Bulletin of the National Research Centre

RESEARCH

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In vitro propagation of *Dianthus barbatus* L. and cytokinin-mediated changes in its phenolic content and antioxidant capacity

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Abstract

Background *Dianthus barbatus* is a valuable ornamental and medicinal species with potential for the production of bioactive compounds, yet information on the integrated effects of plant growth regulators (PGRs) on its in vitro propagation and phytochemical responses remains limited. Thus, the effects of PGR treatments on in vitro seed germination, organogenesis, phytochemical profiles, and antioxidant responses of *D. barbatus* were evaluated.

Results Surface-sterilized seeds for seedling establishment and seedling-derived nodal explants for shoot multiplication were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP), thidiazuron (TDZ), or 6-(γ,γ-dimethylallylamino)purine riboside (2-iP) at 0.25, 0.5, and 1.0 mg L^{-1}. The maximum seed germination rate (66.7%) was achieved with 0.25 mg L^{-1} BAP. During axillary shoot multiplication, 0.25 mg L^{-1} TDZ induced the highest shoot production (11.1 ± 1.3 per explant) and total leaf number (38.3 ± 2.3), whereas PGR-free MS medium produced the longest shoots (3.8 ± 0.9 cm). Multiplied shoots were rooted on MS medium with indole-3-acetic acid (IAA), 4-chlorophenoxyacetic acid (4-CPA), or α-naphthaleneacetic acid (NAA) at 0.25, 0.5, and 1.0 mg L^{-1}. Rhizogenesis was most effective on PGR-free medium, yielding the highest root number (9.6 ± 0.9 per shoot), while 0.25 mg L^{-1} IAA stimulated the longest root growth (4.2 ± 0.3 cm). Conversely, 4-CPA markedly suppressed root development. Rooted plants acclimatized ex vitro, achieving an 85% survival rate after 4 weeks in a peat-and-perlite mixture. Phytochemical analyses revealed that 0.25 mg L^{-1} BAP promoted the highest mean total flavonoid content, condensed tannin content, and total antioxidant capacity, whereas 0.25 mg L^{-1} TDZ elicited the highest mean total phenolic content in aerial parts of the plant.

Conclusions These results demonstrate that cytokinin and auxin type and concentration differentially regulate *D. barbatus* development and phytochemical accumulation, providing an integrated basis for its in vitro culture and further evaluation of its bioactive potential.

Keywords Auxin, Caryophyllaceae, Cytokinin, Ornamental plants, Secondary metabolites

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Introduction

Dianthus barbatus L. (Sweet William) is a perennial ornamental plant in the family Caryophyllaceae that exhibits winter hardiness (Sharma et al. 2015). This species originates from Europe and Asia and has subsequently spread to North America and many other geographical regions (POWO, 2026). While it is generally used as a bedding plant for landscaping and cut flower production, its flowers have a delicate, pleasant, spicy aroma (Abdushaeva 2021). Furthermore, some *Dianthus* species have significant merit as environmental protection and greening plants against soil erosion due to their abiotic stress tolerance and high reproduction rates (Szarvas et al. 2025). The plant is also widely used as a flower bed and garden border plant and is therefore the subject of agronomic studies aimed at improving its cultivation conditions (Chen et al. 2024).

Beyond their ornamental value, members of the family Caryophyllaceae are also rich in bioactive medicinal compounds. Species of the genus *Dianthus* have attracted considerable attention because of their long-standing use in traditional medicine and diverse phytochemical profiles (Chandra and Rawat 2015). In particular, *D. superbus* and *D. chinensis* have been traditionally employed for conditions including urinary disorders, inflammation, and menstrual disturbances, while experimental studies have demonstrated antioxidant, antimicrobial, antiviral, anti-inflammatory, antitumor, and analgesic activities. These pharmacological effects are associated with a diverse array of secondary metabolites, with triterpenoid saponins, flavonoids, phenolic acids, phenylpropanoids, anthraquinones, and volatile constituents representing important phytochemical classes (Liu et al. 2022). Notably, recent investigations of endemic Turkish *Dianthus* species identified rutin, phloridzin, ferulic acid, and chlorogenic acid as prominent phenolic constituents and demonstrated substantial antioxidant, antibacterial, and antimutagenic activities (Celik et al. 2024).

