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Callus proliferation, morphological and somatic embryogenesis responses of bread wheat (Triticum aestivum L.) to salinity stress in vitro

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I. Abstract

Plant tissue culture is a unique tool for studying plant responses to stress at the cellular level and monitoring their development under controlled conditions. The current study aimed to investigate the callus response wheat exposed to salt stress and to monitor the development of the callus into somatic embryogenesis. The study involved using mature seeds as explants to induce callus by culturing them on MS medium supplied with a concentration of 6 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) for four weeks in the dark under controlled conditions. The callus formed was then exposed to levels of NaCl salt (0, 50, 100, and 150 mM) for four weeks in nutrient MS medium free of 2,4-D in the dark. After that, the cultures were transferred to fresh MS medium free of 2,4-D containing the same salt concentrations for four weeks in the light. The results of the study indicated that the response of the callus tissue to salt stress conditions varied according to the concentration of NaCl, where the concentration of 50 mM caused the best response of the callus tissue with regard to growth parameters (fresh and dry weight), stimulation of somatic embryogenesis and their differentiation into plantlets, as it achieved the highest rate of growth and number of somatic embryos and plantlets compared to the other treatments. While high concentrations of NaCl caused a deterioration in callus growth and development parameters, as embryonic callus differentiation decreased, the number of somatic embryos decreased, and necrosis occurred when treated with 150 mM NaCl, despite this, the callus cells were able to survive under the influence of salt stress, which indicates their ability to acquire the ability to withstand induced stress in their growth medium.

Keywords : *Plant tissue culture; Wheat plant; callus induction; Somatic embryogenesis; Salt stress*

II. Introduction

Wheat (Triticum aestivum L.) is a staple food crop and one of the most widely cultivated and important crops worldwide, playing a key role in food security. It is also widely used in many food products (Khalid et al., 2023). With the world's population expected to reach 9 billion by 2050, demand for wheat is projected to increase by 60% (Malik et al., 2021). However, wheat production faces environmental pressures, particularly soil salinity(Naeem et al.,2022). As global climate change worsens, characterized by decreased rainfall and rising temperatures, salt accumulation in the soil increase (Al Maamory et al,2017). Wheat growth is affected by salt stress, which causes numerous physiological and biochemical changes. Salinity negatively impacts water and nutrient absorption due to osmotic and ionic disturbances, thus negatively affecting crop yield (Yaycili & Alikamanoğlu, 2012). Since salt tolerance is a complex trait controlled by many genes, plants exhibit different responses to salt stress depending on their gene content (Gupta& Huang ,2014).

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Tissue culture provides an ideal environment for studying the effect of salinity on cell growth and is also used to select salt-tolerant cell lines. The response of wheat tissue culture is influenced by several factors, including the genotype, the type of tissue (explants) used, the components of the nutrient medium, and the interactions between these factors (Benderradji, 2012). Both mature and immature wheat embryos have been used as explants in tissue culture programs; however, mature embryos are available year-round, making them the preferred choice for callus induction (Özgen,1996). Callus is defined as a collection of rapidly dividing parenchyma cells, and can form in several patterns in tissue culture or as a result of wounds (Xu & Huang, 2014). Studies conducted on rice, a model monocotyledonous plant, indicate the possibility of inducing callus formation from regenerative cells within the plant under tissue culture conditions. In rice, epidermal cells in the scutellum (embryonic cotyledon) can act as callus-initiating cells (Hu et al., 2017).

Callus tissue can be induced from many plant families capable of forming it through in vitro tissue culture. Cereals are of particular interest in this field due to the difficulty of inducing callus and inducing somatic embryogenesis in vitro, as well as plant regeneration. Callus can be induced from many explants including vegetative, reproductive and embryonic parts (Kruglova et al.,2021). When provided with suitable laboratory conditions, explant cells demonstrate the ability to form not only different types of tissues and organs, known as pluripotency, but also to form a complete plant organism through various morphogenetic pathways, known as totipotency. The success of callus formation is determined by a complex set of interconnected internal and external factors(Kruglova et al.,2021; Zinatullina,2020; Fehér, 2019). The internal factors for callus formation can be considered as the presence of morphogenetically qualified cells (which can be defined as "primary callus cells") in the explants, while the external factors can be considered as inducers of in vitro callus formation from these cells (Kruglova et al., 2021).

