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Innovative applications of flamingo explants and TDZ in *Digitalis purpurea* L. organogenesis

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Abstract: *Digitalis purpurea* L. is a valuable medicinal and ornamental species, but its conventional propagation is constrained by low seed germination and high genetic variability. The present study aimed to develop an efficient and reproducible *in vitro* regeneration system for *D. purpurea* through both direct and indirect organogenesis by optimizing explant type and plant growth regulator (PGR) combinations. Among the explants evaluated, hypocotyl-cotyledon (flamingo) explants exhibited the highest morphogenic competence, whereas leaf and root explants showed no regenerative response under any treatment. In direct organogenesis, MS medium supplemented with 0.25 mg L⁻¹ thidiazuron (TDZ) was the most effective, producing the maximum shoot length (6.3 mm) and shoot number (8.6 shoots per explant). Indirect organogenesis was strongly influenced by auxin type, with callus induction reaching 100% in flamingo explants cultured on media containing 0.5-1 mg L⁻¹ NAA, while 2,4-D was markedly less effective. Shoot regeneration from callus was significantly enhanced by cytokinin-polyamine interactions, with the highest shoot proliferation (up to 28 shoots per callus) and superior shoot elongation obtained on MS medium supplemented with 0.25-0.5 mg L⁻¹ BAP in combination with 50 mg L⁻¹ putrescine. Root induction was optimized on half-strength MS medium containing IBA, where 0.25 mg L⁻¹ IBA resulted in the highest rooting frequency (91%), maximum root number, and well-developed roots. Overall, this study highlights the critical role of explant selection and tailored PGR-polyamine combinations in establishing a high-efficiency micropropagation protocol for *D. purpurea*, providing a reliable platform for large-scale propagation and future biotechnological applications.

1. Introduction

Digitalis purpurea L. (foxglove) is a commercially significant biennial

plant, valued both as an ornamental and a vital medicinal herb. It is the primary source of cardiac glycosides, such as digoxin and digitoxin, which are crucial in the treatment of heart conditions (Bourgau *et al.*, 2001). These compounds inhibit the Na⁺/K⁺-ATPase pump, increasing myocardial contraction (Gavidia and Pérez-Bermúdez, 1997; Bourgau *et al.*, 2001). However, their narrow therapeutic index demands precise dosage, highlighting the need for standardized cultivation to ensure consistent metabolite levels (Gavidia and Pérez-Bermúdez, 1997).

Conventional propagation of *D. purpurea* via seeds faces significant challenges, including low natural germination rates ($\leq 2\%$) and high genetic variability in seed-grown populations, which complicate the production of uniform plant material (Mastenbroek, 1985). These limitations hinder reliable cultivation for both pharmaceutical and horticultural purposes. Moreover, inconsistent seed-based propagation constrains large-scale commercial production, especially where uniformity in bioactive compound profiles is critical for regulatory compliance.

Plant tissue culture techniques offer a robust solution by enabling the rapid, large-scale production of genetically uniform plants under controlled conditions (Abbaszadeh *et al.*, 2023). For medicinal species, efficient *in vitro* regeneration systems are essential for mass propagation, germplasm conservation, and future genetic improvement. While somatic embryogenesis is a powerful method for such goals (Quiroz-Figueroa *et al.*, 2006), successful protocol development is highly genotype-dependent (Blasco *et al.*, 2013). In the case of *D. purpurea*, existing studies primarily emphasize callogenesis and secondary metabolite accumulation, resulting in a noticeable research gap regarding dependable and reproducible whole-plant regeneration systems. Research on *in vitro* regeneration of *D. purpurea* remains limited, with studies often focusing on secondary metabolite production in callus cultures (Jan *et al.*, 2023) rather than efficient whole-plant regeneration pathways like organogenesis.

Given the global expansion of the medicinal plant industry and the emerging demand for standardized, high-quality raw material, establishing a robust regeneration platform for *Digitalis* spp. has become particularly timely. Given Iran's long history and significant role in medicinal plant cultivation within

the Middle East, developing advanced biotechnological protocols for key species is of strategic importance. A reproducible, genotype-independent regeneration approach would not only support commercial propagation but also facilitate downstream applications such as metabolic engineering, conservation programs, and genetic transformation aimed at enhancing cardiac glycoside biosynthesis.

