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GENOME SIZE ESTIMATION AND PHYSIOLOGICAL RESPONSES IN WHEAT (*TRITICUM AESTIVUM* L. VAR. YECORA ROJO) UNDER SALT STRESS FOR IN VITRO SELECTION OF TOLERANT LINES

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Abstract

In vitro callus culture of wheat (*Triticum aestivum* L. var. Yecora rojo) was established on MS medium supplied with different concentrations of 2, 4-D, and NAA (0.5-10 µM). The maximum callus growth was observed in MS media with 5.0 µM 2,4-D on a fresh weight basis. The effects of salt stress were studied on the callus of wheat. We analyzed biochemical markers and the response of the antioxidant system in callus. The callus fresh and dry weight were found to be maximum (fresh wt.1.01 g and dry wt. 0.14 g on MS+50.0 mM NaCl). As the concentration of salt increased, the content of soluble protein, proline, and TBARS were higher in comparison to control. However, all these parameters decreased at the highest and enhanced salt concentration (200 mM NaCl) as of control. Similar observations were made in the case of antioxidant enzymes under salinity stress which were found to be highest at 150 mM NaCl as of control. The flow cytometry analysis exhibited no significant differences in genome size in treated and control calli, hence it was genetically stable with their genome size (2C DNA content) like control. The high salt tolerance of calli was evident from the accumulation of solute in the cell as of control.

Key words: Salinity stress, Callus, Biochemical markers, Genetic fidelity, Genome size

Introduction

Wheat (*Triticum aestivum* L.) is one of the most significant cereal crops globally, cultivated across diverse regions (Briggle & Curtis, 1987). It has earned the moniker "King of Cereals" due to its extensive cultivation, high yield, and prominent role in international grain trade (Anon., 2005). According to the NCBI Taxonomy Browser, wheat belongs to the family Poaceae, subfamily Poodeae, and tribe Triticeae.

A pressing issue that is likely to intensify in the future is salt stress resulting from soil salinization. This problem is primarily caused by over-fertilization, poor irrigation practices, and other factors. Statistics from the Food and Agriculture Organization (Anon, 2008) indicate that over 800 million hectares of land worldwide are currently affected by salinization, encompassing both saline and sodic soils, which constitute more than 6% of the global land area. The ongoing salinization of arable land is anticipated to have a substantial global impact, leading to a projected loss of 30% of agricultural land within the next 25 years and a 50% loss by 2050.

Wheat (*Triticum aestivum* L.) plays a crucial role in global food security, providing approximately 30% of the world's total cereal harvest and ranking second only to rice in terms of human calorie provision (Anon., 2006). Beyond its significant contribution of carbohydrates, fats, minerals, and vitamins, wheat offers a more fiber- and nutrient-rich dietary staple compared to meat-based diets (Šramková *et al.*, 2009). With the global population projected to approach 9 billion within the next 40 years (DESA UN, 2015), the demand for wheat is expected to increase substantially. While wheat is cultivated across diverse agroecosystems, its productivity is frequently constrained by a range of biotic and abiotic stresses. The area planted for wheat in Saudi Arabia has varied over time. According to the International Grains Council (IGC), Saudi Arabia's wheat production in 2019-20 will be 700,000 tons, up from 500,000 tons in 2018-19. Saudi Arabia's per capita wheat consumption is currently estimated to be 107 grams per day or approximately 39 kilograms per year. According to the Saudi Arabia Grains Organization (SAGO), Saudi Arabia consumed approximately 3.5 million tons of wheat in 2017-18. In addition, in 2017-18, 2.8 million tons of wheat flour were consumed. 2020 (World Grain). Saudi Arabia is a net wheat importer, purchasing an average of 1.1 million tons per year. Yecora rojo Aksada 59, and Aksada 67 are the most cultivated varieties.

Therefore, it is highly recommended to develop stress-tolerant and high-yielding varieties capable of growing in the shortest possible time. Tissue culture and gene transformation technology used in crop improvement. Overall, it is estimated that at least 3 ha of farming land is lost every minute worldwide because of soil salinization (Kundzewicz *et al.*, 2007). The total area of salinized soils worldwide is projected to be about 76.3 million ha, of which 41.5 ha are believed severely degraded (Oldeman *et al.*, 1990). Tissue culture procedures have made extensive

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use of both mature and immature embryos; mature embryos were a better option than immature embryos (Šramková *et al.*, 2009). When it comes to regeneration, immature embryos are a better source for explants; however, they need time and growth facilities (Kundzewicz *et al.*, 2007), while mature embryos are available all year round. Mature embryos can be used directly (Šramková *et al.*, 2009) or dissected (Oldeman *et al.*, 1990). Plant tissue culture techniques provide a potential and feasible approach to developing salt-tolerant plants (Dracup, 1991) and (Tal, 1994) opted for in vitro selection of salt-tolerant cell lines for various species. Although studies on in vitro selection for salt tolerance in wheat have been carried out using mainly somaclonal variants (Barakat & Abdel-Latif, 1996; Karadimova & Djambova, 1993).A very few studies have been conducted on genotypic evaluation for callus production and in vitro selection of salt tolerance.

Wheat plants undergo many biochemical, molecular, and physiological mechanisms to adjust salinity stress at the tissue, cell, and whole plant to optimize the growth and yield by antagonizing the adverse saline environment. The present investigation was carried out to evaluate the effect of salt on wheat calli raised on MS medium using biochemical and molecular approaches.

Material and Methods

The experiments were performed in the growth room and plant tissue culture laboratory, at the Department of Botany and Microbiology, College of Science, King Saud University. Riyadh, KSA.

Plant material: Mature seeds of a bread wheat variety (*Triticum aestivum* L. var. Yecora rojo) were used as a starting explant.