In wheat, callus formation involves the inhibition of early germination of the zygote embryo, followed by the initiation of undifferentiated cell proliferation within it. Callus formation is induced by treating embryos with 2,4-dichlorophenoxyacetic acid (2,4-D). Upon removal of 2,4-D, somatic embryos begin to form from the callus periphery (Caliskan & Cuming, 2004). There are two types of stimulating conditions that allow differentiated cells to transform into undifferentiated cells capable of differentiation: plant growth regulators (internal and/or external at the cell level) and various stress factors such as osmotic shock, heavy metals, and water stress. 2,4-D can act as a stressor as well as a growth regulator, facilitating the transition from embryonic cells to somatic embryos by redirecting the developmental program of cells. The conditions of tissue culture (in vitro) represent an unusual combination of stressors to which plant cells are exposed, such as the oxidative stress product resulting from reactive oxygen species (ROS) as a secondary messenger that contributes to the stress response and stimulates somatic embryogenesis.In addition to the effect of plant growth regulators, the concentration of salts in the medium can be decreased or increased, as can the intensity of light (Fehér, 2005; Begum et al.,2008; Lincy et al.2009; Spinoso & Bello, 2022) . It has been found that creating stress conditions in the growth medium can stimulate somatic embryogenesis (Garcia et al., 2019).These include heavy metal stress, dehydration using osmotic agents, and shocks with high or low temperatures or chemicals (Zavattieri et al., 2010; Fehér, 2019). This was confirmed by several studies, including Vikrant (2015), which indicated that adding PEG or mannitol to the growth medium at a concentration that enhanced somatic embryo formation in the millet Crop. Rajalakshmi, (2024) also used sodium chloride salt at different concentrations to stimulate the formation of salt-tolerant somatic embryos derived from mature rice seed embryos and found that concentrations of (10, 25, 50, and 100) mM supported germination, but a concentration of 150 mM was lethal to both somatic embryos and germination. Therefore, the current study aimed Therefore, the current study aimed to evaluate the callus response induced from mature wheat seeds to salinity stress conditions and to monitor somatic embryo development.

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III. Materials and Methods

The study was conducted in the Tissue Culture Laboratory, Biology Department, College of Education, University of Basra, using the bread wheat(Triticum aestivum L.) Ebaa 99 cultivar. Seeds were obtained from Agricultural Research Department / Al-Mishikhab, Ministry of Agriculture, Najaf, Iraq .

Plant materials

Mature seeds were used as explants for the purpose of inducing callus and somatic embryogenesis.

Surface sterilization of seeds

The wheat seeds were sterilized by placing them in 70% ethanol for 5 minutes with continuous stirring, then rinsing with sterile distilled water. This was followed by leaching sterilization with 50% commercial bleach containing 5% sodium hypochlorite, with the addition of the surfactant Tween-20 and continuous stirring for 30 minutes. The seeds were then washed three times with distilled, sterilized water to remove any residual sterilizing agents. The sterilization process was carried out on a Laminar Air Flow Cabinet table that had been pre-sterilized with 70% ethanol.

Preparation of callus induction medium

Callus induction medium was prepared from the basal salts of MS nutrient medium (Murashige and Skoog, 1962) (MS medium), and supplemented with sucrose at a concentration of 30 g/L, sodium dihydrogen orthophosphate at 170 mg/L, adenine sulfate at 40 mg/L, 0.5 mg/L thiamine-HCl, and 6 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). The solution was placed on a magnetic stirrer hot plate, and the pH was set to 5.8 using a Digital pH meter. The medium was titrated using potassium hydroxide (0.1N) or hydrochloric acid (0.1N). Agar was added at a concentration of (7)g/L, and the mixture was heated to (92)˚C. The culture medium was then distributed into test tubes at 10 ml/tube, sealed with cotton plugs, and wrapped in aluminum foil for sterilization. The tubes were sterilized for 20 minutes in an autoclave at 121 °C and 0.1 MPa. The tubes were removed after sterilization and shaken to homogenize their contents, and left to cool and solidify until the time of culture.

Culture seeds on the induction medium

The pre-sterilized seeds were planted on the prepared, pre-sterilized induction medium at a rate of 4 seeds per tube. The seed-planting process was carried out in a sterile Laminar Air Flow Cabinet. The cultures were incubated in a growth room at 25 ± 2 °C in the dark for four weeks to induce callus formation. Through continuous monitoring, the number of days required for callus induction was calculated, and morphological observations were recorded.

