MOLECULAR WEIGHT AND CONCENTRATION-ASSOCIATED EFFECTS OF CHITOSAN IN *DORYCNIUM GERMANICUM* MICROPROPAGATION

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Abstract

*Micropropagation of habitat-specialised species requires protocols that support shoot multiplication while maintaining plantlet quality. This study evaluated how cytokinins and selected low molecular weight chitosan fractions modify in vitro shoot growth and leaf colour of Dorycnium germanicum. Nodal explants were cultured on Murashige and Skoog medium supplemented with BAP or zeatin, or with chitosan fractions ($CH_{3.33kDa}$ or CH_{8kDa}) applied at 0.5–2.0 mg/L. The highest number of newly formed shoots was observed with BAP at 1.0 mg/L, whereas 2.0 mg/L promoted shoot elongation but resulted in fewer new shoots. The response to chitosan depended mainly on the tested fraction, with concentration-associated differences within each fraction. CH_{8kDa}, particularly at 1.0 mg/L supported shoot elongation, while $CH_{3.33kDa}$ reduced shoot length and shifted CIE L*a*b* parameters away from the control profile. Overall, chitosan should be considered a supportive additive rather than a substitute for cytokinin-based shoot multiplication in preliminary conservation-oriented micropropagation of D. germanicum.*

Keywords: *in vitro culture, conservation biotechnology, low molecular weight chitosan, 6-benzylaminopurine, silky dorycium, CIE L*a*b**

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1. Introduction

The genus *Dorycnium* (including the *Lotus dorycnium* complex) comprises perennial species of the Fabaceae family that inhabit dry xerothermic grasslands of Central Europe [1]. Silky dorycium (*Dorycnium germanicum*) forms low tussocks on calcareous, drought-prone slopes and is characterised by a deep root system, which constitutes a key adaptation to water deficit and poor, skeletal soils. Owing to its habitat specialisation, this species is gaining importance in the context of biodiversity conservation and the restoration of degraded patches of xerothermic grasslands [1, 2]. Such xerothermic and calcareous grasslands are among the most species-rich semi-natural habitats in Europe, but their persistence is strongly dependent on appropriate management. Their decline has been linked mainly to habitat fragmentation, agricultural intensification, abandonment of traditional grazing, secondary succession and gradual overgrowth by shrubs or forest vegetation [3]. Therefore, plant species adapted to these habitats may be locally vulnerable even when they are not uniformly threatened across their entire distribution range. In this context, *ex situ* approaches may provide useful complementary tools for maintaining plant material of habitat-specialised taxa and reducing the need for repeated collection from natural populations [3, 4]. Beyond its ecological value, *D. germanicum* is also of interest as a representative of a genus known to accumulate phenolic compounds, including flavonoids and tannins. Although the phytochemical profile of *D. germanicum* itself remains insufficiently characterised, studies on related *Dorycnium* taxa indicate the presence of biologically active metabolites and report antioxidant, antimicrobial, anti-inflammatory and cytotoxic properties [5, 6]. This provides an additional rationale for developing controlled *in vitro* culture systems, as they may enable the production of uniform plant material for future phytochemical and physiological studies. Importantly, such an approach may reduce the need for harvesting plants from natural or semi-natural habitats, which is particularly relevant for species associated with rare and sensitive grassland ecosystems [3, 4].

In light of the above, *in vitro* cultures may be considered a tool supporting species conservation and the controlled production of biomass for research and reclamation-related applications. Plant tissue cultures of legumes, including species of forage and medicinal importance, are widely used for the mass production of plant material, the preservation of genetic resources (*ex situ*), and the modulation of secondary metabolite profiles under controlled conditions [4]. Despite the lack of dedicated micropropagation protocols for silky dorycium, experience with other representatives of the Fabaceae indicates that effective shoot regeneration can be achieved on MS [7] media supplemented with cytokinin (e.g., 6-benzylaminopurine (BAP), zeatin or thidiazuron (TDZ)), often in combination with auxins (1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA)). Such combinations may improve both multiplication efficiency and regenerant quality [8]. The development of an *in vitro* propagation system for *D. germanicum* may therefore be of practical importance, as it would provide controlled plant material for further studies and reduce reliance on plants collected from natural habitats [8, 9].

Among the potential factors that can improve micropropagation efficiency, chitosan deserves particular attention as a natural biopolymer with biostimulatory activity in plant *in vitro* cultures [10, 11]. It has been shown that chitosan may stimulate cell division and shoot elongation, which are important for both the efficiency of *in vitro* cultures and the maintenance of the bioactive potential of the biomass [8, 9, 12]. Additionally, chitosan activates plant defence-related pathways, including the induction of reactive oxygen species and the increased activity of antioxidant enzymes, which may improve explant tolerance to abiotic stresses under *in vitro* conditions [13]. In studies on other legume

species, chitosan (0.5–2.0 mg/L) added to the culture medium increased shoot multiplication by 20–50%, improved rooting, and enhanced bioactive compound content, making it a promising additive for future protocols for *D. germanicum* [8, 14].

