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Growth and Physiological Characterization in Five Genotypes of *Brassica napus* Callus Culture under Drought–Induced Osmotic Stress

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ABSTRACT

As the demand for edible oil continues to soar, rapeseed cultivation remains pivotal for sustainable and efficient oil production, catering to the nutritional needs of a burgeoning population. Given the substantial impact of environmental stress on plant productivity, this study aimed to explore the in vitro responses of calli-derived hypocotyls from five *Brassica napus* genotypes under varying concentrations of polyethylene glycol (PEG) 6000 (0%, 10%, 20%, 30%, and 40%). The objective was to assess various growth and physiological parameters, including Callus Growth Rate (CGR), Relative Water Content (RWC), Index of Tolerance (INTOL), Relative Growth Rate (RGR), and Proline Content (PC). Results unveiled a significant surge in proline content with increasing levels of PEG, with the highest accumulation observed at 40% PEG concentration. These findings suggest that heightened osmotic stress induced by PEG led to a notable rise in proline accumulation in calli-derived hypocotyls. Furthermore, elevated levels of PEG-induced osmotic stress adversely impacted growth parameters such as RWC, RGR, and CGR. Notably, genotypes Geronimo and Arc5 exhibited enhanced drought resistance in in vitro environments compared to other genotypes. These particular genotypes showcased greater resilience and adaptability to PEG-induced osmotic stress, as evidenced by significantly higher measurements of relative water content (RWC), relative growth rate (RGR), and Callus Growth Rate (CGR). Physiological indices like Relative Water Content (RWC), Relative Growth Rate (RGR), and Callus Growth Rate (CGR) offer valuable insights into a plant's response to environmental stresses like water scarcity. This selection process is crucial for developing more resistant plant varieties that thrive even in challenging environmental conditions.

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1. Introduction

The impact of drought stress on agricultural productivity is particularly severe in arid and semiarid regions, where water availability is limited and rainfed agriculture is common (Seleiman *et al*., 2021). Water stress, including drought-induced water scarcity, significantly affects the metabolic and physiological processes in plants. To cope with limited water availability, plants undergo osmotic adjustment, actively accumulating solutes such as sugars, amino acids, and organic acids (Kapoor *et al*., 2020). This process helps maintain proper cellular turgor pressure and mitigate excessive water depletion from compromised tissues (Moradi, 2016).

Osmotic adjustment in plants aids in maintaining water uptake and reducing water loss by closing stomata, thus lowering transpiration rates during drought conditions. This adaptation conserves water, allowing plants to survive and sustain vital physiological processes. Additionally, solute accumulation stabilizes proteins and cellular structures, protecting them from dehydration-induced damage,

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and maintains enzymatic activity and metabolic processes even in water-limited environments (Turner, 2018).

Ions like potassium (K^+), sodium (Na^+), and chloride (Cl^-) help in osmotic adjustment by increasing solute concentration within cells. Soluble sugars such as sucrose, glucose, and fructose serve as osmolytes while also providing energy and carbon for metabolic processes. Quaternary ammonium compounds like glycine betaine and proline betaine accumulate as osmolytes in some plants and microbes, aiding in osmotic adjustment and protecting cellular structures and enzymes during water stress. Accumulation of these osmolytes maintains cellular hydration, turgor pressure, and cell integrity, enhancing the ability of plants and microbes to endure drought conditions.

Proline accumulation in plants enhances stress tolerance and cellular homeostasis through multiple mechanisms (Bajji *et al*., 2000; Errabii *et al*., 2006). Firstly, it acts as a compatible solute, aiding in osmotic adjustment and maintaining cellular hydration and turgor pressure during stress. Secondly, proline stabilizes proteins, membranes, and subcellular structures, protecting them from denaturation and maintaining functionality. Additionally, proline serves as an effective scavenger of reactive oxygen species (ROS), preventing oxidative damage to cellular components (Kaur and Asthir, 2015). Overall, proline's multifunctional roles contribute to plants' ability to tolerate and adapt to various stressors, enhancing stress resistance (Hayat *et al*., 2012; Mohammadrezakhani *et al*., 2023). Tissue culture systems provide a valuable platform for studying plant responses to environmental stresses. By controlling and manipulating stress conditions in a reproducible manner, researchers gain insights into stress tolerance mechanisms and develop strategies for enhancing crop resilience (Hussain *et al*., 2012). The uniformity and synchronization of cells in tissue culture enable accurate stress tolerance evaluations, as any observed variations can be attributed to genetic or treatment differences rather than environmental factors. This precise control allows for better analysis of stress-related responses, simplifies data collection, and contributes to the development of stress-tolerant crop varieties (Pérez-Clemente and Gómez-Cadenas, 2012).

