

Optimization of *In Vitro* PEG-induced Drought Stress and Evaluation of Morpho-physiological and Biochemical Response in *Coffea arabica* L.

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Abstract

Coffee (*Coffea arabica* L.) has low drought tolerance and is therefore vulnerable to prolonged water deficits under changing climatic conditions. This study aimed to optimize an *in vitro* drought stress protocol using PEG 6000 and evaluate the morpho-physiological and biochemical responses of coffee regenerants. Zygotic embryos were cultured on Murashige and Skoog (MS) medium, and after three months, regenerants were subcultured onto MS media supplemented with 0, 2, 4, and 6% (w/v) PEG 6000. The experiment was arranged in a Completely Randomized Design with three replications. Results indicated that increasing PEG concentrations impaired Arabica coffee regenerants, reducing growth, photosynthetic pigments, and water relations, while increasing membrane permeability, reflecting overall cellular stress. Stomatal density decreased, suggesting reduced gas exchange capacity, whereas leaf temperature increased, implying reduced transpirational cooling. Shoot and root growth were also reduced, although soluble sugar content increased, suggesting osmotic adjustment. Antioxidant activity and carotenoid content remained statistically unchanged. Overall, higher dosages of PEG 6000 effectively simulated drought stress *in vitro*, with 6% being the most effective. The optimized protocol provides a reliable and cost-effective tool for drought screening *in vitro* to enhance climate resilience in coffee production.

Keywords: Arabica coffee, climate resilience, embryo culture, PEG 6000, osmotic stress

Introduction

Coffee is one of the most valuable agricultural commodities and the second most widely traded product worldwide, after oil. Among the commercially cultivated species, Arabica (*Coffea arabica* L.) and Robusta (*Coffea canephora* Pierre ex A. Froehner) dominate global production, with Arabica being highly valued for its superior flavor and quality. In 2022, Arabica accounted for approximately 93.87 million bags of global production (International Coffee Organization [ICO], 2022). In the Philippines, Arabica contributed approximately 23% of the total coffee production in 2018, primarily grown in highland regions such as Davao del Sur, Sultan Kudarat, Bukidnon, and the Cordilleras (Philippine Statistics Authority [PSA], 2018).

Despite its economic importance, Philippine coffee production has been declining. Over the past decade, yields have declined by an average of 3.5% annually, while national consumption has increased steadily by 2.1% between 2018 and 2020 (Department of Agriculture [DA], 2021). Between 2015 and 2020, coffee supply declined sharply, with yields falling from 0.64 MT ha⁻¹ to 0.54 MT ha⁻¹. Globally, the International Coffee Organization (2022) reported a 4.2% reduction in production from 2019 to 2020, attributed to poor germination, limited availability of quality planting materials, pests and diseases, and climate change-induced stresses, particularly drought.

Drought is one of the most critical abiotic stresses affecting arabica coffee production, leading to reductions in vegetative growth, yield, and bean quality (Camargo, 2010; DaMatta & Ramalho, 2006). Studies have reported significant decreases in leaf size, stomatal conductance, and photosynthetic rate under drought conditions (Vu et al., 2018; Leon-Burgos et al., 2022). In the Philippines, arabica coffee farmers, such as those in Bansalan, Davao del Sur, have reported that water deficit negatively affected their plantations.

To address these challenges, *in vitro* tissue culture offers a promising method for understanding plant responses to water-deficit conditions and developing climate-resilient genotypes. Polyethylene glycol (PEG 6000), an osmotic agent, is widely used to simulate drought

stress *in vitro* by lowering water potential (Hajihashemi & Ehsanpour, 2014). Although PEG 6000 effectively simulates drought, optimal concentrations differ among species (Khayatnezhad et al., 2010; Vuksanović, 2019).

Despite the relevance of PEG-based stress assays, a standardized *in vitro* protocol for arabica coffee is currently lacking. To address this gap, the present study aimed to optimize *an in vitro* drought stress protocol using PEG 6000 and evaluate *Coffea arabica* L. morpho-physiological and biochemical responses. The findings provide a foundation for genotype selection, breeding programs, and drought-mitigation strategies. These contribute to the United Nations Sustainable Development Goals, particularly SDG 2 (Zero Hunger) and SDG 13 (Climate Action), by improving coffee productivity and climate resilience.