To address the propagation constraints of *D. purpurea*, this study was designed to establish a reliable *in vitro* regeneration protocol. Specifically, we focused on direct organogenesis as a rapid and mutation-minimizing pathway for regenerating true-to-type plantlets. The present study aims to optimize an *in vitro* regeneration protocol for *D. purpurea* through direct organogenesis, with the following specific objectives: (a) to determine the optimal combination of PGRs and culture medium for inducing direct shoot organogenesis from seed-derived explants, and (b) to identify the most effective culture conditions for subsequent shoot elongation and root development to produce complete plantlets.

2. Materials and Methods

Plant material

Cultivated seeds of *D. purpurea* L. were collected from the Agricultural Sciences and Natural Resources University of Khuzestan (31°59' N, 48°88' E; 14 m above sea level) during early spring. Seeds were collected within a single growing season and were used immediately for experimental purposes. A voucher specimen (No. KHAU993) was prepared and deposited in the Herbarium of the Agricultural Sciences and Natural Resources University of Khuzestan for future reference.

Decontamination

Mature seeds were washed under running tap water for 20 min with the addition of one drop of liquid detergent. Surface sterilization was carried out by immersion in 70% (v/v) ethanol for 15 s, followed by treatment with 5% (v/v) sodium hypochlorite solution (commercial bleach, Clorox®) supplemented with one drop of Tween-20 for 5 min. Sterilized seeds were rinsed six times with sterile distilled water under aseptic conditions and cultured on a germination medium consisting of half-strength

Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar. The pH was adjusted to 5.7 prior to autoclaving. Cultures were maintained in 100 mL glass jars containing 25 mL of medium. Contamination frequency during seed germination was less than 2%.

Explant preparation

Four explant types were used: hypocotyls with one attached cotyledon (commonly referred to as “flamingo bill” explants due to their characteristic shape), leaves, nodes, and roots. Explants were excised aseptically from *in vitro*-germinated seedlings aged 10-15 days. The variation in seedling age reflected natural differences in germination timing. Explants were cut immediately prior to culture and transferred to media without delay. Leaf and node explants were approximately 7 mm in length, whereas root and hypocotyl-cotyledon explants were approximately 10 mm long. Slight variation in explant size was unavoidable due to the curved morphology of certain tissues and manual excision. All explants were cultured on full-strength MS basal medium containing MS salts and vitamins, 30 g L⁻¹ sucrose, and 8 g L⁻¹ agar, with pH adjusted to 5.7 before autoclaving.

Direct organogenesis

For direct organogenesis, explants were cultured on MS basal medium supplemented with different concentrations of 6-benzylaminopurine (BAP) or thidiazuron (TDZ), as detailed in Table 1. A plant growth regulator (PGR)-free MS medium served as the control. Media were dispensed into 100 mL glass jars (25 mL per jar) and autoclaved at 121°C for 15 min. Cultures were maintained in a growth chamber at 25 ± 1°C under a 16 h photoperiod provided by

cool-white fluorescent lamps with a photosynthetic photon flux density (PPFD) of 50 µmol m⁻² s⁻¹. Each treatment consisted of three biological replicates, with 10 explants per replicate (n= 30). After six weeks of culture, regeneration responses were evaluated by recording (1) the percentage of explants producing shoots, (2) the mean number of shoots per responsive explant, and (3) the mean length (cm) of the longest shoot. The experiment followed a factorial design with explant type and PGR concentration as experimental factors.

Indirect organogenesis via callus culture

Callus induction. For callus induction, explants were cultured on MS basal medium supplemented with various combinations of α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and BAP, as shown in Table 2. A PGR-free MS medium was used as the control. Cultures were maintained at 25 ± 1°C in continuous darkness (24 h). Each treatment consisted of three biological replicates, with 10 explants per replicate. After six weeks, callus induction frequency (%) and mean callus fresh weight (mg) per responsive explant were recorded. This stage followed a factorial experimental design with explant type and PGR treatment as factors.

