Physiological and Biochemical Responses of Chrysanthemum × morifolium to Salinity Stress under In-vitro and in vivo 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. The present study investigated the induction, multiplication, rooting, and salinity tolerance of *Chrysanthemum morifolium* under both in-vitro and in-vivo conditions. Maximum shoot induction (89.66%) and proliferation (13.03 shoots per explant) were obtained on half-strength MS media supplemented with 2.0 mg/L BAP. To evaluate salinity tolerance, eight NaCl concentrations (0–300 mM) were tested in-vitro, showing highest regeneration at 25 mM and survival up to 30 days at 100 mM, indicating moderate tolerance. Salt primed microshoots were rooted efficiently (90.40%) on half-strength MS media containing 0.2 mg/L IBA, with the longest roots (12.30 cm) at 0.4 mg/L IBA. During the hardening phase, the highest survival rate was obtained using a potting mixture of coco peat, sand, vermicompost, and garden soil in a 2:1:1:1 ratio. In-vivo pot culture studies revealed that exposure to 100 mM NaCl resulted in moderate stress symptoms, whereas 200 mM NaCl caused pronounced growth inhibition, significant biomass reduction, leaf scorching, and necrosis. Physiological and biochemical analyses revealed that moderate salinity (25–100 mM) enhanced chlorophyll, carotenoids, and prolin, whereas severe salinity (≥ 200 mM) led to a sharp decline in pigments, elevated proline accumulation, and activation of antioxidant enzymes (SOD, CAT) to mitigate oxidative stress. This integrated in-vitro and in-vivo approach provides a robust platform for screening and developing salt-tolerant *C. morifolium* genotypes suitable for cultivation under saline conditions.

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 supplementing the media with sodium chloride (NaCl), providing a controlled system to study cellular responses (Shibli et al., 2007). The studies have demonstrated species-specific variations in *in vitro* salt tolerance in sugarcane tolerated up to 212–254 mM NaCl with stability confirmed under greenhouse conditions (Laksana et al., 2023), *Pistacia vera* L. showed highest performance at 120 mM NaCl (Raoufı et al., 2021), and nodal explants of *Limnophila aromatica* (Lamk.) Merr. and *Bacopa monnieri* (L.) Wettst. tolerated up to 100 mM NaCl (Dogan, 2020). The present study aimed to optimize NaCl concentrations for tissue-cultured chrysanthemums under in vitro conditions and to apply the optimized levels to in vitro-developed plants under polyhouse conditions. The plants were exposed to varying NaCl concentrations to assess their effects on growth and stress tolerance, along with associated physiological and biochemical responses during development.

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 Source of explant and surface sterilization: Healthy donor plants of *Chrysanthemum × morifolium*, a perennial herb maintained through vegetative propagation, were collected from the greenhouse of the Ornamental Nursery at department of Floriculture, Banda University of Agriculture and Technology, Banda (UP). Nodal segments were rinsed with running tap water for 30 min. The surface sterilization of

explant was done under aseptic condition using different concentration of biocides, fungicides, and sodium hypochlorite, followed by $HgCl_2$ as surface sterilization for *in vitro* culture (Table S1).

2.2 **Preparation of Culture Media**: Murashige and Skoog (MS) basal media salt (Murashige and Skoog, 1962) (Hi-medis, India) was prepared with sucrose (30 g/L) as carbon source, and solidified with agar (7 g/L). The pH was adjusted to 5.7–5.8 before autoclaving at 121°C and 1.05 kg cm − 2 pressure for 20 min. All the cultures were incubated in culture room at 25 ± 1°C with light intensity 40 $\mu mol^{-2} s^{-1}$ for 16 h/8 h light/dark photoperiod provided by cool white, fluorescent tubes (Philips, India).**2.3 In vitro shoot induction and multiplication**: For shoot induction and multiplication, *C. morifolium*, nodal explants (3–4 cm) were cultured for assessing the effects of different culture media for *in vitro* shoot multiplication. A total of eighteen either full or half MS strength culture media supplemented with various concentrations of 6-Benzylaminopurine (BAP), kinetin (Kn), Thidiazuron (TDZ) were used for the present study. Full or half strength basal MS media free from plant growth regulators was used as control. Observations were recorded for percentage shoot induction, number of shoots per explant, and shoot length. The details of different culture media are presented in Table S1.

2.4 Sub-Culturing and Root Induction Under Salt Stress: To evaluate salinity effects, sterile NaCl stock solutions (25–300 mM) were added to MS media (pH 5.7–5.8) prior to autoclaving. The microshoots (3–5 cm) were subcultured on NaCl-supplemented media. Plant growth was regularly monitored for signs of salt stress, including 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. Healthy microshoots (2–5 cm) were inoculated onto half-strength MS media supplemented with IBA or IAA (0.2–1.0 mg/L) (Table S2).

2.5 Establishment of In-vitro propagated plants for hardening

Rooted plantlets were gently removed from culture bottles, washed to eliminate agar, treated with a mild fungicide, and subjected to primary hardening for two weeks in a cocopeat, perlite, and vermiculite mixture (2:1:1) under poly-house conditions without salt stress. Subsequently, the acclimatized plants were transferred to pots containing vermicompost, garden soil, sand, and cocopeat (2:1:1:1) for secondary hardening and maintained under controlled poly-house conditions. During this period plants were then irrigated with NaCl solutions at 25, 50, 100, 200, and 400 mM, while normal soil of BUAT, Banda (So^-) and vermicompost, garden soil, sand, and cocopeat (2:1:1:1) (So^+) plants served as controls. Physiological and biochemical responses, including growth rate, chlorophyll and carotenoid content, protein concentration, proline accumulation, and antioxidant enzyme activity, were monitored under both stress and control conditions. Growth traits were recorded at 15, 30, and 45 DAT, and data were analysed statistically using ANOVA at a 5% significance level

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:

2.6.2.1.1. 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.

2.6.2.1.2. 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). The leaves 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.