The ornamental plant industry widely employs plant tissue culture for rapid, large-scale multiplication of selected genotypes with desirable traits. Recent work on endangered *Dianthus* species has further demonstrated the value of optimized in vitro propagation systems for *ex situ* conservation and recultivation (Farkas et al. 2025). However, regeneration responses are highly species- and genotype-dependent, limiting the direct transfer of established protocols among *Dianthus* species (Farkas et al. 2025). In particular, comparative information on the effects of different cytokinins and auxins on organogenesis in *D. barbatus* remains limited.

Despite the recognized phytochemical diversity of *Dianthus* species, the phytochemical and antioxidant characteristics of in vitro-grown *D. barbatus* remain poorly understood. While propagation experiments determine conditions that promote seedling establishment, shoot multiplication, and rooting, plant growth regulator treatments can also alter phytochemical composition. Exogenous cytokinins upregulate key secondary metabolic pathways and promote tissue differentiation in vitro, thereby significantly increasing total phenolic, flavonoid, and tannin accumulation and enhancing overall antioxidant activity (Gomulski et al. 2026). Thus, this study investigated the effects of exogenous cytokinin treatments on tissue differentiation and secondary metabolism in *D. barbatus*. It was hypothesized that these treatments would enhance in vitro growth and significantly increase the accumulation of total phenolics, flavonoids, and tannins, thereby improving overall antioxidant capacity of the species.

Materials and methods

Seed surface sterilization, culture medium preparation, and culture establishment

A commercial seed mixture of double-flowered *Dianthus barbatus* L. (commonly designated as 'Double Mix') containing a variety of flower colors was used as the plant material. The seeds were obtained from a commercial supplier (Genta Tarım, Türkiye) and stored in their original packaging at 22 °C until use. The seeds' surface was sterilized in 1% (v/v) NaOCl for 8 min, then rinsed several times in sterile distilled water. Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose was used for seed germination and organ development experiments. During culture establishment, 1.0 mL L^{-1} Plant Preservative Mixture (PPM; Plant Cell Technology) was added to the medium to prevent microbial contamination. The pH was adjusted to 5.7–5.8, and the medium was solidified with 7 g L^{-1} agar (Duchefa Biochemie). The media were autoclaved at 121 °C and 118 kPa for 20 min. The seeds were then inoculated onto the respective cytokinin-supplemented media and cultured in GA-7 vessels (Magenta LLC) containing 30 mL of medium per vessel.

In vitro seed germination, shoot multiplication, and rooting

Surface-sterilized *D. barbatus* seeds were inoculated onto MS medium supplemented with 6-benzylaminopurine (BAP; Caisson Labs), thidiazuron (TDZ; Duchefa Biochemie), or 6-(γ,γ-dimethylallylamino)purine riboside (2-iP; Duchefa Biochemie) at 0.25, 0.5, and 1.0 mg L^{-1}. MS medium without plant growth regulators (PGRs) was used as the control. Germination was recorded throughout the culture period, and germinated seedlings were subsequently used for organ development experiments.

For shoot multiplication, axillary shoots were excised from 30-day-old in vitro-grown seedlings and sectioned into approximately 1–2 cm long nodal segments, each

containing at least one node. The explants were cultured on MS medium supplemented with BAP, TDZ, or 2-iP at 0.25, 0.5, or 1.0 mg L^{-1}. PGR-free MS medium was used as the control. After the multiplication period, well-developed shoots were transferred to the rooting stage.

For rooting, the basal leaves were removed from the shoots, which were subsequently cultured on MS medium supplemented with 4-chlorophenoxyacetic acid (4-CPA; Duchefa Biochemie), indole-3-acetic acid (IAA; Sigma-Aldrich), or α-naphthaleneacetic acid (NAA; Sigma-Aldrich) at 0.25, 0.5, and 1.0 mg L^{-1}. PGR-free MS medium was used as the control. Root number and root length were recorded at the end of the rooting period. Rooted shoots were subsequently transferred to ex vitro conditions for acclimatization.