Table 2 - PGR combinations for callus induction media

Treatment code	NAA (mg L ⁻¹)	2,4-D (mg L ⁻¹)	BAP (mg L ⁻¹)	Basal medium
C1 (Control)	0.0	0.0	0.0	MS
C2	0.5	0.0	0.0	MS
C3	1.0	0.0	0.0	MS
C4	0.5	0.0	0.5	MS
C5	1.0	0.0	0.5	MS
C6	0.0	0.5	0.0	MS
C7	0.0	1.0	0.0	MS
C8	0.0	0.5	0.5	MS

Table 1 - Composition of media for direct shoot organogenesis

Treatment code	BAP (mg L ⁻¹)	TDZ (mg L ⁻¹)	Basal medium
D1 (Control)	0.00	0.00	MS
D2	0.25	0.00	MS
D3	0.50	0.00	MS
D4	1.00	0.00	MS
D5	0.00	0.25	MS
D6	0.00	0.50	MS
D7	0.00	1.00	MS

Shoot regeneration from callus. Friable and actively proliferating callus obtained from the most effective induction treatment was selected for shoot regeneration experiments. Approximately 100 mg of callus was transferred to MS basal medium supplemented with different combinations of BAP, NAA, and putrescine, as detailed in Table 3. Cultures were maintained under the same environmental conditions as described for direct organogenesis. The experiment followed a completely randomized one-

Table 3 - PGR combinations for shoot regeneration media from callus

Treatment code	BAP (mg L ⁻¹)	NAA (mg L ⁻¹)	Putrescine (mg L ⁻¹)	Basal medium
SR1	0.25	0.00	0.00	MS
SR2	0.50	0.00	0.00	MS
SR3	0.25	0.10	0.00	MS
SR4	0.50	0.10	0.00	MS
SR5	0.25	0.00	50.00	MS
SR6	0.50	0.00	50.00	MS

factor design based solely on PGR treatments. After six weeks, shoot regeneration efficiency was evaluated by recording (1) the percentage of callus clumps producing shoots, (2) the mean number of shoots per regenerating callus, and (3) the mean length (cm) of the longest shoot. No subculturing was performed during the regeneration period due to the absence of tissue browning or growth limitation.

Root induction

Shoots regenerated through both direct and indirect organogenesis (1.5-2.0 cm in length) were excised and transferred to rooting media. The basal medium consisted of half-strength MS salts and vitamins, 15 g L⁻¹ sucrose, and 8 g L⁻¹ agar (pH 5.7). Media were supplemented with indole-3-butyric acid (IBA) or NAA at the concentrations listed in Table 4. A PGR-free medium served as the control. Cultures were maintained under the same growth chamber conditions as described previously. Each treatment consisted of three biological replicates with 10 shoots per replicate (n = 30). After six weeks, rooting frequency (%), mean number of roots per plantlet, and mean root length (cm) were recorded.

Acclimatization and greenhouse transfer

Rooted plantlets were gently removed from the culture vessels, and residual agar was washed off with sterile distilled water. Plantlets were transplanted individually into sterile plastic pots (9

cm diameter × 8 cm height) containing an autoclaved substrate mixture of cocopeat and perlite (1:1, v/v). Pots were transferred to a greenhouse maintained at 25±2°C under a 16/8 h light/dark photoperiod, relative humidity of 85±5%, and a PPFD of approximately 55 µmol m⁻² s⁻¹. For the first two weeks, plantlets were covered with transparent polyethylene bags to maintain high humidity and were irrigated every four days with one-quarter-strength MS salt solution. The covers were gradually perforated and removed over the following two weeks. Acclimatization was conducted as a standard non-comparative step to complete the regeneration cycle. Survival rates were recorded 60 days after transfer.

Experimental design and statistical analysis

All experiments were conducted using a completely randomized design. Each treatment consisted of three independent biological replicates, with 10 explants, callus clumps, or shoots per replicate. Percentage data were arcsine square-root transformed prior to analysis. Data were subjected to analysis of variance (ANOVA). Factorial ANOVA was applied where appropriate (direct organogenesis and callus induction stages), while one-way ANOVA was used for subsequent experiments involving a single factor. Mean comparisons were performed using the Least Significant Difference (LSD) test at p<0.05, which is commonly applied in plant tissue culture studies. Statistical analyses were conducted using SPSS software (version 16.0), and results are presented as mean ± standard error (SE).