2.7. Experimental Design and Statistical analysis

The experiments were conducted using a completely randomized design (CRD) with three replications per treatment. Data on shoot induction, proliferation, and rooting were recorded at defined intervals, expressed as mean ± standard error (Mean ± SE), and analyzed by one-way ANOVA using Microsoft Excel to assess the significance of treatment effects

Result

3.1 In vitro shoot induction and multiplication:

The study investigated the effect of sterilizing agent, 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 *C. 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 media, 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 media 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 S2).

3.2. Sub-Culturing and Root Induction in *C. morifolium* under Salt Stress:

To evaluate the effect of salinity stress on shoot regeneration, eight NaCl concentrations (0, 25, 50, 75, 100, 125, 150, 200, and 300 mM) were used to establish a gradient from mild to severe stress. These concentrations were incorporated into half-strength MS medium supplemented with 2 mg L^{-1} BAP for subsequent culture experiments. Under control conditions (T0), the regeneration frequency was same as described above (Figure S1a&b and Table 1). 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 (Fig. 1 **(a-x), y & z; Table S3).** The result showed that the salt stress caused a substantial decline in regeneration frequency and shoot length, with the inhibitory effects increasing with higher NaCl concentrations. The healthy microshoot of *C. morifolium* grown at salt stress of 150 mmol of shooting media after 30 day stress were transferred to NaCl-free, half-strength MS incorporated with 0.2-1.0 mg/L IBA/IAA media. In which 0.2 mg/L IBA gave the highest rooting (90.4%, 11.66 roots/shoot), while 0.4 mg/L IBA produced the longest roots (12.30 cm). IAA showed lower efficiency, and higher auxin levels (1.0 mg/L) suppressed rooting (Fig. 2a &b and table S4).

3.3. 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 (Fig. 3a). Proline, significantly increased with salinity, at 300 mmol/L NaCl (92.33 µg/g) (Fig. 3b).

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: 3c-3e**).** 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 **(**Fig. 3a-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 (Fig. 3a-e).

3.4. Histochemical detection of Superoxide and H_2O_2 by NBT and DAB staining of C. morifolium 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 (Fig. 4A (T_0-T_8)), while NBT staining showed dark blue spots, indicating superoxide build up (Fig. 4B (T_0-T_8)).

3.5. 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 S2). 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.

3.6. Establishment of *In-vitro* propagated plants of *C. morifolium* for hardening

The tissue culture-raised, salt stress-primed and rooted *C. morifolium* plants were subjected to sequential acclimatization and hardening. In the primary hardening stage, the plants exhibited significant growth in a media 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 comprising vermicompost, garden soil, sand, and cocopeat in a 2:1:1:1 ratio, where they were allowed to establish well under controlled polyhouse conditions. After one month of establishment, tissue culture-raised *(*TCR*) C. morifolium* plants were subjected to *in vivo* screening under different salt concentrations. Treatments included a negative control (field soil from BUAT, Banda), a positive control (designed soil mixture), and NaCl solutions at 25, 50, 100, 200, and 400 mM, applied in distilled water as irrigation three times at 15-day intervals following primary and secondary hardening (Figure S3). The effects of these treatments were assessed at 15, 30, and 45 days after transplanting (DAT) under controlled polyhouse conditions (Fig. 5a–b). Significant effects were observed on parameters such as the number of leaves (Fig. 5c), plant height (Fig. 5d), secondary shoots (Fig. 5e), and plant spread (Fig. 5f), (Fig. 5c-f and Table S5). The highest number of leaves and plant height were recorded in treatments S2 and S3 (Fig. 5c). Secondary shoots at S3 (19.25 at 30 DAT) and S5 (14 at 15 DAT), while plant spread was greatest in S1 (18.45 cm at 30 DAT). Shoot diameters were maximized under S4 and S3, respectively (Table S5). The higher NaCl concentrations generally suppressed plant growth, with S1 and S3 performing best across most parameters. Negative controls demonstrated significantly reduced responses compared with positive controls.

Root length measurements at the end of the experiment (45 DAT) revealed the longest roots under S1 salt treatment (15.22 cm), followed by S2. Negative controls produced shorter roots compared to positive controls (Figs. 6A,B). Soil conductivity (E.C.) and pH were also influenced by NaCl levels (Supplementary table S6). At 45 DAT, 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 S6).

3.7. 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) (Figure. 7a). The chlorophyll A/B ratio increased under higher salt stress, at S5 (2.09) (Figure. 7a). 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 (Fig. 7a). Proline, an osmoprotectant, increased markedly under high salt stress, at S5 with 98.63 µg/g of fw, compared to the lowest content in S1 (40.88 µg/g of fw) (Fig. 7b). Antioxidant enzyme activities, including SOD and catalase, were significantly elevated in response to salt stress, with catalase activity at 143.87 µmoles of H_2O_2 decomposed/min/mg of protein in S5 (Fig. 7c), and SOD activity reaching a maximum of 113.19 unit/min/mg of protein in S5 (Fig. 7d). 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) (Fig. 7e). 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.

3.8. Histochemical Detection of superoxide and H_2O_2 by NBT and DAB Staining of In-vivo raised tissue cultured plants C. morifolium

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 DAT, 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 S4).

3.9. Principle component analysis:

The Principal Component Analysis (PCA) biplot reveals the relationships between physiological and biochemical variables and treatments 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 (Figure S5). 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.

Discussion

4.1. Induction and multiplication of *C. morifolium* under without and with salt stress condition

This study focuses on identifying optimal NaCl concentrations that do not adversely affect *C. morifolium* growth in both *in-vitro* and *in-vivo* (pot culture) conditions. Field-grown explants face microbial contamination challenges in *in-vitro* cultures. The explants under goes 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* (Verma et al. 2011) and yielded 65.08% survival in *Chrysanthemum* (Anjum et al. 2023). For shoot induction, half-strength of MS media with 30 g/L sucrose, 2.0 mg/L BAP, and 7 g/L agar achieved 89.66% induction. Comparable rates were reported in *C.* morifolium *at* 76.66%, 2.0 mg/L BAP (Yesmin et al. 2014), 83.30% with 2.0 mg/L BAP + 0.5 mg/L IAA (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, et al.,) 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 media supplemented with 2.0 mg/L BAP. Similar findings were reported in *Chrysanthemum* (7.7 shoots) (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).

