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# Optimization of Callus Induction and Cell Suspension Culture for the Endemic Species *Verbascum hasbenlii*

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## Abstract

*Verbascum hasbenlii* Aytaç & H. Duman is a narrowly distributed endemic species native to Çanakkale, Türkiye. This study aimed to establish and optimize callus induction and cell suspension culture systems for *V. hasbenlii*. Leaf explants obtained from 10-week-old seed-derived in vitro plants were cultured on six Murashige and Skoog (MS) media containing different combinations of α-naphthaleneacetic acid (NAA; 0.5 or 1.0 mg/L) and 6-Benzylaminopurine (BAP; 0.5, 1, 2 or 3 mg/L). After four weeks, callus induction was achieved in all treatments (96–100%), although significant differences were observed in explant browning, callus biomass, diameter, and morphology. The medium supplemented with 0.5 mg/L BAP + 0.5 mg/L NAA produced the highest callus biomass (1.245 g) and diameter (5.06 mm), while maintaining low explant browning and a compact-friable texture suitable for suspension culture establishment. Cell suspension cultures exhibited a typical growth pattern with lag, exponential, and stationary phases. On day 9, cultures showed increased growth parameters, including packed cell volume (PCV: 7.50%), fresh weight (FW: 0.0580 g), and dry weight (DW: 0.0052 g), with relatively high cell viability (80.72%). Biomass accumulation reached maximum levels between days 18–21, while cell viability decreased to 66.82%. These findings provide an optimized in vitro culture system for future studies on secondary metabolite production in *V. hasbenlii*.

**Keywords:** auxin; cell inoculum; cytokinin; growth regulators; in vitro culture; Mullein

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## 1. Introduction

The genus *Verbascum* L. is represented in Türkiye —one of the major centers of global biodiversity—by 257 species, of which 203 are endemic [1]. *Verbascum hasbenlii* Aytaç & H. Duman ("Benli" or "Spotted" Mullein) is a local endemic species restricted to a narrow area in Çanakkale, Türkiye and it grows exclusively on large metamorphic rock formations. Morphologically, this species is characterized by a dense indumentum composed of glandular and non-glandular trichomes, and a yellow corolla bearing purple-brown spots on the two upper lobes [2].

Worldwide, numerous species of the genus *Verbascum*, particularly *V. thapsus* L., are widely used in traditional medicine for the management of respiratory, dermatological, circulatory, and infectious disorders [3]. Phytochemical investigations have revealed that *Verbascum* species contain a wide array of structurally diverse secondary metabolites, including aucubin, ajugol, verbascoside, forsythoside, apigenin, luteolin, quercetin, protoverbine,

caffeic acid, and *p*-coumaric acid. These compounds have been reported to exert significant antioxidant, anti-inflammatory, mucolytic, antimicrobial, analgesic, cytoprotective, neuroprotective, antitumor, anti-aging and membrane-stabilizing activities, highlighting the considerable pharmacological potential of the genus and supporting its ethnopharmacological relevance [4–7]. Due to their chemically diverse secondary metabolites, *Verbascum* species represent a promising resource for pharmacology and the pharmaceutical industry. Therefore, the sustainable production of bioactive compounds derived from these species is of considerable importance. In this context, in vitro culture systems offer an effective approach for the controlled production of valuable secondary metabolites.

Plant tissue culture represents an important biotechnological strategy that enables the controlled and sustainable production of valuable secondary metabolites. Among in vitro systems, callus and cell suspension cultures are widely used for secondary metabolite production [8,9]. Callus cultures consist of unorganized and rapidly proliferating masses of parenchymatous cells that generally develop in response to tissue injury or stress and are induced from explants on solid media supplemented with appropriate plant growth regulators [10]. In particular, friable calli can be transferred to liquid media to establish cell suspension cultures, which offer several advantages, such as improved nutrient uptake, enhanced aeration, and precise control of environmental conditions, making them suitable for large-scale production of secondary metabolites [11]. Furthermore, in vitro culture systems reduce dependence on natural plant resources and overcome geographical and environmental limitations, allowing the standardized production of bioactive compounds [12]. However, the efficient production of secondary metabolites largely depends on the optimization of culture conditions, including culture medium composition, plant growth regulators, and other culture parameters [13]. Therefore, the effective utilization of in vitro culture systems and the optimization of culture parameters play a critical role in the efficient and scalable production of secondary metabolites.