To the best of our knowledge, no dedicated *in vitro* propagation procedure has yet been described for *D. germanicum*. Establishing such a system may be important as an initial *ex situ* tool for maintaining and multiplying plant material of this habitat-specialised species, while also providing uniform biomass for further ecological, physiological and phytochemical investigations [3, 4]. However, the optimisation of micropropagation should not be limited only to shoot number but should also consider shoot elongation and visual quality, which may influence the usefulness of regenerants for subsequent culture stages and acclimatisation. The integration of classical growth regulators with selected biostimulatory additives, such as low molecular weight chitosan fractions, may therefore represent a useful approach for preliminary protocol development [8, 14]. In the present study, BAP and zeatin were selected as representative cytokinin treatments to stimulate shoot development, whereas two low molecular weight chitosan fractions were included as natural biostimulatory and elicitor-like additives with potential effects on shoot growth and visual quality. Accordingly, the aim of this study was to evaluate the effects of selected cytokinins and two low molecular weight chitosan fractions on shoot multiplication, shoot elongation, and leaf colour parameters of *D. germanicum* under *in vitro* conditions.

2. Materials and Methods

2.1. Plant Material

In vitro shoot cultures of silky dorycium (*Dorycnium germanicum* L.) were used as plant material. The initial plant material originated from the collection of the Centre for Research and Conservation of Biological Diversity in Kielce, Poland, and was subsequently established and maintained under *in vitro* conditions. The taxonomic identity of the material was confirmed before the establishment of the *in vitro* cultures on the basis of morphological characteristics consistent with the species description. Stem segments (15–18 mm) excised from previously established *in vitro* cultures maintained under the same conditions served as primary explants for all experimental variants.

2.2. Chitosan Preparation and Characterisation

The chitosan used in the experiment was not a commercial preparation, but it was obtained from shrimp shells according to the procedure described by Bartkowiak [15]. Low molecular weight chitosan fractions were prepared by oxidative depolymerisation through the gradual addition of hydrogen peroxide at a dose of 0.8–6.4 mM per g of polysaccharide to a 2.5% (w/w) chitosan solution adjusted to pH 3.5–4.0 and maintained at 80 °C. The resulting preparations were obtained as chloride salts and characterised by a high degree of deacetylation (> 95%) and a relatively uniform molecular weight distribution.

The molar mass of the chitosan fractions was determined by HPLC/GPC using a Knauer SmartLine system equipped with a refractive index detector (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany), following the previously reported procedure [15]. The fractions of 3.33 and 8.00 kDa average molar masses were coded as $CH_{3.33kDa}$ and CH_{8kDa}, respectively. The viscosity of the chitosan preparations was not determined. After autoclaving, the molecular weight and viscosity of the chitosan preparations were not re-determined.

2.3. Culture Medium Composition and Sterilisation

Explants were aseptically placed on MS basal medium, containing macro- and micronutrients as well as vitamins according to the original Murashige and Skoog [7] formulation, under a laminar-flow cabinet. All media variants were supplemented with 3% (w/w) sucrose (Chempur, Piekary Śląskie, Poland), 0.8% (w/w) agar (Biocorp, Warsaw, Poland), and 100 mg·/L myo-inositol (Duchefa Biochemie, Haarlem, The Netherlands).

The MS medium composition was supplemented according to the experimental design, which comprised the following variants:

- 0.1 or 0.2 mg/L of zeatin (Zea) (Sigma-Aldrich, St. Louis, MO, USA);
- 0.5, 1.0, 1.5 or 2.0 mg/L of 3.33 and 8 kDa molecular weight chitosan (CH);
- 1.0 or 2.0 mg/L of 6-benzylaminopurine (BAP) (Sigma-Aldrich, St. Louis, MO, USA)

The control treatment consisted of plain MS medium without cytokinin or chitosan supplementation.

The tested concentrations were selected based on the available literature and the authors' previous studies on the use of plant growth regulators and chitosan in *in vitro* cultures [8, 11, 13]. Cytokinin concentrations were chosen to represent levels commonly used for shoot induction and multiplication, whereas chitosan concentrations were selected within a low-dose range previously reported to show biostimulatory effects without causing excessive growth inhibition.

Before sterilisation, the required amounts of chitosan preparations were dissolved in water and added directly to the MS medium to obtain the final concentrations specified above. The pH of all media was adjusted to 5.7–5.8. After complete dissolution (under heating), 30 mL aliquots of medium were dispensed into 450 mL culture vessels. The media, including those containing chitosan, were sterilised by autoclaving at 121 °C and 0.1 MPa for 20 min. This sterilisation time was applied to the 30 mL medium volume used in each culture vessel. After sterilisation, no visible precipitation or loss of solubility was observed in the chitosan-containing media.