For development of salt-tolerant plants through *in-vitro* techniques, a total of eight NaCl treatments (0/control, 25, 50, 75, 100, 125, 150, 200, and 300 mM) were selected to cover a broad salinity gradient ranging from mild to severe stress. The Fig. 1a (A-X) and graph (B&C) indicates a progressive decline in shoot regeneration frequency with increasing NaCl concentration. The highest regeneration ocurred at 25 mmol/L followed by a steady reduction as salinity increased. At 100 mmol/L, plants still survived up to 30 days, suggesting moderate tolerance. However, at 150 mmol/L, regeneration of plant up to 20 day

after that growth dropped sharply, showing that this concentration marks the onset of severe stress. Further increases to 200–300 mmol/L resulted in a drastic reduction in regeneration, with the lowest value at 300 mmol/L, indicating near-complete growth suppression under extreme salinity. Similar high-salt treatments have been successfully employed in *C. morifolium* by Hossain et al. 2007 and Li et al. 2024, confirming that concentrations in this range are suitable for screening salinity tolerance. Salt stress generally reduces plant growth, with slower growth often linked to better stress survival (Queiros et al., 2007). Similar to result in Chrysanthemum also reported by (Thongpukdee et al., 2012; Hossain et al., 2007) and in *Bacopa monnieri* by (Sable et al., 2018). Comparable tolerance has been observed in sugarcane at 212–254 mM NaCl by Laksana et al., 2023, in *Pistacia vera* at 120 mM; by Raoufi et al., 2021, and in *Limnophila aromatica* and *Bacopa monnieri* at 100 mM by Dogan, 2020, indicating that moderate salinity can support acclimation, whereas higher levels strongly inhibit regeneration.

The microshoot grown at 100 mmol Nacl, MS media upto 30 days transfer to NaCl free rooting media contained different concentration of IAA and IBA. The root induction was highest (90.40%) with half-strength MS media + 0.2 mg/L IBA as supported by studies of Yesmin et al. 2014; Rashid et al. 2009; Waseem et al. 2011, Sushmarani et al. 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 Yesmin et al. 2014, and Deltalab et al. 2024.

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; Hossain et al. 2007)).

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. In the present study, tissue culture-raised *C. morifolium* plants were exposed to a range of NaCl concentrations (25, 50, 100, 200, and 400 mM) to evaluate salinity tolerance under controlled polyhouse conditions. At Lower concentrations (25–100 mM) was TCR *C. morifolium* growth showed mild to moderate stress effects, whereas at 200mM – 400 mM represented severe stress conditions. A decline in growth parameters was observed with increasing NaCl concentrations, with the most significant biomass reduction at 45 DAT in S5 (400 mmol/L NaCl). Li et al. (2024) reported that 200 mM NaCl provides a consistent and measurable level of salinity stress in chrysanthemum plants without causing complete lethality, particularly when transferring a CmDOF18 gene. Leaf number, plant height, and secondary shoot numbers varied across treatments, with S1, S2, and S3 showing the highest values at different stages. Plant spread was highest in S1, while primary stem diameter in S4 and secondary shoot diameter in S2, S3, and S4 at different DAT 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, in S5 (400 mmol/L NaCl) at 45 DAT. In contrast, soil pH remained highest in S1 and S2 at different time points.

4.4 Effect of NaCl on physiology, biochemical and histochemical 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 DAT 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).

Conclusion

The present study successfully established a reproducible and efficient protocol for the in-vitro regeneration and salt stress screening of *C. morifolium*. The standardized protocol ensures the production of true-to-type plants, which are otherwise difficult to obtain for this species, and can be used for large-scale multiplication to meet the growing demand for high-quality planting material. Half-strength MS medium supplemented with 2.0 mg/L BAP was found optimal for shoot induction and proliferation, while 0.2 mg/L IBA achieved the highest rooting efficiency.

In-vitro and in-vivo salinity experiments revealed that *C. morifolium* exhibits moderate tolerance up to 100 mM NaCl, beyond which growth, biomass, and physiological performance declined sharply. Severe salinity stress (≥ 200 mM NaCl) caused marked reductions in root development, chlorophyll and carotenoid content, and photosynthetic efficiency, accompanied by membrane damage, necrosis, and biomass loss. Biochemical and histological analyses confirmed that salinity imposes both osmotic and ionic stress, disrupting cellular homeostasis, impairing photosynthetic apparatus, and restricting nutrient accumulation. Plants exposed to moderate salinity showed enhanced chlorophyll, carotenoids, and protein content, suggesting a priming effect, while elevated proline accumulation and upregulated antioxidant enzymes (SOD, CAT) under higher salinity indicated activation of stress mitigation pathways that protect against oxidative damage caused by Na^+ and Cl^- ions. These findings highlight the adaptive mechanisms employed by *C. morifolium* under salt stress and provide a robust platform for screening, selecting, and propagating salinity-tolerant genotypes. The outcomes of this work have significant commercial implications for sustainable floriculture and the development of salt-tolerant chrysanthemum cultivars for cultivation in saline-affected soils.

Declarations

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Table 1

Table 1 is not available with this version.