A review of the literature indicates that in vitro callus culture protocols have been successfully established for several *Verbascum* species, including *V. thapsus* using 0.5 mg/L BAP and 1 mg/L NAA, *V. scamandri* Murb. using 0.5–3 mg/L Kinetin and 0.5 mg/L 2,4-dichlorophenoxyacetic acid, and *V. biledschikianum* Bornm. using 2 mg/L Kinetin [14–16]. However, despite the increasing interest in biotechnological approaches for secondary metabolite production in this genus, no studies to date have reported the establishment of in vitro culture systems for the endemic species *V. hasbenlii*. Therefore, the objectives of the present study were (i) to determine the optimal callus induction protocol for *V. hasbenlii*, and (ii) to establish a stable and rapidly proliferating cell suspension culture and characterize its growth dynamics.

## 2. Materials and Methods

### *2.1. Plant Material and Culture Conditions*

*V. hasbenlii* seeds were collected from their natural habitat under the required legal permits and taxonomically authenticated by Prof. Dr. Ersin KARABACAK (Department of Biology, Faculty of Science, Çanakkale Onsekiz Mart University) (Figure 1a). The collected seeds were brought to the Plant Tissue Culture and Genetics Research Laboratory of Çanakkale Onsekiz Mart University and stored at +4 °C until in vitro cultures were initiated.

Seed surface sterilization and germination were carried out following Cambaz and Çördük [15]. For germination, the seeds were placed in glass Petri dishes (100 × 20 mm). In vitro germinated plants were sub-cultured to fresh Murashige and Skoog (MS) basal medium at 4-week intervals [17]. All in vitro cultures were incubated under growth room

conditions (25 ± 2 °C, 50 ± 5% relative humidity, 16 h light/8 h dark photoperiod, and light intensity of 72 µmol $m^{-2}s^{-1}$).

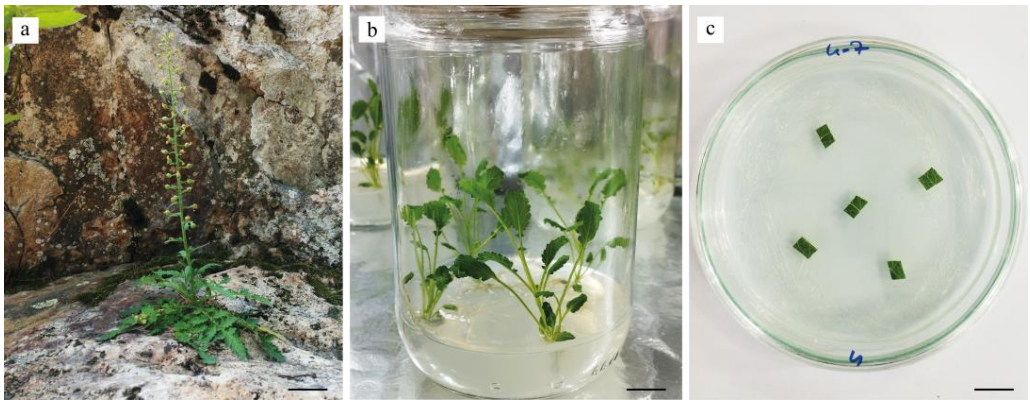



**Figure 1.** Plant samples of *V. hasbenlii*: (**a**) in its natural habitat, (**b**) 10-week-old seed-derived in vitro plants, (**c**) explants excised from rosette leaves (scale bar = 1 cm).

### 2.2. Callus Induction Culture

For callus induction, plant growth regulator combinations were selected based on previous studies [15,16,18]. MS medium supplemented with 30 g/L sucrose and 8 g/L agar was amended with NAA and BAPat the following concentrations: 0.5 mg/L BAP + 0.5 mg/L NAA, 3 mg/L BAP + 0.5 mg/L NAA, 0.5 mg/L BAP + 1 mg/L NAA, 1 mg/L BAP + 1 mg/L NAA, 2 mg/L BAP + 1 mg/L NAA, and 3 mg/L BAP + 1 mg/L NAA. Rosette leaf explants (~0.5 × 0.5 cm) were excised from the parts of the leaf away from the central vein of 10-week-old seed-derived in vitro plants (Figure 1b). These explants were cultured for 4 weeks, with each treatment consisting of 10 replicate Petri dishes (5 explants per dish, total 50 explants) (Figure 1c). Callus induction efficiency was assessed using quantitative and qualitative parameters, including callus induction rate (CIR), explant browning rate (EBR), number of wound sites forming calli (WS; counted in the four cut regions of the explants), callus diameter (CD; measured with caliper), callus color, callus texture, callus browning intensity, callus fresh weight (FW) and callus dry weight (DW; dried at room temperature in the shade for 48 h). All chemicals used in plant tissue culture, including MS medium, plant growth regulators, agar, and sucrose, were obtained from Duchefa Biochemie (Haarlem, The Netherlands). CIR and EBR were calculated according to the following formulas:

$$CIR(\%)\frac{Callus\ induced\ explants}{Total\ explants} \times 100 \quad (1)$$

$$EBR(\%)\frac{Browning\ explants}{Total\ explants} \times 100 \quad (2)$$

### 2.3. Cell Suspension Culture

Following callus induction, calli derived from the optimal treatment were selected for the establishment of cell suspension cultures and subsequent evaluation of growth dynamics. Cell suspension cultures were established to determine growth dynamics by inoculating callus at density of 1 g per 100 mL into 250 mL flasks containing the optimal callus induction medium (pH 5.75), with six replicates. Cultures were incubated for 21 days on an orbital shaker (110 rpm; Daihan Scientific SHO-2D, Wonju, South Korea) under the previously described growth room conditions, except in darkness. Growth dynamics were monitored at 3-day intervals by analyzing 5 mL suspension samples to determine packed cell volume (PCV), fresh weight (FW), and dry weight (DW). Following centrifugation at 4000 rpm (Elektromag M19, İstanbul, Türkiye) for 5 min to record the PCV, the supernatant

was discarded to immediately measure the pellet's FW, after which samples were air-dried under shaded conditions at ambient room temperature for 48 h to constant weight for DW determination.

Cell viability was determined using the methylene blue exclusion assay (0.01%; ISOLAB Laborgeräte GmbH, Eschau, Germany). After mixing the cell suspension with the dye solution (1:1) and incubating for 5 min, at least 6000 cells per sample were counted microscopically (Motic BA21A, Xiamen, China); unstained cells were considered viable. PCV and viability were calculated according to the following formulas:

$$PCV(\%) \frac{Pellet\ volume\ (mL)}{Total\ suspension\ volume\ (mL)} \times 100 \quad (3)$$

$$Viability(\%) \frac{Number\ of\ unstained\ cells}{Total\ number\ of\ cells} \times 100 \quad (4)$$

### *2.4. Statistical Analysis*

All experimental data were analyzed using the R statistical environment (version 4.5.2; R Core Team, Austria). The data were checked for normality and homogeneity of variances using the Shapiro–Wilk and Levene tests, respectively, before analysis. Differences among treatments were evaluated by One-Way Analysis of Variance (ANOVA), and mean comparisons were performed using Duncan's Multiple Range Test (DMRT; $p < 0.05$). Relationships among quantitative parameters were assessed using Spearman's rank correlation. Additionally, simple linear regression analyses were conducted to determine specific relationships between CB and CD, as well as between CB and EBR. Principal component analysis (PCA) was used to evaluate multivariate patterns among treatments. The PCA included five key variables (CIR, EBR, CB, CD, and WS), and the dataset was standardized (centered and scaled) to ensure equal weighting of all parameters.

## 3. Results

### *3.1. Callus Induction Culture*

The callus induction responses of rosette leaf explants from *V. hasbenlii* were assessed after 28 days of culture on six different media containing various combinations of BAP and NAA (Table 1).

**Table 1.** Qualitative evaluation of callus induction responses in *V. hasbenlii*.

| Medium | Callus Color | Callus Texture | Callus Browning Intensity |
|---|---|---|---|
| 0.5 mg/L BAP + 0.5 mg/L NAA | Yellow-Greenish | Compact-Friable | + |
| 3 mg/L BAP + 0.5 mg/L NAA | Yellow-Greenish | Compact | ++ |
| 0.5 mg/L BAP + 1 mg/L NAA | Yellow-Greenish | Compact | + |
| 1 mg/L BAP + 1 mg/L NAA | Yellow-Brown | Compact | +++ |
| 2 mg/L BAP + 1 mg/L NAA | Yellow-Brown | Compact-Friable | +++ |
| 3 mg/L BAP + 1 mg/L NAA | Yellow-Brown | Compact-Friable | +++ |

Callus Browning Intensity; +: slight, ++: moderate, +++: severe.

Callus induction rate was high across all tested media, ranging 96% to 100%, and no statistically significant differences were detected among treatments for this parameter ($p > 0.05$). Therefore, callus induction rate alone was not considered sufficient for the selection of an optimal culture medium. Instead, explant browning rate, callus biomass, callus diameter, number of wounds forming calli, and callus morphology (color, texture, and callus browning intensity) were used as key selection criteria.