2.4. Culture Conditions and Experimental Design

Cultures were maintained in a growth chamber under a 16 h photoperiod at 24 ± 2 °C and a photosynthetic photon flux density (PPFD) of 40 µmol/(m^2·s). Each experimental variant, defined as a specific medium composition, was established in eight culture vessels, each considered a replicate, with four explants per vessel. Thus, each experimental variant comprised 32 explants. After 35 days of culture, the following parameters were evaluated: shoot length (cm), number of newly formed shoots (per explant), and leaf colour attributes.

2.5. Leaf Colour Measurement

Leaf colour was measured using a CM-700d spectrophotometer (Konica Minolta, Tokyo, Japan) in the Cie L*a*b* colour space, using a 10° standard observer and D65 illuminant [16]. Measurements were taken on leaf tissue collected from the middle portion of shoots. 32 independent replicates were analysed for each experimental variant [12]. The overall colour difference, ΔE^*_{ab}, was calculated descriptively for each treatment in relation to the control MS medium using mean L*, a* and b* values according to the formula:

$$\Delta E^*_{ab} = \sqrt{\left(\Delta L^*\right)^2 + (\Delta a^*)^2 + \left(\Delta b^*\right)^2} \quad (1)$$

where ΔL^*, Δa^*, Δb^* represent differences between each treatment and the control MS medium. Because ΔE^*_{ab} was calculated from mean values, it was used as a descriptive indicator of total colour difference and was not subjected to separate statistical analysis.

2.6. Statistical Analysis

All data were analysed using Statistica 13.0 software (StatSoft, distributed in Poland by StatSoft Poland, Kraków). Prior to inferential testing, data were evaluated for normality and homogeneity of variance. Differences among treatments were assessed by analysis of variance (ANOVA), and mean separation was performed using Tukey's honestly significant difference (HSD) test at a significance level of $p < 0.05$.

3. Results and Discussion

Table 1 presents the phenotypic response of *D. germanicum* explants to different categories of medium additives tested in this preliminary optimisation experiment. Cytokinin treatments were included to assess classical shoot multiplication responses, whereas chitosan treatments were evaluated as potential supportive biostimulatory additives. The highest mean number of newly formed shoots per explant was obtained on MS medium supplemented with 1.0 mg/L BAP (3.83), indicating that this treatment was the most effective for shoot multiplication. In turn, increasing the BAP concentration to 2.0 mg/L was associated with an increase in mean shoot length (3.26 cm), accompanied by a reduction in the number of newly formed shoots (2.25) (Figure 1, Table 1), suggesting that within the tested range, the response shifted from intensive proliferation towards stronger elongation. This pattern is consistent with previous studies on Fabaceae species showing that morphogenetic responses *in vitro* are strongly influenced by the type and concentration of cytokinins, as well as by the competence of the cultured explants. In *Vigna subterranea*, different cytokinins, including BAP, zeatin, and TDZ, produced distinct organogenic responses depending on explant characteristics and genotype [17]. Similarly, in *Ceratonia siliqua*, zeatin and BAP differed in their effects on shoot elongation and shoot formation [18], whereas in *Albizia amara*, BAP was more effective than zeatin in terms of shoot response, shoot number, and shoot length [19]. This confirms that cytokinin treatments affected not only the intensity of shoot formation but also the balance between multiplication and elongation. Such a pattern is consistent with the typical role of cytokinins in promoting axillary shoot proliferation, where increased multiplication may occur at the expense of individual shoot length. In the present study, zeatin also increased shoot formation at 0.1 mg/L (2.5), although its effect was weaker than that of BAP under the tested conditions.

It should also be noted that not all cytokinin treatments differed significantly from the control medium for all analysed traits. This suggests that the response of *D. germanicum* to exogenous cytokinins was strongly concentration-dependent and that some of the tested concentrations may not have been optimal for this species or for the evaluated stage of culture. Therefore, the lower effectiveness of zeatin compared with BAP should not be interpreted as a general feature of zeatin, but rather as a response observed under the specific experimental conditions of this study. Further optimisation of cytokinin type and concentration would be required to determine whether other zeatin levels or combinations with auxins could improve the balance between shoot multiplication and elongation.

Table 1. Effect of different medium composition on morphological traits of *Dorycnium germanicum* L. plants under *in vitro* conditions after 35 days of culture (n = 32 shoots per treatment).