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Callus culture under NaCl stress

After 4 weeks of seed culture on the induction medium and callus formation, the callus (150 mg) was transferred to a new MS medium free of the auxin (2,4-D). This was done to stimulate somatic embryogenesis and included the same components added to the callus induction medium, while creating salt stress conditions by adding NaCl (0, 50, 100, 150 mM) at a temperature of (25±2) °C, incubated in the dark for four weeks. Callus tissues were transferred to a fresh medium of the same composition with the same applied salt concentrations and incubated under the previously mentioned growth conditions in the presence of light for four weeks. Then measurements were taken (fresh weight, dry weight, number of somatic embryos formed, number of plantlets from embryonic callus formed, and morphological observations).

Statistical Analysis

Using the SPSS statistical software, the data were analyzed, and significant differences between treatments were determined using analysis of variance (ANOVA), with differences between means compared using the least significant difference (L.S.D.) test at the 5% significance level.

IV. Results and Discussion

Three days after the mature seeds were cultured as plant parts in the stimulation medium to which 6 mg/L of 2,4-d was added, callus induction was observed on the 7 day. The callus was friable, watery, and non-glandular (primary non-embryonic callus), as shown in Figure 1-A. At the end of the 4 week of culture, a friable, Pale yellowish callus was observed, with some nodules (embryonic callus) (Figure 1-B).

When the callus was transferred to fresh MS medium free of the growth regulator 2,4-D and containing different concentrations of NaCl, after four weeks of culture, the callus morphology differed depending on the salt concentration. The nodules on the periphery of the callus were clearly (multi-nodular) with a greenish-yellow color in the case of the control treatment and the low concentration of NaCl (50) Mm(Figure 1-C,D). When treated with concentrations of 100 and 150 mM, the callus changed morphologically from friable, nodular, yellow to non-nodular, smooth, brown callus with necrosis (Figure 2-E,F). It also caused induced salt stress by added NaCl in the callus growth medium at concentrations of 0, 50, 100, and 150 mM resulted in a significant decrease in average fresh weight (Figure 2), particularly at 100 mM and 150 mM compared to the control. The 50 mM treatment also achieved the highest significant increase in average fresh weight, reaching 0.21 g, compared to the 100 mM and 150 mM treatments.

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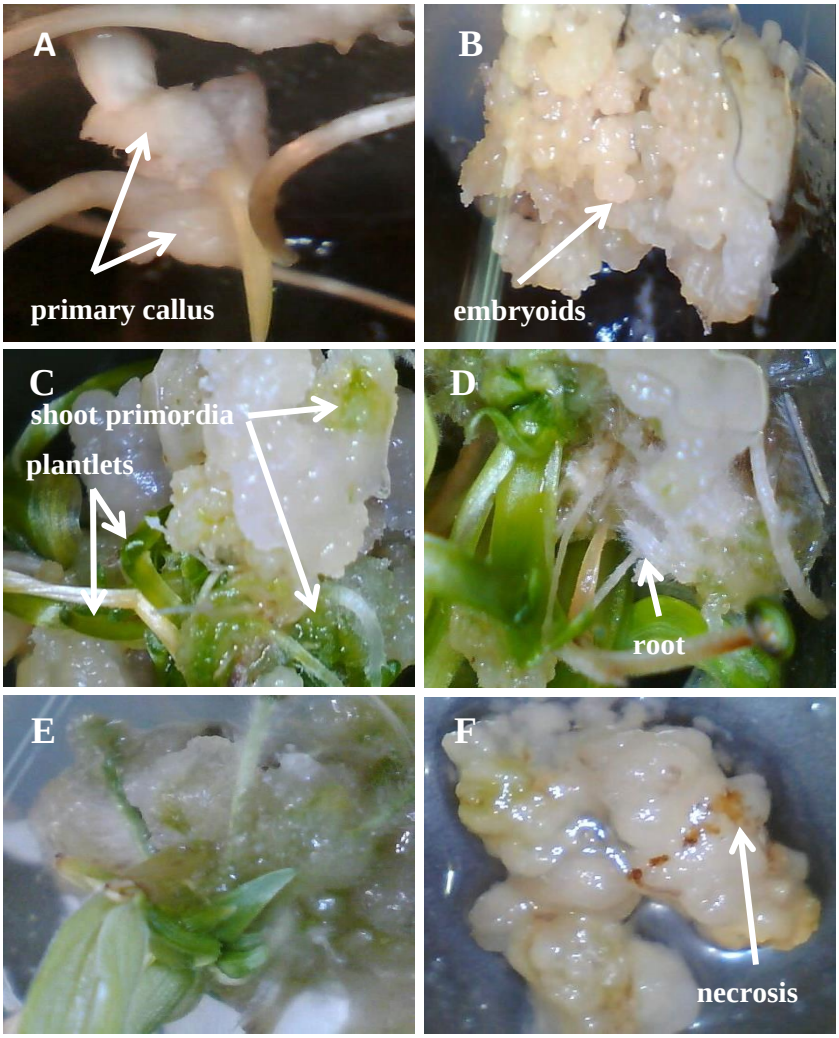