One of the prominent oilseed crops cultivated in Iran is rapeseed (*Brassica napus*). Rapeseed is widely grown for its high oil content, which is extracted and used for cooking, as well as in the production of biodiesel. Iran has been working towards increasing rapeseed cultivation to meet its domestic oil demand and reduce dependence on imports (Rostami Ahmadvandi and Faghihi, 2021). Rapeseed (*B. napus*) is a prominent oilseed crop in Iran, cultivated for its high oil content. Iran aims to increase rapeseed cultivation to meet domestic oil demand and reduce import dependency. The oil extracted from rapeseed is utilized for cooking and biodiesel production, making it economically significant for the country (Rostami Ahmadvandi and Faghihi, 2021).

Plant breeders and agronomists have developed oilseed cultivars and practices adapted to semi-arid regions, leading to increased oilseed production. Promoting oil crop cultivation in water-deficient areas can reduce oil imports and enhance domestic production, benefiting food security and economic growth in Iran. Canola rotation with wheat improves soil health, weed control, and crop productivity, contributing to sustainable agriculture. Drought stress affects rapeseed growth and yield, emphasizing the need for drought-tolerant genotypes. Research on water deficit effects during canola growth stages informs breeding programs for resilient varieties (Liu *et al*., 2019; Rostami Ahmadvandi and Faghihi, 2021; Schillinger and Paulitz, 2018; Aboodeh *et al*., 2024).

By studying these genotypes, we can gain valuable knowledge about their performance under drought stress and potentially identify genotypes that exhibit superior drought tolerance compared to others. This information could help contribute to breeding programs and the development of new varieties that are better suited to water-limited environments. Studying these genotypes offers valuable insights into their performance under drought stress, potentially identifying those with superior drought tolerance. Such findings could significantly contribute to breeding programs, facilitating the development of new varieties better adapted to water-limited environments.

2. Materials and methods

2.1. Plant material

In this study, five genotypes of *B. napus* (SLM 046, ARC5, Dante, Sunday, and Geronimo) were utilized. The seeds were sourced from the Agricultural Faculty of Razi University, Iran. In the year 2007-2008.

2.2. Seed sterilization

The surface sterilization process of the seeds was conducted using 1.5% sodium hypochlorite for 10 min, and then thoroughly rinsed thrice via sterilized distilled water for 10 min.

2.3. Germination medium

To germinate the seeds, we placed 50 sterilized seeds per glass on MS medium (supplemented with 3% sucrose and 0.8% agar, pH 5.8) and kept them in a 16-hour light / 8-hour dark photoperiod at 25°C. After 5-7 days hypocotyl explants were excised (size about 1 cm) from seedling and cultured on callus induction medium supplemented with some phytohormones.

2.4. Callus induction

The MS callus induction medium was supplemented with NAA (0.5 mg/l), 2.4.D (0.5 mg/l), and BAP (0.5 mg/l), 3% sucrose, and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 using a 0.1 N solution of HCL and autoclaved at 121°C for 20 minutes. The hypocotyls were placed on the medium at a density of 20-25 hypocotyls per plate. Every two weeks, the explants were subcultured on the same medium. After four weeks, the data on the calli were recorded.

2.5. Determination of index tolerance (INTOL)

The index of tolerance (INTOL) is a suitable method for comparing the response of different cultivars to stress conditions. It is calculated based on the relative growth rate (RGR) of plants under various treatments compared to control conditions. The formula for calculating INTOL is as follows (Equation 1):

(1) INTOL = RGR treatment / RGR control

In this equation, RGR treatment represents the relative growth rate of plants under stress conditions, while RGR control represents the relative growth rate of plants under control (non-stress) conditions.