Materials and Methods

Time and place of the study

The experiment was conducted at the Plant Tissue Culture Laboratory of the Department of Horticulture, Faculty of Agriculture and Food Science, Visayas State University, Baybay City, Leyte, Philippines (10.7435° N, 124.7929° E). The study period covered November 2024 to March 2025.

In vitro plantlets and drought stress induction

Under *in vitro* conditions, arabica coffee (*Coffea arabica* L. var. Catimor) zygotic embryos, obtained from sterilized seeds collected from Sitio Balutakay, Bansalan, Davao del Sur, were initially cultured individually in Murashige and Skoog's (MS) basal medium supplemented with vitamins, Fe-EDTA, 30 g L⁻¹ sucrose, and 5 g L⁻¹ agar. Cultures were maintained at 25°C under cool-white fluorescent light (2,500 lux) with an 8-h photoperiod. After four weeks, germinated embryos were subcultured

onto fresh MS medium containing 2.0 mg L⁻¹ BAP for two consecutive passages, at four-week intervals, to establish plantlets. Polyethylene glycol (PEG 6000; Sigma-Aldrich, USA) was prepared by dissolving it in a small volume of warm distilled water to ensure complete dissolution, sterilized, and then incorporated into the experimental medium at the respective concentration. Three-month-old regenerants of similar size and extent of shoot and root proliferation were selected and transferred to PEG-supplemented MS media without plant growth regulators. The selected regenerants were then subjected to four levels of PEG 6000 treatments, namely MS alone (control), MS + 2% PEG 6000, MS + 4% PEG 6000, and MS + 6% PEG 6000. The experiment was laid out in a Completely Randomized Design with three replications and 15 samples per replicate.

Data gathered

Data collection was conducted in two phases for morphological characteristics: before drought (BD) and after drought stress induction (AD). For stomatal and physiological parameters, an initial general baseline was obtained before the drought. Since these measurements required the samples to be removed from culture bottles, this approach minimized the handling of regenerants and reduced contamination risks in the *in vitro* system. These baseline values served as reference points but were not included in the statistical analysis. In contrast, biochemical parameters and photosynthetic pigment contents were measured only after the induction of drought stress. The following data were gathered:

1. Morpho-anatomical characteristics

Shoot and root lengths were measured with a tape measure. Foliage senescence was scored using the rating scale of Maliro et al. (2008). For stomatal traits, the method of Matias et al. (2024) was adapted with some modifications; samples from the third fully expanded leaf from the top were collected to analyze stomatal characteristics. Transparent nail polish was applied to create impressions of the leaves. These impressions

were magnified at 40x using a light microscope. A calibrated micrometer slide was used to determine the field of view (FOV) area, and a cell counter was employed to count the stomata in each microscopic image. Sample images were taken, and the ImageJ software was used to measure the stomatal aperture (μm), length (μm), and width (μm).

2. Physiological characteristics

Leaf gas exchange parameters, including transpiration rate (E , $\text{mmol m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$), and leaf temperature ($^{\circ}\text{C}$), were measured using a portable photosynthesis system (CI-340 Handheld Photosynthesis System; CID Bio-Science, Camas, WA, USA). Relative water content (RWC) was determined following Yamasaki and Dillenburg (1999). Relative water content (RWC) was determined following Yamasaki and Dillenburg (1999) by recording fresh weight (FW), turgid weight (TW), and dry weight (DW) of the leaves. Membrane permeability was evaluated through electrolyte leakage using the Blum and Ebercon (1981) method with slight modifications. Six uniform leaf segments were incubated in 10 mL deionized water for 12 hours, autoclaved, and the electrical conductivity 1 and 2 was measured using a Biobase Multiparameter Meter (Model PH-900/P).

