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Response of rice (*Oryza sativa* L.) callus under influence of polyethylene glycol

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Abstract

Water stress resulting from water scarcity is a major factor limiting crop production in Iraq. The present study aimed to evaluate the effect of adding Polyethylene Glycol-1500 (PEG-1500) used to induce water stress on the growth of embryogenic callus in the rice cultivar 'Anbar 33', as well as on some physiological and biochemical traits associated with water stress tolerance *in vitro*. PEG-1500 was added to the MS growth medium at concentrations of (0, 5, 10, and 20) g/L to investigate its impact on callus growth, relative water content, somatic embryogenesis, cellular membrane integrity (electrolyte leakage), proline and carbohydrate content. The study found that increasing the PEG concentration resulted in a significant reduction in the fresh weight of embryogenic callus; at a concentration of 20 g/L, the weight recored to 0.14 g compared to 0.22 g in the control treatment. A significant decrease in relative water content was also observed with PEG treatments at 10 and 20 g/L, reaching 73.4% and 67.8% respectively, compared to 94.2% in the control. Furthermore, the results indicated that PEG-induced water stress at concentrations of 10 and 20 g/L negatively affected somatic embryo formation, as the number of somatic embryos decreased from 30.6 in the control treatment to 18.6 and 14, respectively. Increased water stress levels led to a significant rise in electrolyte leakage at concentrations of 10 and 20 g/L compared to the control, indicating greater damage to cell membranes. Conversely, PEG treatment stimulated the accumulation of both proline and carbohydrates. The results indicate that water stress limits embryogenic callus growth and the efficiency of somatic embryogenesis, while enhancing the accumulation of osmolytes compounds such as proline and carbohydrates, reflecting their role in adaptation to water deficient conditions in the growth medium,

Keywords: *Oryza sativa*, water stress, Polyethylene glycol, Callus, Somatic embryogenesis

Introduction

Plant growth and development are negatively affected by exposure to biotic and abiotic stresses, and resulting decrease in the yield of economically important crops leads to significant losses on a global scale (Wijerathna & Hiti-Bandaralage, 2023). Water scarcity stress is considered one of the most influential abiotic stresses on crops, affecting several stages of the plant life cycle, from seed germination, vegetative growth and reproductive development, which ultimately leads to a decrease in the quantity and quality of crops, especially in arid and semi-arid regions (Leng & Hall, 2019) [4]. Water availability is one of the most important factors affecting crop production in Iraq, as the quality and productivity of rice plants are closely linked to their ability to withstand drought (Abdul-Qadir & Al-Ka'aby, 2011; Khokhar *et al.*, 2021) [2, 21]. Under conditions of water scarcity stress, many processes at the molecular, biochemical, physiological and morphological levels in plants are affected, as a result of the activation of many stress response factors. Consequently, plant productivity and quality decrease in water scarcity conditions (Sehgal *et al.*, 2018) [32]. Plant tissue culture is a valuable tool for developing and improving plant stress tolerance, examining this tolerance, and studying the physiological and biochemical changes that occur during stress exposure. Selection under controlled laboratory conditions in specific environments and areas is highly efficient and less expensive (Wijerathna & Hiti-Bandaralage, 2023). Plant tissue culture techniques also contribute to understanding the mechanisms of stress tolerance in order to develop resistant plant varieties. The use of *in vitro* tissue cultures to study stress responses is based on the fact that cells grown *in vitro* exhibit behavior similar to that of whole plant cells when exposed to conditions of water scarcity and salt stress (Al-Taha, 2013) [5]. In plant tissue culture, several osmotic agents are