3. Results

Direct organogenesis

The results for both shoot length and number from direct organogenesis reveal consistent trends across explant types and cytokinin treatments. The flamingo (hypocotyl-cotyledon) explant demonstrated the highest regenerative capacity. For shoot length, the optimal treatment was D5, yielding 6.3 mm. This same treatment also produced the greatest number of shoots (8.6 shoots) in the flamingo explant. Shoot number generally increased with D3 (6.3 shoots) and peaked with TDZ before declining at the highest concentration (D7). Node explants showed a moderate response, with maximum shoot length (4.3 mm) and number (4.3

Table 4 - Auxin treatments for root induction media

Treatment code	NAA (mg L ⁻¹)	IBA (mg L ⁻¹)	Basal Medium
R1 (Control)	0.00	0.00	½ MS
R 2	0.25	0.00	½ MS
R3	0.50	0.00	½ MS
R4	0.00	0.25	½ MS
R5	0.00	0.50	½ MS

shoots) also achieved with D5. In stark contrast, leaf and root explants exhibited no regeneration (0 value for all parameters) under any treatment, including the hormone-free control. This confirms the absolute necessity of exogenous cytokinins and the critical explant-specific competence for direct organogenesis in *D. purpurea* (Fig. 1).

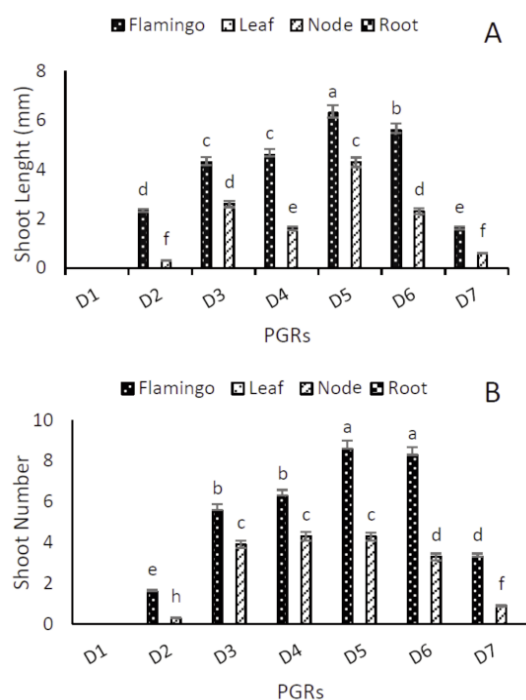


Fig. 1 - Effect of explant types and PGRs on shoot length (A) and number (B) in direct organogenesis of *D. purpurea* L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test ($P < 0.05$), with LSD values of 0.167 and 0.598 for shoot length and number, respectively.

Indirect organogenesis

The callus induction data reveal distinct responses based on explant type and PGR treatment. The flamingo explant was consistently superior, achieving 100% callus formation with NAA (C2 and C3) and its combinations with BAP (C4 and C5). This high induction percentage corresponded with the greatest callus fresh weight (4.6 g) on medium containing 0.5 mg L⁻¹ NAA alone (C2). For the flamingo explant, the addition of BAP to auxin treatments generally reduced callus weight, and the use of 2,4-D, either alone (C6 and C7) or with BAP (C8), resulted in lower induction percentages and markedly reduced biomass. Root explants showed the second-highest competency for callogenesis, with induction reaching 80% using NAA. However, the resultant callus weight

was moderate (~2.3 g). In contrast, leaf and node explants exhibited low responsiveness across most treatments, with callus induction rarely exceeding 25% and producing minimal biomass. For all explant types, the control treatment (C1) resulted in little to no callus formation, underscoring the essential role of exogenous auxins. Notably, the synthetic auxin 2,4-D was consistently less effective than NAA in promoting robust callus growth across all explants tested (Fig. 2).

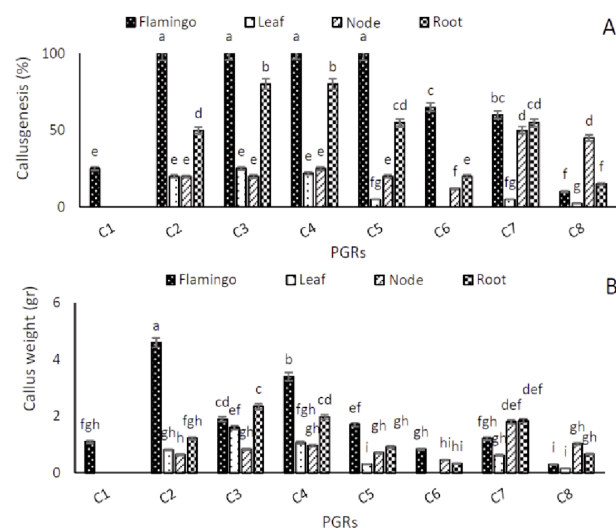


Fig. 2 - Effect of explant types and PGRs on callusgenesis (A) and callus weight (B) in indirect organogenesis of *D. purpurea* L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test ($P < 0.05$), with LSD values of 0.167 and 5.981 for callusgenesis and callus weight, respectively.