Induction of callus from mature embryo: Fully developed embryos were extracted from the seeds of the selected bread wheat variety. The culture of mature embryos was carried out following the methods of (Salama *et al.*, 2013), Initially, the seeds underwent sterilization with 70% ethanol for a duration of 5 minutes. Subsequently, they were disinfected with Clorox, a commercial bleach solution containing 5% sodium hypochlorite, for 15 minutes. This disinfection process was enhanced by the addition of a few drops of Tween 80 to the solution. All procedures were conducted under sterile conditions within a Laminar Air Flow cabinet. Following disinfection, the seeds were thoroughly rinsed three times with sterile distilled water to eliminate any residual bleach and detergent. The seeds were then immersed in sterile distilled water and maintained at room temperature (22-25°C) in complete darkness for an overnight period. The following day, embryos were carefully excised from the soaked seeds using sterile scalpels and forceps. These embryos were then cultured on a variety of callus induction media to facilitate further experimentation. The callus induction media was MS salts and vitamins (Murashige & Skoog, 1962) supplemented with 3% (w/v) sucrose, solidified using 7.5 g l-1 agar, and three replicates of different 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations ranging from 1.0 to 10 µM for callus induction. After adjusting all media to a pH of 5.7±0.1, they were autoclaved for 20 minutes at 121°C. Each Petri plate contained five embryos, which were then subcultured every four weeks while being kept in the dark at 25±2°C for a month. Weighing fresh callus tissue after four weeks of culture allowed us to calculate callus fresh weight (CFW), which is a measure of callus growth. The following formula is used to calculate growth, which is expressed as relative growth (RG): W - W0/W0 × 100 = RG (Chen *et al.*, 2006; Meredith, 1978) . W0 indicates the callus's initial fresh weight, and W denotes the fresh weight after the culture passage.

Salt stress on callus cultures: One-month-old calluses were employed to initiate *in vitro* experiments for the selection of salt tolerance. For this purpose, the optimal callus induction medium was identified and supplemented with four concentrations of sodium chloride (NaCl) at 50, 100, 150, and 200 mM, with three biological replicates per treatment. Cultures were maintained in complete darkness at a temperature of 25 ± 2°C for one month. Callus fresh weight (CFW) was measured before and after salt treatment to evaluate the effects of salt stress on callus growth. Additionally, callus necrosis (CN) and *in vitro* callus tolerance (INTOL) were assessed using the following formula:

CN=(number of necrotic calli total number of calli)×100 CN = (Briggle & Curtis 1987) total number of calli}} \right) \times 100CN=(total number of calli number of necrotic calli) × 100 (Bouiamrine & Diouri, 2012). INTOL= RGR treatment / RGR control (Al-Khayri & Al-Bahrany, 2004). Where RGR, the relative growth rate captured by the formula: CRG = [LnW2-LnW1] / GP (Birsin & Ozgen, 2004). Where W1 and W2 are the initial and final callus weights, respectively, and GP is the growth period.

Biochemical parameters

Proline content estimation: The proline content of the callus (0.25 g) was estimated using the technique adopted by . (Hanson *et al.*, 1979). In five milliliters of 3% aqueous sulphosalicylic acid, the fresh samples were ground. The samples were centrifuged for 10 minutes at 5,000 rpm, and the supernatant was gathered to estimate the amount of proline. To the 2 ml of supernatant that was produced in the previous step, 2 ml of each of the acids ninhydrin and acetic acid were added. The mixture was incubated for one hour in boiling water before the reaction was halted in an ice bath. The mixture above was vortexed after 4 milliliters of toluene was added. Toluene containing the chromatophore was separated from the proline-containing mixture. By measuring the absorbance of a chromatophore containing toluene at 520 nm, the proline content of the samples was determined (Model UB-1800, Shimadzu, Japan).

Thiobarbituric acid reactive substances (TBARS): The Cakmak and Horst method was used to evaluate the amount of TBARS in new calli (Cakmak & Horst, 1991). The 0.25 g callus samples were homogenized in 0.1% (w/v) trichloroacetic acid (TCA). To determine TBARS, the supernatant was extracted at 5,000 rpm. TBA was added to the above mixture at a rate of 0.5% (w/v) and left in a water bath at 90°C for 30 minutes. After stopping the reaction with ice, it was centrifuged for 10 minutes at

8,000 rpm. After collecting the supernatant, TBARS was measured using a spectrophotometer to measure absorbance at 600 and 532 nm wavelengths.

$$\text{TBARS (nmolg} - 1/fw) = \frac{(A532 - A600) \times V \times 100}{155 \times \text{extinction coefficient} \times w \times 1}$$

A532 represents absorbance at 532 nm, A600 represents absorbance at 600 nm, V = extraction volume, and W = fresh weight of tissue.

Estimation of antioxidant enzymes: Catalase was estimated to be one of the antioxidant enzymes in fresh calli. (EC 1.11.1.6) (Aebi, 1984) Superoxide dismutase (EC 1.15.1.1) (Dhindsa *et al.*, 1981), Ascorbate peroxidase (EC 1.11.1.11) (Nakano & Asada, 1981), and Glutathione reductase (EC 1.6.4.2) (Rao, 1992) . The CAT, SOD, APX, and GR were recorded at 240nm, 550 nm, 290 nm, and 340 nm, according to a UV-vis spectrophotometer. The unit of enzyme was measured in mg−1 protein min−1.

Assessment of genetic stability: By using flow cytometry to measure the genome size (2C DNA content) of calli under salinity stress and comparing it to the genome size of control calli, the genetic stability of the organisms was evaluated.

Nuclei isolation Buffers: MB01 buffer developed by (Sadhu *et al.*, 2016) was used for the extraction of nuclei. It consists of different components including 20 mM MOPS; 25 mM Na_2 EDTA; 0.7 mM spermine.4HCl; 80 mM KCl; 20 mM NaCl; 1% (w/v) PVP; 0.5% (v/v) β-mercaptoethanol; 0.2% (v/v) and Triton X-100. The young calli was used to extract nuclei. Young leaves from the germination of the *Vicia faba* seed were used as an external reference. The seeds were a kind gift from Prof. J. Dolezel ˇ (Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc Research Center, Czech Republic).