Figures

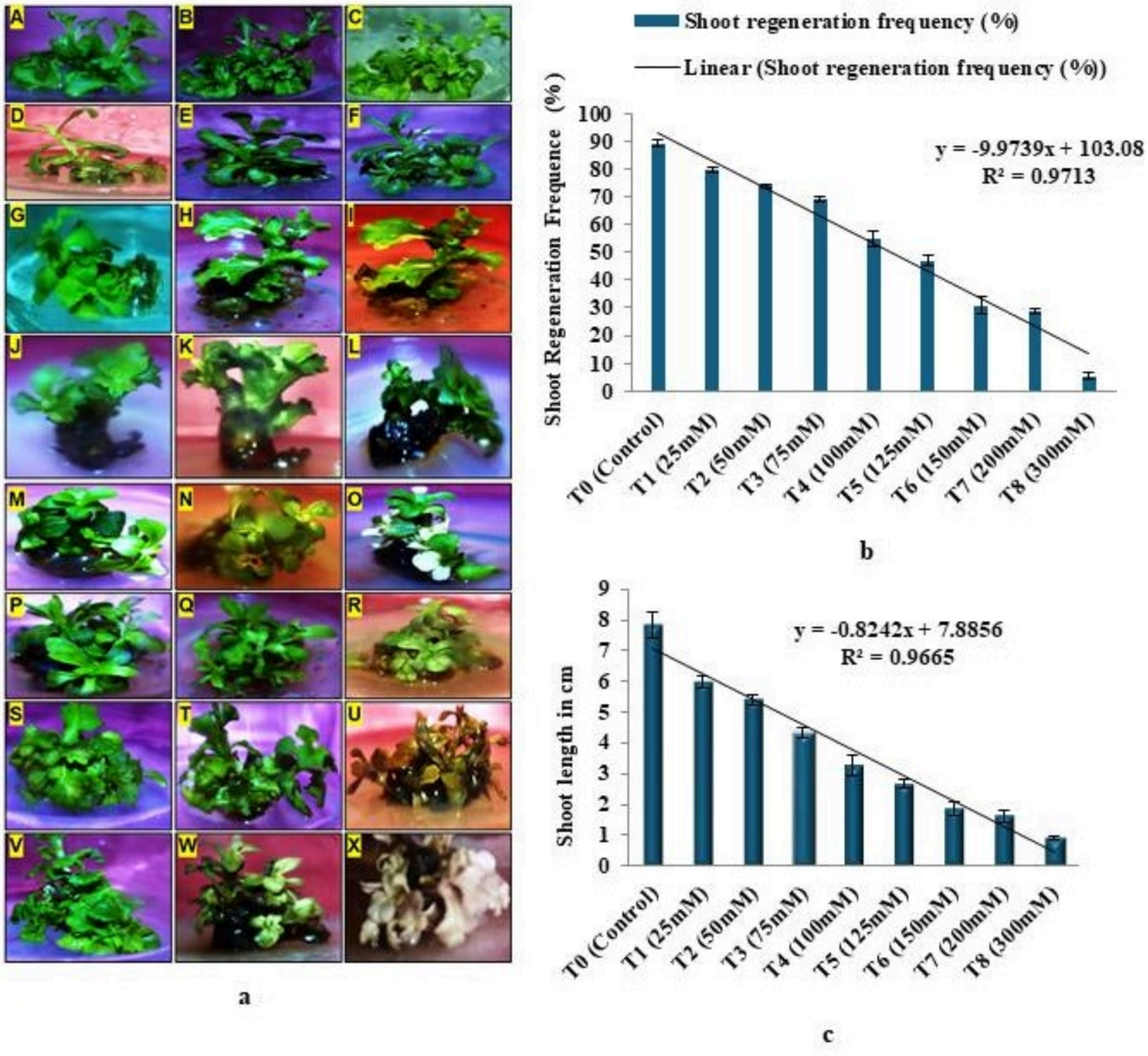
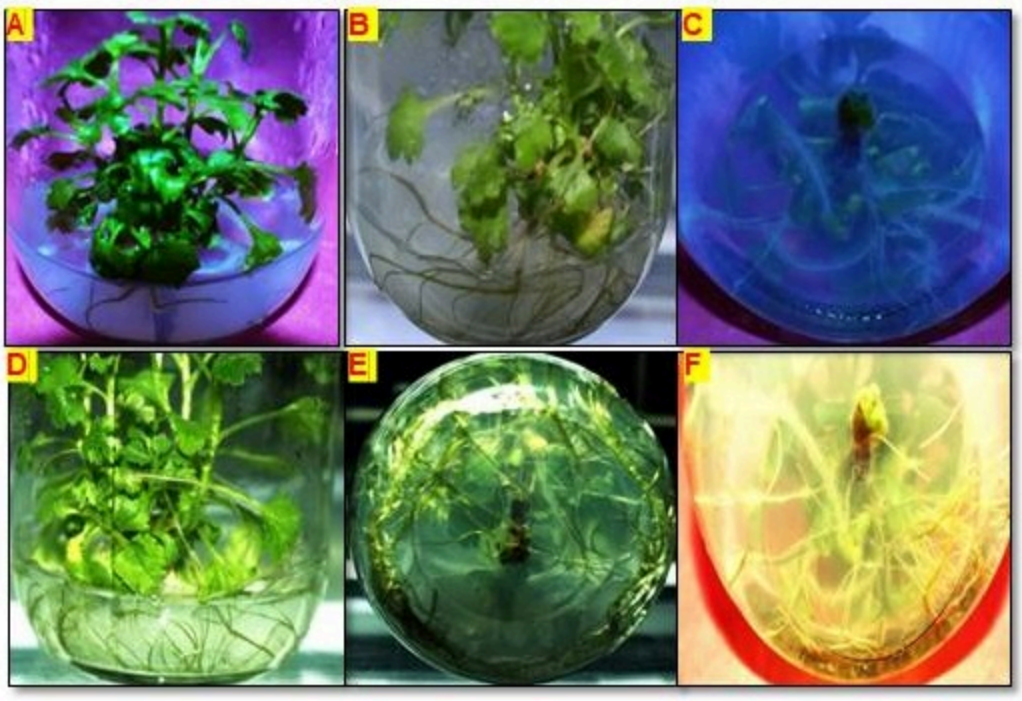