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Used to induce water stress, such as polyethylene glycol (PEG) and mannitol. PEG is the most widely used because it is a high molecular weight, non-ionic, non-toxic osmotic agent with high water solubility (Newton *et al.*, 1989) [26]. Furthermore, it is not absorbed by the plant due to its large molecular size, making PEG suitable for creating artificial water stress conditions that mimic those found in the plant's natural environment (Hamayun *et al.*, 2010) [16]. Under *in vitro* culture conditions, callus tissue can be induced, redifferentiated, exposed to different levels of PEG, and then tissues capable of surviving under water stress conditions can be selected (Rashid *et al.*, 1996) [29]. *In vitro* selection occurs at the callus stage, which consists primarily of undifferentiated cell masses and serves as a starting material for *in vitro* treatment. Furthermore, callus development is more controlled and faster compared to field methods. Polyethylene glycol (PEG) is used as a non-toxic compound in selection processes under drought stress. Its addition to growth media stimulates cellular responses that enhance plant adaptation to stress and activates drought response mechanisms (Yustiningsih *et al.*, 2021). The somatic callus is targeted because of its ability to regenerate and form complete plants. Its characteristics include a white to yellowish color, a friable texture, and high weight and diameter. The white color indicates high cell division activity (Humaria & Amien, 2019). Somatic embryogenesis is a technique that can be used in *in vitro* selection and is of great value, because the selected traits can be passed on to subsequent generations if they are hereditary in nature (Larkin & Scowcroft, 1981) [22]. Several studies have used compounds such as PEG, sorbitol, and mannitol to examine water stress tolerance and selection *in vitro* for many plants such as durum wheat, wheat and Sugarcane (Kacem *et al.*, 2017; Mahmood *et al.*, 2012; Errabii *et al.*, 2006) [13, 19, 25]. Therefore, the current study aimed to evaluate the response of callus, a local rice cultivar (Anbar 33) grown under water stress induced by poly ethylene glycol (PEG-1500).

Materials and methods

The experiment was conducted in the Plant Cell and Tissue Culture Laboratory / Department of Biology/ College of Education for Pure Sciences / University of Basra on rice plants, cultivar Anbar 33, where the seeds were brought from the Al-Mishkhab Research Station / Najaf Governorate for the purpose of using them as explants in inducing callus. The experiment included the following :

1- Seed vitality test

Seed viability was checked by germination percentage testing, where one hundred seeds were grown in four Petri dishes with 5 ml of distilled water added to filter paper inside each dish and incubated in the dark at a temperature of 25°C. Then the germination percentage of the seeds was calculated after 72 hours according to the following equation:

Germination (%) = Number of germinated seeds/ Total number of seeds * 100

2- Surface sterilization of seeds

The surface of the dehulled seeds was sterilized with 70% ethanol for 5 minutes, then washed with sterile distilled water three times to remove the alcohol. After that, 0.1% mercuric chloride HgCl₂ solution was used for 10 minutes, then rinsed three times with sterile distilled water to remove

traces of the sterilizing agent. This process was carried out inside a laminar airflow cabinet.

3- Preparation of callus induction medium

To prepare 1 liter of callus induction medium, dissolve 4.33 g/L MS (Murashige and Skoog, 1962) [25] nutrient medium salts in distilled water along with 30 g/L sucrose and place it on a plate equipped with a magnetic stirrer. After dissolution, add the growth regulators 2,4-D at a concentration of 4 mg/L and BA at a concentration of 1 mg/L, then bring the volume up to 1 liter and adjust the pH to 5.8. Then add agar at a concentration of 8 g/L. Next, heat the solution to 92°C. Dispense the nutrient medium into test tubes at a concentration of 10 ml per tube and seal them with cotton plugs and aluminum foil. Sterilize the tubes in a sterilizer at a temperature of 121°C and a pressure of 0.1 MPa for 15 minutes. After sterilization, remove the tubes, shake to homogenize their contents, and allow them to cool and solidify. The dehulled and sterilized seeds were cultured on a pre-sterilized induction medium at a rate of 4 seeds per tube and incubated in the dark at 25°C for four weeks. The callus tissue formed after four weeks of incubation was transferred to a new medium with the same components as the callus induction medium for four weeks of propagation. Following this, 100 mg of the callus tissue was transferred to a new nutrient medium with the addition of different levels of PEG (0, 5, 10, and 20 g/L) to create water stress conditions in the growth medium for four weeks. The following measurements were then taken:

1- Fresh weight

2- Number of Somatic Embryos Formed

3- Relative Water Content (RWC) Estimation

The relative water content of embryonic callus tissue was estimated by directly measuring the fresh weight (FW) and placing it in test tubes containing distilled water. The tubes were left in the dark for 4 hours, after which the samples were removed, the water was discarded using filter paper, and their weights were remeasured. This represents the turgid weight (TW) of the callus. Dry weights (DW) were then calculated after drying in an electric oven at 70°C for 48 hours. The relative water content was estimated using the following equation (Barr & Weatherly, 1962) [6].

