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Growth Dilatory Effects of PEG and Sucrose on *Geranium wallichianum*: An *In Vitro* Approach for Conservation

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Received: 16 December 2024; Accepted: 12 February 2025; Published: 31 March 2025

ABSTRACT: The medicinal herb *Geranium wallichianum* belongs to the family Geraniaceae. The East Asian Himalayas are its primary habitat. Overexploitation and overharvesting pose a threat to this plant, given its extensive ethnomedical utilization in the community. In Pakistan, its population has already declined by over 75%. Given its critical medicinal importance, urgent conservation efforts are needed to prevent extinction. The aim of the current research was to determine the effectiveness of sucrose and polyethylene glycol (PEG) in decelerating the growth of this medicinally important species. Nodal segments were utilized as explant with varying levels of polyethylene glycol (PEG) (0.5% and 1.5%) and sucrose (4% and 8%) as treatments on different growth attributes. Over a 45-day period, PEG treatments (0.5% and 1.5%) proved more efficient compared to sucrose in suppressing *in vitro* growth, with reduced shoot lengths (0.92 and 0.57 cm), dry weight (0.04 and 0.02 g), and fresh weight (0.06 and 0.04 g). PEG also significantly enhanced phenolic levels (0.96 and 0.19 mg/g) and soluble sugars (4.07 and 4.12 mg/g) while reducing total protein level (2.38 and 2.32 mg/g) and total chlorophyll content (0.17 and 0.14 mg/g). The Pearson correlation analysis showed a negative association between phenolic levels and total chlorophyll content, chlorophyll a, and chlorophyll b. The length of the shoot had a negative relationship with phenolic level but a positive relationship with both fresh and dry weight. The principal component analysis revealed that total sugars and phenolics that were grouped separately from other variables led to stronger responses to osmotic stress. These outcomes suggested that higher PEG was successful in reducing *in vitro* growth throughout short-term preservation in comparison to sucrose. The outcomes of this research could be applied for the *in vitro* conservation of medical plants in the future.

KEYWORDS: Indigenously threatened plant; *G. wallichianum*; osmotic stress inducer; *in vitro* conservation

1 Introduction

In developing nations like Pakistan, over 80% of population depends upon herbal remedies for primary healthcare, with nearly 25% of recommended pharmaceuticals originating from wild plants [1]. Worldwide, the demand of medicinal plants continues to grow due to the rising interest in secondary metabolites and natural health products obtained from these plants [2,3]. Pakistan's diverse climate, geography, and



phytogeographical zones support around 6000 species and 1572 genera of wild plants, predominantly found in the Karakoram, Indus, and Himalayan provinces, including 428 species that are endemic [4]. The northern areas of Pakistan are abundant in medicinal plants, which are deeply rooted in traditional use by local populations. For many residents, these plants serve as their sole primary income source. However, Pakistan's plant biodiversity faces numerous threats, including habitat destruction, overgrazing, desertification, unauthorized trade, industrial contamination, excessive need for natural resources, and a lack of essential awareness [5].

Geranium wallichianum, a plant of the family Geraniaceae, is widely named “Ratanjot” in Pakistan and normally known as “Shepherd's needles”. Other names for this plant include “Wallach cranesbill”, “Sra zeal” [6], “ratinloog”, and “rattenjot” [7]. *G. wallichianum* is a tall, upright plant featuring multiple branches, that predominantly occur in the Himalayan region at elevations of 7000–10,000 feet, extending from Nepal to Kashmir. In Pakistan, it grows in areas such as Hazara, Swat, Murree Hills, Lowari Top, Gilgit, Chitral, Dir, Shogran Valley, and Baluchistan. Globally, its range spans the East Asia-Himalayan region, encompassing Afghanistan, Bangladesh, Nepal, India, and Bhutan [8]. *G. wallichianum* holds significant ethno botanical importance. Its floral extract is utilized to treat vision impairments and purify the blood, while the leaves are beneficial for liver ailments. The plant rhizomes are used to address various health concerns, including ear infections, toothache, constricting, and eye problems [9]. The whole plant (*G. wallichianum*) is used to treat stomach acidity, stomach pain, backache, stiffness, and gastric complications [10]. *G. wallichianum* is seen as facing an indigenous extinction in Pakistan [5]. This species is endemic to Pakistan, and its population has declined by more than 75% (91). The main risks to this plant stem from over-harvesting and over-exploitation, due to its considerable ethnomedicinal significance [11].

Various conservation initiatives have been implemented to protect vital medicinal plants. However, to effectively conserve threatened and endangered species, it is crucial to adopt long-term and diverse strategies. Both traditional methods, such as *in situ* and *ex-situ* conservation, and recent biotechnological techniques, including cryopreservation, *in vitro* conservation, and micropropagation, are valuable tools for preserving plant biodiversity [12]. *Ex-situ* conservation of plants relies on factors like species, techniques employed, and the intended duration of storage, each involving various costs, challenges, and scientific limitations. Conversely, *in vitro* conservation facilitates the concurrent safeguarding of plant genetic diversity, ensures year-round access to healthy planting material for farmers, and enables the monitoring of gene integrity through molecular analysis [13]. Among *in vitro* conservation strategies, slow growth approaches, especially those involving fewer subcultures, minimize the rate of germplasm loss due to handling mistakes like contamination. These strategies also mitigate the risks associated with genetic instability and reduce the demand for labor and storage space in the preservation of germplasm.

This method is often referred to as “slow growth storage” owing to the utilization of specific chemical, nutritional, or physical conditions that restrict the growth of plantlets [13]. In micropropagation facilities, the slow growth technique serves as a successful method for storing plant materials in a small space for short to medium terms. This technique enables the affordable production of premium cultivars at a reduced cost, and meets the market demand [14]. Microplants are cultured onto Murashige and Skoog [15] (MS) medium enriched with growth inhibitors or osmotic stress agents, then maintained within controlled conditions of reduced light intensity, low temperature, and adjusted photoperiods to minimize sub-culturing frequency [16]. Slow-growth methods have been applied to preserve plants like *Glycyrrhiza glabra* [17], *Coleus forskohlii* [18], *Ullucus tuberosus* [19], *Rhazya stricta* and *Lavandula dentata* [20].

The slow growth storage approach for *in vitro* preservation can be implemented through various methods, such as applying growth inhibitors and osmotic agents [21], dark storage with controlled light spectra [22], encapsulation [23], adjusting growth media via lowering sucrose and media concentrations [24],

combining diluted media with growth inhibitory agents [25,26] and utilizing specific light spectra [22]. Altering the media through lowering sugar, minerals, growth regulators, or osmotic agent levels (e.g., mannitol and sorbitol) can suppress cell division, considerably hindering callus development and shoot growth. Diluting MS medium to reduce macronutrients is one method to slow *in vitro* culture growth [25]. To prolong the interval between subcultures, methods for *in vitro* plant storage are utilized [27]. While studies on *in vitro* micropropagation to boost explant development and the production of seedlings are ongoing, advancing *in vitro* storage is equally important. Therefore, research into the *in vitro* preservation of ornamental plants is crucial for preserving germplasm, maintaining sterile cultures, and conserving rare and exotic species.