Shoot regeneration was significantly influenced by the type and combination of PGRs in the regeneration medium (Fig. 3). Media supplemented with BAP alone (SR1 and SR2) resulted in a relatively low to moderate number of regenerated shoots. The lowest shoot number (3 shoots per callus) was observed on MS medium containing 0.25 mg L⁻¹ BAP (SR1), whereas increasing the BAP concentration to 0.5 mg L⁻¹ (SR2) markedly enhanced shoot proliferation, yielding an average of 12 shoots per callus. The addition of a low concentration of NAA (0.1 mg L⁻¹) to BAP-containing media further improved shoot regeneration (SR3 and SR4). Calli cultured on MS medium supplemented with 0.25 mg L⁻¹ BAP plus 0.1 mg L⁻¹ NAA (SR3) produced an average of 12 shoots per callus, while increasing BAP to 0.5 mg L⁻¹ in combination with NAA (SR4) resulted in a higher regeneration response, with up to 14

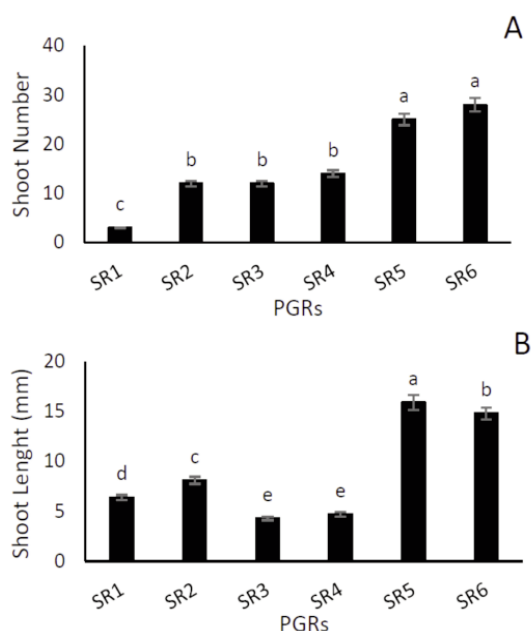


Fig. 3 - Effect of PGRs on number (A) and length (B) formed shoot in indirect organogenesis of *D. purpurea* L. after 6 weeks. Columns within each graph marked with the same letter are not significantly different according to the LSD test ($P < 0.05$), with LSD values of 2.61 and 1.38 for shoot number and length weight, respectively.

shoots per callus. The highest shoot regeneration efficiency was achieved in media supplemented with putrescine in combination with BAP (SR5 and SR6). The inclusion of 50 mg L^{-1} putrescine significantly enhanced shoot proliferation, producing 25 and 28 shoots per callus when combined with 0.25 and 0.5 mg L^{-1} BAP, respectively (SR5 and SR6). These treatments were significantly superior to all other PGR combinations tested (Fig. 3A). Shoot elongation exhibited a pattern distinct from shoot number. Media containing BAP alone promoted moderate shoot elongation, with mean shoot lengths of 6.4 and 8.1 mm at 0.25 and 0.5 mg L^{-1} BAP, respectively (SR1 and SR2). In contrast, the addition of NAA to BAP reduced shoot elongation, resulting in shorter shoots (4.3-4.7 mm), despite increased shoot numbers. The longest shoots were obtained on putrescine-containing media. Calli cultured on MS medium supplemented with 0.25 mg L^{-1} BAP plus 50 mg L^{-1} putrescine (SR5) produced shoots with a mean length of 15.9 mm, while a slightly shorter but comparable shoot length (14.8 mm) was recorded for the combination of 0.5 mg L^{-1} BAP and 50 mg L^{-1} putrescine (SR6). These results indicate a strong promotive effect of putrescine on shoot elongation

as well as shoot proliferation during indirect organogenesis.