Acclimatization of in vitro-rooted plants

To facilitate acclimatization, the lids of the culture vessels were replaced with ventilated lids, and the rooted plants were maintained for an additional week under the same environmental conditions applied during seed germination. The plants were then removed from the vessels, and residual culture medium was carefully washed from the roots under running tap water. The plants were transferred to pots containing a 3:1 (v/v) mixture of peat and perlite and covered with transparent plastic bags to maintain high humidity. The plants were irrigated with tap water every three days. After one week, ventilation holes were gradually made in the bags, and their number was progressively increased to facilitate adaptation to ex vitro conditions. The plastic bags were completely removed after 4 weeks of acclimatization.

Culture conditions, incubation periods, and parameters measured

The cultures were incubated in a growth chamber at 23 ± 1 °C, under a 16/8 h light/dark photoperiod, and at a light intensity of 60 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps. The incubation period was set at 30 days for all stages of the study. At the end of each incubation period, germination percentage, shoot number, shoot length, leaf number, root number, and/or root length were evaluated, as appropriate for each stage. In addition, the survival rate of plants during acclimatization was calculated.

Preparation of extracts from aerial parts

Aerial parts, including shoots and leaves, were harvested from culture vessels at the end of the shoot multiplication period, dried at 55 °C for 24 h, and then finely powdered. Extracts were prepared with slight modifications according to the method described by Redhwan et al. (2025). Briefly, 50 mg of the powdered plant material was extracted using 1 mL of 80% (v/v) aqueous ethanol (EtOH) in an ultrasonic bath (ISOLAB, 300 W, 50 kHz) at 60 °C for 60 min. After centrifugation at 4400 × g for 10 min, the supernatants were collected and used directly in the biochemical assays.

Determination of total phenolic, flavonoid, and condensed tannin contents

Total phenolic content (TPC) was determined following the method described by Johari and Khong (2019). Gallic acid (Alfa Aesar) was used as the standard at concentrations of 0.1–0.5 mg mL^{-1}. TPC was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW).

Total flavonoid content (TFC) was determined following the method described by Phuyal et al. (2020). Quercetin (TCI) was used as the standard at concentrations of 0.2-1.0 mg mL^{-1}. TFC was expressed as mg quercetin equivalents per gram of dry weight (mg QE g^{-1} DW).

Total condensed tannin content (TTC) was quantified following the protocol described by Fadda and Mulas (2010). Catechin (TCI) was used as a standard, at 1–100 µg mL^{-1}. The condensed tannin content was expressed as mg catechin equivalents per gram of dry weight (mg CE g^{-1} DW).

Determination of antioxidant capacity

Total antioxidant capacity (TAC) was determined using the phosphomolybdenum method described by Nartey et al. (2021). Absorbance was recorded at 695 nm. Ascorbic acid was used as the reference standard, and TAC was expressed as µg ascorbic acid equivalents per mL of extract (µg AAE mL^{-1} extract).

Statistical analyses and data visualization

For the seed germination experiment, nine seeds were cultured in each vessel, with five vessels per treatment, resulting in 45 seeds per treatment. Each culture vessel was considered an independent replicate. For the shoot multiplication and rooting experiments, 20 explants or shoots were used per treatment, with five explants per replicate and four independent replicates. The biochemical assays were conducted in triplicate. Data were expressed as mean ± standard deviation (SD). The data were subjected to one-way analysis of variance (ANOVA), and differences among means were assessed using Tukey's honestly significant difference (HSD) test at $P < 0.05$. All statistical analyses were performed using IBM SPSS Statistics 22. During acclimatization, at least three plants were transferred to each pot, and 60 plants were evaluated in total. Acclimatization success was calculated as the percentage of surviving plants relative to the total number of plants transferred to ex vitro conditions. The morphological data were subjected to min-max normalization. Hierarchical cluster analysis based

on Euclidean distance and complete linkage was subsequently performed, and clustered heat maps were generated using the Interactive Clustered Heat Map Builder (Ryan et al. 2020).