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

4- Cell membrane injury estimation

Cell membrane damage was assessed by measuring electrolyte leakage according to the method described by Yadav *et al.* (2018). A 100 mg sample of embryogenic callus was washed with distilled water and transferred to test tubes containing 10 mL of distilled water. The tubes were incubated at room temperature for one hour and then stored at 4°C for 24 hours; subsequently, electrical conductivity was measured and recorded as (L1). The samples were then placed in an autoclave at 120°C for 60 minutes to release all electrolytes present in the samples. After cooling the samples to 25°C, the final electrical conductivity reading was taken and recorded as (L2). The electrolyte leakage percentage was calculated using the following equation:
$$\text{Electrolyte Leakage} = \frac{L1}{L2} \times 100$$

5- Determination of proline amino acid content

A 500 mg sample of embryogenic callus was homogenized in 10 mL of 3% sulfosalicylic acid and filtered. Subsequently, 2 mL of the filtrate was added to a mixture

consisting of 2 mL of ninhydrin and 2 mL of glacial acetic acid. The mix was incubated in a water bath at 100°C for one hour, followed by cooling in an ice bath. Extraction was then performed by adding 4 mL of toluene to separate the colored (chromophore) phase from the aqueous phase. Finally, the intensity of the resulting color was measured using a spectrophotometer at a wavelength of 520 nm, following the method described by Bates *et al.*, (1973)^[7].

6- Determination of carbohydrate concentration

A 0.5 g sample of fresh embryogenic callus was taken and ground with 2 mL of 1N hydrochloric acid, then heated in a boiling water bath for 20 minutes. The sample was allowed to cool to room temperature and subsequently centrifuged at 2000 rpm for 5 minutes. Next, 0.5 mL of the supernatant was mixed with 0.5 mL of a 5% phenol solution, followed by the addition of 2.5 mL of concentrated sulfuric acid. Finally, the carbohydrate concentration was determined by measuring the intensity of the resulting color using a spectrophotometer at a wavelength of 490 nm, in accordance with the method described by Edith and Uripek (1989).

Statistical Analysis

The obtained results were statistically analyzed using SPSS software. Significant differences between treatments were determined using analysis of variance (ANOVA), and mean differences were compared using the Least Significant Difference (L.S.D) test at a 5% probability level.

Results

1-Fresh weight of embryonic callus

The results presented in Table (1) indicate that treating rice callus with varying concentrations of PEG (0, 5, 10, and 20) g/L elicited differing responses. These ranged from no significant effect at concentrations of 5 and 10 g/L where fresh weights of 0.26 g and 0.17 g were recorded, respectively to a negative impact on embryogenic callus growth, specifically at the 20 g/L concentration. At this highest concentration, the lowest mean fresh weight (0.14 g) was observed, representing a significant difference compared to the control, which had a mean fresh weight of 0.22 g.

2- Relative water content (R.W.C)

A significant negative effect on the water content of embryonic callus tissue was observed at concentrations of 10 and 20 g/L; the mean relative water content was 73.4% and 67.8%, respectively, compared to 94.2% in the control. In contrast, the 5 g/L concentration did not cause any significant effect on relative water content, as shown in Table 2.

Table 1. Fresh weight of embryonic callus

PEG concentrations (g/l)	0	5	10	20
Means	0.22	0.26	0.17	0.14
SD	0.019±	0.035±	0.011±	0.015±
L.S.D at level 5% 0.078				

Table 2. Relative water content (R.W.C)

PEG concentrations (g/l)	0	5	10	20
Means	94.2	90.3	73.4	67.8
SD	3.09 ±	0.85 ±	1.8 ±	1.17 ±
L.S.D at level 5% 10.3				

3- Somatic embryogenesis

The results appeared in Table (3) and Figure (1) indicate the formation of a friable texture, nodular, yellowish-white embryogenic callus tissue eight weeks after culture initiation. Furthermore, the study revealed that treating the embryogenic callus growth medium with PEG to induce water stress at concentrations of 10 and 20 g/L negatively affected the formation of somatic embryos; their numbers decreased significantly, recording 18.6 and 14 embryos, respectively, compared to the control treatment, which exhibited the highest rate of somatic embryo formation 30.6 somatic embryos.

