumulation of SOD

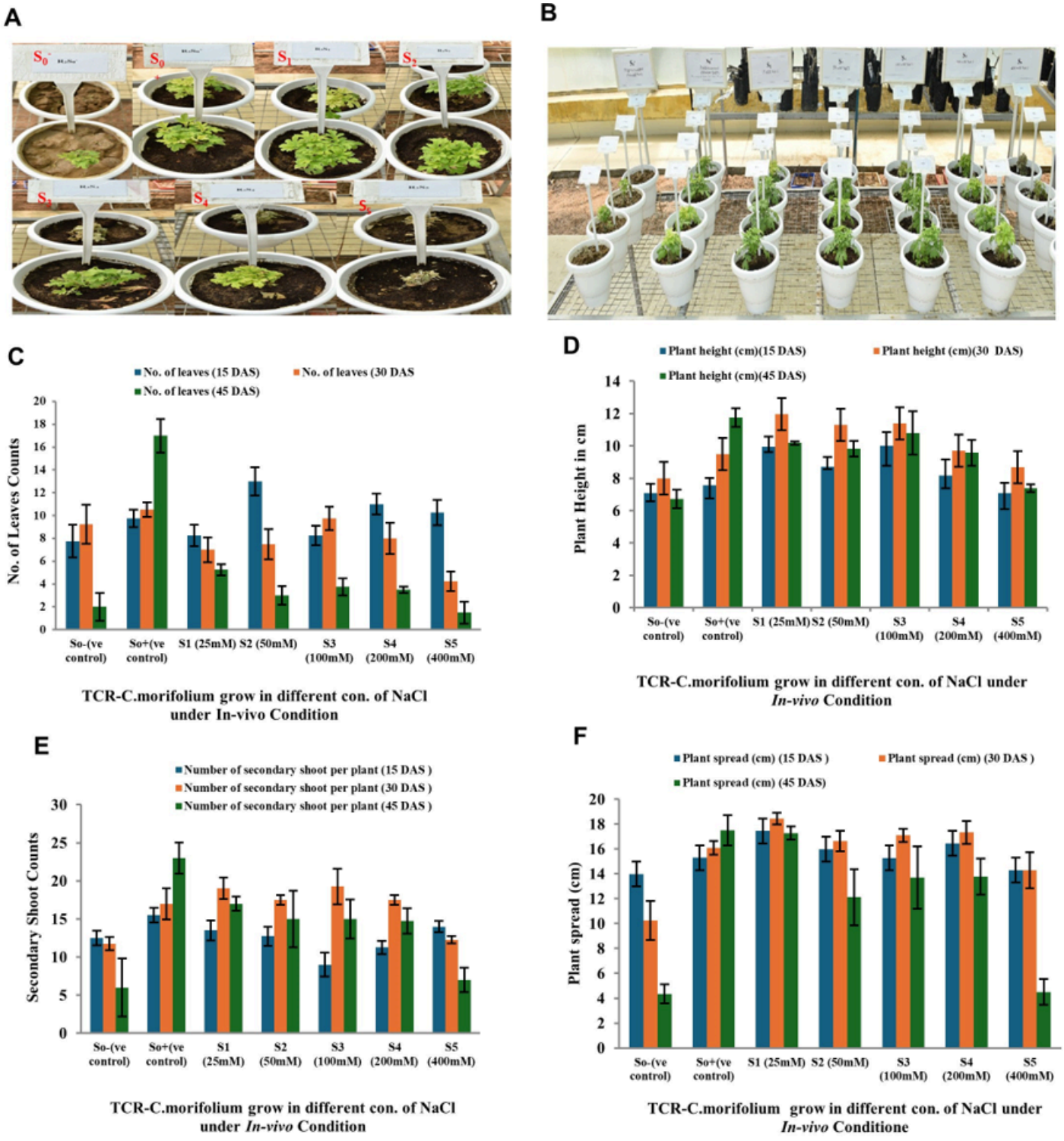

Figure 4

TCR-*C. morifolium plants* transferred to media having different concentration of NaCl (0 – 400 mmol) under *In-vivo*conditions a) after 15 days b) after 45 days; (**c)**Number of leaves; (**d)** Plant height (cm); (e**)** Number of secondary shoots; (**f)** Plant spread (cm) at 15, 30 and 45 DAS.