Based on explant browning rate results, media supplemented with 0.5 mg/L BAP in combination with either 0.5 or 1 mg/L NAA exhibited the lowest browning levels (14–18%). In contrast, media containing 1, 2 or 3 mg/L BAP combined with either 0.5 or 1 mg/L NAA showed significantly increased explant browning rates (64–68%; $p < 0.05$). This trend was consistent with callus browning intensity, and morphological observations confirmed more pronounced browning in calli cultured on media with elevated BAP concentrations (Table 1 and Figure 2).

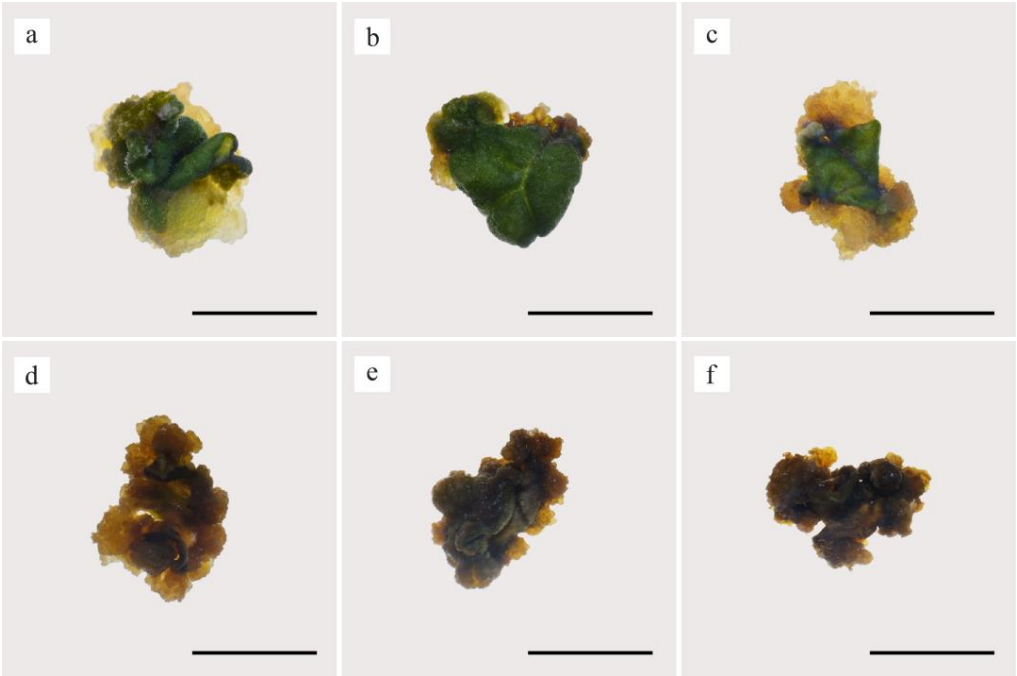



**Figure 2.** Representative images of *V. hasbenlii* leaf explants cultured on different callus induction media after 28 days of culture. Cultures were established from 10-week-old seed-derived in vitro plant explants: (**a**) 0.5 mg/L BAP + 0.5 mg/L NAA, (**b**) 3 mg/L BAP + 0.5 mg/L NAA, (**c**) 0.5 mg/L BAP + 1 mg/L NAA, (**d**) 1 mg/L BAP + 1 mg/L NAA, (**e**) 2 mg/L BAP + 1 mg/L NAA, (**f**) 3 mg/L BAP + 1 mg/L NAA (scale bar = 1 cm).

Statistical analyses further revealed significant differences among culture media in terms of callus biomass, callus diameter, and number of wounds forming calli ($p < 0.05$; Figure 3a–c). Evaluation of callus biomass and diameter showed that the medium supplemented with 0.5 mg/L BAP + 0.5 mg/L NAA (medium no. 1) produced the highest values (1.245 g and 5.06 mm, respectively) and differed statistically from the other media. In contrast, media containing higher BAP concentrations exhibited marked reductions in both callus biomass and callus diameter.

Spearman correlation analysis (Figure 4a) demonstrated a strong and statistically significant positive relationship between callus biomass and callus diameter ($\rho = 0.70$, $p < 0.001$). The number of wound sites also showed significant positive correlations with both parameters ($\rho = 0.51$–$0.67$, $p < 0.001$). In contrast, explant browning rate was significantly and negatively correlated with callus biomass and diameter ($\rho = -0.51$ and

−0.45, respectively; $p < 0.001$). Regression analysis (Figure 4c) further revealed that biomass variation was largely explained by callus diameter ($R^2 = 0.89$, $p = 0.017$).