This study employed osmotic regulators to deaccelerate growth and prolong subculturing intervals. Osmotic stress agents in culture media primarily function by reducing photosynthesis via the closure of stomata, lowering water potential, and decreasing turgor pressure when applied at optimal concentrations. Effective osmotic regulators include mannitol, sorbitol, sucrose, ribose, 2,2-dimethyl hydrazide, and polyethylene glycol (PEG) [28]. Sucrose serves as the ideal energy supply for *in vitro* culture [29] and is also employed as an osmotic regulator to slow the mature explants' development. Elevated sucrose content acts to decelerate the development of various plant species cultured *in vitro* [30]. Furthermore, PEG is widely used in *in vitro* and *in vivo* osmotic stress research as an optimal, inert, and non-ionic substance. It lowers tissue's water potential by binding the water molecules and remains localized due to its inability to penetrate cell walls [31].

This research focused on studying the capability of PEG and sucrose in slowing down *G. wallichianum* growth, thereby developing an *in vitro* conservation system to facilitate reduced growth. The study's objectives were to investigate the effectuation of PEG and sucrose on the growth factors of *G. wallichianum in vitro* and to analyze the influence of these osmotic agents on crucial biochemical parameters of *G. wallichianum*. This is essential since the rising requirement for *G. wallichianum's* therapeutic value has significantly reduced its population, endangering its survival. This conservation system will help to preserve viability and genetic stability by extending the period between *in vitro* subcultures and lowering the need for space, plant growth regulators, and other growth retardants.

2 Materials and Methods

2.1 Plant Material

The *G. wallichianum* nodal segments (Fig. 1) served as explants for this study, which was conducted at the Department of Biotechnology, COMSATS University Islamabad, Abbottabad Campus, Pakistan. The plant was gathered through its flowering phase from Galyat, Pakistan, verified by a taxonomist' plant, and accustomed in the department's nursery.

2.2 Surface Sterilization of Explants

The protocol of Munir et al. [32] was followed to surface sterilize the nodal explants, with few adjustments, as indicated in Table 1. The explants were divided into minute slices, subjected to a 1-minute treatment with 70% ethanol (Sigma-Aldrich, Saint Louis, MO, USA) and a 5-min treatment with 2% sodium hypochlorite (Sigma-Aldrich, Saint Louis, MO, USA), and washed thrice with sterile distilled water. The explants were subsequently positioned onto autoclaved filter paper in a Petri plate for 1 min to allow them to air dry prior to being fully inoculated into the culture medium. Within aseptic conditions, the explants were placed on a basic MS medium [15] following surface sterilization. In order to establish the culture, the culture medium was kept at 25°C ± 2°C and a photoperiod of 16 h in a controlled environment chamber. The light intensity was

3000 lx. After one week of culture, various parameters, including contamination, explant mortality (blackening or browning), and survival rate, were documented (Table 1).



Figure 1: *Geranium wallichianum* at the nursery of the Department of Biotechnology, CUI Abbottabad Campus

Table 1: Impact of sterilizing agents on nodal explants of *Geranium wallichianum*

Sterilizing agent		Nodal segment		
Ethanol 70%	Sodium Hypochlorite 2%	Survival %	Mortality %	Contamination %
1 min	4 min	54.16	12.5	33.33
1 min	5 min	83.33	8.33	8.33
1 min	6 min	58.33	12.5	29.16
1 min	7 min	33.33	37.5	29.16

Note: The percentage mean of three replicates represents the values.

2.3 PEG and Sucrose Treatments

The PEG and sucrose are osmotic stress inducers and are applied to limit the *in vitro* growth of *G. wallichianum*. Two weeks after explants growth on MS [15] media, a well-established culture was subcultured on MS media enriched with sucrose and PEG (CAS 25322-68-3) at variable concentrations. Sucrose accounted for the sole carbon source that was introduced into the medium. For the preparation of simple MS culture media, 3% sucrose (control) and 10 mg/mL agar (Sigma-Aldrich, Saint Louis, MO, USA, bacterial grade) were used for solidification. The solution pH was fixed at 5.6, and the mixture was kept for 20 min in an autoclave (121°C) at 15-psi pressure. In order to examine the impact of high sucrose concentrations and polyethylene glycol on the *in vitro* reduced growth of *G. wallichianum*, the MS culture media was enriched with varying concentrations of sucrose (4% and 8%) and PEG (0.5% and 1.5%). The sterile culture medium, treated with PEG and sucrose, was where the explants were moved. They were subsequently placed in a growth chamber with controlled conditions, subjected to a 16-hour light/dark cycle, and maintained at 23°C $\pm$ 2°C. The culture was morphologically and biochemically evaluated after 15, 30, and 45 days following PEG and sucrose treatments.

2.4 Morphological Analysis

The length of the shoot (cm), survival rate (%), and fresh and dried weight (g) of nodal explants were documented at specific durations of 15, 30, and 45 days of culturing. The nodal explants' heights were assessed visually using a ruler. The explants were gently extracted from the culture medium, dried with tissue paper, and weighed to determine both their fresh and dried weight. The electronic balance was employed to record the explant fresh weight. After weighing, the explants were set onto fresh aluminium foil and oven-dried at 100°C for 24 h. The dried explants were measured for dry weight with the same electronic balance. Explant cultures showing signs of death or brownish nodal explants were classified as non-surviving, while those exhibiting healthy growth were considered to be surviving. The explant survival rate was computed by dividing the surviving explants' number by the counts of explants initially cultured, then multiplying by 100.

2.5 Biochemical Analysis

The analysis of soluble sugars, total phenolics, protein content, and chlorophyll was conducted 15, 30, and 45 days post-cultivation using the experimental procedures outlined below.

2.5.1 Estimation of Total Soluble Sugars

The nodal explant extracts were prepared utilizing ethanol (80%). These extracts were then mixed with 25 mL of anthrone reagent, which was a solution of 0.15 g anthrone (Merck, Darmstadt, Germany) in 100 mL of 72% H₂SO₄ (Sigma-Aldrich, Saint Louis, MO, USA). The absorbance was quantified spectrophotometrically at 625 nm utilizing the IRMECO Model U2020, after the mixture was heated for 15 min at 97°C. Using a glucose standard, the total soluble sugars was assessed [33].