Extraction of nuclei: Nuclei were extracted from the fresh callus of wheat using the protocol reported by (Sadhu *et al.*, 2016). The calli (25 mg) were sliced with a sharp blade in a 600 µl in cold nuclei MB01 extraction buffer. The suspension of nuclei was pipetted slowly to prevent the formation of bubbles and then filtered through a 20 µm double nylon mesh filter. A 50 µg/ml PI stain was applied to the nuclei suspension (Propidium iodide, Sigma, USA) for 10 min and stored on ice before analysis.

Flow cytometric analysis: The amount of nuclear DNA in the wheat calli was determined using the method of Dolezel ˇ *et al*., (2007). The flow cytometer FACS Muse cell analyzer (Sigma, USA) was used to estimate the fluorescence of at least 5,000 propidium iodide-stained nuclei. The wheat genome size was calculated using the external reference *Vicia faba* (2C = 26.9 pg). The capillary's minimum flow rate was set at 0.12 µl/s. Muse cell analyzer software packages (Muse 1.8 analyses, USA) were used to compute the produced histograms. The following formula was used to determine the DNA content of the sample: $2C\ DNA\ content\ of\ Triticum = \frac{\text{Florescence mean intensity of Triticum}}{\text{Floresence mean intensity of standard}}$ x 2C DNA content of standard. According to Bennett & Smith (1976), the equivalent of 1 pg DNA = 965 megabase pairs was used to determine the number of base pairs per haploid genome.

Statistical Analysis

SPSS software was used to statistically analyze the data. To ensure the accuracy of the results, the data was used in triplicate. They were then subjected to a one-way ANOVA and examined by Duncan's test. Different alphabet letters were used to indicate significant difference levels at $p \leq 0.05$.

Results

Studies on callus induction were conducted by testing two plant growth regulators, 2,4-D and NAA, at varying concentrations ranging from 0.5 to 10 µM. The effects of 2,4-D and NAA were assessed by culturing mature embryos of *Triticum aestivum* var. Yecora rojo on MS medium. Embryo growth and callus were initiated after a week of inoculation on all the PGRs and concentrations. Induction of callus and callus growth was calculated after 6 weeks of culture. Maximum induction and callus growth on a fresh weight basis (FW=0.95g) as compared to control (FW=0.22g) was observed on MS medium containing 5.0 µM 2,4-D (Fig. 1) whereas in other concentrations slow growth was noted and increasing the concentration reduced the callus proliferation (Plate 1 A, B, C). In the case of NAA treatment, maximum FW (0.75g) was increased and noted at 5 µM concentration and lowest in control FW (0.16g) (Fig. 2). The growth of calluses was found to be inhibitory at higher concentrations of both the PGRs evaluated. The callus cultures were maintained on an MS medium supplied with 5 µM of 2,4-D for subsequent investigations.

The effect of salinity stress was studied on calli raised on MS media containing 5.0 µM of 2,4-D under different concentrations of salt (50, 100, 150, and 200 mM NaCl). The impact of salt stress was investigated through biochemical and molecular approaches. Maximum fresh and dry weights were recorded at varying concentrations of NaCl (Plate 1D-I, Fig. 3). The proline and TBARS contents were found to be significantly higher at 150 mM NaCl compared to the control (Figs. 4 and 5). However, both parameters declined as the NaCl concentration increased further in the MS medium. At the highest salt concentration (200 mM NaCl) on MS media, all studied parameters (soluble protein, TBARS, proline, and antioxidant enzymes, Figs. 5-10) were decreased, however, all these parameters remained higher than the controls significantly. The addition of 50 and 100 mM NaCl to the media resulted in a higher accumulation of soluble protein in the calli compared to the control, although the difference between the two treatments was non-significant. The activities of CAT, SOD, APX, and GR peaked at 150 mM NaCl but declined as the NaCl concentration increased further to 200 mM in the MS medium. CAT activity at 50 mM NaCl showed no significant difference compared to the control. Similarly, GR activity at 100 and 150 mM NaCl also exhibited non-significant results.