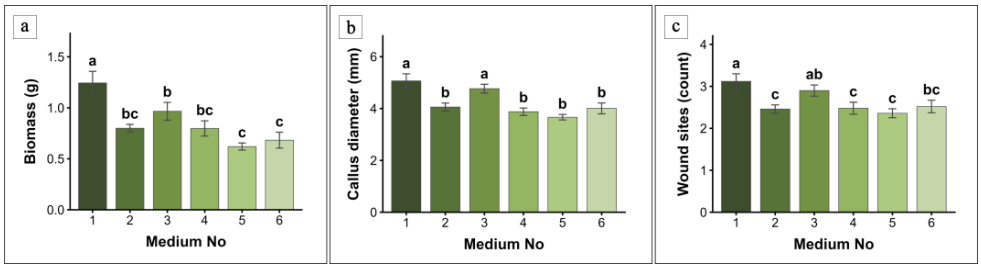



**Figure 3.** Effects of different callus induction media on (**a**) callus biomass, (**b**) callus diameter, (**c**) number of wounds forming calli in *V. hasbenlii* (Medium no 1: 0.5 mg/L BAP + 0.5 mg/L NAA; 2: 3 mg/L BAP + 0.5 mg/L NAA; 3: 0.5 mg/L BAP + 1 mg/L NAA; 4: 1 mg/L BAP + 1 mg/L NAA; 5: 2 mg/L BAP + 1 mg/L NAA; 6: 3 mg/L BAP + 1 mg/L NAA). Bars represent mean values ±SE ($n = 10$ biological replicates). Different letters above the bars indicate significant differences according to DMRT ($p < 0.05$).