Root induction

Root induction of regenerated shoots was significantly affected by both the type and concentration of auxin applied to the rooting medium (Fig. 4). Shoots cultured on auxin-free half-strength MS medium (control) exhibited a low rooting response, with only 30% of shoots forming roots. In addition, root development in the control treatment (R1) was weak, producing an average of

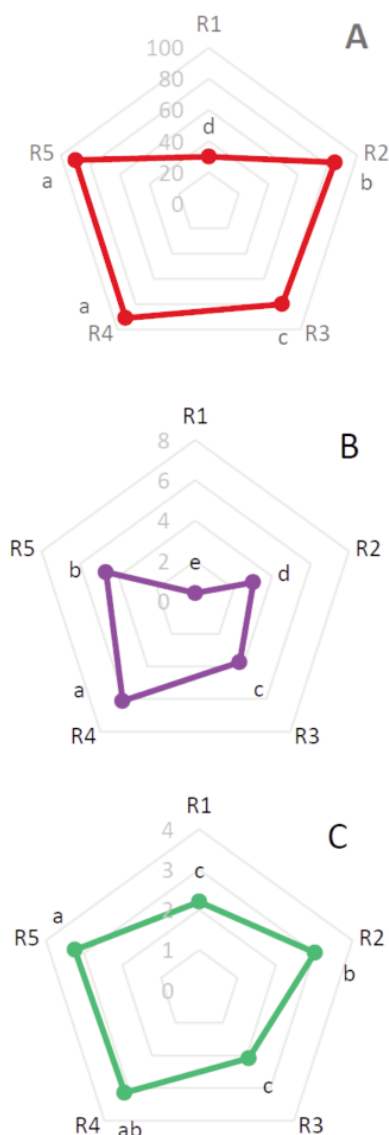


Fig. 4 - Effect of PGRs on the percentage (A), number (B), and length (C) of roots formed in root genesis of *D. purpurea* L. after 6 weeks. In each graph, points marked with the same letter are not significantly different according to the LSD test ($P < 0.05$), with LSD values of 6.61, 0.37 and 0.33 for percentage, number and length, respectively.

0.4 roots per shoot with a mean root length of 2.2 cm. The inclusion of NAA in the rooting medium markedly enhanced rooting efficiency. At a concentration of 0.25 mg L⁻¹ NAA (R2), rooting frequency increased to 85%, accompanied by the formation of an average of 3.02 roots per shoot with a mean length of 3.02 cm. Increasing NAA concentration to 0.5 mg L⁻¹ (R3) resulted in a slightly lower rooting percentage (80%) but a higher number of roots per shoot (3.74), although root elongation was reduced, yielding shorter roots with a mean length of 2.09 cm. IBA proved to be more effective than NAA in promoting both root induction and root development. The highest rooting frequency (91%) and the greatest number of roots per shoot (6.12) were obtained on medium supplemented with 0.25 mg L⁻¹ IBA (R4). Shoots cultured under this treatment also developed well-elongated roots with a mean length of 3.15 cm. A comparable rooting frequency (90%) was observed at 0.5 mg L⁻¹ IBA (R5); however, the number of roots per shoot decreased to 4.65, while the longest mean root length (3.24 cm) was recorded at this concentration. Overall, among the auxins tested, IBA at 0.25 mg L⁻¹ was the most effective treatment for inducing a high rooting frequency combined with superior root number and adequate root elongation, indicating its suitability for efficient root induction in regenerated *D. purpurea* plantlets. In addition, a strong positive correlation was observed between rooting frequency and the number of roots per shoot ($r = 0.89$), indicating that treatments enhancing root induction also promoted root proliferation. Root length showed moderate positive correlations with rooting percentage ($r = 0.67$) and root number ($r = 0.65$), suggesting partially independent regulation of root initiation and elongation (Fig. 5).

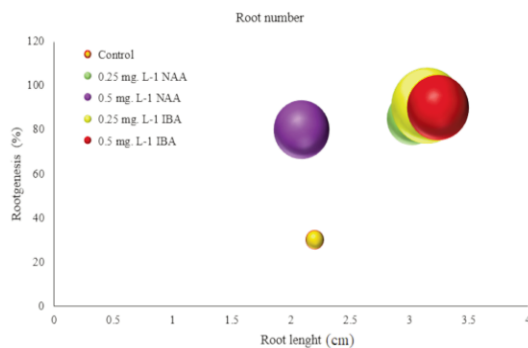


Fig. 5 - Analysis of root formation in *D. purpurea* L.: Correlation of percentage, number, and length of roots.