2.6. Determination of RWC (Relative Water Content)

The relative water content (RWC) is a measure of the water content in plant tissues and is often used to assess the water status of cells or tissues. The RWC of callus samples was calculated as below (Equation 2) (Mullan and Pietragalla, 2012):

(2) RWC = [(Fresh weight - Dry weight) / Dry weight] × 100

To calculate the RWC using Equation 2, both fresh and dry weights of the callus samples were required. First, the callus samples were weighed when fresh (before drying) to acquire the fresh weight. The callus samples underwent dehydration in an oven at a temperature of 60°C for 24 hours to eradicate any remaining moisture. Subsequently, upon completion of the drying process, the callus samples were subjected to reweighing to determine their dry weight. With the fresh weight and dry weight values, RWC was calculated using Equation 2.

2.7. Determination of RGR (Relative Growth Rate)

Calli were weighed after four weeks of growth and then transferred to different treatments: the same MS medium without stress and MS medium supplemented with different concentrations of the stressing agent PEG6000 (0%, 10%, 20%, 30%, and 40%). For each treatment, six calli were used. The callus relative growth rate (RGR) was measured using the following formula (Equation 3) (Loveys *et al*., 2002):

(3) RGR = [(Final weight - initial weight) × 100] / initial weight

RGR was calculated using the initial weight and final weight of callus. First, the calli were weighed at the beginning of the experiment to obtain the initial weight. After the four-week growth period, the calli were weighed again to obtain the final weight. After determining the initial and final weight, RGR was calculated using Equation 3. (Errabi *et al*., 2006).

2.8. Determination of proline concentration in callus under stress condition

Fifty mg of callus fresh matter was weighed into a 1.5 ml centrifuge tube. To precipitate protein, 1.2 ml of 3% sulphosalicylic acid was added to the tube. The samples were mixed and then centrifuged at 18,000 g for 10 minutes. After centrifugation, the upper phase, which contains the choromophore-containing compound proline, was extracted using ninhydrin acid reagent (Bates *et al*., 1973). The ninhydrin acid reagent reacts with proline to form a colored product. To measure the concentration of proline, the chromophore-containing proline was extracted into 2

ml of toluene. Then, the extracted solution was measured using a spectrophotometer at a wavelength of 520 nm. The spectrophotometer detects the absorbance of the colored product formed by the reaction of proline with ninhydrin acid reagent. By measuring the absorbance at 520 nm, you can determine the concentration of proline in the callus samples.

2.9. Determination of CGR (Callus Growth Rate)

To calculate CGR using Equation 4, first, the callus diagonal mean and the time over which the measurement was taken were determined. The diagonal length of the callus was measured. In irregularly shaped callus, this was done by taking the average of multiple diagonal measurements. The period when the callus was measured was determined. This could be the duration of the experiment or a specific time interval. The callus diagonal mean was divided by time to calculate CGR. In Equation 4, "Callus diagonal mean" represents the average diagonal length of the callus, and "time" refers to the duration over which the measurement was made.

(4) CGR = Callus diagonal mean/time

The experiment was set up as a completely randomized 5×5 factorial design with three replications. Data were subjected to the analysis of variance (ANOVA) and the mean comparison was conducted using Duncan's test (Duncan, 1955) at 5% and 1% significance. All statistical analyses were performed using MSTATC and EXCEL.

3. Results and discussion

The results of variance analysis, as presented in Table 1, indicated that there is a highly significant difference among genotypes, drought levels, and their interaction ($p<0.01$). This finding suggests that the variations in the studied trait, which could be any trait under investigation, are influenced by the different genotypes used in the experiment. It also indicates that the imposed drought levels have a substantial influence on the trait. Furthermore, the interaction between genotypes and drought levels further contributes to the observed differences in the trait. In our study, we compared RGR, RWC, and CGR for five different genotypes under four osmotic pressure levels: 0%, 10%, 20%, 30%, and 40%. Our results indicated that there were substantial differences between the genotypes. Furthermore, we found that some of the genotypes exhibited a high RGR. This suggests that these specific genotypes had a greater rate of growth compared to the others under the given osmotic pressure levels. The differences in RGR among the genotypes highlight the genetic variability and potential variations in growth performance under different stress conditions.