3. Photosynthetic pigments

Chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids (mg/g) were determined following the method of Hiscox and Israelstam (1979) with minor modifications. Leaves were collected, cut into thin strips, and incubated in a water bath containing 80% ethanol at 70°C until the tissues were bleached. The absorbance of the solution was measured using a spectrophotometer at 645 and 663 nm for chlorophylls and at 470 nm for carotenoids. Pigment concentrations were calculated using the equations of Arnon (1949) for chlorophylls and the formula of Lichtenthaler (1987) for carotenoids.

4. Biochemical properties

Total soluble sugars (mg/g) were extracted from fresh leaf tissues using 80% ethanol and quantified with the Anthrone reagent method, following the protocol of Yemm and Willis (1954) with minor modifications. The absorbance was measured at 620 nm using a spectrophotometer. Antioxidant activity, expressed as free radical scavenging activity (% FRSA), was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay following the procedure of Salas et al. (2015). The absorbance of the resulting solutions was measured at 517 nm with a UV–Vis spectrophotometer, using ethanol as the reference.

Statistical Analysis

Data were recorded, consolidated, tabulated, and statistically analyzed through analysis of variance (ANOVA) in a Completely Randomized Design (CRD). The Least Significant Difference (LSD) test was used to determine significant differences among treatment means. Statistical analysis was conducted using the computer software Statistical Tool for Agricultural Research (STAR) version 2.0.1, developed by the International Rice Research Institute (IRRI).

Results and Discussion

Morphological responses of arabica coffee under PEG 6000 simulated drought stress

Table 1 presents the impact of simulated drought stress on PEG 6000 on the root and shoot growth, and foliage senescence of *Coffea arabica* L. regenerants under *in vitro* conditions.

Table 1. Morphological characteristics of *Coffea arabica* L. regenerants under *in vitro* drought stress.

Treatments	Shoot Length (Mm)		Root Length (Mm)		Foliage Senescence
	BD	AD	BD	AD	
MS Alone	22.13	23.53 ^a	27.47	31.07 ^a	1.00 ^c
MS + 2% PEG 6000	23.93	24.57 ^a	25.27	27.60 ^{ab}	2.13 ^b
MS + 4% PEG 6000	23.27	21.87 ^a	21.60	22.27 ^b	2.53 ^{ab}
MS + 6% PEG 6000	18.27	14.73 ^b	20.73	21.33 ^b	2.93 ^a
P-value	0.0531	0.0046	0.2114	0.0478	0.0001
c.v. (%)	10.67	11.51	16.76	15.27	11.39

Means with the same letter in a column are not significantly different (Fisher's LSD, p < 0.05); [BD] before drought; [AD] after drought

After 4 weeks, shoot length was markedly reduced at higher PEG concentrations, with the 6% treatment showing the shortest shoots (14.73 mm). In contrast, growth was maintained in the MS Alone, 2% and 4% PEG treatments (23.53, 24.57, and 21.87 mm, respectively). Root elongation exhibited a similar trend, with 6% PEG regenerants showing the most significant reduction (21.33 mm) compared to the MS Alone (31.07 mm). Reduced growth under PEG stress is attributed to osmotic inhibition of cell elongation, decreased turgor pressure, and limited water uptake in meristematic zones (Farooq et al., 2012; Priyanka et al., 2011).

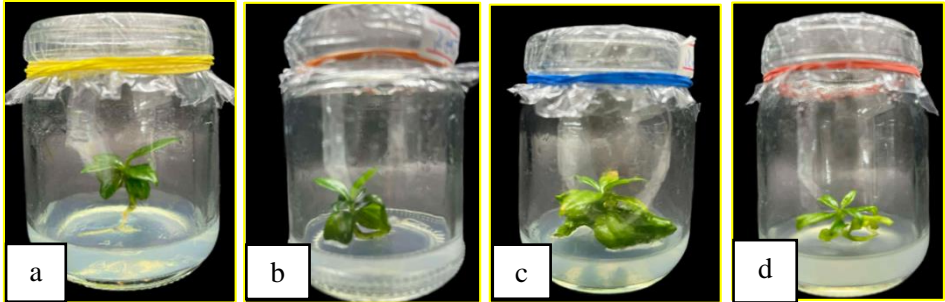


Figure 1. Appearance of *Coffea arabica* L. regenerants after 1 month of incubation in PEG-6000-added medium: (a) MS Alone; (b) 2%; (c) 4%; and (d) 6%.