2.5.2 Determination of Total Phenolics

The technique reported by Ainsworth et al. [34] was utilized to quantify the total phenolic content. The 3-minute centrifugation at 10,000 rpm was undergone by the plant extracts, which were formulated with 80% methanol (Sigma-Aldrich, Saint Louis, MO, USA). Subsequently, 300 µL of extract was combined with 700 µL of 10% Folin and Ciocalteu reagent (Merck, Darmstadt, Germany). The ultimate mixture was completed by incorporating 700 mM sodium carbonate (Na₂CO₃) and incubating at ambient temperature (25°C) for an hour. The phenolic content was determined by measuring its absorbance at 765 nm spectrophotometrically (IRMECO Model U2020) and employing the standard gallic acid (Sigma-Aldrich, Saint Louis, MO, USA) [35,36].

2.5.3 Determination of Chlorophyll Content

To determine the chlorophyll content, the extract was obtained through pulverizing fresh explants within 10 mL acetone (80%, Sigma-Aldrich, Saint Louis, MO, USA) for 12 h. Chlorophyll a and b levels were measured spectrophotometrically (IRMECO Model U2020) at 645 and 663 nm, respectively, after centrifuging the mixture at 10,000 rpm for 3–5 min to acquire the supernatant [37]. The total chlorophyll, along with chlorophyll a and b, was obtained by averaging the readings from three replicates using these equations [38]:

$$\text{Chlorophyll a content (mg g}^{-1}\text{)} = 12.7 (A_{663}) - 2.69 (A_{645}) \times W/1000 \times V$$

$$\text{Chlorophyll b content (mg g}^{-1}\text{)} = 22.9 (A_{645}) - 4.68 (A_{663}) \times W/1000 \times V$$

$$\text{Total chlorophyll content (mg g}^{-1}\text{)} = \text{Chlorophyll (a + b)}$$

In these equations, V represents the whole extract volume (mL), W denotes the sample fresh weight (g), and A denotes the absorbance at the specified wavelengths.

2.5.4 Determination of Total Proteins

The Bradford [39] technique was employed for total protein content estimation. Extracts (0.5 g) underwent homogenization within phosphate buffer (pH 7.4), then underwent 20 min of centrifugation at 8000 rpm. Afterward, the supernatant was mixed with 2.9 mL of Coomassie blue dye (Sigma-Aldrich, Saint Louis, MO, USA) and left to react for an hour at ambient temperature. The standard curve was created using bovine serum albumin (BSA; Sigma-Aldrich, Saint Louis, MO, USA), and absorbance was recorded spectrophotometrically at 600 nm (IRMECO Model U2020).

2.6 Experimental Design and Statistical Analysis

The experiment was conducted using a fully randomized design, incorporating three biological replicates, every consisting of eight explants ($n = 8$). The resulting data were analyzed using a two-way analysis of variance (ANOVA), considering treatments and days as the primary factors, followed by the least significant difference (LSD) test at $p < 0.05$. The Pearson correlation analysis was employed for dependent variables and principal component analysis (PCA) was employed for treatments by utilizing IBM SPSS software (version 26).

3 Results

3.1 Morphological Analysis

The impact of osmotic stress inducers on morphological aspects, comprising shoot length, survival rate, and the shoot's fresh and dry weights, is depicted in Fig. 2.

3.2 Impact of PEG and Sucrose Levels on Shoot Length of Nodal Explant

Fig. 2a illustrates the impact of the concentrations of varying sucrose and PEG on the length of the explant shoot. Analysis of variance was conducted on shoot length data, revealing that treatments significantly affected the results over time. The greatest decline in shoot length due to 8% sucrose was observed to be 0.93, 1.03, and 1.12 cm at 15, 30, and 45 days, respectively (Fig. 3). At intervals of 15, 30, and 45 days, the length of the shoot of explants treated with PEG (0.5%) decreased to 0.84, 0.88, and 0.92 cm, respectively, relative to the control values of 1.52, 1.55, and 1.57 cm. In contrast, an elevated PEG level (1.5%) significantly reduced the shoot length to 0.61, 0.62, and 0.57 cm, respectively (Fig. 3). The medium containing 4% sucrose yielded the most significant length of the shoot (1.45 cm) at the 45-day mark, demonstrating a significant difference from other treatments and time periods. While 1.5% PEG resulted in the shortest (0.60 cm) shoot length.

3.3 Nodal Explant Survival Percentage under Sucrose and PEG Treatments

Analysis of the variance of the survival percentage demonstrated the significant impact of the treatments. The survival rate declined with higher levels of sucrose and PEG. Nodal explants exposed to PEG revealed a higher survival rate compared to those treated with sucrose. Media with sucrose (4%) and PEG (0.5%) yielded the highest survival rates, 83.3% and 87.5% after 15 days while reduced to 79.2% and 83.3% after 30 days. The lowest survival rates were achieved with media containing the maximum concentrations of sucrose (8%) and PEG (1.5%): 62.5% and 66.66% at 15 days, 54.16% and 66.66% at 30 days, and 54.16% and 62.5% at 45 days, relative to the control (Fig. 2b). The data illustrated in Fig. 2b demonstrate that the medium

with 0.5% PEG and a 15-day interval yielded the maximum survival percentages (91.67%). On the other hand, the medium containing 8% sucrose and a 15-day interval resulted in minimal survival percentages (83.33%).