The results of variance analysis, outlined in Table 1, reveal a highly significant difference among genotypes, drought levels, and their interaction ($p<0.01$). This indicates that variations in the studied trait, influenced by different genotypes and imposed drought levels, are substantial. Moreover, the interaction between genotypes and drought levels contributes significantly to observed trait differences. In our study, we examined the Relative Growth Rate (RGR), Relative Water Content (RWC), and Callus Growth Rate (CGR) across five genotypes under four osmotic pressure levels (0%, 10%, 20%, 30%, and 40%). Our findings demonstrate notable differences between genotypes, with some showing high RGR, suggesting superior growth rates under the given osmotic pressure levels. These disparities underscore genetic variability and potential variations in growth performance under diverse stress conditions.

Table 1. Analysis of variance for callus growth parameters under different drought levels and genotypes in *Brassica napus*

S.O.V	df	RWC	CGR	RGR	Proline	INTOL
Genotype	4	1344.54**	0.256**	2301.77**	378.354**	4553.006**
Drought	4	4087.74**	0.414**	8428.17**	2560.662**	624.029**
G × D	16	229.45**	0.005**	216.93**	64.426**	96.193**
E	50	0.854	0.000	0.41	0.802	0.092
CV (%)		1.82	3.89	1.68	1.95	1.73

** Significant at 0.01

Among the five cultivated genotypes, Geronimo displayed elevated values for RGR, RWC, and CGR when subjected to an osmotic pressure of 40%. This observation suggests that the Geronimo genotype exhibits superior growth rate, enhanced water retention capacity, and overall better growth performance compared to the other genotypes under the specific osmotic pressure condition of 40%. These findings imply that Geronimo demonstrates relatively higher tolerance or adaptability to the imposed osmotic stress. The increased RGR indicates that the Geronimo genotype maintains a faster growth rate, showcasing its

ability to sustain or even accelerate growth under osmotic stress conditions. Additionally, the higher RWC implies that Geronimo can retain more water, crucial for maintaining turgidity and essential physiological functions under water-limited circumstances. Lastly, the elevated CGR suggests that Geronimo exhibits superior overall growth over time, reflecting its capacity to sustain growth and development under osmotic stress. These findings demonstrate the negative impact of increasing PEG concentration on various growth and physiological parameters of *B. napus* callus. PEG is often used as an osmotic agent in experiments to simulate water stress conditions. It is important to note that PEG-induced osmotic stress can have varying effects depending on the plant species, genotypes, growth conditions, and the specific experimental setup. Researchers often conduct dose-response experiments to determine the optimal PEG concentration that induces the desired level of stress without causing excessive damage or cell death. The results indicate that higher levels of osmotic stress, induced by increased PEG concentration, negatively affected the callus's growth, water content, and tolerance to the stress (Fig. 1 and 2).

Figure 1. Status of calli in four osmotic pressures (On the left side, the names of the genotypes are written, and on the right side, the drought stress levels in MPa)