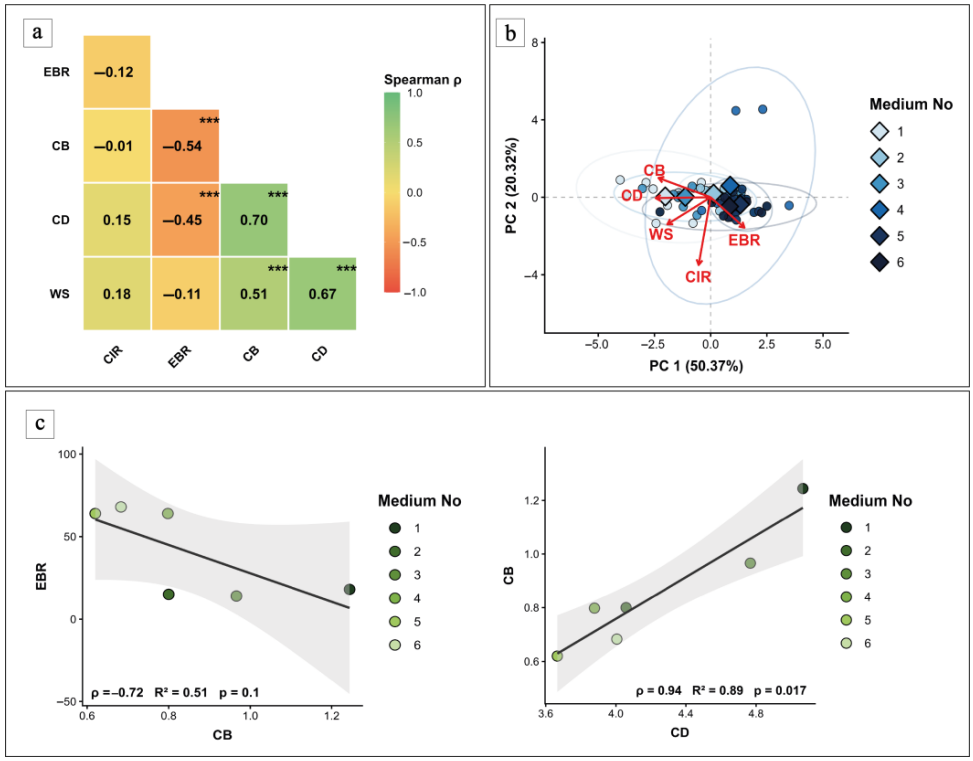



**Figure 4.** Multivariate and regression analyses of *V. hasbenlii* callus induction across six media. (**a**) Spearman correlation matrix, (**b**) PCA biplot with biological replicates (circles, $n = 10$), group means (diamonds), 95% confidence ellipses and variable vectors (red arrows) (circle, diamond, and ellipse colors correspond to Medium nos. 1–6), (**c**) regression analysis of selected variables (CIR: callus induction rate; CB: callus biomass; EBR: explant browning rate; WS: number of wound sites forming calli; CD: callus diameter. Medium no 1: 0.5 mg/L BAP + 0.5 mg/L NAA; 2: 3 mg/L BAP + 0.5 mg/L NAA; 3: 0.5 mg/L BAP + 1 mg/L NAA; 4: 1 mg/L BAP + 1 mg/L NAA; 5: 2 mg/L BAP + 1 mg/L NAA; 6: 3 mg/L BAP + 1 mg/L NAA. Asterisks indicate statistical significance levels: $p < 0.001$: ***).

PCA (Figure 4b) showed that the first two principal components accounted for 70.69% of the total variance and separated media characterized by higher browning from those associated with superior growth-related parameters. Specifically, PC1 (50.37%) was primarily driven by the strong trade-off between callus growth parameters (CB and CD) on the negative axis and EBR on the positive axis. PC2 (20.32%) was mainly influenced by the CIR

and WS responses. The biplot clearly illustrated the distribution of treatments: Media 1, 2, and 3 clustered tightly on the negative side of PC1, aligning strongly with the growth vectors (CB and CD), indicating optimal conditions for callus growth. In contrast, Media 4, 5, and 6 shifted towards the positive side of PC1, following the EBR vector, reflecting suboptimal culture conditions that promote browning and suppress growth.

### *3.2. Cell Suspension Culture*

Growth dynamics of the cell suspension culture exhibited clear temporal changes (Figure 5). Mean cell viability was highest on day 3 (87.41%); however, low PCV (2.33%), FW and DW values (0.0265 g, and 0.0022 g, respectively) at this stage indicated limited proliferation (Figure 6a,b). On day 6, viability decreased to 76.23%, and PCV dropped to 4.16%, suggesting that the culture was still in the adaptation (lag) phase. By day 9, viability remained relatively high (80.72%), while PCV returned to 7.50%, FW and DW (0.0580 g, and 0.0052 g respectively) increased markedly, indicating the onset of active proliferation. Biomass accumulation accelerated after day 12 (FW: 0.0917 g, and DW: 0.0114 g) and reached maximum levels between days 18–21 (FW: 0.1605–0.1842 g and DW: 0.0189–0.0174 g respectively). In contrast, viability progressively declined toward the late culture stage, reaching 66.82% on day 21, reflecting increasing physiological stress.

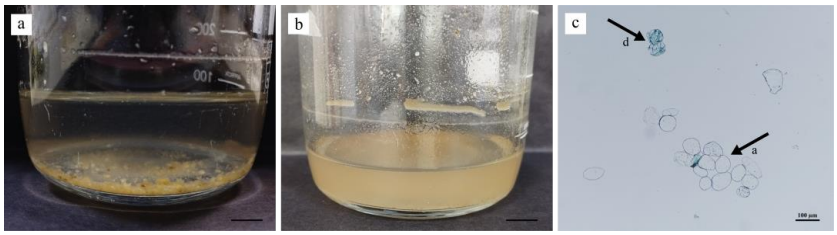



**Figure 5.** Cell suspension culture was incubated under dark conditions. (**a**) Day 0 of culture. (**b**) Day 21 of culture. (**c**) Methylene blue-stained sample showing viable (transparent: a) and non-viable (blue-stained: d) cells (scale bars = 1 cm and 100 µm).