Medium	Shoot length [cm]	Number of new shoots per plant
1. MS	2.89 ± 1.11^{abc}	1.00 ± 0.00^{d}
2. MS+0.1 mg/L Zea	1.90 ± 0.55^{cde}	2.50 ± 0.52^{b}
3. MS+0.2 mg/L Zea	1.37 ± 0.53^{defg}	1.33 ± 0.49^{cd}
4. MS+1.0 mg/L BAP	2.30 ± 0.75^{bcd}	**3.83 ± 1.45^{a}**
5. MS+2.0 mg/L BAP	3.26 ± 0.59^{ab}	2.25 ± 1.06^{bc}
6. MS+0.5 mg/L $CH_{3.33\ kDa}$	1.31 ± 0.20^{defg}	1.00 ± 0.00^{d}
7. MS+1.0 mg/L $CH_{3.33\ kDa}$	0.98 ± 0.26^{efg}	1.58 ± 0.67^{bcd}
8. MS+1.5 mg/L $CH_{3.33\ kDa}$	0.79 ± 0.27^{fg}	1.00 ± 0.00^{d}
9. MS+2.0 mg/L $CH_{3.33\ kDa}$	0.40 ± 0.10^{g}	1.00 ± 0.00^{d}
10. MS+0.5 mg/L CH_{8kDa}	1.48 ± 0.54^{def}	1.42 ± 0.42^{cd}
11. MS+1.0 mg/L CH_{8kDa}	**3.51 ± 1.08^{a}**	2.00 ± 0.64^{bcd}
12. MS+1.5 mg/L CH_{8kDa}	2.62 ± 0.92^{abc}	2.08 ± 0.69^{bc}
13. MS+2.0 mg/L CH_{8kDa}	2.33 ± 0.81^{bcd}	1.83 ± 0.83^{bcd}

Note. Means followed by the same letter do not differ significantly at $p < 0.05$ according to the Tukey multiple range test.

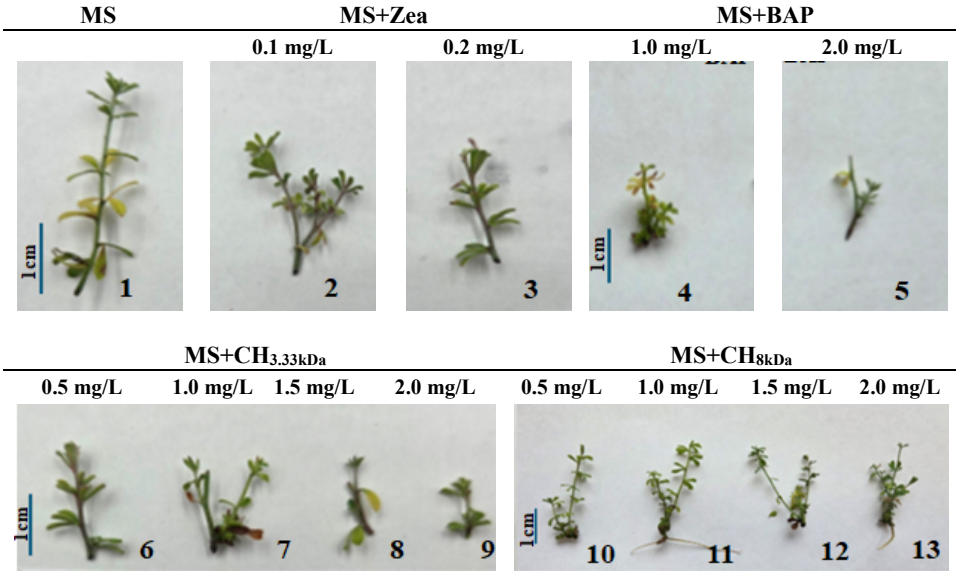

Figure 1. Effects of BAP, Zea and different molecular weight CH at various concentrations on micropropagation of *D. germanicum* L. plants.

The variability of responses to different cytokinins and their concentrations is consistent with reports indicating a strong dependence of morphogenetic responses on the type and dose of PGRs (Plant Growth Regulators), as well as on the physiological competence of the tissues [4].

The variability of responses to different cytokinins and their concentrations is consistent with reports indicating a strong dependence of morphogenetic responses on the type and dose of PGRs (Plant Growth Regulators), as well as on the physiological competence of the tissues [4]. From a practical perspective, within the tested concentration range, BAP proved to be a more effective solution for intensifying shoot multiplication than the applied doses of zeatin, which may be important when designing an *ex situ* protocol aimed at the rapid increase in the number of regenerants and the reduction of pressure on natural populations [1, 4].

In contrast to classical plant growth regulators, chitosan caused a differentiated response that depended mainly on the chitosan fraction, while the effect of concentration was less consistent (Table 1) [10, 11]. Namely, CH with a molecular weight of 3.33 kDa reduced shoot elongation compared with the control, particularly at 1.5 and 2.0 mg/L, and did not significantly increase the number of newly formed shoots. Although the 1.0 mg/L CH3.33 kDa treatment resulted in numerically higher shoot numbers than the control (158% of the control), these differences were neither consistent nor statistically significant. In contrast, CH_{8kDa} gave the highest mean shoot length at 1.0 mg/L (121% of the control). At 1.5 mg/L CH_{8kDa}, shoot length remained statistically comparable with the control (91% of MS) and 2.0 mg/L BAP, whereas shoot number was higher than in the control (of 208%) but lower than in 1.0 mg/L BAP. Thus, CH_{8kDa} supported shoot elongation more effectively than $CH_{3.33kDa}$ under the tested conditions, but the results should be interpreted as a fraction-related response with concentration-associated trends rather than as a strictly dose-dependent effect.