Figure 1. Callus formation (primary callus) from mature seeds 7 days after culturing on the callus induction medium in darkness (**A**). Embryonic callus formed after 4 weeks in callus induction medium in darkness (**B**). Embryonic callus and Plantlets regenerated obtained after 4 weeks of culture on a 2,4-D-free medium in light (control treatment) (**C**). Embryonic callus and Plantlets regenerated under the influence of NaCl salt at a concentration of 50 mM (**D**). Embryonic callus and Plantlets regenerated under the influence of NaCl salt at a concentration of 100 mM (**E**). Callus tissue under the influence of NaCl salt at a concentration of 150 mM (**F**).

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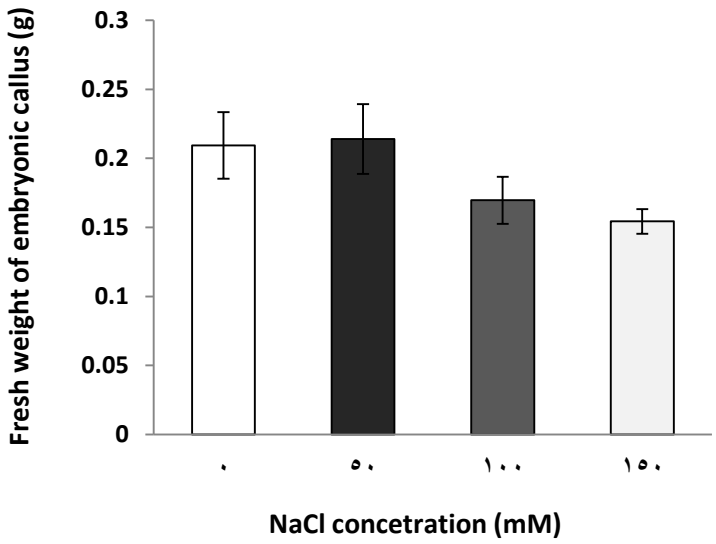

Figure 2. Effect of salt treatment on the fresh weight of callus

Regarding the effect of NaCl on the dry weight rates of callus tissue, it was observed through the results of Figure 3 that increasing the salt concentrations caused a statistically significant decrease at concentrations of 100 and 150 compared to the control treatment, and the lowest dry weight rate was recorded at a concentration of 150 mM, which was 0.015 g. It was also observed that treatment with 50 mM resulted in a significant increase compared to 100 and 150 mM.