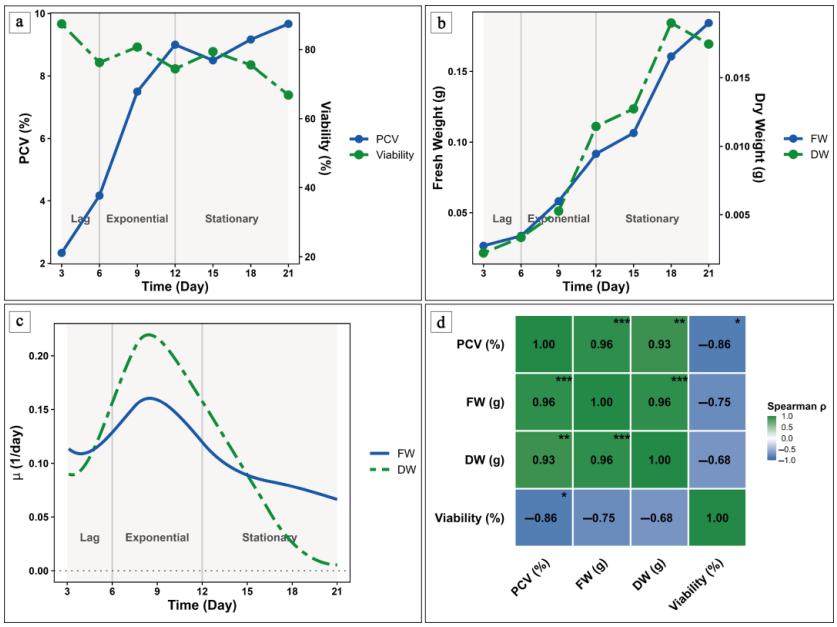



**Figure 6.** Growth dynamics of *V. hasbenlii* suspension cultures over a 21-day period. Time-dependent changes in (**a**) fresh weight and dry weight, (**b**) PCV and cell viability (%), (**c**) specific growth rate (µ) profiles derived from fresh weight and dry weight data, (**d**) Spearman correlation heatmap illustrating the relationships between growth parameters and viability (Asterisks indicate statistical significance levels: $p < 0.05$: *; $p < 0.01$: **; $p < 0.001$: ***).

Specific growth rate profiles showed a typical growth pattern, with a lag phase (days 3–6), an exponential phase (days 6–12) characterized by maximum μ values, and a stationary phase (days 12–21) marked by declining μ despite continued biomass accumulation (Figure 6c). In addition, Spearman correlation analysis revealed strong positive correlations between PCV with FW ($\rho = 0.90$) and DW ($\rho = 0.86$) as well as very strong correlations between FW and DW ($\rho = 0.96$) (Figure 6d). Conversely, viability was negatively correlated with PCV ($\rho = -0.74$), FW ($\rho = -0.75$), and DW ($\rho = -0.68$).

## 4. Discussion

Plant tissue culture techniques, particularly callus and cell suspension cultures, are widely recognized as important biotechnological tools for the sustainable production of valuable secondary metabolites and the conservation of plant species. In this context, in the present study, callus induction and cell suspension cultures were successfully established using rosette leaf explants of *V. hasbenlii*, an endemic species of Türkiye.

Callus formation is largely dependent on the type and concentration of plant growth regulators. In particular, the balance between auxins and cytokinins plays a decisive role in cell division, dedifferentiation, and callus induction [19]. Consistent with the literature, our results showed that auxin–cytokinin combinations markedly influenced callus formation. Callus induction was achieved in all media. However, the highest callus biomass and diameter, together with low browning and a compact-friable morphology, were obtained with 0.5 mg/L BAP + 0.5 mg/L NAA. These results indicate that low and balanced plant growth regulator combinations improve not only callus induction but also callus quality, which is particularly relevant for biotechnological applications because friable calli are more suitable for the establishment of cell suspension cultures [20]. Studies on *Verbascum* species also support our findings. In *V. sinuatum* L., callus was induced in media supplemented with different BA and NAA combinations, with the highest frequency of non-embryonic callus initiation reported on MS media containing 2.5 or 5 mg/L BA + 3 mg/L NAA [21]. In *V. bugulifolium* Lam., 93.33% cream-white, friable compact callus formation was obtained on medium containing 0.25 mg/L BAP + 0.25 mg/L NAA, while high callus induction was also observed in combinations of 0.25 mg/L BAP with 0.5–2 mg/L auxin [22]. These results indicate that low and balanced hormone combinations positively influence not only callus induction but also the morphological quality of calli. By contrast, in *V. scamandri* and *V. biledschikianum* efficient callus proliferation has been achieved under Kinetin- or Kinetin + 2,4-dichlorophenoxyacetic acid-based treatments [15,16]. Similarly, in *V. thapsus*, the highest callus production was recorded with 0.5 mg/L BAP, whereas the BAP + 2,4-dichlorophenoxyacetic acid combination promoted increases in both fresh and dry callus weight [23]. Taken together, these findings and our results clearly indicate that callus induction in *Verbascum* is strongly dependent on both genotype and the plant growth regulator regime. Although auxin–cytokinin combinations generally promote callus formation, the optimal type and concentration of plant growth regulators appear to vary among species. In the present study, all quantitative, qualitative, and statistical data consistently demonstrate that MS medium supplemented with 0.5 mg/L BAP + 0.5 mg/L NAA was the most suitable callus induction medium for *V. hasbenlii*, as it resulted in low explant browning, high callus biomass, diameter, compact-friable texture suitable for cell suspension culture. In contrast, our study observed that higher BAP concentrations (1–3 mg/L) did not improve callus induction frequency, but instead promoted phenolic oxidation and tissue necrosis, thereby limiting callus quality and growth. Kieber and Schaller [24] reported that cytokinin oxidase enzymes in plant tissue exhibit oxidation activity at high cytokinin levels, which can weaken the effects of cytokinin like BAP, thereby inhibiting callus formation. Previous studies on different plant species have also shown that high BAP concentrations