This type of response is consistent with the dual role of chitosan as a biostimulatory and elicitor-like compound. At an appropriate concentration, chitosan may support cell division, cell elongation, and developmental processes, whereas higher concentrations may intensify stress-related responses and reduce growth. Similar non-linear responses have been reported in plant tissue cultures, where low or intermediate chitosan concentrations promoted morphogenesis or plantlet growth, while higher concentrations produced no additional stimulation or even inhibitory effects. Rahmah *et al.* [20] showed that in *Dendrobium* cultures, 1 mg/L chitosan promoted protocorm-like body proliferation, whereas higher concentrations were less effective or inhibitory. Likewise, in micropropagated *Dendrobium* plantlets, a lower chitosan concentration enhanced shoot, leaf, and root formation, while higher concentrations did not produce a stimulatory effect. Therefore, the pronounced response of *D. germanicum* to 1.0 mg/L CH_{8kDa} may reflect an effective concentration range in which the biostimulatory effect of chitosan was expressed without inducing excessive elicitor-related growth limitation.

The present results indicate that the response of *D. germanicum* to chitosan was associated with both the applied concentration and the molecular weight of the tested fractions. However, other physicochemical properties of chitosan, such as degree of polymerisation, degree of acetylation/deacetylation, solubility and charge density, were not directly analysed in this study. According to previous reports [13, 9, 14], these parameters may influence chitosan interactions with the plant cell surface, including the cell wall and plasma membrane, and may affect whether the plant response is expressed primarily as growth stimulation or as an elicitor-like stress response. Therefore, the observed differences between $CH_{3.33kDa}$ and CH_{8kDa} should be interpreted with caution as phenotypic responses associated with the tested chitosan fractions, rather than as direct evidence of the specific mechanisms underlying their biological activity [13, 9, 14]. Chitosan and its oligomers can induce ion fluxes, membrane-related responses, ROS

formation, callose deposition, activation of defence-related enzymes and changes in phenylpropanoid metabolism. At the same time, under appropriate conditions, chitosan may support plant growth by affecting cell division, cell elongation, nutrient uptake and hormone-related processes [21]. In the present study, no biochemical traits, stress markers, antioxidant enzyme activity, or pigment contents were measured. Consequently, the data support only a phenotypic interpretation of the response, not a direct confirmation of the physiological mechanisms involved. The stronger shoot elongation observed after application of 1.0 mg/L CH_{8kDa} may indicate that this fraction and concentration provided a more effective balance between biostimulatory activity and the absence of excessive elicitor-like growth limitation. In contrast, the 3.33 kDa fraction may have been less suitable for supporting shoot elongation in *D. germanicum* under the tested conditions. Overall, the obtained results indicate that the response of *D. germanicum* explants was strongly dependent on the type of medium supplement and the analysed trait. Cytokinin-containing media primarily affected shoot multiplication, whereas the response to chitosan was more fraction-specific and less consistently concentration-dependent. From an applied perspective, the results also suggest that a two-step system could be worth testing in future studies, with BAP used during the multiplication stage and selected chitosan treatments evaluated subsequently as a conditioning step to improve shoot quality. Since such a sequential protocol was not directly tested here, this possibility should be regarded as a working hypothesis for future optimisation rather than as a confirmed recommendation [8, 9, 12].

In micropropagation studies, leaf colour analysis may provide supplementary information on plantlet quality and phenotype stability. Changes in leaf colour can reflect differences in pigment-related traits, tissue development, physiological status, or stress-related responses under *in vitro* conditions [10, 12, 22]. Although colour measurements do not replace direct biochemical analyses of chlorophylls, carotenoids, or stress markers, they can serve as a non-destructive indicator of whether regenerated shoots maintain a visually stable and potentially healthy phenotype. Therefore, in the present study, colour parameters were assessed as additional quality-related traits that complemented the morphological evaluation of shoot multiplication and elongation. Leaf colour was evaluated using the Cie L*a*b* system, which enabled separate analysis of L* (lightness), a* (green-red coordinate), and b* (blue-yellow coordinate). The observed differences are discussed below as instrumentally measured changes in individual colour parameters rather than as general visual changes. This distinction is important because differences detected by spectrophotometric measurements may not necessarily correspond to changes that are clearly visible to the naked eye.