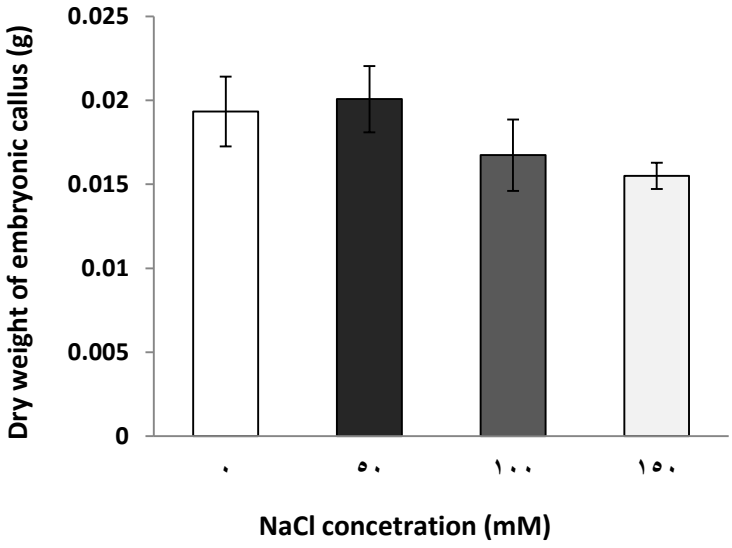

Figure 3. Effect of salt treatment on the dry weight of callus

One important factor in initiating in vitro culture and regenerating plants from embryos is the composition of the nutrient medium, especially the hormonal balance (Jiang et al., 1998). Auxin 2,4-D is widely used to initiate callus stimulation, either alone or in combination with cytokinins (Castillo et al., 1998).

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The callus induction stage is of great importance in the success of in vitro grain cultivation, and 2,4-D auxin is the most suitable plant growth regulator for callus induction in wheat plants. (Pour et al., 2020; Khokhar et al., 2016). The results of the study showed the observation of callus tissue on the seventh day of culturing mature seeds on MS medium supplied with 6 mg/L of the growth regulator 2,4-D, which is the most efficient regulator for inducing and promoting callus tissue. Several studies used 2,4-D, including the study by Benderradji et al. (2007) at a concentration of 10 mg/L in MS medium for inducing callus from mature seeds of bread wheat and plant regeneration. Sakr & Sayed (2018) also used 2,4-D at concentrations of 2 and 2.5 mg/L to induce callus formation in mature rice seed embryos. The time required to induce callus from mature and immature wheat embryos varies. For example, Elshahhat et al. (2017) found that the time required to stimulate callus ranged between 2-3 days in MS medium supplied with 2 mg/L 2,4-D. Saha et al. (2017) indicated that callus induction began after 7 days of cultivation on MS medium containing 2.5 mg/L 2,4-D. This result is similar to the results we obtained.

The results obtained showed morphological changes in the callus tissue formed during its various growth stages, as well as differences in tissue color, where the colors ranged from white at the beginning of callus formation to pale yellowish and greenish-yellow, then to brown, and from non-nodular callus (primary non-embryonic callus) to friable nodular callus (embryonic callus). The addition of NaCl salt to the nutrient medium also caused changes in the callus texture and color according to the degree of stress. These changes in the color and texture of the callus indicate transformations in the growth stages and its ability to regenerate plants, as the white and yellow color indicates the activity of division and differentiation (Yasmin et al., 2001). Callus tissues differ in their appearance, texture, gene expression pattern, as well as the ability of their cells to divide and the ability to regenerate plants (Luciani et al.,2006). The type of callus that forms is affected by many factors such as the type of plant, the plant part used, the composition of the culture medium, and growth regulators (Luciani et al.,2006; Zhou et al.,2018).

The results obtained from the study indicated that the NaCl salt treatment at concentrations of 100 and 150 mM led to a decrease in the average fresh and dry weights compared to the control treatment. The current study also found that treatment with a concentration of 50 mM led to a significant increase in the average fresh and dry weights. This is an interesting result as it resulted in growth improved which is consistent with what several studies have confirmed.

Many studies have concluded that exposure of callus tissue to salt stress results in negative effects on growth, including decrease both fresh and dry weights. One such study is Klay & Slim (2024) which aimed to evaluate the tolerance of wild wheat varieties to stress caused by increased sodium chloride levels. The same study also indicated that a low concentration of 50 mM resulted in improved callus growth, a result similar to the results of our study. Al Hattab et al. (2015) also found that salt treatment with different concentrations of NaCl (6, 8, 12, or 14 dSm-1) caused a decrease in the average fresh and dry weights of bread wheat callus with an increase in stress intensity. Notably, the 8 dSm-1 concentration resulted in the highest average dry weight. The addition of NaCl negatively affected callus growth and development at concentrations of 100 and 150 mM. This is likely due to water deficit and ionic toxicity resulting from the absorption of Na^+ and Cl^- ions, leading to reduced water availability in callus cells, energy consumption, and consequently, a reduced growth rate(Ghane et al.,2014).