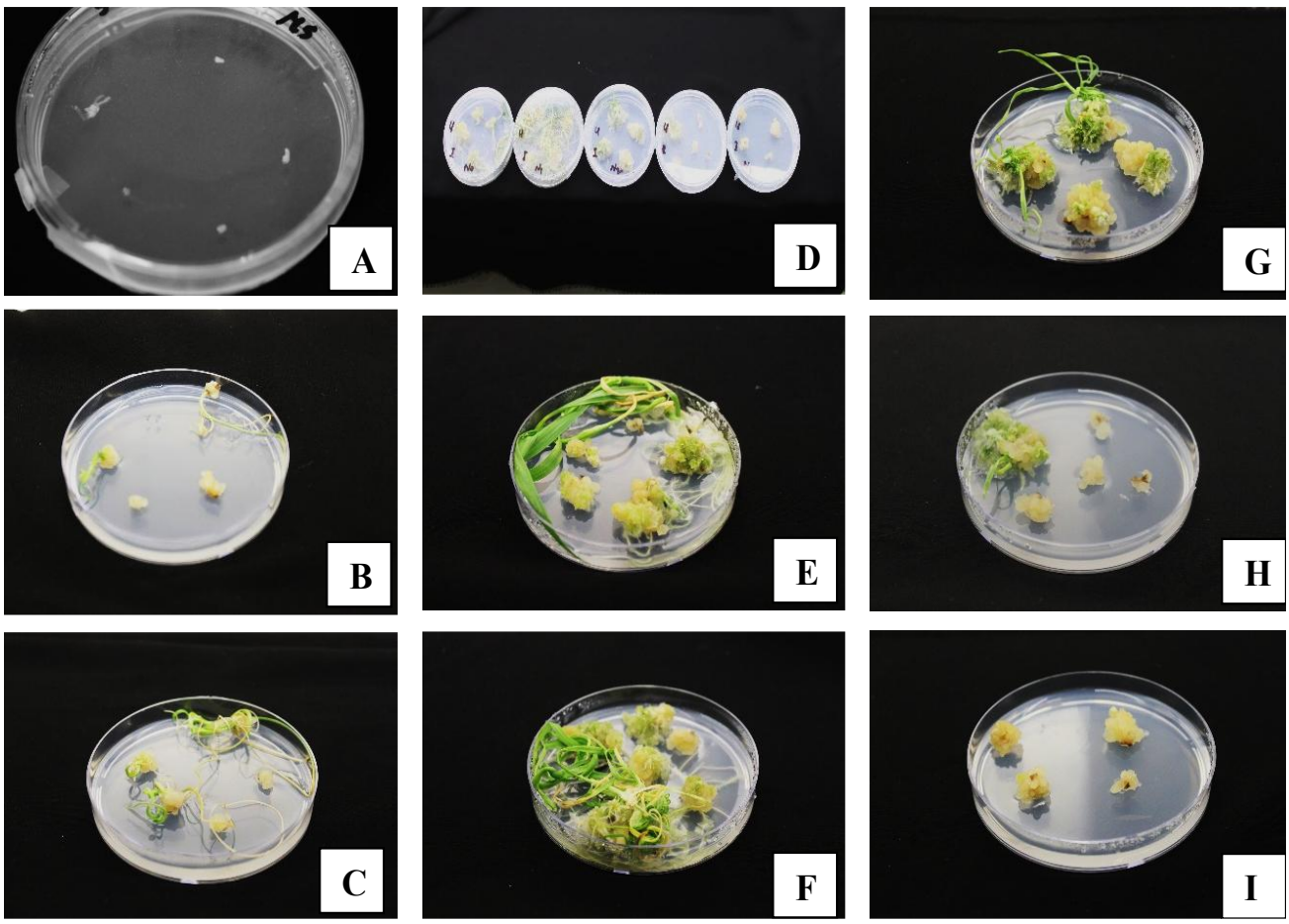

Plate 1. Embryo culture, callus induction and salt stress to callus of wheat **A)** Embryo culture on MS media as control; **B)** Callus on 5 µM NAA; **C)** Callus on 5 µM 2,4-**D)** Callus on 5 µM 24-D with different salt concentration; **E)** Callus on 5 µM 2, 4-D control for salt stress; **F)** Callus on 50 mM NaCl; **G)** Callus on 100 mM NaCl; **H)** Callus on 150 mM NaCl; **I)** Callus on 200 mM NaCl

The genetic stability was assessed in the calli under salinity stress by measuring the genome size (2C DNA content) with flow cytometry following the protocol of Sadhu *et al*., (2006). The nuclei were extracted from both treated and untreated calli along with reference plants for genome size (2C DNA content) estimation (Fig. 11). The extracted nuclei with MB01 buffer showed a sharp peak as shown in the generated histogram (Fig. 11**)**. The estimated genome size (2CDNA content) in treated and untreated callus was almost similar (Fig. 12).

Discussion

The overall impact of 2,4-D on the observed outcomes suggests that increasing concentrations of 2,4-D or NAA negatively affect callus growth and associated processes. This phenomenon may be linked to the accumulation of supra-optimal levels of NAA and 2,4-D within the tissues, which can impair growth performance. Consequently, MS media supplemented with 2,4-D yielded the highest mean values for callus induction. This observation may be explained by the mechanism through which it influences callus induction; for example, (Irfan Hafeez *et al.*, 2012) and (Ashraf & Osama, 2005) It has been reported that the significant challenge of inducing and sustaining callus in wheat, in particular, has been partially addressed by employing 2 mg/l of 2,4-D. In terms of callus induction and callus weight, mature embryos demonstrated a higher frequency of callus formation and produced larger calluses (Tyankova & Zagorska, 2001). In mature embryo culture, the embryos exhibited a greater capacity to form callus structures. The increased hormone concentration correlated with enhanced callus production in mature tissue cultures (Kintzios *et al.*, 1996; Stals & Inzé, 2001).

In general, the induction of wheat callus in tissue cultures is influenced by several key factors, including the composition of the culture medium, the type of explant used, and the genetic characteristics of the genotype. (He *et al.*, 1989; Maddock *et al.*, 1983; Özgen *et al.*, 1998). In this study, our findings demonstrate that 2,4-D alone was effective in inducing yellow compact callus from mature embryos of wheat. The efficacy of 2,4-D for wheat callus induction has also been supported by earlier research (Abdrabou & Moustafa, 1993). Moreover, in other studies (Arzani & Mirodjagh, 1999; Barro *et al.*, 1998) 2,4-D has been widely recognized as the most commonly used growth regulator for callus induction and maintenance in wheat. Among the various concentrations of 2,4-D tested in this study, 5.0 µM was found to yield the optimal results in terms of callus weight (Table 1). These outcomes align with the conclusions of (Yu *et al.*, 2008), It has been reported that the frequency of wheat callus induction reached its maximum with 2.0 mg/l of 2,4-D in the induction medium. In contrast, the response to NAA exhibited an inverse relationship, as higher NAA concentrations generally resulted in reduced performance across the traits studied.

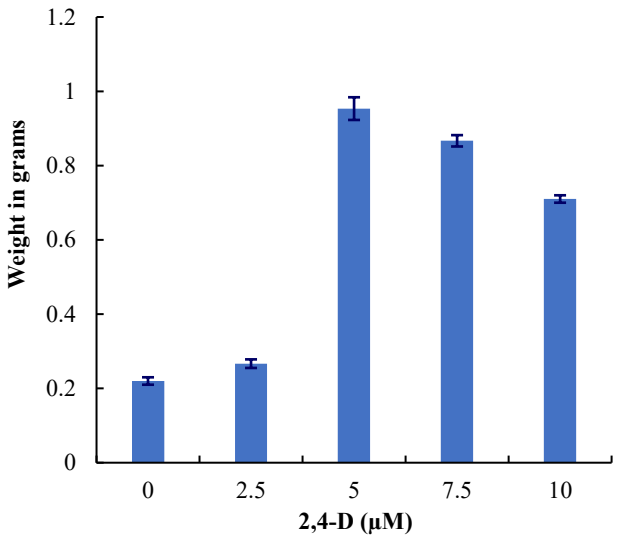

Fig. 1. Variation in callus weight at different concentration of 2, 4 -D after six weeks of culture.