Figure 1

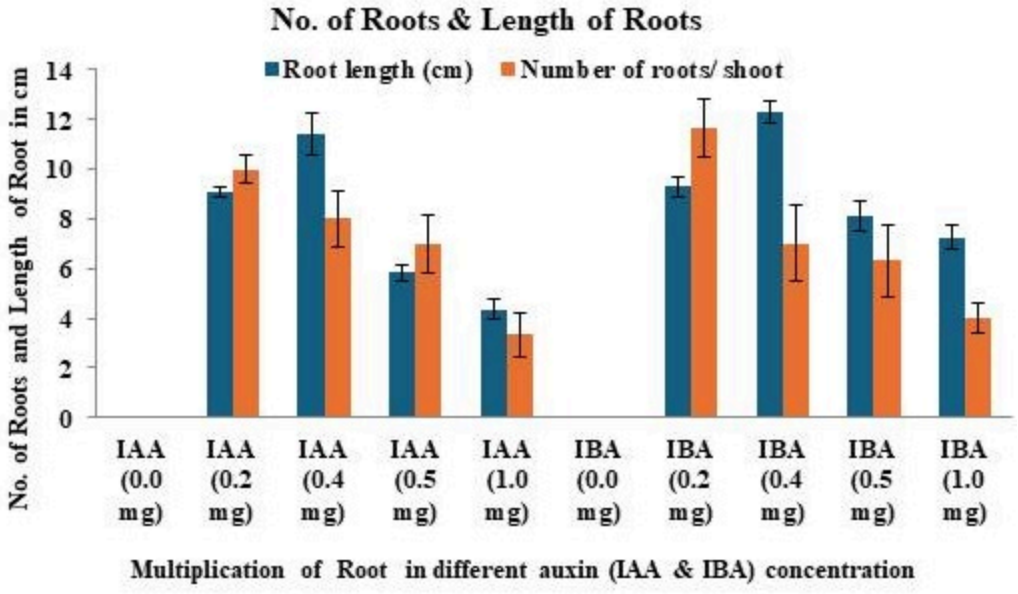
a) Shoot multiplication in Murashige and Skoog (MS) media under different concentrations of NaCl salt stress: 25 mM (A - 10 days, B - 20 days, C - 30 days), 50 mM (D - 10 days, E - 20 days, F - 30 days), 75 mM (G - 10 days, H - 20 days, I - 30 days), 100 mM (J - 10 days, K - 20 days, L - 30 days), 125 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).

b) Graphical representation of Shoot regeneration frequency at different concentration of NaCl (25mM, 50mM, 75mM,100mM,125mM,150mM, 200mM,200mM) in half MS media.

c) Graphical representation of Shoot length at different concentration of NaCl (25mM, 50mM, 75mM,100mM,125mM,150mM, 200mM,200mM) in half MS media.



a



b

Figure 2

(A) Healthy, prime microshoots of *C. morifolium* subjected to 100 mmol NaCl stress for 30 days were transferred to root induction and multiplication media with varying auxin concentrations (0.2–1.0 mg/L IAA (a–c) and IBA (d–f)) **(B)** Graphical representation of root response under, showing both root number and root length (cm).