Results

Effects of cytokinins on seed germination and organ development

Seeds started to germinate after the 4th day of culture. The medium supplemented with 0.25 mg L^{-1} BAP increased the germination rate, giving the maximum value for the parameter (66.7 ± 7.9%). The control medium resulted in the lowest germination rate (44.4 ± 7.9%) among all treatments (Table 1).

Shoot production varied greatly in response to different cytokinin treatments in a concentration-dependent manner. Among the cytokinin treatments, 0.25 mg L^{-1} BAP and 2-iP at 1.0 mg L^{-1} produced the highest mean shoot number (1.0 ± 0.3), whereas 0.5 mg L^{-1} TDZ resulted in the lowest value (0.5 ± 0.1). Shoot length varied with cytokinin treatment. The longest shoots (1.4 ± 0.6 cm) were obtained on MS medium supplemented with 0.25 mg L^{-1} BAP, while the shortest shoots (0.3 ± 0.1 cm) were recorded in the presence of 0.5 mg L^{-1} TDZ. The TDZ treatments reduced mean shoot length (Table 1).

Leaf production generally followed the same pattern as shoot production (Table 1). However, all results were statistically similar, although numerical differences in mean leaf number were observed. The numerically highest mean leaf number was obtained on MS medium supplemented with 0.25 mg L^{-1} BAP (5.2 ± 1.0), whereas the lowest value was recorded on 0.5 mg L^{-1} TDZ (2.8 ± 0.5). Treatments containing 2-iP produced relatively high leaf numbers, while TDZ treatments, particularly at 0.5 mg L^{-1}, resulted in reduced leaf formation.

BAP and 2-iP treatments generally promoted root formation, but TDZ reduced it (Table 1). The highest mean root number was obtained with 0.25 mg L^{-1} BAP (2.1 ± 0.4), whereas the lowest mean value was recorded at 0.5 and 1.0 mg L^{-1} TDZ (0.3 ± 0.1). A similar trend was observed for root length. The longest roots were produced on 0.25 mg L^{-1} 2-iP (1.2 ± 0.6 cm), while the shortest roots were recorded in response to TDZ treatments (0.2 ± 0.1 cm).

Effects of cytokinins on shoot multiplication

Different types and concentrations of cytokinins showed statistically significant effects on shoot multiplication in *D. barbatus* (Fig. 1A-D). BAP at 0.25 mg L^{-1} reduced mean shoot number, whereas increasing concentrations gradually increased the number of shoots. TDZ treatments generally promoted shoot production, and the average number of shoots obtained in the medium containing 0.25 mg L^{-1} TDZ was the highest among all treatments. However, no statistically significant difference in shoot number was observed between the 0.25 and 0.5 mg L^{-1} TDZ treatments. In contrast, 2-iP treatments reduced shoot production. The lowest shoot numbers were obtained in media containing 0.5 and 1.0 mg L^{-1} 2-iP. The control medium produced the longest shoots (3.8 ± 0.9 cm). All phytohormone variants produced significantly shorter shoots compared with the control. Among them, 0.25 mg L^{-1} TDZ produced the shortest shoots (1.0 ± 0.2 cm), and no statistically significant difference was found between the 0.25 and 0.5 mg L^{-1} TDZ treatments (Table 2).

The highest total leaf number was recorded in shoots grown in the medium with 0.25 mg L^{-1} and 0.5 mg L^{-1} TDZ, whereas the lowest was recorded in the medium with 1.0 mg L^{-1} 2-iP. Although TDZ reduced shoot length, it significantly increased leaf production, which may indicate greater node density per shoot and, consequently, more potential explants for subsequent subcultures (Table 2).