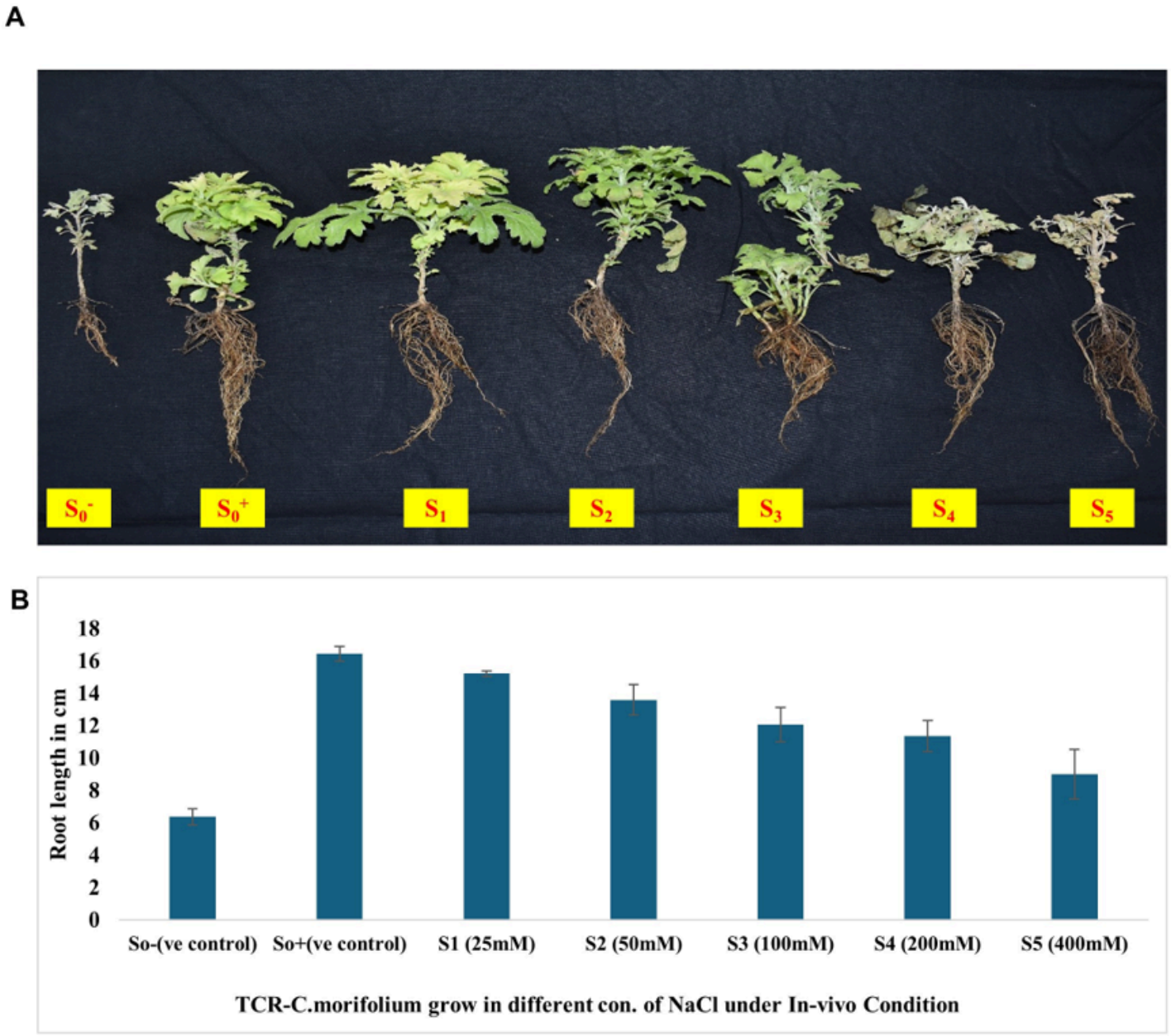

Figure 5

(A) Root observation after different salt stress under *In-vivo*conditions at 45 DAS and b) Graphical representation of the root length (cm) at 45 DAS