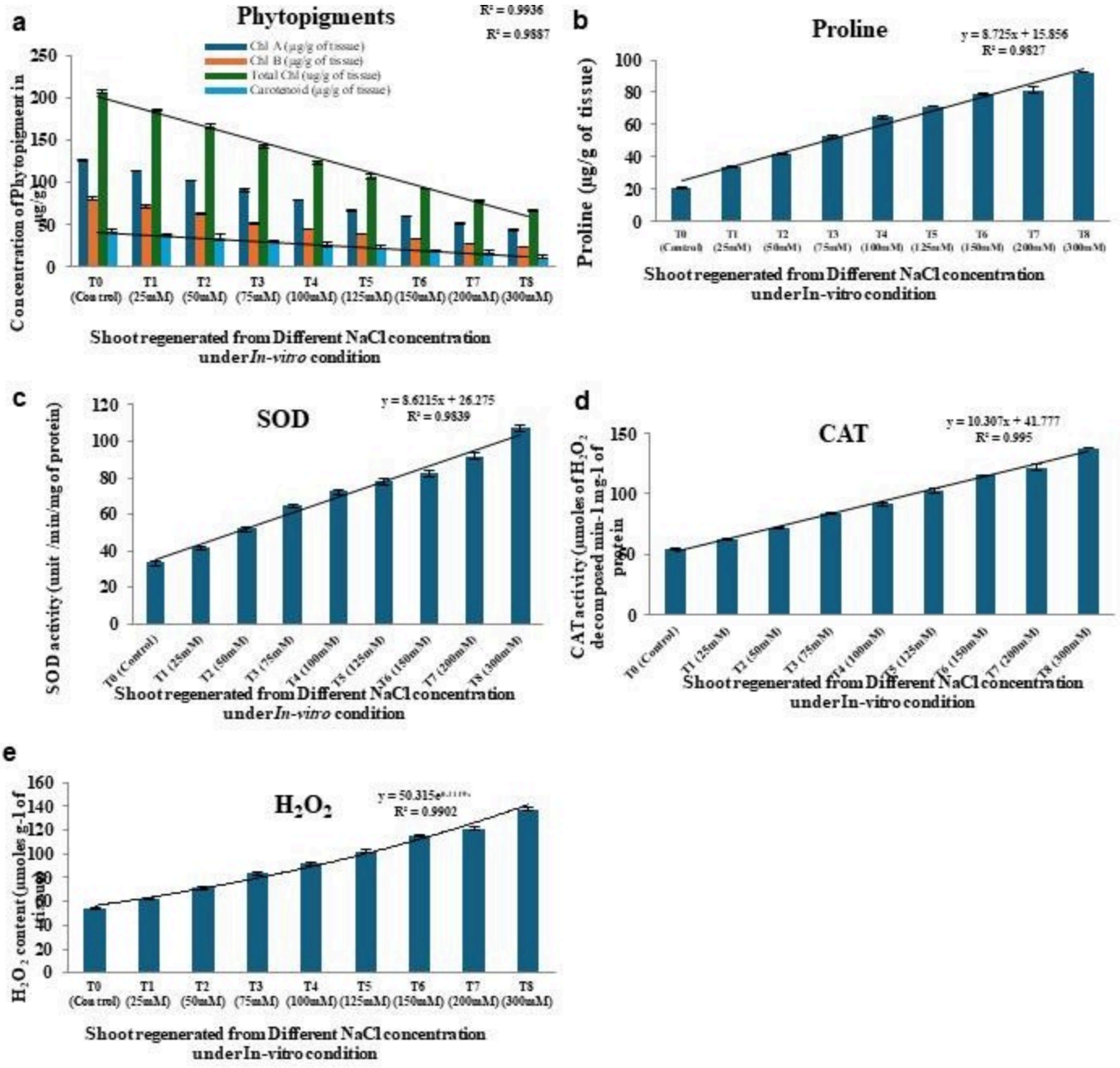


Figure 3

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)** Proline c) SOD d**)** CAT e**)** H_2O_2 estimation by spectrophotometer methods.

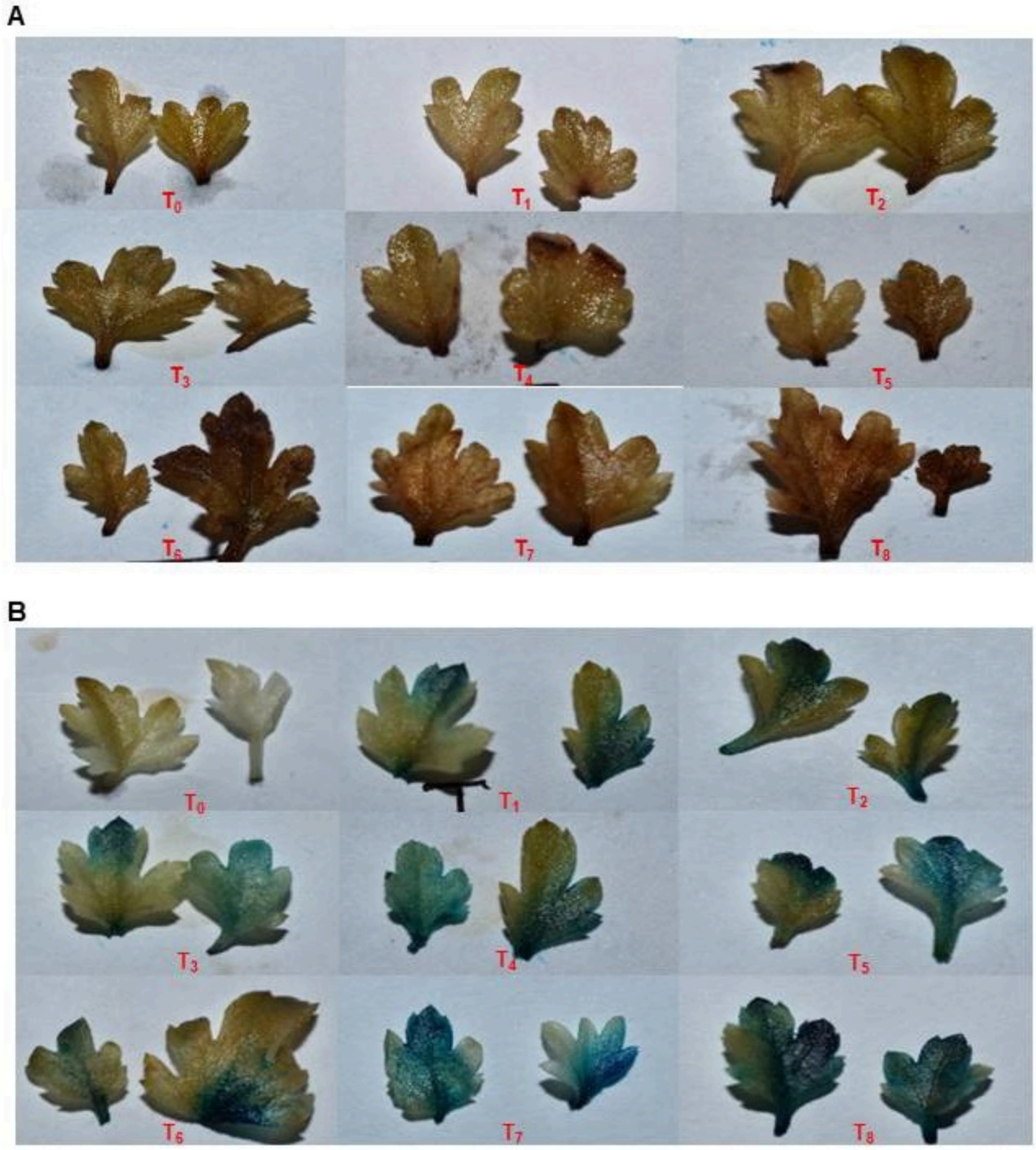


Figure 4

The histochemical analysis of *C. morifolium* leaves grown under tissue culture half MS media supplemented with different concentration Control (T0) 25mM (T1), 50mM T2)75mM(T3),100mM(T4),125mM(T5),150mM(T6),200mM(T7),300mM(T8) of NaCl :) a) DAB Staining showed the accumulation of H2O2 b) NBT Staining showed the accumulation of SOD