Table 3: Numbers of somatic embryos formed

PEG concentrations (g/l)	0	5	10	20
Means	30.6	32.3	18.6	14
SD	3.05 ±	1.52 ±	2.51 ±	2.64 ±
L.S.D at level 5% 3.52				

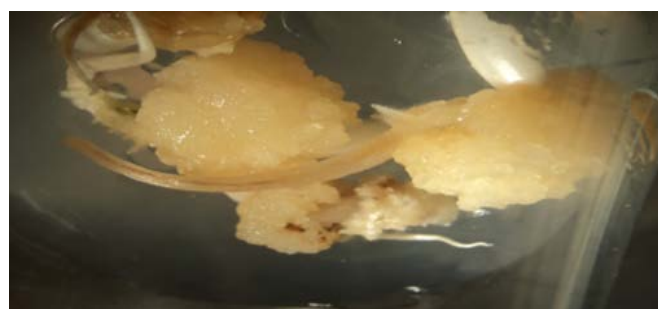


Fig 1: Embryonic callus formation from seeds after eight weeks cultured on induction medium

4- Cellular membranes injury

The results showed in Table (4) indicate that osmotic stress induced in the callus growth medium caused a significant increase in the electrolyte leakage rate, specifically at concentrations of 10 and 20 g/L, where leakage rates were 19.2% and 26.6%, respectively. This contrasts with the control, which recorded the lowest electrolyte leakage rate at 10.2%. The highest rate was observed with the addition of PEG at 20 g/L, whereas no significant difference in electrolyte leakage was recorded at the 5 g/L concentration compared to the control.

Table 4: Electrolyte leakage (%)

PEG concentrations (g/l)	0	5	10	20
Means	10.4	12.44	19.2	26.6
SD	1.52 ±	0.77 ±	1.15 ±	1.58 ±
L.S.D at level 5% 3.2				

5- Proline content

Table (5) shows an increase in the proline amino acid content of embryonic callus tissue, a rise that corresponded with increasing levels of water stress specifically, raising the PEG concentration from 5 g/L to 10 g/L and 20 g/L resulted in a significant, gradual increase in proline levels reaching 0.67, 0.89, and 0.93 mg/g, respectively compared to the control, which exhibited the lowest level of proline accumulation at 0.48 mg/g.

6- Carbohydrate concentration

The results indicated that adding PEG to the culture medium led to increased carbohydrate accumulation in the embryogenic callus tissue, as shown in Table (6).

Significant increases in carbohydrate accumulation were observed with PEG treatments at concentrations of 5, 10, and 20 g/L recording values of 0.73, 0.96 respectively, compared to the control 0.59 mg/g with the highest rate of carbohydrate accumulation 1.2 mg/g recorded at a PEG concentration of 20 g/L

Table 5: Proline content (mg/g FW)

PEG concentrations (g/l)	0	5	10	20
Means	0.48	0.67	0.89	0.93
SD	0.07 ±	0.04 ±	0.05 ±	0.02 ±
L.S.D at level 5% 0.17				

Table 6: Carbohydrates concentration (mg/g FW)

PEG concentrations (g/l)	0	5	10	20
Means	0.59	0.73	0.96	1.2
SD	0.2±	0.15 ±	0.24 ±	0.25 ±
L.S.D at level 5% 0.12				

Discussion

The results in Table (1) indicate the negative effect of adding PEG to the callus growth medium, as it caused a reduction in fresh weight, this effect was observed at a concentration of 20 g/L. The reduction in callus fresh weight is a natural response in many plants (Ehsanpour & Razavizadeh, 2005; Sakthivelu *et al.*, 2008) [12, 30]. The reduction in callus fresh weight in response to PEG may be attributed to water deficit, which affects the growth and development of callus cells. The addition of PEG to the growth medium lowers the water potential leading to reduced turgor pressure thereby negatively impacting cell division and inhibiting callus growth (Sakthivelu *et al.*, 2008) [30]. Cell division and growth are responsible for the increase in the fresh weight of the callus. Reduced growth parameters have been recorded in various plants such as rice growing under water-stress conditions (Khokhar *et al.*, 2021) [21]. The decrease in callus fresh weight was accompanied by a significant reduction in relative water content (Table 2) at concentrations of 10 and 20 g/L compared to the control. The water potential gradient established between the callus tissue cells and the surrounding nutrient medium resulting from the addition of PEG leads to reduced water content and cellular dehydration. Consequently, a correlation was observed between the decrease in relative water content and increasing PEG levels. A reduction in callus tissue water content has been documented in various plant species, such as the date palm and durum wheat callus (Al-Khayri & Al-Bahrany, 2004; Bouiamrine & Diouri, 2012) [3, 4, 8]. Prior to further treatment, the formation of a friable, nodular, yellowish-white embryogenic tissue was observed a tissue characterized by its capacity to produce somatic embryos. The color and texture of the callus induced on the growth medium reflect cellular activity; a white callus is considered high-quality, indicating the proliferative potential of the callus cells, whereas a brown color signifies senescence and cell death resulting from the accumulation of phenolics (Saputro *et al.*, 2025) [31]. PEG-induced water stress reduced the callus's capacity for somatic embryogenesis; the ability to form embryos was linked to the stress level, with a significant decline observed as PEG concentrations increased to 10 and 20 g/L compared to the control treatment. This result aligns with the findings of Wani *et al.* (2010) regarding PEG-6000 induced drought stress