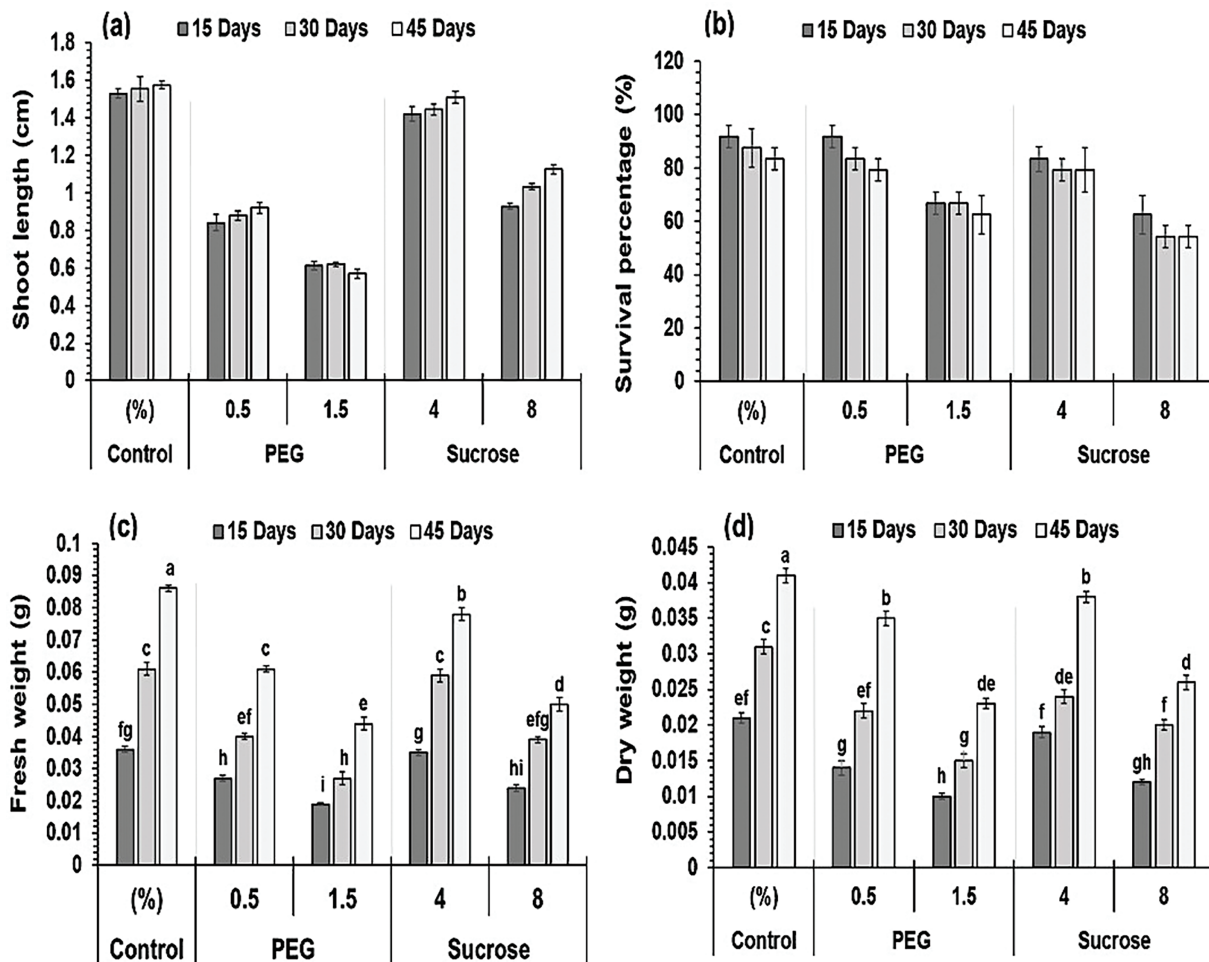


Figure 2: Impact of treatments and duration on the nodal explants' growth. (a) Shoot length, (b) Survival rate, (c) Fresh weight, (d) Dry weight. Data are expressed as mean $\pm$ SE from three independent experiments, illustrated as bars with error bars. Bars with distinct letters designate a significant difference following the LSD test at $p < 0.05$

3.4 Impact of Sucrose and PEG Levels on the Nodal Explant Weight

Fresh and dry weights were evaluated by analysis of variance, revealing a significant influence of treatments over time. Fig. 2c,d illustrates a gradual decline in the explant dry and fresh weights as sucrose and PEG concentrations increase. Over 15 days, the explant's maximum fresh and dry weights were recorded at 0.03 and 0.01 g, respectively, with 0.5% PEG in the medium. On the contrary, the explant's minimum dry and fresh weights recorded were 0.01 and 0.02 g, respectively, at 1.5% PEG. After 15 days, the explant's fresh and dry weights followed a similar trend, with 0.03 and 0.02 g at 4% sucrose medium, while 8% sucrose medium showed the minimum values at 0.02 and 0.01 g, respectively.

The control and 4% sucrose media produced satisfactory results following 45 days. The control medium gave the greatest fresh (0.08 g) and dry weights (0.02 g), while 4% sucrose medium resulted in 0.09 and 0.04 g for fresh and dry weights. The minimum fresh and dry weights observed were 0.04 and 0.02 g,

respectively, in PEG (1.5%) enriched medium. The explant's dry and fresh weights in the medium enriched with sucrose (8%) were 0.03 and 0.05 g, respectively. The data in Fig. 2c,d reveal that the medium enriched with 4% sucrose induced the maximum fresh (0.09 g) and dry (0.04 g) weights of nodal cultures, after 45 days. The media with 0.5% and 1.5% PEG at a 15-day interval produced the minimum fresh and dry weights, measured at (0.02 vs. 0.03 g) and (0.01 vs. 0.02 g), respectively.

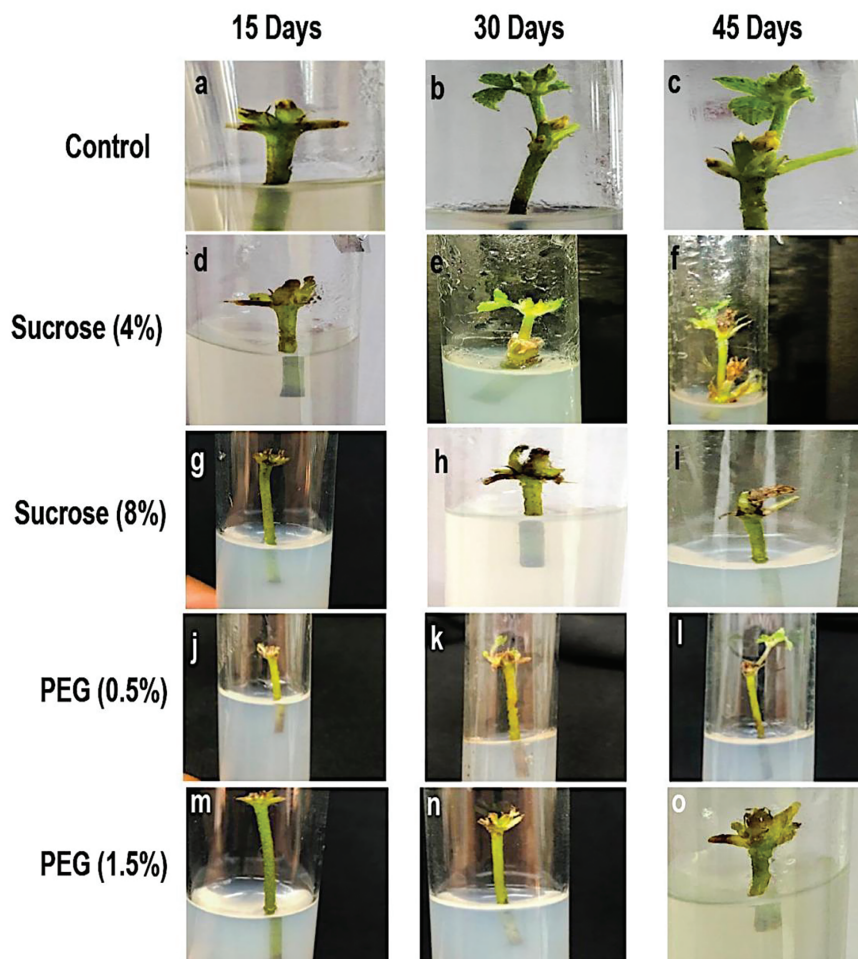


Figure 3: Impact of PEG and sucrose levels on the nodal explant morphology at 15-, 30- and 45-day. Control: (a, b, c); 4% sucrose: (d, e, f); 8% sucrose: (g, h, i); 0.5% PEG: (j, k, l) and 1.5% PEG: (m, n, o)

3.5 Biochemical Analysis

3.5.1 Total Soluble Sugars

Fig. 4a displays the total soluble sugar analysis results. The analysis of variance on total soluble sugars showed significant impacts from the durations, treatments, and their interaction. After 15, 30, and 45 days, the explants cultured in a 1.5% PEG-enriched medium-exhibited higher total soluble sugars (3.96, 4.06, and 4.12 mg g⁻¹). Explants cultured on media containing 8% and 4% sucrose, showed increases in total soluble sugars (2.64, 2.95, and 3.20 mg g⁻¹) and (2.61, 2.77, and 2.95 mg g⁻¹), respectively, in comparison to the control group. These increases happened over a period of 15 days, 30 days, and 45 days. Total soluble sugars

also increased significantly (3.63, 3.93, and 4.07 mg g⁻¹) in a 0.5% PEG-enriched medium at 15, 30, and 45-day intervals.