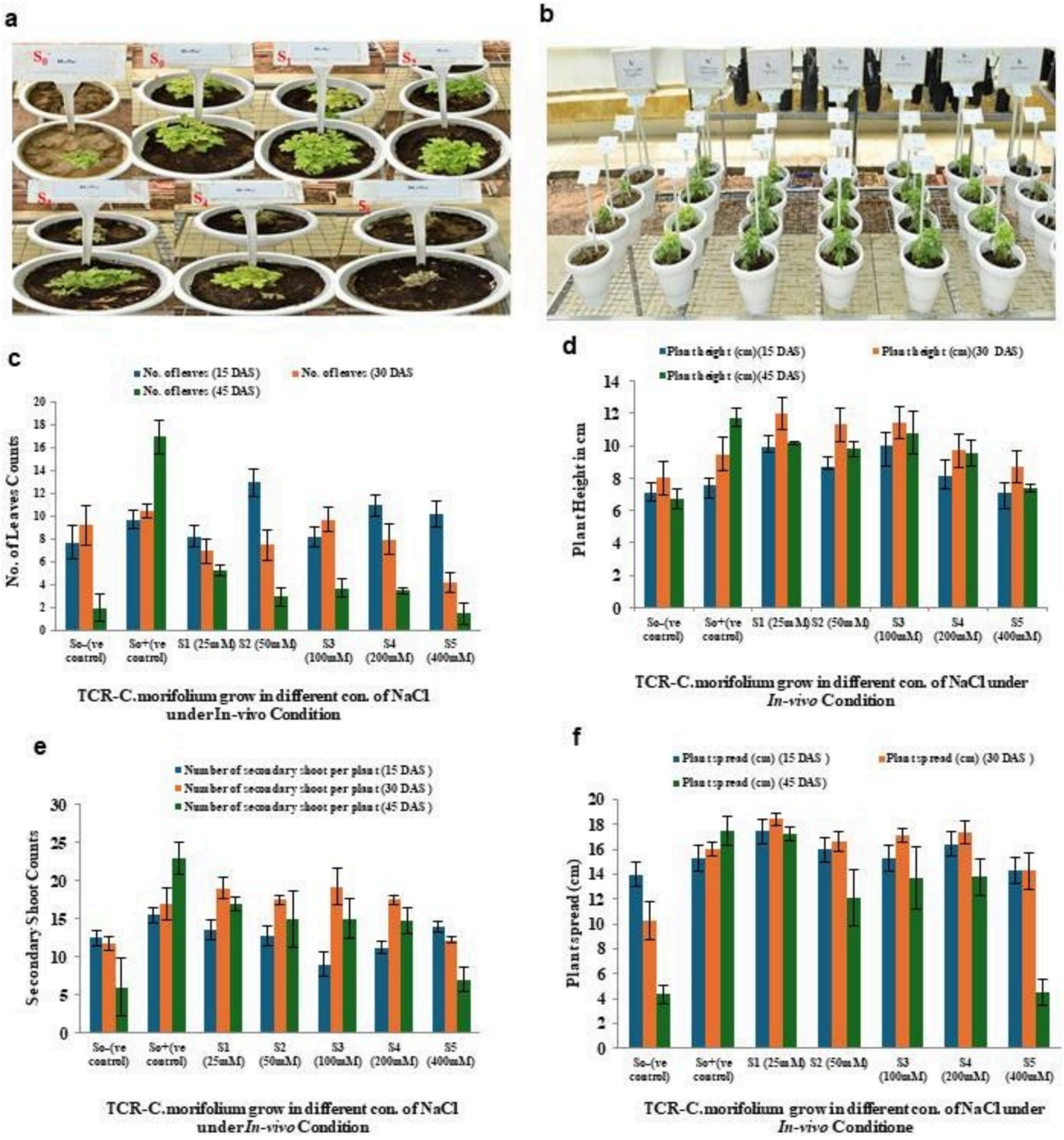


Figure 5

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 DAT.