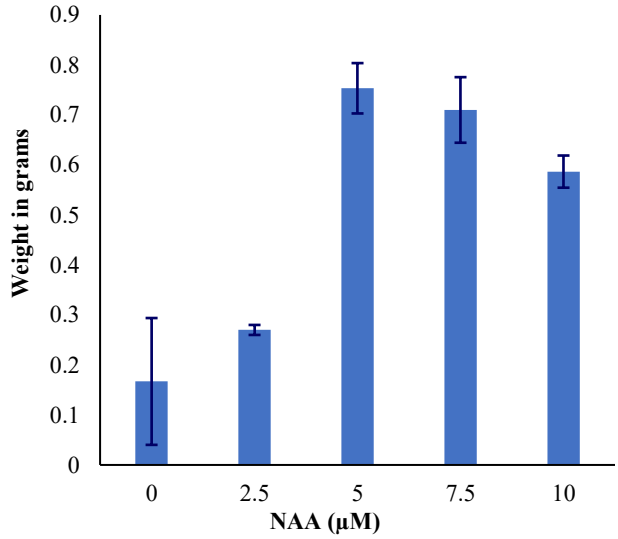

Fig. 2. Variation in callus weight at different concentration of NAA after six weeks of culture.

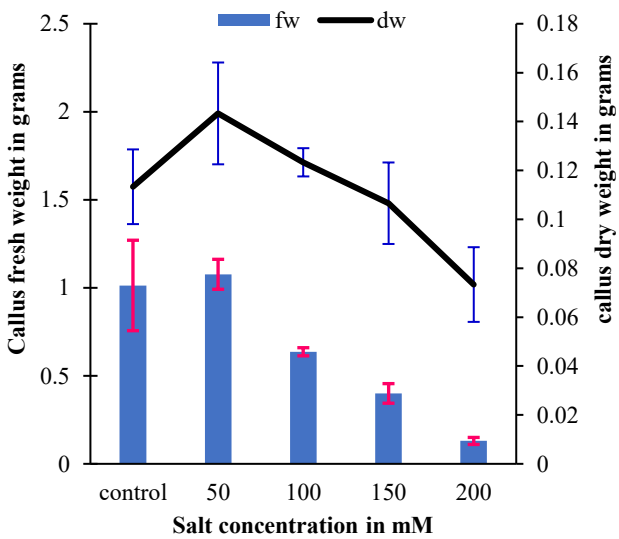

Fig. 3. Callus fresh and dry weight on different concentrations of salt after six weeks of culture.

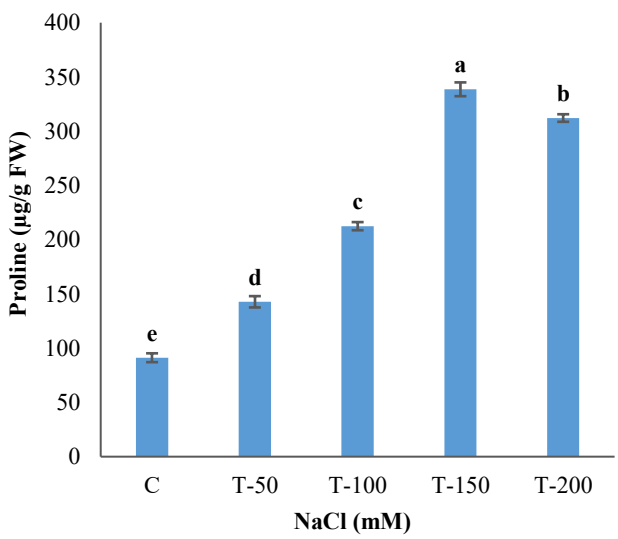

Fig. 4. Proline content in calli under different concentrations of salt and control.

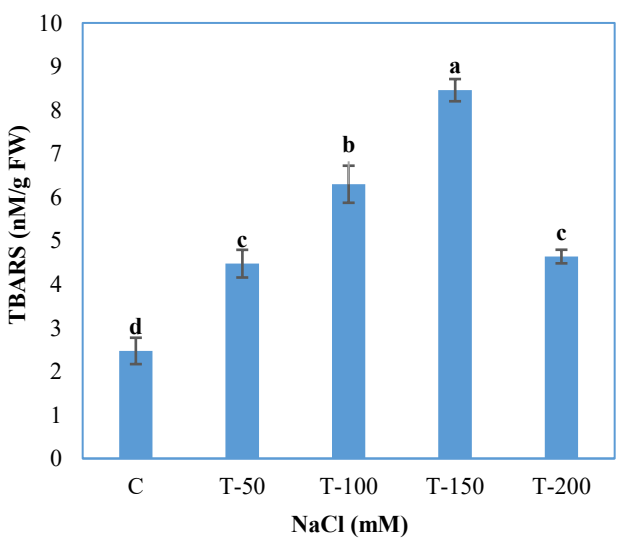

Fig. 5. TBARS content in calli under different concentrations of salt and control.

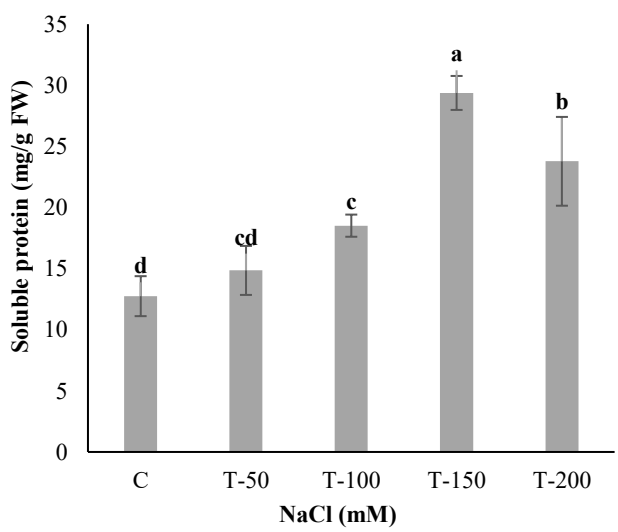

Fig. 6. Soluble protein content in calli under different concentrations of salt C (control), T-50 (50 mM NaCl), T-100 (mM NaCl), T-150 (150 mM NaCl), T-200 (200 mM NaCl).