When the induced callus was transferred from the stimulation medium to a medium free of 2,4-D, the formation of clusters of cell groups in the periphery of the callus (somatic embryos) was observed, and

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the number of these embryos varied depending on the salt concentration in the nutrient medium. The results shown in Figure 4 indicate the negative effect of adding NaCl on the number of somatic embryos after 8 weeks of culture, as it recorded the lowest rate of 5.67, with a significant difference compared to all treatments 0, 50, and 100, which achieved the highest rate of somatic embryos of 15.00, 15.33, and 14.33, respectively. The results also indicated that, in Figure 5, the addition of NaCl also affected the number of resulting plantlets, as the number of plantlets decreased significantly at concentrations of 100 and 150 mM, reaching 4.33 and 2.67 plantlet, respectively, compared to the control treatment. The concentration of 50 mM achieved the highest rate of plantlets, reaching 9.33 plantlet.

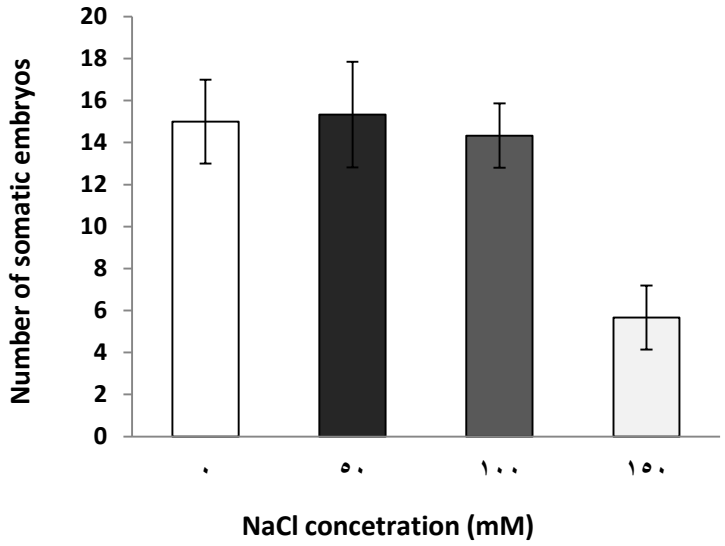

Figure 4. Effect of salt treatment on the number of somatic embryos

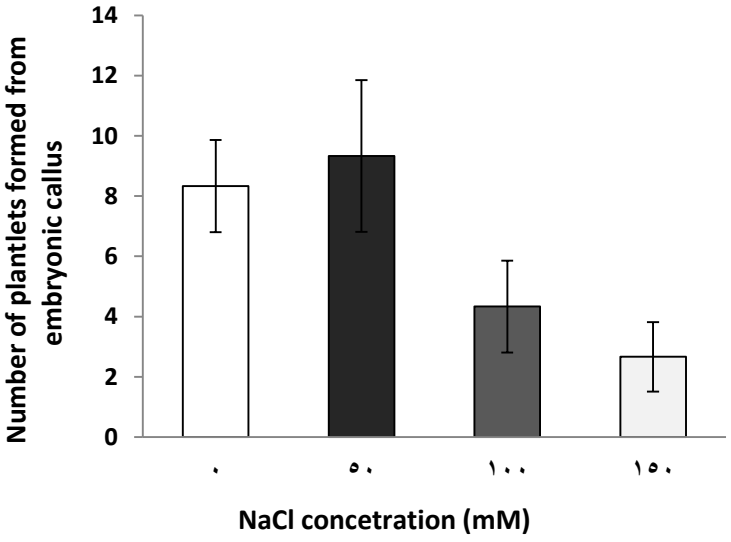

Figure 5. Effect of salt treatment on the number of plantlets formed from embryonic callus

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Color and textural changes were also observed in callus tissue cultured on nutrient media with varying concentrations of NaCl, where the callus transformed from nodular, pale yellowish nodules to soft, brown callus with necrosis at a concentration of 150 mM Figure 1-F.

Regarding the development of callus into embryonic callus, somatic embryogenesis, and plant regeneration, study showed the formation of slightly nodular embryonic callus after four weeks of cultivation on the callus stimulation medium. When transferred to a medium free of 2,4-D, the transformation of callus into nodular embryonic callus (multi-nodular) was stimulated under normal growth conditions (control treatment), Figure 1-B.