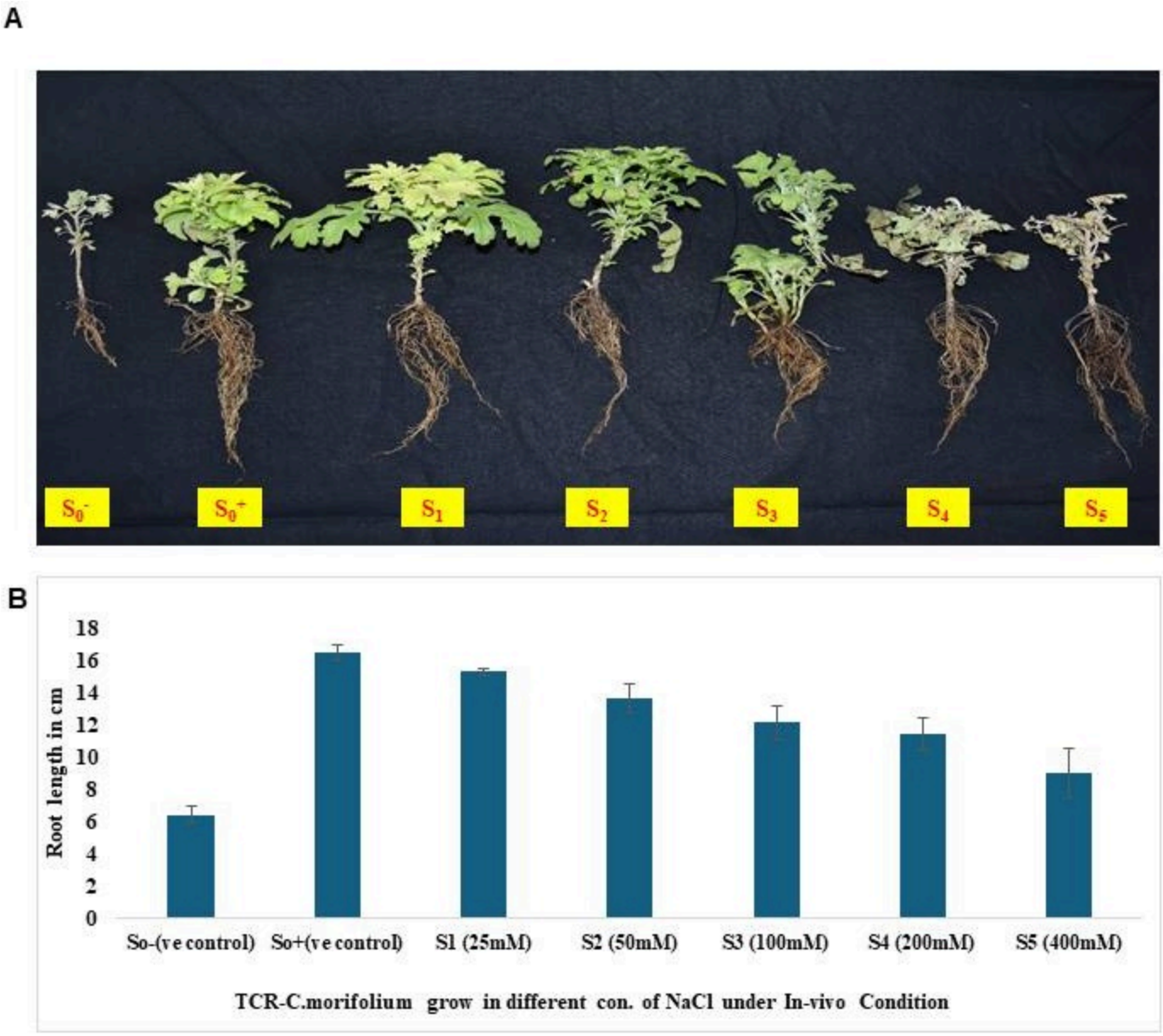


Figure 6

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

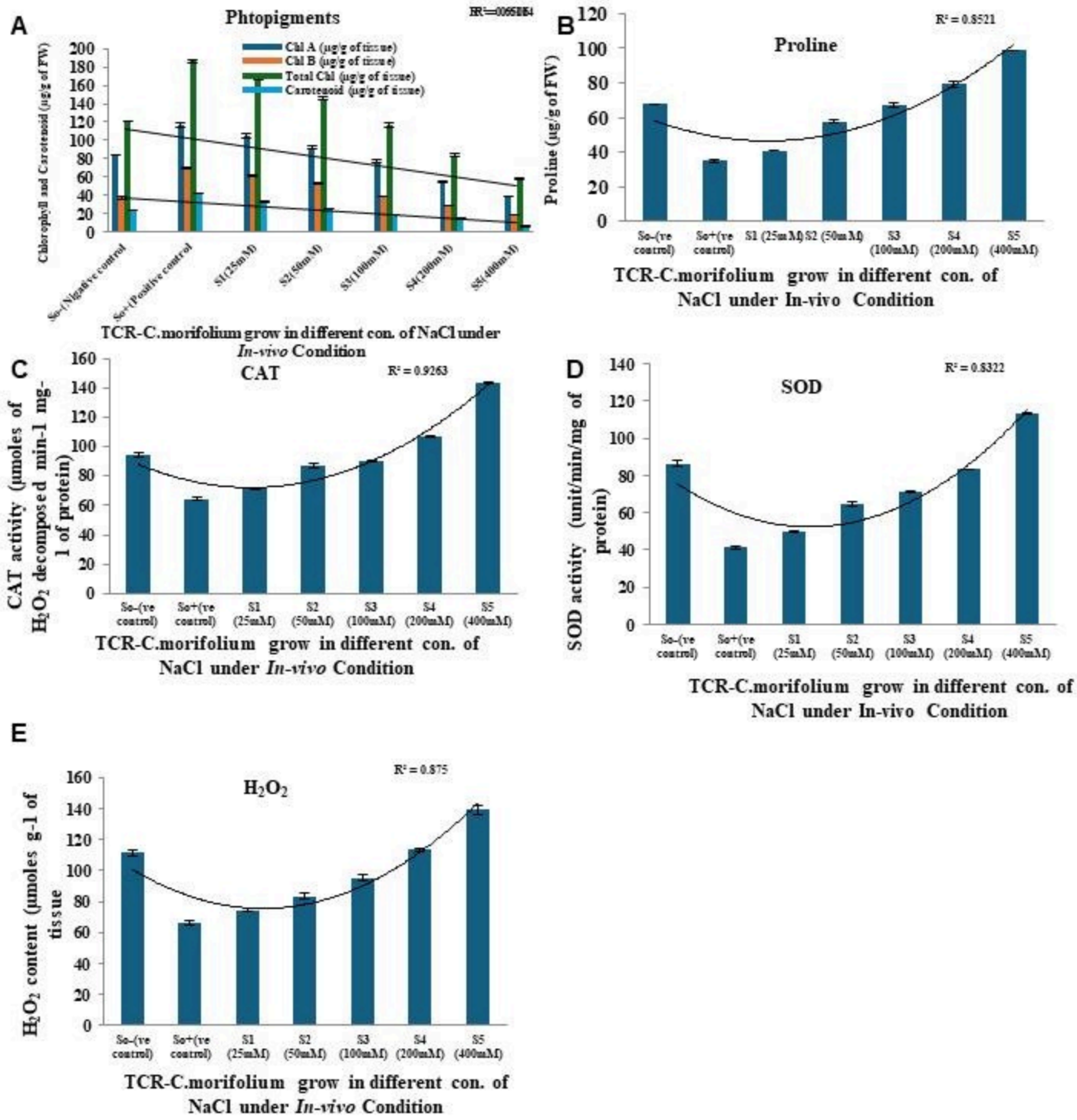


Figure 7

Physiological and biochemical analysis of leaves collected from *in vivo* planted TCR-*Chrysanthemum* plants subjected to different concentrations of NaCl (Control, 25 mM, 50 mM, 75 mM, 100 mM, 150 mM, 200 mM, 400 mM) in a specially designed soil mixture. **A)** Chlorophyll and Carotenoid **B)** Protein **C)** Proline **D)** CAT **E)** H_2O_2 **F)**SOD estimation by spectrophotometer methods.

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