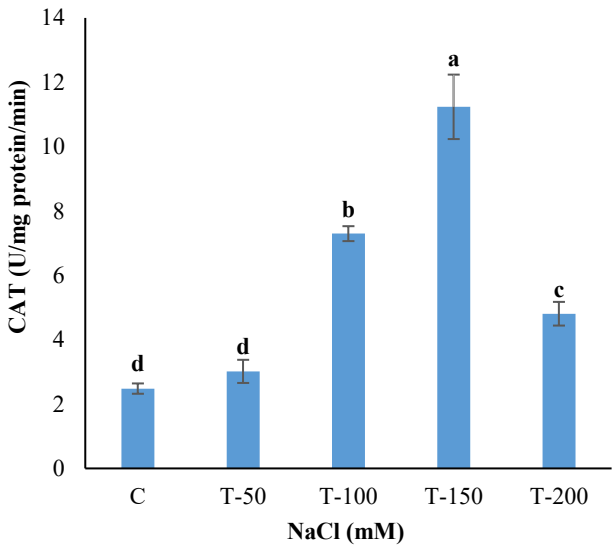

Fig. 7. CAT activity in calli under different concentrations of salt C (control), T-50 (50 mM NaCl), T-100 (mM NaCl), T-150 (150 mM NaCl), T-200 (200 mM NaCl).

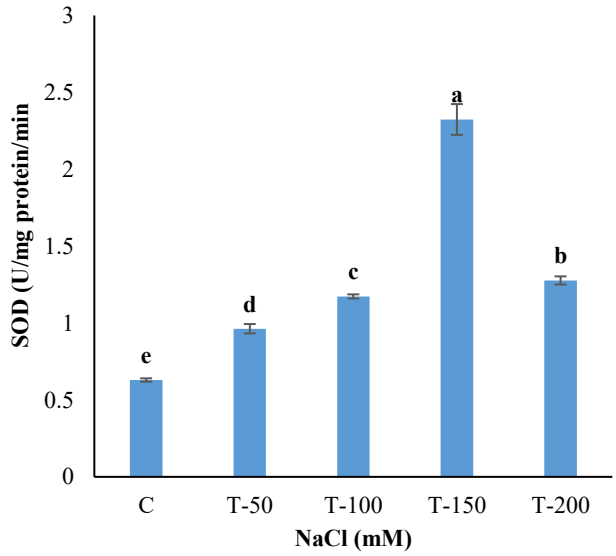

Fig. 8. SOD activity in calli under different concentrations of salt C (control), T-50 (50 mM NaCl), T-100 (mM NaCl), T-150 (150 mM NaCl), T-200 (200 mM NaCl).

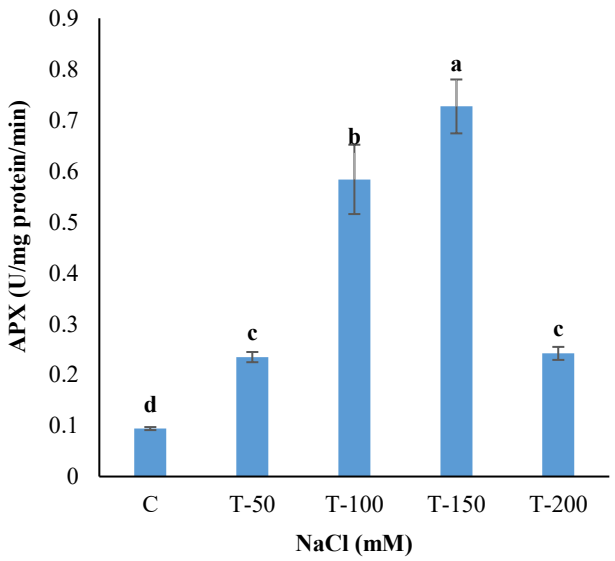

Fig. 9. APX activity in calli under different concentrations of salt C (control), T-50 (50 mM NaCl), T-100 (mM NaCl), T-150 (150 mM NaCl), T-200 (200 mM NaCl).

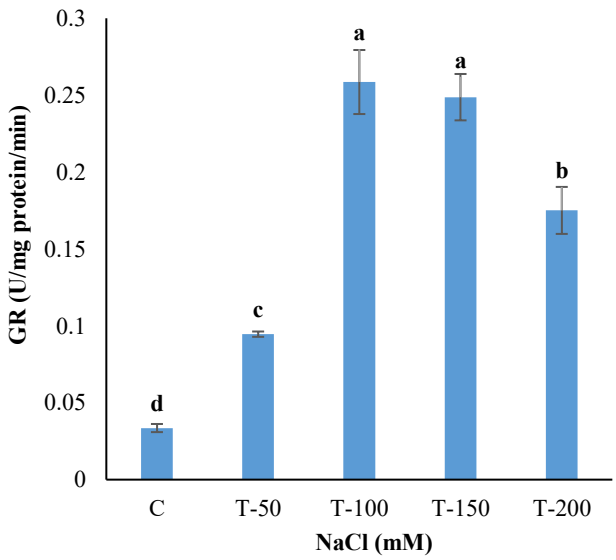

Fig.10. GR activity in calli under different concentration of salt C (control), T-50 (50 mM NaCl), T-100 (mM NaCl), T-150 (150 mM NaCl), T-200 (200 mM NaCl).

Regarding the explant, these results are consistent with findings reported by several researchers, as (Zale *et al.*, 2004) who also demonstrated that mature embryos are capable of generating a sufficient number of regenerated plants. These findings are further supported by (Özgen *et al.*, 1998) according to whom mature embryos exhibit a high frequency of callus induction and strong regeneration potential. Consequently, due to their year-round availability, they are highly suitable for use in wheat tissue culture (Delporte *et al.*, 2001) utilized mature embryos successfully to produce calli, which aligns with the findings of this study. However, rather than employing whole seeds or embryos, they used fragmented embryo sections. (Yu *et al.*, 2008) also employed mature embryos in wheat tissue culture, achieving results consistent with those reported by numerous researchers (Ahmet & Adak, 2007; Bi *et al.*, 2007; Patnaik *et al.*, 2006).