3.5.2 Total Phenolics Contents

Fig. 4b presents the findings from the total phenolic content test, revealing that treatment, time, and their interaction significantly influenced the results. When compared with the control medium, 8%, and 4% sucrose caused the total phenolic content to rise slightly (0.18, 0.33, and 0.65 mg g⁻¹) and (0.15, 0.21, and 0.40 mg g⁻¹), respectively. However, the medium with 0.5% PEG caused the total phenolic level to rise greatly (0.33, 0.78, and 0.96 mg g⁻¹). The medium comprising 1.5% PEG caused the total phenolic content to rise twice as much (0.47, 0.95, and 1.19 mg g⁻¹) compared to the control.

3.5.3 Total Protein Contents

The nodal explant's protein levels were notably influenced by the treatments, days, and their interaction (Fig. 4c). The total protein content at 15-, 30-, and 45-day intervals decreased slightly (2.47, 2.69, and 2.89 mg g⁻¹) and (3.12, 3.39, and 3.60 mg g⁻¹) in the 8% and 4% sucrose-enriched medium relative to the control. Conversely, the medium containing 0.5% PEG caused a great significant decline in total protein levels, recorded at 2.08, 2.28, and 2.38 mg g⁻¹. The medium comprising PEG 1.5% caused a twofold decline in total protein content, with values of 1.96, 2.17, and 2.32 mg g⁻¹, relative to the control.

3.5.4 Chlorophyll Contents

The findings indicated that chlorophyll levels were significantly impacted by treatments, days, and their interaction. The duration did not affect chlorophyll b levels; however, the treatments and their interaction with days exhibited great significance (Fig. 4e,f). Chlorophyll b levels were lowest in the medium with 1.5% PEG (0.12, 0.11, and 0.06 mg g⁻¹), preceded by 0.5% PEG (0.14, 0.12, and 0.07 mg g⁻¹), 8% sucrose (0.12, 0.12, and 0.11 mg g⁻¹), and the highest levels were found in the medium with 4% sucrose (0.19, 0.23, and 0.27 mg g⁻¹) at 15, 30, and 45 days. Similarly, chlorophyll levels were lowest in the 1.5% PEG medium (0.14, 0.12, and 0.08 mg g⁻¹), then 0.5% PEG (0.16, 0.13, and 0.10 mg g⁻¹), 8% sucrose (0.17, 0.15, and 0.15 mg g⁻¹), and second highest in the 4% sucrose medium (0.16, 0.18, and 0.21 mg g⁻¹).

Analysis of variance on the whole chlorophyll content data indicated that treatments, days, and their interaction exhibited great significance. The medium comprising 1.5% and 0.5% PEG had the lowest level of total chlorophyll in comparison with the control (0.27, 0.23, and 0.14 mg g⁻¹) and (0.29, 0.25, and 0.17 mg g⁻¹) at 15-, 30-, and 45-day intervals. Nevertheless, the media with 8% and 4% sucrose had a minimal reduction in total chlorophyll content (0.29, 0.28, and 0.26 mg g⁻¹) and (0.34, 0.40, and 0.47 mg g⁻¹), respectively (Fig. 4d).

3.6 Pearson Correlation Analysis

The data in Table 2 indicate a notable negative association ($p < 0.01$) amid shoot length and total phenolic content. The total phenolic content exhibited a significant negative correlation ($p < 0.01$) with chlorophyll a, b, and total chlorophyll content. The length of the shoot exhibited a positive and significant correlation with both shoot dry and fresh weight ($p < 0.01$).