Foliage senescence increased with PEG concentration, reflecting drought-induced oxidative stress. MS Alone regenerants remained fully green (score = 1.00), whereas 6% PEG caused moderate necrosis and yellowing of upper leaves (score = 2.93). Drought-induced senescence is characterized by the accumulation of reactive oxygen species (ROS), chlorophyll degradation, and accelerated leaf aging, resulting from decreased photosynthesis and oxidative damage (Anjum et al., 2011). As shown in the photosynthetic pigment parameters, chlorophyll declined.

Overall, shoot and root growth reductions, as well as increased foliage senescence, were most pronounced at 6% PEG, indicating the critical threshold for drought stress *in vitro*. These results align with previous studies in Arabica coffee (Dadi, 2017) and other crops, where higher PEG concentrations consistently inhibited growth and induced stress symptoms (Akbarpour et al., 2016; Hajihashemi & Ehsanpour, 2014).

Stomatal characteristics of arabica coffee under PEG 6000 simulated drought stress

Table 2 presents the stomatal responses of *Coffea arabica* L. regenerants under PEG 6000-induced drought stress over a 4-week period. Before drought imposition, stomatal density on the abaxial surface averaged 121 mm², with mean stomatal length, width, and aperture recorded at 28 μm, 39 μm, and 10 μm, respectively.

Table 2. Stomatal characteristics of *Coffea arabica* L. regenerants under *in vitro* drought stress.

Treatments	Stomatal Characteristics			
	Density (Mm ²)	Width (μm)	Length (μm)	Aperture (μm)
Initial (Baseline)*	121.00	28.00	39.00	10.00
MS Alone	112.64 ^a	37.67	36.67	7.33
MS + 2% PEG 6000	118.25 ^a	36.00	36.67	7.67
MS + 4% PEG 6000	72.05 ^b	37.00	36.33	6.00
MS + 6% PEG 6000	57.50 ^b	35.67	39.00	6.33
P- value	0.0001	0.8043	0.2825	0.2099
cv (%)	10.07	7.57	4.66	14.63

Means with the same letter in a column are not significantly different (Fisher's LSD, $p < 0.05$); *Not included in the ANOVA; baseline values before treatment

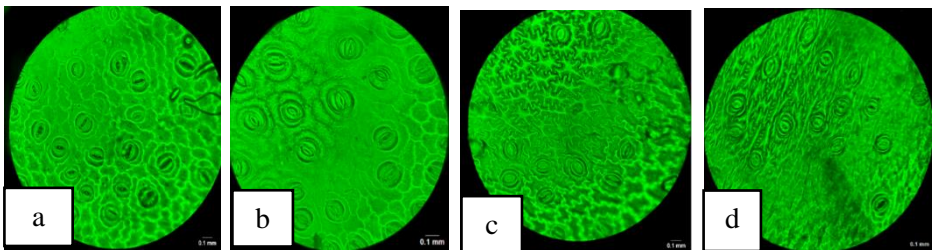


Figure 2. Abaxial leaf stomata of *Coffea arabica* L. regenerants after 1 month of incubation in PEG-6000-added medium: (a) MS Alone; (b) 2%; (c) 4%; and (d) 6%.

PEG-induced drought stress influenced stomatal traits in *Coffea arabica* L. regenerants. After stress, stomatal density remained high in the control and 2% PEG treatments (118.25 and 112.64 mm²) but decreased significantly at 4% and 6% PEG (72.05 and 57.50 mm²), whereas stomatal dimensions (length, width, and aperture) were unaffected ($p > 0.05$). This indicates that *Coffea arabica* L. primarily regulates stomatal number, rather than size, as a protective mechanism to reduce transpirational water loss while maintaining stomatal functionality for rapid recovery.