Modification of the culture medium with growth regulators and chitosan affected the instrumentally measured colour parameters of *D. germanicum* shoots, and the direction of these changes depended on the type of additive, its concentration and, in the case of chitosan, the tested fraction (Figures 2 and 3). These observations refer to the present experiment and describe treatment-related differences in the measured colour coordinates. Previous studies have shown that plant growth regulators and chitosan-based additives may influence pigment-related traits, photosynthetic activity, and stress-related responses in plant tissues [8, 9, 14]. Therefore, the colour changes observed here may be considered supplementary indicators of phenotype modulation, although their physiological basis was not directly analysed in this study. The L* parameter describes leaf lightness, with higher values indicating lighter tissue. The highest L* value was recorded for explants cultured on MS+0.1 mg/L Zea (58.98), whereas the lowest L* values were noted for explants cultured on MS+CH_{8kDa} at 1.0 and 2.0 mg/L content

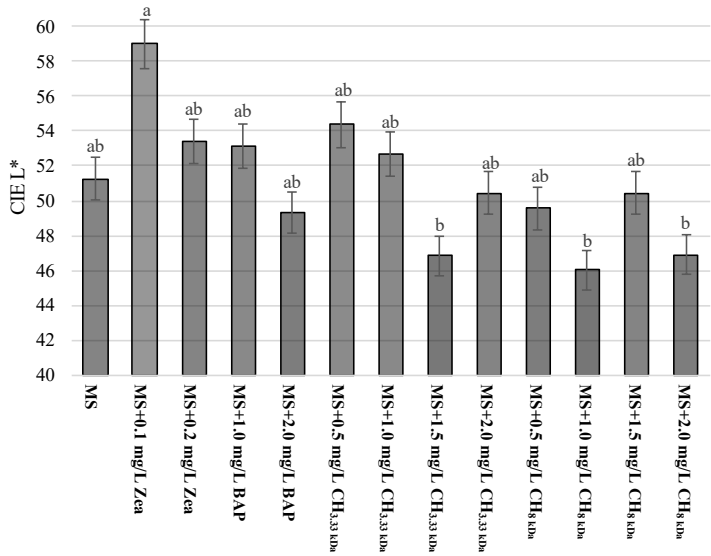

Figure 2. Effect of different medium composition on the lightness (L*) of *D. germanicum* L. leaf colour in CIE L*a*b* colour system at the end of the experiment. Bars followed by the same letter do not differ significantly at $p < 0.05$ according to the Tukey multiple range test.

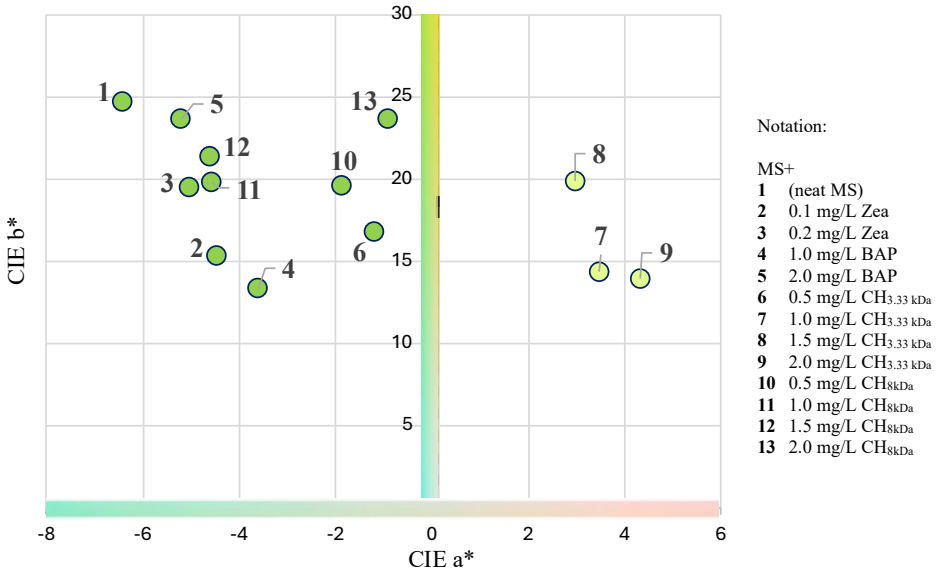

Figure 3. Effect of different medium composition on the a* (red-green axis) and b* (blue-yellow axis) of *D. germanicum* L. leaf colour in CIE L*a*b* colour system at the end of the experiment.

(46.02 and 46.89, respectively) and MS+$CH_{3.33kDa}$ at 1.5 mg/L (46.86) (Figure 2). However, because the control treatment belonged to an overlapping homogeneous group, decreases in L* relative to the control should be interpreted cautiously as treatment-associated tendencies rather than as uniformly confirmed differences. Because L* may be

affected by tissue structure, hydration, and pigment-related traits, it should be treated as a supplementary phenotypic indicator rather than a direct physiological marker.