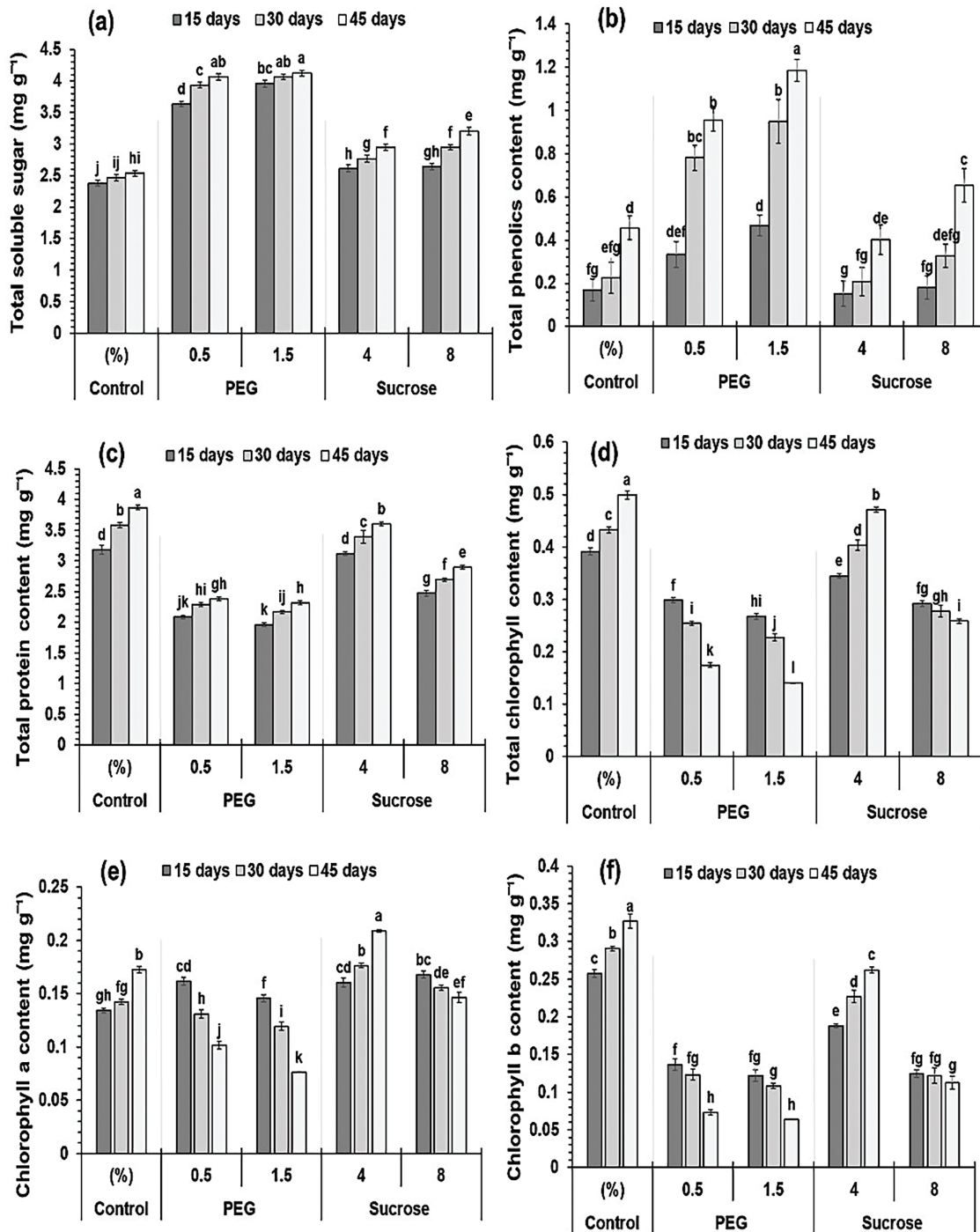


Figure 4: Effect of treatments and days on nodal explants biochemical measures (mg/g). (a) Total soluble sugars, (b) Total phenolic content, (c) Total protein content, (d) Total chlorophyll content, (e) Chlorophyll a content, (f) Chlorophyll b content. Data are expressed as mean $\pm$ SE from three independent experiments, illustrated as bars with error bars. Within each panel, bars with distinct letters designate a significant distinction following the LSD test at $p < 0.05$

3.7 Principal Component Analysis (PCA)

The variance was contributed 99.5% by the first principal component (PC1), while the second component (PC2) contributed 0.4%, as shown in Fig. 5. Together, these two components explain 99.9% of the variance.

Table 2: Pearson correlation analysis between physiological parameters (survival rate, length of the shoot, and dry and fresh weights) and biochemical metrics (protein content, phenolic content, soluble sugars, and chlorophyll levels) of *G. wallichianum*

	SL	FW	DW	SR	TSS	TPh	TP	TCC	Cha	Ch b
SL	1									
FW	0.640**	1								
DW	0.572**	0.950**	1							
SR	0.419**	0.190	0.210	1						
TSS	-0.858**	-0.317*	-0.243	-0.227	1					
TPh	-0.626**	0.025	0.126	-0.245	0.812**	1				
TP	0.934**	0.769**	0.689**	0.288	-0.798**	-0.469**	1			
TCC	0.871**	0.556**	0.446**	0.428**	-0.793**	-0.676**	0.859**	1		
Cha	0.584**	0.288	0.147	0.153	-0.607**	-0.701**	0.532**	0.770**	1	
Ch b	0.877**	0.593**	0.508**	0.483**	-0.769**	-0.585**	0.882**	0.969**	0.587**	1

Note: ** A significant correlation is observed at 0.01; * A significant correlation is observed at 0.05; survival rate % = SR, shoot length = SL, fresh weight = FW, dry weight = DW and biochemical parameters total soluble sugars = TSS, protein content = TPC, phenolic content = TPh, total chlorophyll = TCC, chlorophyll a = Cha and chlorophyll b = Chb.

3.8 Heatmap and Hierarchical Clustering

Heatmap hierarchical clustering was done to define the trait–treatment association (Fig. 6). The treatments were positioned along the X-axis, while the Y-axis displayed the survival rate as well as physiological and biochemical parameters (Fig. 6). The treatments were categorized into two main groups, A and B, each of which was categorized into five smaller groups, numbered 1 to 5 (Fig. 6). The survival rate, and physiological and biochemical parameters under investigation were organized into two primary clusters, which were further sub-grouped into three sub-clusters numbered 1 through 3 (Fig. 6). The results of parameter clustering indicated a distinct separation between the sucrose and PEG samples in comparison to the control group. Both the 0.5% and 1.5% PEG concentrations dramatically decreased the growth attributes compared to the 4% and 8% sucrose concentrations, yet the low concentrations showed a very good survival rate. The *in vitro* cultivated nodal segments may have been experiencing osmotic stress because PEG treatment led to increased levels of soluble sugars and total phenolics compared to sucrose treatment, accompanied by a decrease in protein and chlorophyll levels.

4 Discussion

G. wallichianum is at risk of extinction in northern Pakistan due to overharvesting for its medicinal properties. *Ex situ* conservation methods have been employed to study various endangered plants [40], but knowledge of this species' protection remains limited. The results suggest that osmotic stress inducers can effectively inhibit the growth of nodal explants temporarily, offering the potential for long-term *in vitro* preservation.

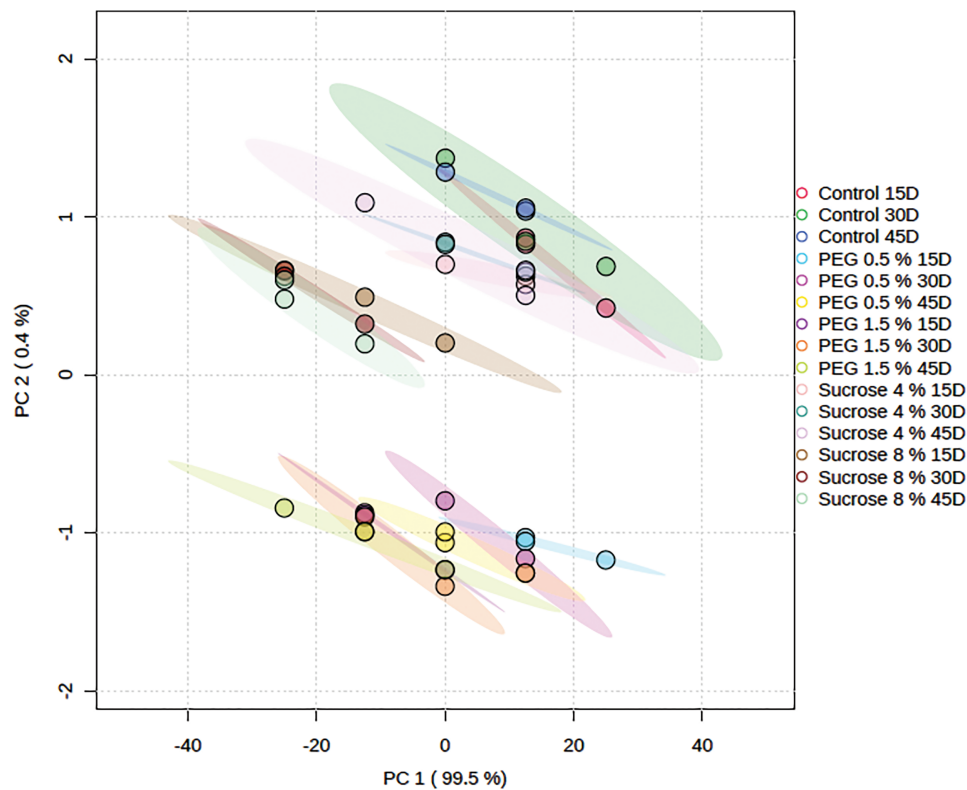


Figure 5: Principal component analysis of studied variables (survival %, length of the shoot, dry and fresh weight) and biochemical metrics (phenolic content (TOC), total soluble sugars (TSS), protein content (TPC) and chlorophyll a and b content) from the *in vitro* culture of *G. wallichianum* in reaction to various PEG and sucrose treatments