Similar patterns have been observed in other species. In *Prunus spp.*, osmotic stress reduced growth and altered anatomical features, with tolerant species maintaining tissue integrity (Rajabpoor et al., 2014). In *Phoenix dactylifera*, PEG stress promoted cuticle thickening, wax deposition, and plastid density, facilitating tighter stomatal control (Din et al., 2020). In *Coffea arabica* L., stomatal density varies with environmental conditions, while size remains stable, demonstrating the plasticity of stomatal traits (Taye & Burkhardt, 2011). As such, these findings, along with the present study, suggest that density modulation is a key adaptive strategy under drought, allowing plants to conserve water without compromising stomatal function.

Physiological characteristics of arabica coffee under PEG 6000 simulated drought stress

To assess the physiological response of regenerants to *in vitro*-induced drought stress, various parameters such as transpiration rate (E , mmol m⁻² s⁻¹), stomatal conductance (g_s , mol m⁻² s⁻¹), leaf temperature (LT, °C), relative water content (RWC, %), and membrane permeability (MP, %) were measured after a 4-week drought period (Table 3).

Table 3. Physiological response of *Coffea arabica* L. regenerants under *in vitro* drought stress.

Treatments	Physiological Characteristics				
	E (mmol m ⁻² s ⁻¹)	g _s (mol m ⁻² s ⁻¹)	LT (°C)	RWC (%)	MP (%)
Initial (Baseline)*	5.07	0.91	29.63	47.17	40.00
MS Alone	5.26 ^a	0.99 ^a	26.78 ^b	41.24 ^a	44.85 ^c
MS + 2% PEG 6000	4.52 ^b	0.72 ^b	27.09 ^b	27.75 ^b	53.83 ^{bc}
MS + 4% PEG 6000	4.42 ^b	0.59 ^c	28.49 ^b	26.26 ^b	75.13 ^{ab}
MS + 6% PEG 6000	3.92 ^b	0.50 ^c	30.87 ^a	22.32 ^b	79.47 ^a
P- value	0.0103	0.0000	0.0083	0.0137	0.0261
cv (%)	7.66	7.13	4.01	18.60	19.73

Means with the same letter in a column are not significantly different (Fisher's LSD, $p < 0.05$); *Not included in the ANOVA, baseline values prior to treatment.

Before PEG treatment, *Coffea arabica* L. regenerants showed initial values of 5.07 mmol m⁻² s⁻¹ (transpiration rate, E), 0.91 mmol m⁻² s⁻¹ (stomatal conductance, g_s), 29.63°C (leaf temperature, LT), 47.17% relative water content (RWC), and 40% membrane permeability (MP). After 4 weeks of PEG exposure, significant physiological changes were observed. Transpiration rate and stomatal conductance declined progressively with increasing PEG concentration, while leaf temperature increased, with 6% PEG reaching 30.87°C. These changes reflect stomatal closure and reduced gas exchange, a classical drought avoidance response (Ping et al., 2015; Santos et al., 2018).

RWC decreased significantly under higher PEG levels, confirming cellular dehydration and turgor loss. Maintaining RWC is critical for drought adaptation, as in pepper and wheat studies, where higher RWC supported photosynthesis and chlorophyll retention under stress (Penella et al., 2014; Arjenaki et al., 2012; Pei et al., 2010).

Membrane permeability increased with PEG concentration, reaching 79.47% in 6% PEG, indicating enhanced cellular leakage and oxidative damage. This aligns with reports in tomato and wheat, where PEG-induced osmotic stress caused lipid peroxidation and elevated hydrogen peroxide and malondialdehyde levels (George et al., 2015; Kocheva et al., 2009).

Overall, the combined changes in transpiration, stomatal conductance, RWC, leaf temperature, and membrane integrity demonstrate that PEG 6000 effectively simulated drought stress *in vitro*, with higher concentrations (4–6%) inducing the most pronounced physiological responses.

Photosynthetic pigments of *arabica* coffee under PEG 6000 simulated drought stress

To assess the photosynthetic pigments of *Coffea arabica* regenerants under *in vitro* induced drought stress, photosynthetic pigments were quantified after a 4-week PEG exposure (Figure 3). Photosynthetic pigments serve as sensitive indicators, and their degradation under stress is associated with impaired pigment biosynthesis or enhanced oxidative damage.