Among the analysed colour coordinates (Figure 3), the a* parameter showed clear treatment-related shifts, particularly in explants cultured on media supplemented with the $CH_{3.33kDa}$ fraction. Negative a* values indicate a stronger green component, whereas values shifted towards zero or positive values indicate a shift towards the red component. Explants cultured on neat MS medium reached the lowest a* values (−6.41), indicating the strongest shift towards the green component among all treatments. In contrast, the application of $CH_{3.33kDa}$ at 1.0 and 2.0 mg/L caused a shift in a* towards positive values (+2.97 and +4.32), indicating a marked increase in the red component. By comparison, shoots cultured on BAP-containing media maintained negative a* values (−3.62 and −5.21), which were closer to the control. The contrast between the two chitosan fractions was also evident. Samples cultured on MS+CH_{8kDa} (samples 10–13) showed clearly negative a* values, whereas those treated using MS+$CH_{3.33kDa}$ exhibited mostly, except MS+$CH_{3.33kDa}$ 0.5 mg/L, positive a*.

From a physiological perspective, changes in a* may reflect differences in chlorophyll-related greenness, but they may also be influenced by other pigment groups, including carotenoids and anthocyanins, as well as by stress-related changes in tissue development. In micropropagated plantlets, negative a* values may be interpreted as an indirect indicator of a more stable green phenotype, which is generally desirable during shoot multiplication and elongation. Previous studies have shown that chitosan may modify plant growth, stress responses, and pigment-related traits depending on plant species, concentration, and experimental conditions. For example, chitosan-related responses were reported in *Petunia* × *atkinsiana* under salinity stress, while recent reviews have emphasised the species- and dose-dependent effects of chitosan in plant stress responses and tissue culture systems [9, 13, 14]. However, without direct analyses of chlorophylls, carotenoids, anthocyanins, or stress-related metabolites, the underlying biological basis of these observed colour shifts remains hypothetical. Thus, the changes in a* should be treated as instrumentally measured indicators of phenotype modulation rather than as direct evidence of specific pigment changes.

Changes in the b* parameter were less consistent than those observed for a* (Figure 3). The highest mean b* value (+24.62) was observed for explants cultured on the MS medium. Comparable values were also recorded for explants multiplied on MS+2.0 mg/L BAP and MS+2.0 mg/L CH_{8kDa} (both reaching +23.65). The lowest mean b* value was observed for *D. germanicum* plants grown on MS+1.0 mg/L BAP (+13.34), indicating a highest reduction in the yellow component in this treatment compared with neat MS. Since b* values may be influenced by carotenoid accumulation, chlorophyll-to-carotenoid balance, leaf maturation, tissue hydration and stress-related metabolic responses, these results suggest that medium composition affected the chromatic balance of *D. germanicum* shoots. Nevertheless, as direct pigment analysis has not been performed, the observed b* changes should be interpreted only as descriptive evidence of colour-parameter modulation.

To comprehensively assess the effect of medium additive on leaf colour differences, the ΔE^*_{ab} was calculated in relation to the control MS (Figure 4). The colour differences analysis generally confirmed the interpretation based on individual CIE L*a*b* parameters. The highest ΔE^*_{ab} values were found for MS+2.0 mg/L $CH_{3.33kDa}$ treatment (15.14), MS+1.0 mg/L $CH_{3.33kDa}$ (14.35), as well as for MS+0.1 mg/L zeatin (12.23), indicating the strongest overall shifts in colour parameters. In contrast, the lowest ΔE^*_{ab}

were recorded for the selected MS+CH_{8kDa} treatment, suggesting that it maintained the overall CIE L*a*b* profile closest to the control.

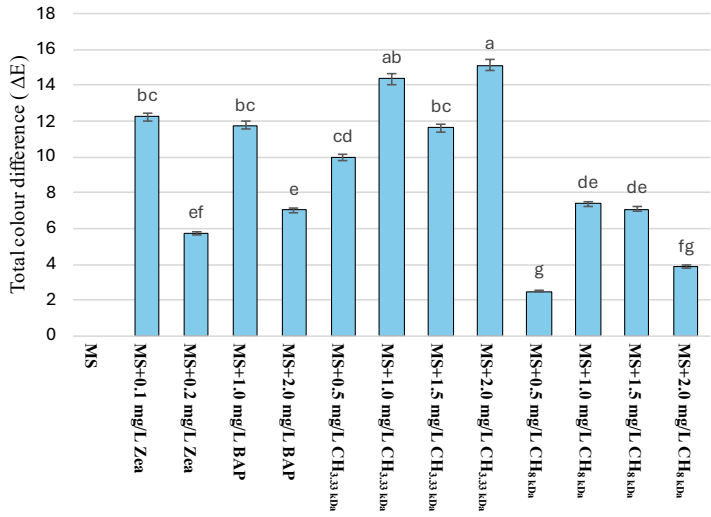

Figure 4. Effect of different medium composition on the difference in *D. germanicum* L. leaf colour in CIE L*a*b* colour system at the end of the experiment. Bars followed by the same letter do not differ significantly at $p < 0.05$ according to the Tukey multiple range test.