Salinity stands as one of the most critical abiotic stress factors, significantly limiting crop productivity by adversely affecting plant growth. It disrupts the activity of antioxidant enzymes essential for nucleic acid and protein metabolism. Previous studies have established a strong correlation between reactive oxygen species (ROS) and plant responses to abiotic stresses. Consequently, understanding plant responses to salt stress, both in vivo and in vitro, is crucial. Under salinity stress, plants generate free radicals that inflict cellular damage. This stress impacts plant growth by modifying lipid composition, cell membrane integrity, and protein structure, while also inducing osmotic and ionic stress. (Cominelli *et al.*, 2013). In response to salinity stress, plants produce certain solutes that counteract free radicals and safeguard cells from damage. This study aimed to evaluate the impact of salt stress on wheat

callus by analyzing morphological, molecular, and biochemical markers. Morphological markers, such as callus size and weight, provide insights into growth rates under salinity stress. Variations in fresh and dry weight of calli were observed across different NaCl concentrations (50–200 mM). Changes in morphological traits of calli under varying salt concentrations may be attributed to the generation of free radicals, which disrupt cellular physiology. The highest biomass of calli, measuring 0.14 g, was observed under 50 mM NaCl treatment, surpassing both other treatments and the control (0.11 g, without salt). Callus growth on MS medium supplemented with 50 mM NaCl was superior to that observed at higher concentrations (100–200 mM NaCl). The reduced growth at 200 mM NaCl is likely due to the production of free radicals, which negatively impacted callus development.

Proline and Thiobarbituric Acid Reactive Substances (TBARS) levels in plants are helpful indicators of their ability to withstand abiotic stressors. Maintaining osmotic balance and shielding the cell structure from stress are the primary roles of osmolytes. (Polash *et al.*, 2019). In comparison to the control, the wheat callus displayed a greater accumulation of different solutes, such as soluble protein and proline contents, as supported by the results of other researchers where these parameters increased under salinity stress (Al-Qurainy *et al*. 2021; Aharaf & Foolad 2007). The accumulation of these compounds helps in maintaining the osmotic balance of the cell and also removes the free radicals from the cell, which are produced under salinity stress. Our outcome is validated. by (Hannachi *et al.*, 2021) who conducted experiments on *Solanum melongena* callus under salinity stress, reported that lipid peroxidase activity increased in calli tolerant to 40 and 80 mM NaCl, but no such increase was observed in callus tolerant to 120 mM NaCl. (Al-Qurainy *et al.*, 2021) demonstrated that the accumulation of TBARS content was higher at 100 mM NaCl compared to 150 mM NaCl in date palm cultivars. Numerous studies have also indicated that proline levels increase significantly in plants subjected to salt stress (Calzone *et al.*, 2019; Vanlalruati *et al.*, 2019). Under salinity stress, the proline levels in *B. monnieri* cultured in tissue culture were six times higher compared to the control (Ali *et al.*, 1999). Proline accumulation is primarily driven by the enzymatic conversion of Pyrroline-5-carboxylate (P5C) to proline, catalyzed by Pyrroline-5-carboxylate reductase (P5CR) (Nazar *et al.*, 2015). Proline plays a crucial role in maintaining the structural integrity of macromolecules, particularly enzymes, by stabilizing hydration levels within the cell cytoplasm. (Foyer & Noctor, 2005).

A similar pattern was observed in the activities of antioxidant enzymes, such as SOD, CAT, APX, and GR, which mirrored the trend of osmolyte accumulation under salinity stress in wheat calli. The levels of these enzymes increased in a concentration-dependent manner, except at 200 mM NaCl, where a decline was noted compared to lower salt concentrations. The antioxidant enzymes produced under abiotic stress detoxify the reactive oxygen species (ROS) from the cell (Gill & Tuteja 2010) and help in maintaining the growth and weight of the plant. Each of these enzymes plays a distinct role in protecting plant cells from reactive oxygen species (ROS) under salinity stress. SOD serves as a critical early indicator of plant sensitivity to ROS, acting as a first line of defense. In numerous NaCl-tolerant plants, increased SOD activity has been reported as a key adaptive response. (Gomez *et al.*, 2004). The antioxidant enzyme, GR plays an important role to ameliorate the tolerance of plants against environmental stresses (Hasanuzzaman *et al.*, 2020). The level of GR was reported to be high under salinity stress (Manai *et al.*, 2014). APX and CAT both enzymes overcome the toxic level of H_2O_2 in plants (Foyer & Noctor, 2005; Gomez *et al.*, 2004). The ROS inhibition system and plants' ability to withstand alkaline and salinity stressors are both significantly impacted by APX. The plant's high CAT activity demonstrated its tolerance as a result of increased H2O2 detoxification. (Mittova *et al.*, 2002). The CAT activity depends on many parameters such as metabolic status and growth of the plant, time, and intensity of stresses (Srinieng *et al.*, 2015). The CAT and SOD activity was increased in NaCl-tolerant calli of *Solanum melongena* under salinity stress (Hannachi *et al.*, 2021).

Regardless of the information that is encoded, it is believed that the size of the genome affects plant characteristics at the subcellular and organismal levels. (Bennett, 1971) and can interact with environmental stressors to affect both plant responses and traits (Suda *et al.*, 2015) (Bennett & Leitch, 2005). In our study, the genome size (2C DNA content) was observed to be the same in treated and untreated calli and no significant difference was to be detected. The histograms generated from treated and untreated calli using the MB01 buffer were almost similar (Figs. 11 and 12). Thus, the applied concentration of salt on calli grown on MS media has not been observed any genotoxic effect on genome size (2C DNA content). However, the growth of calli at the highest concentration of salt (200 mM NaCl) was reduced along with variation in biochemical parameters, and hence the growth of calli was inferior to the control and in other salt treatments.