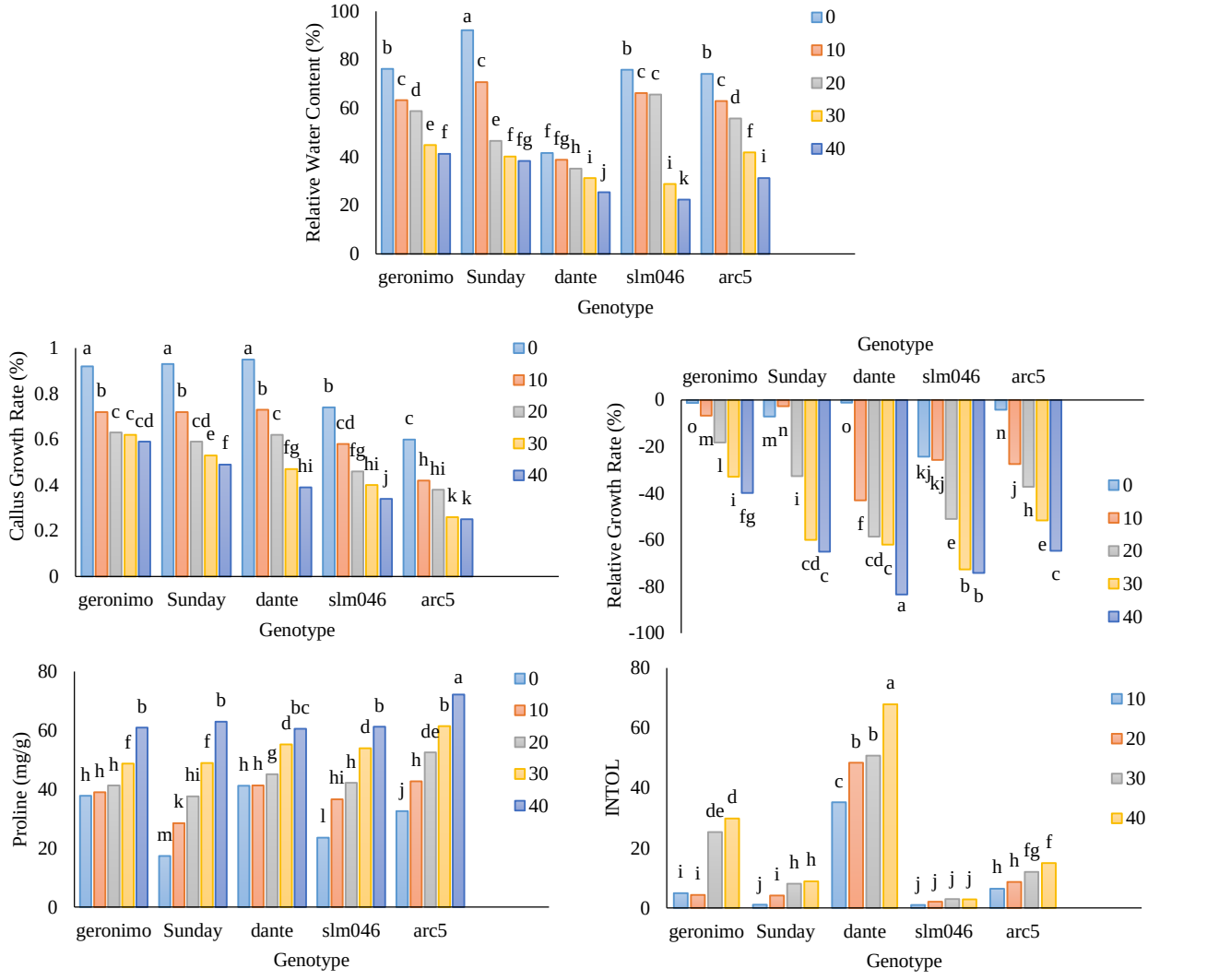

Figure 2. Effect of polyethylene glycol concentration (0 (Control), 10%, 20%, 30% and 40% on RWC, CGR, RGR, proline and INTOL of callus culture in five *b.napus* genotypes.

These findings underscore the adverse effects of escalating PEG concentration on various growth and physiological parameters of *B. napus* callus. PEG is commonly utilized as an osmotic agent in experiments to simulate water stress conditions. However, it's important to recognize that the impact of PEG-induced osmotic stress can vary depending on factors such as plant species, genotypes, growth conditions, and experimental setups. Researchers often conduct dose-response experiments to determine the optimal PEG concentration that induces the desired level of stress without causing excessive damage or cell death. The results indicate that heightened levels of osmotic stress, induced by increased PEG concentration, detrimentally impacted the callus's growth, water content, and stress tolerance (Fig. 1 and 2).

Erabii et al. (2006) noted a remarkable influence of genotype on RGR. The authors exposed calli obtained from two sugarcane cultivars (R570 and CP59-73) to various levels of osmotic stress and found significant differences among the cultivars. Interestingly, it was demonstrated that cultivars with high RGR under high osmotic pressure were slightly more drought-resistant than those with low RGR (Erabii *et al*., 2006). Similar results were reported in several other species such as *Triticum durum* (Bajji *et al*., 2000; Lutts *et al*., 2004), *Oryza sativa* (Lutts *et al*., 1996) *Tagetes minuta* (Mohamed *et al*., 2000) and Arabidopsis (Fletcher *et al*., 2022).