Effects of auxins on rooting

Rooting frequency reached 100% in the control, NAA-, and IAA-enriched medium, whereas it decreased to 96%

Table 1 Effects of cytokinins on in vitro seed germination and organ development in *Dianthus barbatus*

Cytokinins (mg L^{-1})	Seed germination (%)	Number of shoots	Length of shoots (cm)	Number of roots	Length of roots (cm)	Number of leaves
Control	44.4 ± 7.9 d	0.6 ± 0.1 ab	1.3 ± 0.7 ab	1.3 ± 0.3 abc	1.0 ± 0.5 ab	3.1 ± 1.0 a
TDZ 0.25	57.8 ± 4.9 a-d	0.8 ± 0.3 ab	0.5 ± 0.2 bc	0.5 ± 0.2 d	0.2 ± 0.1 d	5.0 ± 1.2 a
TDZ 0.5	46.7 ± 9.3 cd	0.5 ± 0.1 b	0.3 ± 0.1 d	0.3 ± 0.1 d	0.2 ± 0.1 d	2.8 ± 0.5 a
TDZ 1.0	51.1 ± 6.1 a-d	0.6 ± 0.1 ab	0.5 ± 0.7 bc	0.3 ± 0.1 d	0.2 ± 0.1 d	3.5 ± 1.2 a
BAP 0.25	66.7 ± 7.9 a	1.0 ± 0.3 a	1.4 ± 0.6 a	2.1 ± 0.4 a	0.9 ± 0.3 abc	5.2 ± 1.0 a
BAP 0.5	57.8 ± 4.9 a-d	0.8 ± 0.3 ab	1.3 ± 0.4 ab	2.0 ± 0.7 a	0.9 ± 0.3 abc	5.0 ± 2.1 a
BAP 1.0	57.7 ± 9.3 a-d	0.7 ± 0.1 ab	0.6 ± 0.2 abc	0.7 ± 0.2 cd	0.3 ± 0.1 cd	4.1 ± 1.3 a
2-iP 0.25	64.4 ± 9.3 ab	0.9 ± 0.2 ab	1.2 ± 0.5 ab	2.0 ± 0.6 a	1.2 ± 0.6 a	4.6 ± 1.2 a
2-iP 0.5	62.2 ± 6.1 abc	0.9 ± 0.2 ab	1.2 ± 0.3 ab	1.7 ± 0.5 ab	1.1 ± 0.1 a	5.1 ± 1.2 a
2-iP 1.0	48.9 ± 9.9 bcd	1.0 ± 0.3 a	0.6 ± 0.3 abc	0.9 ± 0.3 bcd	0.4 ± 0.2 bcd	4.8 ± 1.4 a

Data are expressed as mean ± standard deviation. Different letters within the same column indicate significant differences according to Tukey's Honestly Significant Difference (HSD) Test ($P < 0.05$)



Fig. 1 Effects of cytokinins and auxins on in vitro shoot multiplication and rooting of *Dianthus barbatus*. The images in the panels represent axillary shoot multiplication on MS medium supplemented with (**A**) 0.25 mg L^{-1} TDZ, (**B**) 0.25 mg L^{-1} BAP, (**C**) 0.25 mg L^{-1} 2-iP, and (**D**) the control medium, and rooting on (**E**) control medium, (**F**) MS medium supplemented with 0.5 mg L^{-1} 4-CPA, (**G**) 0.5 mg L^{-1} IAA, (**H**) 0.25 mg L^{-1} NAA, and (**I**) acclimatized plants. Scale bars = 1 cm

Table 2 Effects of cytokinins on in vitro shoot multiplication and leaf development in *Dianthus barbatus*

Cytokinins (mg L^{-1})	Number of shoots	Length of shoots (cm)	Total number of leaves
Control	5.2 ± 0.7 cd	3.8 ± 0.9 a	33.4 ± 2.3 b
TDZ 0.25	11.1 ± 1.3 a	1.0 ± 0.2 d	38.3 ± 2.3 a
TDZ 0.5	10.3 ± 1.3 a	1.1 ± 0.1 cd	38.2 ± 1.7 a
TDZ 1.0	5.5 ± 0.9 cd	1.6 ± 0.4 bcd	21.7 ± 1.2 f
BAP 0.25	4.2 ± 1.3 cd	2.0 ± 0.7 bcd	25.6 ± 2.9 de
BAP 0.5	6.3 ± 1.6 bc	2.1 ± 0.3 bc	29.5 ± 1.3 c
BAP 1.0	7.8 ± 1.0 b	1.8 ± 0.1 bcd	27.9 ± 1.3 cd
2-iP 0.25	3.8 ± 0.6 d	1.9 ± 0.3 bcd	27.1 ± 1.6 cd
2-iP 0.5	3.4 ± 0.7 d	2.4 ± 0.7 b	23.3 ± 1.0 ef
2-iP 1.0	3.4 ± 0.4 d	1.9 ± 0.4 bcd	20.6 ± 1.4 f