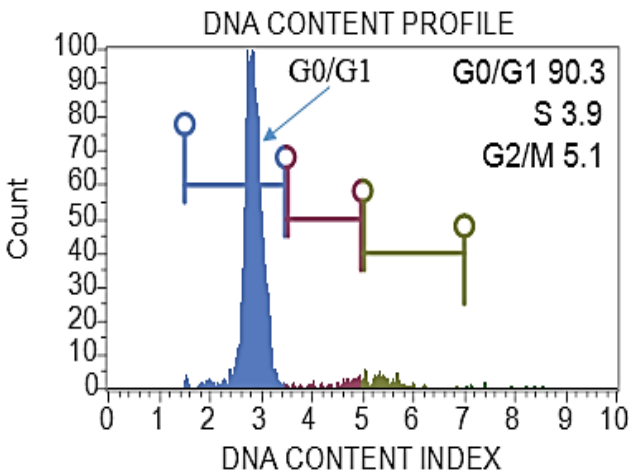

Fig. 11. Histogram generated from untreated calli by flow cytometry using the MB01 buffer.

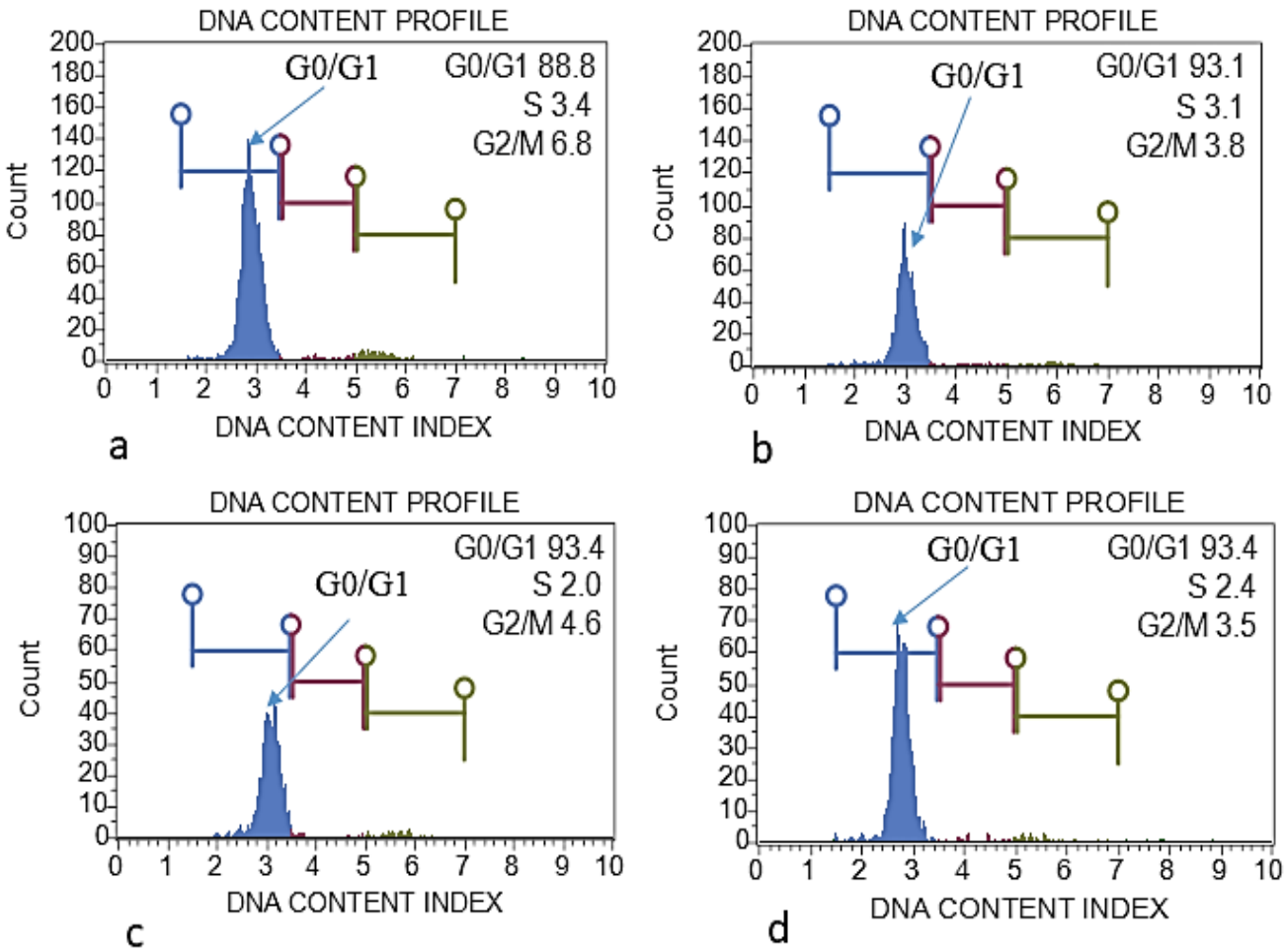

Fig. 12. Histogram generated from calli under different concentrations of salt treatment (a: 50 mM NaCl; b: 100 mM NaCl; c: 150 mM NaCl; d: 200 mM NaCl) by flow cytometry using the MB01 buffer.

Conclusion

In comparison to NAA, MS media supplemented with 2,4-D demonstrated superior callus growth, as evidenced by higher fresh weight gain after one month of culture. Salt stress experiments were subsequently conducted on this vigorously growing callus. The results revealed that salinity stress in the culture medium significantly restricted callus growth, as evaluated through morphological, biochemical, and molecular markers. All analyzed parameters, including soluble protein content, TBARS, proline levels, and antioxidant enzyme activities, were elevated compared to the control. Genetic fidelity in the callus under salinity stress was assessed by measuring genome size (2C DNA content) using flow cytometry, which showed no significant variation across salinity treatments compared to the control callus. These findings highlight the need for further research to evaluate salinity tolerance in wheat calli, which could contribute to the development of salt-tolerant wheat varieties in future studies

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