tolerance in rice callus, where they observed a decrease in callus formation and regeneration capabilities as stress levels increased. Similarly, Rajalakshmi (2025) [28], reached similar conclusions in his study of black rice, finding a decrease in somatic embryogenesis and germination under PEG- and mannitol-induced drought stress conditions. A decrease in the callus regeneration rate of two rice cultivars was also observed with increasing PEG levels (Chakraborty *et al.*, 2018) [9]. Water stress also caused damage to the cell membranes of the callus tissue, evidenced by increased electrolyte leakage specifically at concentrations of 10 and 20 g/L. This increased membrane damage may be attributed to a significant reduction in relative water content, which leads to the oxidation of unsaturated fatty acids and protein degradation; this compromises plasma membrane permeability and integrity, resulting in the leakage of electrolytes from the cytoplasm (Petrov *et al.*, 2018) [27]. A decrease in turgor pressure and changes in plasma membrane tension in callus tissue cells, resulting from osmotic stress, may be a source of plasma membrane damage caused by water stress (Shi *et al.*, 2023; Guo *et al.*, 2024) [14]. The increased rate of electrolyte leakage may be attributed to oxidative stress resulting from water stress, which damages plant cell membrane systems through reactive oxygen species (Fu *et al.*, 2024). These results align with findings from numerous studies, whether at the whole-plant level such as the study by Wang *et al.* (2020) on winter wheat or at the cellular level, as seen in the study by Venkateswarlu & Ramesh (1993) [34] on peanuts, which established an inverse relationship between the level of PEG-induced water stress and cell membrane stability.

Water stress causes changes in metabolic indicators associated with plant tolerance to water-deficit conditions; the accumulation of free amino acids and soluble carbohydrates in plant tissues is considered an adaptive response (Devi & Sujatha, 2014) [11]. The results of the current study indicated that treating callus tissue with varying concentrations of PEG (0, 5, 10, and 20 g/L) elicited differential responses regarding proline and carbohydrate accumulation. A gradual increase in proline content was observed, correlating with rising levels of water stress; similarly, carbohydrate accumulation increased at concentrations of 10 and 20 g/L. The accumulation of proline in plant tissues subjected to osmotic stress helps maintain turgor pressure and the water potential gradient, thereby facilitating effective water uptake within the callus cells and tissues (Al-Bahrany, 2002) [3]. Proline biosynthesis enhances the tolerance of rice plants to water stress; consequently, the capacity for proline accumulation can serve as a criterion or biomarker for evaluating drought resistance potential in rice cultivars (Abdula *et al.*, 2016) [1]. In addition to proline accumulation, water stress leads to increased accumulation of carbohydrates, which act as osmo-protectants to maintain cell stability and turgor pressure, thereby helping the plant withstand stress (Jarin *et al.*, 2024) [18]. Numerous studies have demonstrated the accumulation of proline and carbohydrates at the cellular level in plants subjected to water stress across various species, such as sweet potato cell cultures (Wang *et al.*, 1999), soybean callus (Kaviani, 2008), and date palm (Al-Khayri & Al-Bahrany, 2004) [3, 4, 20].

Conclusion : The results of this study indicate that inducing water stress using polyethylene glycol (PEG) had a negative

impact on somatic callus growth and differentiation efficiency, as well as on physiological and biochemical traits. Increased PEG concentrations in the culture medium led to reductions in fresh weight, water content, and the number of somatic embryos formed, demonstrating the adverse effect of water deficit on callus growth and its capacity for regeneration and differentiation. Water stress also caused an increase in electrolyte leakage, reflecting damage to cell membranes. Conversely, PEG treatments induced a gradual accumulation of proline and carbohydrates as stress intensity increased, indicating the role of these compounds in osmotic adjustment, the maintenance of cellular homeostasis, and the mitigation of the adverse effects of water stress.

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