4. Discussion and Conclusions

The present study demonstrates that direct shoot organogenesis in *D. purpurea* is strictly dependent on both explant type and the presence of exogenous cytokinins. Among the explants tested, flamingo (hypocotyl-cotyledon) explants showed the highest regeneration capacity, whereas leaf and root explants failed to respond under all treatments, including hormone-free media. This confirms that organogenic competence in *D. purpurea* is highly tissue-specific. The superior response of flamingo explants may be attributed to their juvenile developmental stage and higher cellular plasticity, which has been associated with increased responsiveness to plant growth regulators (Guo *et al.*, 2017).

Low concentrations of TDZ were particularly effective, with 0.25 mg L⁻¹ TDZ (D5) producing the greatest shoot number and shoot length, especially in flamingo explants. This result supports previous reports emphasizing the importance of optimizing cytokinin concentration to avoid inhibitory effects at higher levels (Kairuz *et al.*, 2021). TDZ is known to exhibit both cytokinin- and auxin-like activity, influencing endogenous hormone balance and promoting shoot induction at low concentrations (Novikova and Zaytseva, 2018). In contrast, higher TDZ levels can suppress organized shoot development. Although leaf explants of *D. purpurea* have been reported to regenerate shoots under specific hormonal conditions (Bansal *et al.*, 2024), their lack of response in the present study suggests that additional optimization of PGR composition or explant physiological status is required. These findings highlight that medium composition and explant selection are critical determinants of direct organogenesis, as previously reported for *D. purpurea* (Kairuz *et al.*, 2021).

Callus induction responses differed markedly among explant types and auxin treatments. Flamingo explants consistently exhibited the highest callus induction frequency and biomass, achieving 100% induction on media containing 0.5-1 mg L⁻¹ NAA, either alone (C2 and C3) or in combination with BAP (C4 and C5). This strong callogenic response indicates a high capacity for cellular dedifferentiation, which is known to be promoted by auxin-mediated signaling and stress-related pathways (Ikeuchi *et al.*, 2013; Feher, 2005; Jimenez, 2005). In contrast, node, leaf, and root explants showed limited callus formation,

reflecting inherent physiological and biochemical differences among tissues (Guo *et al.*, 2017).

Among the auxins tested, NAA was more effective than 2,4-D in promoting both callus induction and biomass accumulation. Although 2,4-D is commonly used for callogenesis (Isah, 2016; Abbaszadeh *et al.*, 2023), its lower efficiency in this study suggests that it may not support sustained callus growth in *D. purpurea*. Similar variability in auxin effectiveness has been reported across species, including cashew, mango, and woody plants (Nadgauda and Gogate, 2005; Ara *et al.*, 2005; Klimek-Chodacka *et al.*, 2020). The synergistic effect observed between NAA and BAP further supports the established role of auxin-cytokinin balance in regulating cell division and callus development (Novikova and Zaytseva, 2018).

Shoot regeneration from calli was significantly enhanced by cytokinin-polyamine combinations. The highest shoot numbers and longest shoots were obtained on media containing BAP in combination with putrescine, confirming a strong synergistic effect. Polyamines are known to regulate cell division, stabilize membranes, and modulate ion transport, thereby supporting both shoot initiation and elongation (Hagos and Gebremdhin, 2015; Taheri *et al.*, 2021). Similar promotive effects of polyamines on shoot regeneration have been reported in *Bacopa monnieri* and several horticultural species (Dey *et al.*, 2019; Nowakowska *et al.*, 2022). In contrast, the addition of NAA to BAP increased shoot number but reduced shoot elongation, reflecting the antagonistic interaction between auxins and cytokinins during shoot growth (Müller and Leyser, 2011; Abbaszadeh *et al.*, 2023). These results highlight the importance of carefully balancing PGR ratios to optimize both shoot proliferation and elongation.