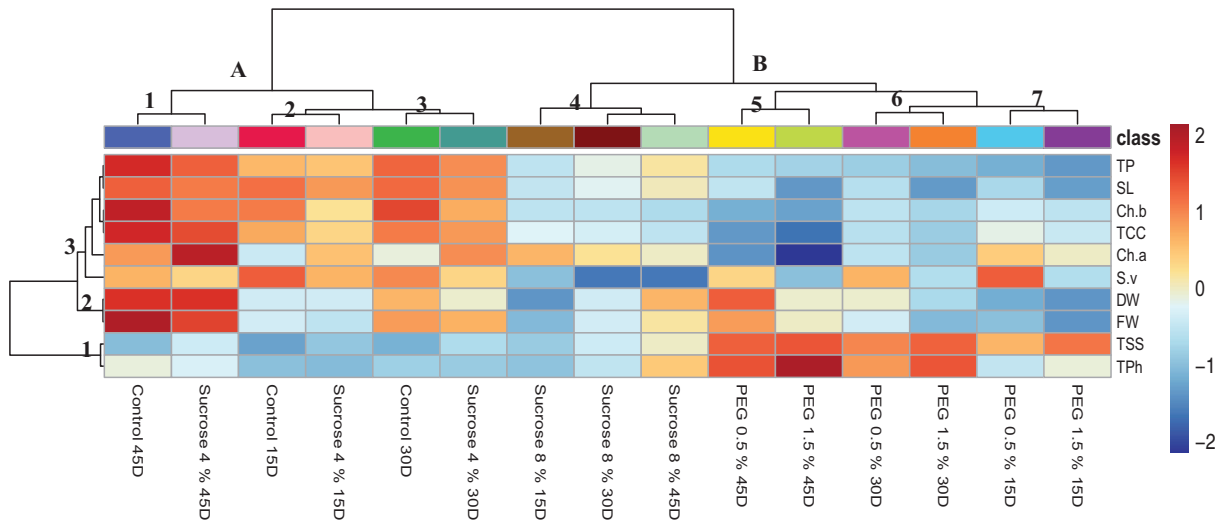


Figure 6: Hierarchical clustering and heatmap of investigated physiological parameters survival rate % (SR), dry weight (DW), fresh weight (FW) and shoot length (SL), alongside biochemical metrics (total soluble sugars (TSS), protein content (TPC), phenolic content (TPh), total chlorophyll (TCC) and chlorophyll a and b contents) from the *in vitro* culture of *G. wallichianum* in response to various treatments of sucrose and PEG at 15, 30, and 35 days. Red and blue shades represent strong and weak associations, respectively, between treatments and investigated parameters

In this study, osmotic stress was induced using PEG and sucrose, both of which decreased the nodal explants' growth rate along with their fresh and dry weights. Notably, these reductions were consistent across various day intervals (15, 30, and 45 days), aligning with findings by Sakthivelu et al. [41]. Growth rate and fresh and dry weight were markedly lower at PEG concentrations of 0.5% and 1.5% compared to sucrose levels of 4% and 8%. This decline can be attributed to a significant rise in osmotic pressure in the medium, diminishing nutrient and water absorption by reducing turgor pressure and severely inhibiting cell elongation. A comparison between PEG and sucrose suggests that PEG exerts a stronger impact on *G. wallichianum* growth compared to sucrose. Comparable findings have been reported for *Lathyrus sativus* [42], Milk thistle [43], and several durum wheat varieties [44]. Razavizadeh et al. [45] indicated that incorporating PEG into the culture medium triggered osmotic stress in *Thymus vulgaris* explants. This stress led to reduced fresh weight and explant length, mirroring observations in other plants exposed to drought conditions. Similar trends have also been documented in studies on various aromatic plants [46–48]. Stressed plants often exhibit decreased growth traits, such as weight and height, which may result from changes in their metabolic processes [49]. Research indicates that plants adjust their physiological, anatomical, and morphological traits to improve stress tolerance triggered by PEG. Similarly, *G. wallichianum* decreased its entire biomass to conserve energy and enhance water absorption, as observed in this research. The reduced fresh weight during drought conditions can be linked to a marked decline in canopy structure, photosynthesis, and phytohormones that regulate stomatal closure, leading to reduced transpiration and lower carbon assimilation [49,50]. Its *in vitro* application has been effective to controlling the *in vitro* growth of plants [51].

Hessini et al. [52] proposed that higher levels of total soluble sugars in plants may enhance stress tolerance. Plants' ability to manage osmotic balance and water regulation during stress conditions leads to the build-up of various nutrients, including organic acids, proline alcohols, soluble sugars, and glycine betaine. Increased water pressure could elevate the soluble sugars in different plant species [53]. Soluble sugars primarily serve to sustain turgor pressure and boost their concentration, which enhances starch breakdown through the action of hydrolytic enzymes during stress [54]. Additional stress factors comprise producing non-photosynthetic sugars, decreased leaf transport, and elevated sugar levels in leaves during water stress [55]. According to Ramak et al. [56], sugar accumulation within the stressed plant's shoots intensifies drought impact through maintaining osmotic balance and preventing dissociation, as observed in *Onobrychis goneifoli* and *O. radiate*. Kumari et al. [57] reported comparable outcomes for medical plants like *Sinopodophyllum hexandrum*, as well as in beans studied by Subbarao et al. [58], and soybeans by Niakan et al. [59] and Rikan et al. [60]. Our research confirms these outcomes, showing markedly elevated soluble sugars in PEG-treated *G. wallichianum* relative to the control. During osmotic stress, the elevated levels of entire soluble sugars may be attributed to the interruption of starch and the activity of amylase [61]. The build-up of sugars and osmotic regulators helps regulate growth under stress via reducing reactive oxygen species (ROS) through a reinforced antioxidant system [62,63]. This study found an increase in soluble sugars during osmotic stress, likely resulting from elevated fructose and glucose from the enhanced sucrose and starch hydrolysis. Soluble sugars also serve other vital roles, including an energy source and osmotic protectors, helping plants withstand stress.