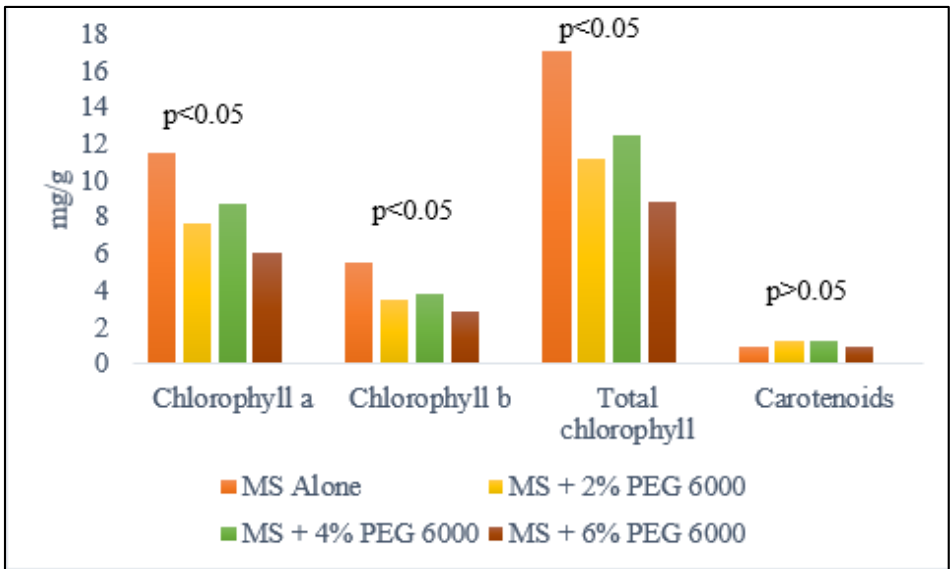


Figure 3. Photosynthetic pigments of *Coffea arabica* L. regenerants after 1 month of incubation in PEG-6000-added medium.

As observed, PEG 6000-treated regenerants exhibited lower photosynthetic pigments. The MS + 2, 4, and 6% PEG 6000 had 7.70, 8.72, and 6.03 mg/g chlorophyll *a*. On the other hand, such treatments had 3.49, 3.80, and 2.82 mg/g chlorophyll *b*, respectively. For total chlorophyll, the MS + 2, 4, and 6% PEG 6000 had 11.19, 12.53, and 8.85 mg/g. These findings suggest that severe drought stress impairs chlorophyll accumulation, possibly due to chloroplast structural damage, pigment photooxidation, or inhibited biosynthetic pathways. Meanwhile, carotenoid levels did not differ significantly between treatments, indicating that while chlorophyll pigments declined, carotenoids, which are often involved in ROS scavenging and photoprotection, were maintained.

These results are consistent with previous studies. PEG-induced drought reduced chlorophyll in *Agave americana* (Enrique et al., 2023) and *Passiflora edulis* (Qi et al., 2023), accompanied by increased oxidative stress markers and altered antioxidant activity. Similarly, drought-sensitive wheat and soybean genotypes showed significant chlorophyll declines under PEG stress, whereas tolerant genotypes maintained higher pigment levels (Peršić et al., 2022; Basal et al., 2020). The progressive chlorophyll declines in Arabica coffee regenerants under increasing PEG concentrations imply impaired pigment biosynthesis and compromised photosynthetic function.

Biochemical properties of arabica coffee under PEG 6000 simulated drought stress

Biochemical properties in plants are reliable indicators of the degree of drought stress and the plant's corresponding coping mechanisms. Figure 4 presents the antioxidant activity (% FRSA) and total soluble sugars (mg/g) of *Coffea arabica* L. regenerants subjected to PEG-induced drought stress under *in vitro* conditions.