Under the influence of salinity stress (NaCl stress), a significant decrease in the number of somatic embryos was observed, along with a reduction in the callus ability to regenerate plants, accompanied by increasing stress levels. This was in addition to the previously mentioned changes in callus color and texture. Various studies have indicated that the growth regulator 2,4-D induces somatic embryo formation from the callus of many plant species such as rice a wheat millet Crop , and when transferred to a nutrient medium free of 2,4-D, somatic embryos develop from the callus periphery (Caliskan et al.,2004; Yasmin & Arfan, 2001; Sakr & Sayed, 2018; Vikrant, 2015). The color changes observed in callus tissue during the 12-week study indicate changes in growth phase and the callus tissues ability to regenerate. White, yellow, and green colors indicate continued active cells division, while brown and black colors indicate signs of cell aging (Nasution & Nasution, 2019). The visible color of callus tissue reflects the level of cell activity, and the color change results from the synthesis of phenolic compounds on the callus tissue surface (George & Sherrington,1984). The browning caused by high salt treatments (150 mM) is likely due to the negative effects of salt stress, including increased accumulation of toxic phenolic compounds that inhibit growth (Hayati, 2010). In general, the callus formed varies depending on the plant species, the components of the nutrient medium, growth regulators, and growing conditions(Jiang et al., 1998). In general, the callus formed varies depending on the plant species, the components of the nutrient medium, growth regulators, and growing conditions. The friable, pale yellowish callus has the ability to divide and differentiate its cells into somatic embryos (Benderradji et al.,2007). Regarding the necrosis observed in callus tissues exposed to a high level of stress (150 mM), this may be due to an ionic imbalance, particularly in the sodium/potassium ratio, leading to impaired cell growth and necrosis. Furthermore, the ability of callus growing under the influence of NaCl to produce somatic embryos and regenerate the plant was negatively affected, as a decrease in the number of embryos and plantlets was observed when 150 mM NaCl was added.The highest number of somatic embryos and plantlets was recorded in the control and 50 mM treatments. The reduced ability of embryonic callus to somatic embryogenesis and produce plantlets when treated with high concentrations of sodium chloride is due to water deficit and ionic toxicity resulting from sodium and chloride ions, thus reducing water availability and nutrient absorption in the growth medium, leading to decreased growth rates(Wani et al.,2010; Ghane et al.,2014). The results are consistent with Abdel-Hady,(2006) findings in his study on wheat, in which he concluded that increased salt stress leads to decreased callus growth and reduced plant regeneration. Vikrant (2015) also found that treating millet crop with sodium chloride at 50, 100, and 250 mM elicited distinct responses, depending on the culture medium salt concentration. At 250 mM, embryonic callus growth was reduced, and this concentration was lethal to the embryonic response. This slowdown in growth is attributed to the fact that a portion of the total energy available for metabolic processes is directed towards stress tolerance(Cushman et al.,1992).

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Another study indicated the effect of NaCl at concentrations of 10, 25, 50, 100, and 150 mM on somatic embryogenesis and plant regeneration in black rice. A concentration of 100 mM NaCl decreased somatic embryo formation and plant regeneration, whereas 150 mM NaCl was lethal to both processes (Rajalakshmi, 2024). Conversely, treatment with a low concentration of 50 mM NaCl resulted in increased stimulation of somatic embryogenesis. One of the interesting observations made by Klay et al.,(2024) is that salt stress induced by different concentrations of NaCl (0, 50, 100, 150 mM) has a different response depending on the concentration applied. Several studies have confirmed that moderate stress promotes somatic embryo differentiation and plant regeneration in many plant species, including black rice and millet, under various stress conditions, including salt, water, and heavy metal stress (Rajalkshmi,2024; Vikrant ,2015). Somatic embryogenesis can be stimulated by abiotic stress, a characteristic that is among its most prominent, as changes in water availability, through osmotic stress, affect the development of embryonic tissues. This stimulation may be related to an increase in endogenous abscisic acid levels as a response to osmotic stress. Oxidative stress caused by free radicals such as ROS can also act as a secondary messenger that contributes to stimulating somatic embryogenesis (Spinoso & Bello, 2022). It is suggested that moderate stress disrupts the protoplasmic connections (plasmodesmata) between pre-embryonic cells, thereby isolating the cells physiologically and facilitating the differentiation of a large number of cells (Patnaik et al,2005).

V. conclusion

In conclusion, plant tissue culture technology can be used as an effective tool to study the effect of abiotic stresses on plant response and to produce wheat callus cell lines with the ability to survive salt stress, as indicated by the study results, where the cells were able to survive under salt stress conditions.

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