The organic compounds, known as osmolytes, contribute to maintaining cellular osmotic balance and shielding cellular structures and functions during challenging circumstances, like drought or high salinity, in plants. These compounds consist of proline, glycine betaine, sugars, and polyols. Different plant species or genotypes may have different preferences or capacities for synthesizing or accumulating specific osmolytes. The potential for osmolytes to function as useful storage compounds for reducing power and providing a source of carbon and/or nitrogen after stress relief is a fascinating idea. Osmolytes are known to accumulate against different types of stresses as part of the plant's adaptive response. These compounds may act as compatible solutes, helping to maintain cellular hydration and stabilize proteins and membranes (Suprasanna *et al*., 2016; Sharma *et al*., 2019; Choudhary *et al*., 2023). Many plant species have well-documented responses to stress, including cellular proline accumulation. Under various stress circumstances including drought and salt, plants may exhibit considerable amounts of proline accumulation, reaching up to 80% of the amino acid pool, compared to only around 5% under normal conditions. The increase in proline levels is primarily attributed to enhanced synthesis and reduced degradation. Proline actively serves as an osmoprotectant, aiding in the maintenance of cellular osmotic balance and stabilization of proteins and membranes in stressful situations. Its accumulation is considered a part of the plant's adaptive response to handle environmental challenges. In Arabidopsis, for instance, proline accounted for up to 20% of the free amino acid pool following sodium chloride (salt) stress. This accumulation is believed to be involved in osmotic tolerance and protect cellular structures from damage produced by high salt concentrations.

Plants under osmotic stress conditions demonstrate a range of roles for proline, including stabilizing proteins, membranes, and subcellular structures. This compound serves as a compatible solute, aiding in preserving cellular integrity and functioning during periods of stress. Proline also acts as an antioxidant, clearing out ROS and shielding cellular components from oxidative harm. Researchers have explored manipulating proline levels in plants as a means of improving stress tolerance. By overexpressing genes related to proline biosynthesis, they seek to increase the amount of proline available for stress-related functions. This method has shown encouraging outcomes in enhancing plant resilience to abiotic stresses like drought, salinity, and extreme temperatures (Kavi Kishor *et al*., 2005). Osmotic stress can cause an excess of ROS, which are extremely reactive molecules that can inflict oxidative damage upon cellular components. Proline functions as an effective antioxidant, directly scavenging ROS and preventing their harmful effects. By reducing ROS levels, proline helps maintain redox balance and safeguards plants from suffering oxidative stress-induced damage (Kaur and Asthir, 2015; Hayat *et al*., 2012; Liang *et al*., 2013; Kavi Kishor *et al*., 2022).

It has also been reported that proline and betaine function as scavengers for free radicals and/or protectors for enzymes, as well as serving as compatible solutes (Okuma *et al*., 2002; Hoque *et al*., 2008). The physiological response of higher plants to

water deficits and salinity stress, commonly known as proline accumulation, has been extensively studied and discussed in numerous reviews over the past two decades (Kavi Kishor *et al*., 2005; Kavi Kishor *et al*., 2022; Ghasempour *et al*., 2007).

The observation that proline content is higher under higher osmotic pressure (-1.69 MPa) and water stress conditions aligns with previous research (Fig. 3). Proline accumulation is a well-known response of plants to osmotic stress, including drought stress. Proline functions as an osmoprotectant or compatible solute in order to sustain cellular osmotic equilibrium and safeguard cellular structures and functions during times of strain. In times of water shortage, plants commonly enhance the synthesis and accumulation of proline as a means of alleviating the detrimental consequences of dehydration. By measuring proline content at the callus stage, researchers can potentially identify and select genotypes with higher drought tolerance. The higher proline content in the callus under water stress conditions suggests that these genotypes have a more effective response mechanism to cope with drought stress.