The overall effects of cytokinin-containing media on colour-related parameters may be linked to the known influence of cytokinins on chloroplast development, chlorophyll accumulation, and photosynthetic activity in *in vitro* shoots [22]. Similarly, chitosan may affect photosynthetic performance, antioxidant defence, secondary metabolism, and stress-related signalling in plants [14]. Therefore, the present results do not allow a direct conclusion that a given colour profile itself was responsible for improved growth. However, comparison of these traits may help to identify treatments that combine desirable morphogenetic features with limited changes in instrumentally measured colour parameters. In this context, CH_{8kDa}, which generally resulted in long shoots (Table 1), with the highest length noted for MS+1.0 mg/L CH_{8kDa}, resulted simultaneously in the lowest ΔE^*_{ab}. In contrast, MS+$CH_{3.33kDa}$, particularly at 1.0 and 2.0 mg/L, mostly resulted in reduced (vs MS) shoot length with simultaneous shift of a* towards higher (positive) values, resulting in higher overall colour differences vs MS. This suggests that the two chitosan fractions differed not only in their effects on shoot growth but also in their influence on colour-related phenotype parameters. These findings are relevant to micropropagation, as successful propagation depends not only on multiplication rate but also on the quality of the regenerated plantlets. Shoot length, colour stability, the absence of abnormal pigmentation, and the maintenance of a green phenotype may influence the suitability of regenerated shoots for subsequent culture stages, rooting, and acclimatisation [10, 12]. Thus, instrumentally measured leaf colour parameters may serve as supplementary, non-destructive indicators of plantlet quality, although they should ideally be combined with direct measurements of photosynthetic pigments, nutrient status and stress markers in future studies.

From a conservation-oriented perspective, the distinction between treatments that promote rapid multiplication and those that may support selected quality-related traits is particularly important. For a rare or habitat-specialised species such as *D. germanicum*, an efficient multiplication phase is essential to reduce collection pressure on natural populations and to establish a reproducible *ex situ* propagation route. In this respect, 1.0 mg/L BAP should be regarded as the most effective treatment tested for shoot multiplication. Chitosan, especially CH_{8kDa}, may still have practical value in further protocol development, but its role appears supportive rather than central at this stage. The present findings therefore provide a useful framework for further refinement of *D. germanicum* micropropagation, while also indicating that different protocol components may need to be optimised for different objectives, such as multiplication efficiency, elongation, or phenotype stability.

4. Conclusions

The present study showed that the *in vitro* response of *D. germanicum* explants varied depending on the type of medium additive and the trait analysed. Among the cytokinin treatments, MS medium supplemented with 1.0 mg/L BAP produced the highest mean number of newly formed shoots and was therefore the most effective treatment for shoot multiplication within the tested range. However, not all cytokinin-containing media differed significantly from the control for all analysed parameters, indicating that the response was concentration-dependent and that further optimisation of cytokinin type and dose is required.

The effect of chitosan was not uniformly stimulatory. The 8 kDa chitosan fraction, particularly at 1.0 mg/L, supported shoot elongation under the tested conditions, whereas the 3.33 kDa fraction reduced shoot length, especially at higher concentrations, and did not significantly improve shoot multiplication compared with the control medium. These results indicate that chitosan should not be regarded as a direct substitute for cytokinin-based shoot induction. Instead, selected chitosan fractions may have potential as supportive additives, but their usefulness depends on fraction properties and concentration.

Instrumentally measured Cie L*a*b* parameters showed that medium composition also influenced selected colour-related phenotype traits of regenerated shoots. The highest overall colour differences from the control, expressed descriptively as ΔE^*_{ab}, were observed in selected $CH_{3.33kDa}$ treatments, whereas selected CH_{8kDa} treatments showed a colour profile closer to the control.

Overall, these results provide a preliminary basis for further optimisation of *D. germanicum* micropropagation. For practical protocol development, BAP appears suitable for the multiplication stage, while selected CH_{8kDa} treatments may be considered in future studies as potential conditioning or elongation-supporting components. Further work should include rooting, acclimatisation, pigment and stress-marker analyses, and a broader range of cytokinin and chitosan treatments to verify the usefulness of these additives in a complete *ex situ* propagation system.

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6. Author Contributions

Conceptualisation, M.K.-M. and I.O; methodology, M.K.-M. and I.O; software, I.O.; validation, M.K.-M. and I.O; formal analysis, M.K.-M. and I.O; resources, M.K.-M. and I.O; writing—original draft preparation, M.K.-M. and I.O.; writing—review and editing, M.K.-M. and I.O; visualisation, M.K.-M. and I.O. All authors have read and agreed to the published version of the manuscript

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