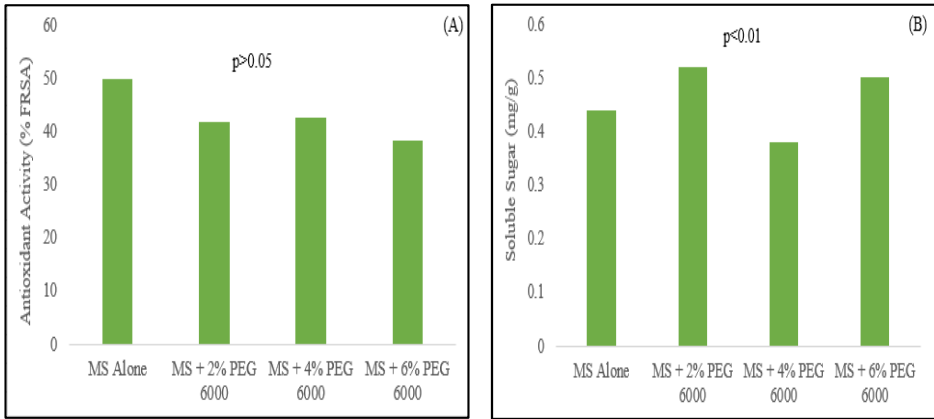


Figure 4. Biochemical properties of *Coffea arabica* L. regenerants after 1 month of incubation in PEG-6000-added medium: (a) Antioxidant activity and (b) Soluble sugar.

Antioxidant activity (%), measured by free radical scavenging activity (FRSA), showed no statistically significant differences across treatments. The lack of statistical significance suggests that the antioxidant defense response was not strongly triggered under the simulated drought levels. The result may reflect the limited capacity of Arabica coffee tissues to generate or activate non-enzymatic antioxidant systems in response to osmotic stress. Interestingly, the results may also indicate that while non-enzymatic antioxidant systems remained unresponsive, enzymatic antioxidant pathways such as superoxide dismutase (SOD), catalase (CAT), and peroxidases (e.g., APX, POD) might have been activated without being quantified in this study. For example, in Tartary buckwheat seedlings, enzymatic antioxidant activities increased significantly under PEG treatments (Hossain et al., 2024). Therefore, the unchanged FRSA in this study could mask an underlying enzymatic response not captured by the non-enzymatic antioxidant assay.

In contrast, soluble sugar content varied significantly among treatments. The highest accumulation was recorded at 2% PEG 6000 (0.52 mg/g), followed closely by 6% PEG (0.50 mg/g). These findings indicate that osmotic stress induced by PEG promoted sugar accumulation, which

may represent an early response mechanism to maintain turgor and protect cellular structures. Soluble sugars play a crucial role in osmotic adjustment, serving as compatible solutes that stabilize proteins and membranes and mitigate oxidative stress (Hennion et al., 2019). PEG-induced drought stress likely increased soluble sugar content in *Coffea arabica* L. regenerants, indicating activation of osmotic adjustment mechanisms. Similar responses have been reported in drought-tolerant genotypes of *Vigna aconitifolia* (Priyanka et al., 2011), sorghum (Zhang et al., 2023), and rice (Dien et al., 2019), where sugar accumulation contributed to osmotic balance, antioxidant protection, and reduced membrane injury.

However, despite this biochemical response, elevated sugar levels in coffee regenerants were insufficient to counteract PEG-induced stress fully. This was evidenced by increased membrane permeability, decreased relative water content, reductions in photosynthetic pigments, and the impact on its morphological characteristics. Severe water deficit likely induced reactive oxygen species (ROS), disrupting membrane integrity and cellular compartmentalization (Blokhina et al., 2003). Thus, the morphological, physiological, and biochemical responses collectively demonstrate the progressive impact of PEG-induced drought on arabica coffee *in vitro*.

Conclusion and Recommendations

Coffea arabica var. Catimor regenerants exhibit variable morpho-physiological and biochemical responses to *in vitro* drought stress induced by PEG 6000. Higher dosages of PEG effectively simulated *in vitro* drought conditions, with 6% producing the most pronounced effects on growth, water status, and physiological and biochemical parameters. This optimized protocol provides a reliable and cost-effective tool for drought screening *in vitro*. It can serve as a foundation for breeding programs and extension initiatives to develop climate-resilient coffee varieties. Further studies are recommended to assess its applicability across diverse arabica coffee genotypes, refine the protocol for large-scale

screening, and explore potential interactions with other stress-alleviating treatments.

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