(0.5–1.5 mg/L) lead to increased phenolic compounds and browning in plant or callus tissues, while low BAP concentrations (0.3–0.5 mg/L) help maintain viability and promote higher rates of callus formation [25–27]. Our findings further emphasize the importance of determining the optimal BAP concentrations.

In plant cell suspension cultures, analysis of the growth pattern is essential for optimizing biomass accumulation and secondary metabolite production [28,29]. Identification of growth phases allows determination of the optimal harvest time and enables elicitation of metabolite biosynthesis at the most appropriate stage [30,31]. In the present study, the growth dynamics of the cell suspension culture showed marked temporal variation. During the early days of our cell suspension culture (up to day 3), cell viability was high, but fresh weight, dry weight, and PCV were low. However, by day 6, a slight decrease in cell viability was observed, accompanied by an increase in both PCV and biomass. This indicates that the cells were in the process of adapting to nutrient sources. During the log phase (days 6–12), logarithmic increases in biomass and PCV were observed, indicating that cell division accelerated and biomass accumulation occurred. In the stationary phase (days 12–21), while biomass continued to increase until day 21, cell viability decreased. However, dry weight decreased on day 21. This suggests that the cells underwent stress due to nutrient limitations, which likely increased their water retention capacity. In contrast, the decline in cell viability during the stationary phase indicates that increasing physiological stress began to limit growth. This growth pattern is generally consistent with the general growth model described in the literature [32] and those reported for other plant cell suspension cultures. Cell suspension cultures of different plant species exhibit distinct growth patterns. For example, in *V. thapsus* suspension cultures [14], settled cell volume increased rapidly after an initial 4-day lag phase, reached nearly sixfold of the initial level by day 12, and then slightly declined during later incubation, supporting the growth pattern observed in the present study. In addition, in *Hylocereus costaricensis* (F.A.C.Weber) Britton & Rose, the exponential phase began on day 16 and continued until day 28 without entering a stationary phase [33]. *Origanum vulgare* L. showed a growth cycle of 16 days, with the exponential phase beginning on day 8 [34]. In *Linum usitatissimum* L., fresh weight was reported to double after day 5 of a 14-day suspension culture, and greatest increase in biomass was observed between days 7 and 10 of culture [35]. Overall, these findings indicate that the growth pattern of *V. hasbenlii* cell suspension cultures is consistent with that reported for other medicinal and horticultural plant species and provides a useful basis for further optimization of culture conditions and metabolite production. Based on the integration of growth dynamics and statistical analyses, day 9 was selected as the optimal time point for elicitor treatments, as this stage corresponds to the early exponential growth phase, characterized by accelerating biomass accumulation, increasing PCV values, and relatively high cell viability.

## 5. Conclusions

In this study, an efficient callus induction and cell suspension culture system was established for the endemic species *V. hasbenlii*. Among six different MS media containing various concentrations and combinations of plant growth regulators, the combination of 0.5 mg/L BAP + 0.5 mg/L NAA was identified as the most suitable for callus induction, providing low explant browning, high callus biomass, diameter, and a compact-friable morphology favorable for suspension culture establishment. The suspension culture exhibited a typical growth pattern consisting of lag, exponential, and stationary phases. Based on growth dynamics and cell viability, day 9 was determined as the most suitable time point for elicitor treatments in future studies. Overall, the in vitro culture systems established in

this study provide a useful platform for further studies on secondary metabolite production and biotechnological applications in *V. hasbenlii*.

**Author Contributions:** Conceptualization, E.C. and N.Ç.; methodology, E.C. and N.Ç.; software, E.C.; validation, E.C.; formal analysis, E.C.; investigation, E.C.; resources, E.C.; data curation, E.C.; writing—original draft preparation, E.C.; writing—review and editing, E.C. and N.Ç.; visualization, E.C.; supervision, N.Ç.; project administration, E.C. and N.Ç.; funding acquisition, E.C. and N.Ç. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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