Root induction was strongly influenced by auxin type and concentration, with IBA outperforming NAA in all rooting parameters. The highest rooting frequency, root number, and satisfactory root elongation were achieved with 0.25 mg L⁻¹ IBA (R4). The superior performance of IBA has been attributed to its metabolic stability, resistance to degradation, and efficient conversion to IAA within plant tissues (Nakhooda *et al.*, 2012; Marquez *et al.*, 2016; Fattorini *et al.*, 2017). Similar findings have been reported in various plant species, including *Panax ginseng*, where IBA was more effective than NAA in promoting lateral root development (Damodaran and Strader, 2019).

The weak rooting response observed in auxin-free media confirms that exogenous auxins are essential for rhizogenesis, consistent with previous reports emphasizing the importance of auxin-cytokinin balance in root formation (Nakhooda *et al.*, 2012; Depaepe *et al.*, 2024). The positive correlations observed between rooting percentage, root number, and root length further indicate coordinated regulation of root initiation and development.

In conclusion, this study establishes an efficient and reproducible *in vitro* regeneration system for *D. purpurea* based on both direct and indirect organogenesis. Regeneration efficiency was strongly explant-dependent, with flamingo (hypocotyl-cotyledon) explants consistently exhibiting the highest morphogenic competence across all stages. Low concentrations of TDZ (0.25 mg L⁻¹, D5) were optimal for direct shoot induction, highlighting the importance of precise cytokinin regulation. For indirect organogenesis, NAA was the most effective auxin for callus induction, while the combination of BAP and putrescine markedly enhanced shoot regeneration and elongation, demonstrating the synergistic role of cytokinins and polyamines. Root induction was most efficiently achieved using low concentrations of IBA, which produced high rooting frequencies and well-developed root systems (Fig. 6). Despite the effectiveness of the established protocols, this study was limited to a restricted range of explant types and PGR concentrations, and endogenous hormone dynamics were not investigated. Future studies integrating broader hormonal regimes and molecular or physiological analyses would provide deeper insight into the mechanisms governing explant-specific responses. Nevertheless, the regeneration system described here offers a practical and reliable framework for the large-scale micropropagation of *D. purpurea*. Given the medicinal and economic importance of this species, the optimized protocols presented in this study can support sustainable propagation, conservation strategies, and future biotechnological applications.

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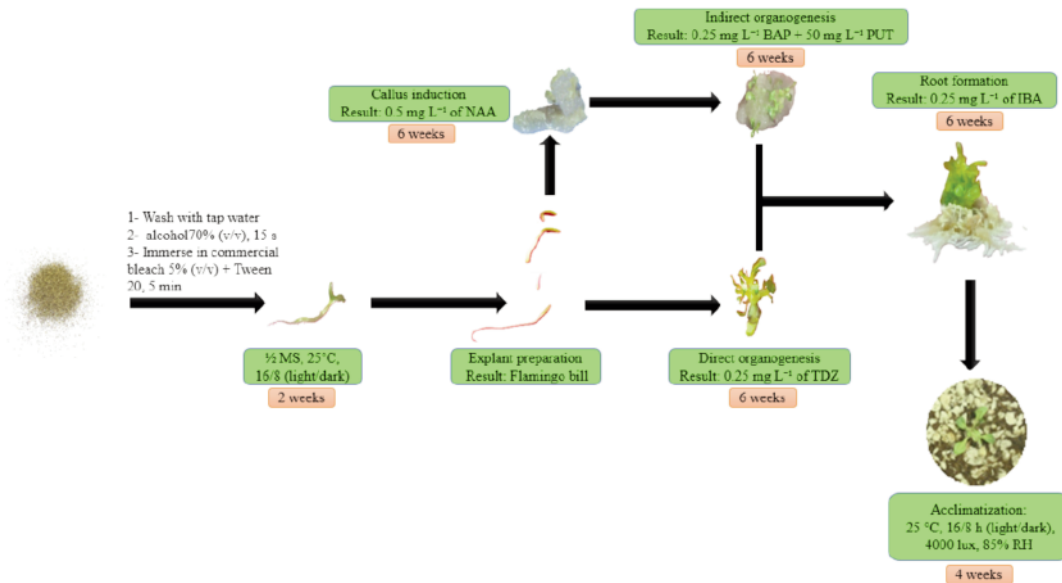


Fig. 6 - Direct and indirect organogenesis techniques for foxglove plant regeneration and optimal media culture and time for each step.

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