Notably, proline content alone may not be the sole determinant of drought tolerance. The characteristic of drought tolerance is a multifaceted trait that is influenced by a combination of genetic and physiological factors. Therefore, proline content at the callus stage can be used as an indicator but should be considered alongside other traits and physiological responses to accurately assess drought tolerance in genotypes. Further studies and validation experiments are necessary to confirm the relationship between proline content at the callus stage and drought tolerance in different plant species and genotypes. This information could potentially contribute to the development of more drought-tolerant crop varieties via marker-assisted breeding or genetic engineering approaches.

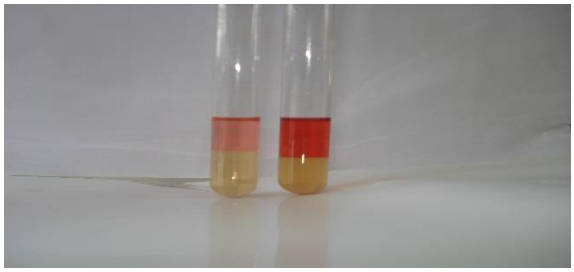

Figure 3. Proline contents in 0 (left) and 40% (Right) osmotic pressure in calli

According to these results, Geronimo was the most drought-resistant genotype compared to other cultivars. The identification of Geronimo as the most drought-resistant genotype suggests that it possesses genetic traits or mechanisms that enable it to better cope with drought stress compared to other cultivars. These traits could include enhanced water-use efficiency, improved stomatal regulation, deeper root systems, efficient osmotic adjustment, or a combination of various physiological and molecular adaptations. It's important to note that the evaluation of drought resistance is a complex process and involves assessing multiple traits and physiological responses. Proline content, as mentioned earlier, can serve as an indicator of drought tolerance, but it is only one aspect of the overall picture. Other factors, such as leaf wilting, biomass accumulation, relative water content, and photosynthetic efficiency, should also be considered to comprehensively evaluate drought resistance.

The ion and proline accumulation in sugarcane callus cultures were studied under drought stress followed by its subsequent relief (Errabii *et al*., 2006; Abdelsalam *et al*., 2021). The findings illustrated that the alterations prompted by the effects of dry spell strain can be reversed, at least on a cellular basis, in different varieties of sugarcane.

4. Conclusion

In conclusion, our study highlights significant differences among genotypes, drought levels, and their interaction, as indicated by the results of variance analysis. These findings underscore the substantial influence of genotypes and imposed drought levels on the studied traits, emphasizing the importance of genetic variability in plant responses to environmental stressors. Examining Relative Growth Rate (RGR), Relative Water Content (RWC), and Callus Growth Rate (CGR) across different genotypes and osmotic pressure levels revealed notable disparities, suggesting varying levels of stress tolerance and adaptability among genotypes. Particularly, the Geronimo genotype demonstrated superior growth performance, enhanced water retention capacity, and overall better adaptation to osmotic stress, especially at higher pressure levels. However, it's essential to consider the potential limitations of PEG-induced osmotic stress, as its effects can vary depending on various factors. Further research, including dose-response experiments, is

warranted to better understand the optimal stress conditions for studying plant responses and to explore potential genetic mechanisms underlying stress tolerance. Overall, these findings contribute valuable insights into the physiological responses of *B. napus* callus to environmental stress and pave the way for future studies aimed at enhancing stress tolerance in crop breeding programs.

Abbreviation

BAP: 6-Benzylaminopurine; CGR: Callus Growth Rate; 2, 4-D: 2, 4-dichlorophenoxyacetic acid; INTOL: Index Tolerance; MS: Murashige and Skoog medium; NAA: α-Naphthalene acetic-acid; PEG: Polyethylene glycol; RGR: Relative Growth Rate; RWC: Relative Water Content.

Conflict of interests

All authors declare no conflict of interest.

Ethics approval and consent to participate

No human or animals were used in the present Research.

Consent for publications

All authors read and approved the final manuscript For publication.

Availability of data and material

All the data are embedded in the manuscript.

Authors' contributions

All authors had an equal role in study design, work, Statistical analysis and manuscript writing.

Informed consent

The authors declare not to use any patients in this Research.

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