Phenolic compounds are crucial in maintaining osmotic balance and notably build-up during osmotic stress [64], as seen in this study. The phenolic content in plants contributes to their stress tolerance. *Citrus sinensis* exhibited a notable rise in antioxidant activity and phenolic content [65]. Razavizadeh et al. [45] reported that PEG treatment in *T. vulgaris* cultures significantly affected phenolic compound production, as these compounds are vital for plant defense. It is suggested that stress conditions may stimulate phenolic biosynthesis because of oxidative stress [66]. Additionally, Zhuang et al. [67] noticed a significant association

between 1,1-diphenyl-2-picrylhydrazine and phenolics, and among total phenolics and antioxidant activity or reducing iron. In our study, explants exhibited the highest phenolic levels upon treatment with both PEG (0.5% and 1.5%) concentrations, in comparison with sucrose treatments. The notable build-up of phenolics may serve as a defensive mechanism, helping to maintain stable levels of stable protein and ROS.

Chlorophyll accumulation in plant tissues serves as a reliable marker for the detrimental impacts of various stressors. Intense stress leads to the build-up of hydrogen peroxide, which may result in a reduction of protein and chlorophyll levels. Additionally, stress can trigger the breakdown of chloroplast structure by activating chlorophyllase [68]. The decline in chlorophyll levels may be linked to heightened enzyme activity or damage to the plastid envelope and thylakoids caused by ROS. Stressed plants tend to produce less chlorophyll, a sign of oxidative stress. As a result, the disruption of ion balance limits chlorophyll synthesis, leading to a declined chlorophyll concentration and metabolism [69]. In our study, explant cultures treated with PEG (0.5% and 1.5%) showed a marked reduction in chlorophyll compared to those treated with sucrose (4% and 8%). Moreover, Tátrai et al. [70] reported that PEG supplementation into the culture media led to the generation of ROS, which contributed to chlorophyll degradation through lipid peroxidation. It has also been demonstrated that PEG enrichment negatively impacts the *in vitro* cultures' physiological, phytochemical, and morphological features, including *Tagetes erecta* seedlings [71], *Hordeum vulgare* [72], and *Allium hirtifolium* [73].

According to Sobhkhizi et al. [74] stress affects chlorophyll a and b in nodal cultures by triggering proteolysis of chloroplasts, leading to the degradation of chlorophyll, a key factor. Water stress is accompanied by reduced chlorophyll levels in plants like *Carthamus tinctorius* [75], *Paulownia imperialis* [76], and beans [77]. As per Martínez-Santos et al. [49], higher PEG levels reduced the chlorophyll levels in the shoots of *Vanilla planifolia*. Similarly, in an *in vitro* Orchidaceae culture, Gao et al. [48] noticed reduced chlorophyll levels in *Dendrobium officinale* shoots treated with 0%, 1%, 3%, and 5% PEG. Suis et al. [78] observed that exposing *Aranda Broga* Blue Bell protocorm-like bodies to PEG (15%) led to decreased chlorophyll levels. *Ex vivo* research has indicated decreased chlorophyll levels in *V. planifolia* exposed to osmotic stress [79]. Osmotic stress impairs the efficiency of photosynthesis by disrupting the flow of energy between photosystem I (PSI) and photosystem II (PSII) [80].

In our study, cultures treated with PEG (0.5% and 1.5%) showed lowered protein levels compared to those exposed to sucrose. Previous research has indicated that PEG treatment decreases the soluble protein levels in *Vigna unguiculata* and several *Nicotiana tabacum* species [81]. PEG triggers oxidative stress in plants, leading to damage to membranes and active proteins, thereby reducing the total protein content in *G. wallichianum*. As per Martínez-Santos et al. [49] findings, as PEG concentration increased, soluble protein levels in *V. planifolia* shoots declined, though protein levels were still elevated in PEG treatments compared to controls. The increased soluble proteins at 1% PEG can be attributed to the production of hydrophilins [82], aquaporins [83], dehydrins [84], antioxidant enzymes [85], and chaperonin [86], which help plants manage oxidative and osmotic stress [87]. In crops like rice [88], legumes [89], soybean leaves [59], and onions [90], a reduction in protein content in shoot tissues is closely linked to rising stress levels. Razavizadeh et al. [45] reported reduced protein levels in seedlings of *T. vulgaris* exposed to 2%, 4%, 6%, and 8% PEG. Likewise, Gao et al. [48] reported reduced protein levels in *D. officinale* shoots experiencing osmotic stress from 1%, 3%, and 5% PEG. Our study explored the biochemical and morphological reactions of *G. wallichianum* nodal cultures to varying sucrose and PEG concentrations within a short duration (45 days). Growth analysis showed that lower sucrose (4%) and PEG (0.5%) concentrations did not adversely affect growth, while higher PEG levels notably inhibit growth compared to the control. However, the current research work focused on short-term control of the *in vitro* growth of *G. wallichianum*, which can be considered its limitation. Therefore, this

research could be expanded to assess the long-term osmotic stressors' impacts on the *in vitro* preservation of medicinal plants with slow growth.

5 Conclusion

Our study attempts to develop a strategy for *in vitro* conservation by slowing down the *G. wallichianum* growth in a regulated setting utilizing osmotic stress agents like PEG and sucrose in response to the threat of its extinction from overharvesting and utilization. PEG concentrations (0.1% and 0.5%) significantly reduced morphological growth attributes relative to sucrose (4% and 8%). PEG treatments increased the amounts of soluble sugars and total phenolics compared to the sucrose treatment which confirms its stress inducer role. Following this, there was a decline in chlorophyll and total protein levels, indicating that the nodal segments cultured in the lab were experiencing osmotic stress. The findings revealed that PEG is more useful in reducing *G. wallichianum in vitro* growth than sucrose. However, there is a need to explore further the role of PEG and sucrose on long-term *in vitro* growth and conservation of *G. wallichianum*, which can be extended to other medicinally important plants.

Acknowledgement: The authors wish to express their sincere gratitude to the Deanship of Scientific Research and the Vice Presidency for Graduate Studies and Scientific Research at King Faisal University, Saudi Arabia, for their support of this project through grant number KFU242733.

Funding Statement: This work was financially supported by the Deanship of Scientific Research, Vice Presidency for Graduate Studies and Scientific Research, King Faisal University, Saudi Arabia, grant number KFU242733.

Author Contributions: Conceptualization: Zubair Ashraf and Yasar Sajjad; Methodology: Zubair Ashraf; Formal analysis and investigation: Sabaz Ali Khan and Gulzar Akhtar; Writing—preparation of the original draft: Yasar Sajjad, Zubair Ashraf, Ahmed Mahmoud Ismail and Tarek A. Shalaby; Writing—review and editing: Yasar Sajjad, Bader Alsubaie and Othman Al-Dossary; Funding acquisition: Ahmed Mahmoud Ismail; Resources: Zubair Ashraf; Supervision: Yasar Sajjad. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: Available data are provided in the manuscript.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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