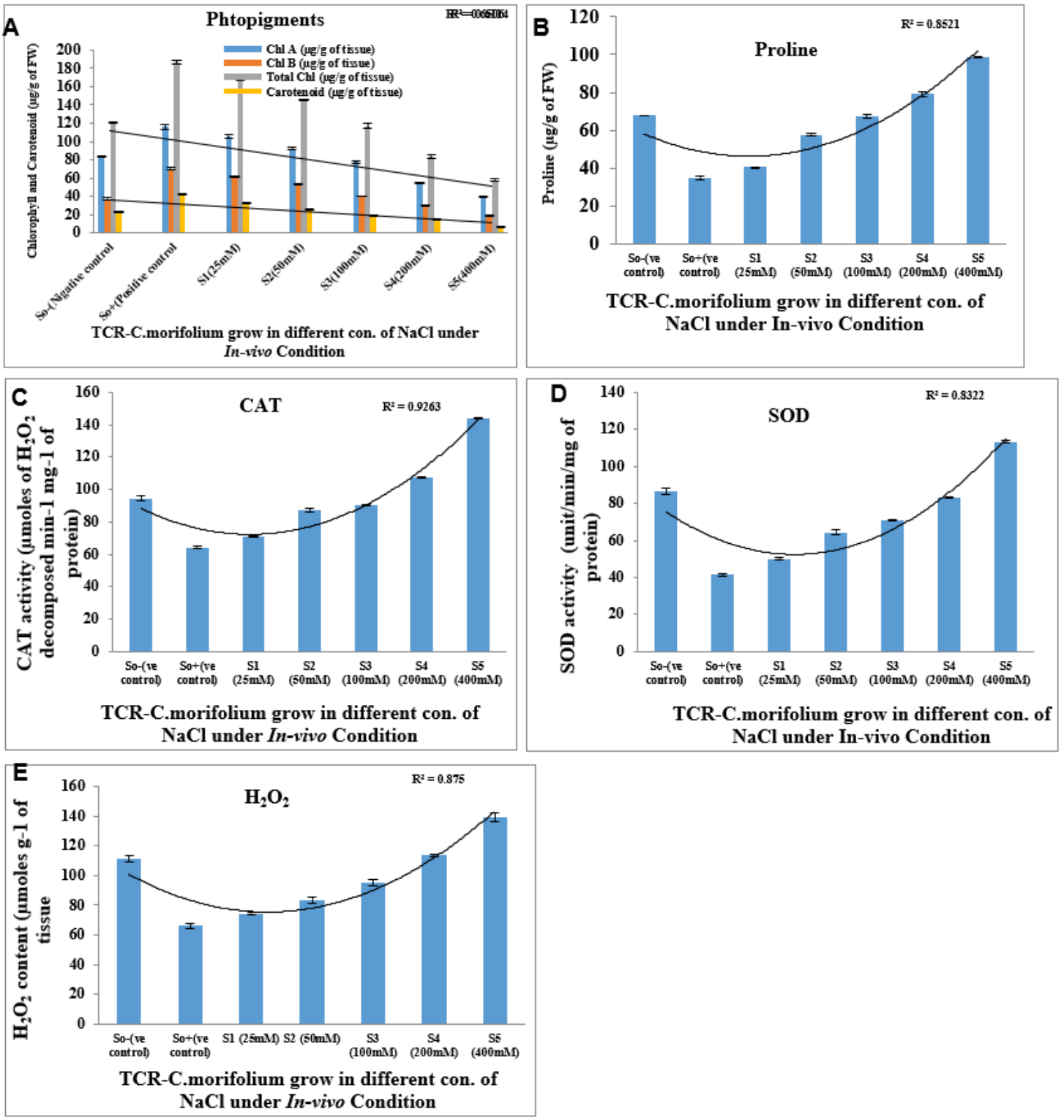

Figure 6

Changes in (A) Phytopigments (B) Proline, (C) CAT, (D) SOD and (E) H2O2 content of *C. morifolium* under *In-vivo* salt stress and control conditions

Supplementary Files

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