Data are expressed as mean ± standard deviation. Different letters within the same column indicate significant differences according to Tukey's Honestly Significant Difference (HSD) Test ($P < 0.05$)

Table 3 Effects of auxins on in vitro rooting of *Dianthus barbatus* shoots

Auxins (mg L^{-1})	Rooting (%)	Number of roots	Length of roots (cm)
Control	100 ± 0 a	9.6 ± 0.9 a	3.1 ± 0.4 ab
NAA 0.25	100 ± 0 a	6.0 ± 0.9 b	2.5 ± 0.6 b
NAA 0.5	100 ± 0 a	4.5 ± 0.6 c	2.6 ± 0.7 b
NAA 1.0	100 ± 0 a	4.0 ± 0.8 c	2.7 ± 0.6 b
IAA 0.25	100 ± 0 a	8.4 ± 0.9 a	4.2 ± 0.3 a
IAA 0.5	100 ± 0 a	8.5 ± 0.8 a	3.3 ± 0.9 ab
IAA 1.0	100 ± 0 a	8.2 ± 0.4 a	3.3 ± 0.6 ab
4-CPA 0.25	100 ± 0 a	4.9 ± 0.7 bc	1.0 ± 0.4 c
4-CPA 0.5	96 ± 8.9 a	4.9 ± 0.6 bc	0.7 ± 0.2 c
4-CPA 1.0	80 ± 14.1 b	1.9 ± 0.3 d	0.7 ± 0.2 c

Data are expressed as mean ± standard deviation. Different letters within the same column indicate significant differences according to Tukey's Honestly Significant Difference (HSD) Test ($P < 0.05$)

and 80% in response to 0.5 and 1.0 mg L^{-1} 4-CPA treatments, respectively (Table 3). The control medium produced the numerically highest mean root number, but this was not significantly different from the IAA treatments and was significantly higher only than the NAA and 4-CPA treatments (Fig. 1E-H). Among the auxins tested, 0.5 mg L^{-1} IAA produced the numerically highest mean root number, whereas 4-CPA exerted the strongest inhibitory effect, with the lowest value recorded from the medium containing 1.0 mg L^{-1} 4-CPA.

Among the auxin treatments, the medium with 0.25 mg L^{-1} IAA produced the longest roots. The control treatment showed root lengths statistically similar to those of 0.5 and 1.0 mg L^{-1} IAA treatments, whereas NAA moderately reduced mean root length. 4-CPA treatments markedly suppressed root growth, resulting in the shortest roots at both 0.5 and 1.0 mg L^{-1} (Table 3).

Acclimatization success

During the initial acclimatization phase, the lids of the culture containers were replaced with filtered lids, and the plants continued to grow without loss. The plants were then transferred to a peat-perlite mixture and acclimated to soil under controlled conditions. After 4 weeks of acclimatization, the overall survival rate was 85%, and the surviving plants continued to develop successfully (Fig. 1I).

Hierarchical cluster analysis of the normalized data

Cytokinin effects on in vitro seedling establishment were grouped into 4 distinct main clusters, each having two subclusters. All TDZ treatments and 2-iP and BAP treatments at 1.0 mg L^{-1} fell into the same main cluster, while the control group formed a standalone subcluster in the other main cluster, which also included BAP and 2-iP treatments at 0.25 and 0.5 mg L^{-1} as a subcluster (Fig. 2A). Effects of cytokinins on shoot multiplication were placed into 3 main clusters. The control group formed a standalone subcluster, while TDZ treatments at 0.25 and 0.5 mg L^{-1} were grouped in the same subcluster (Fig. 2B). Auxins' effects were also grouped into 3 main clusters. 4-CPA at 1.0 mg L^{-1} formed a main cluster by itself, while all IAA treatments and the control group were grouped in the same subcluster (Fig. 2C).

Effects of cytokinin type on phenolic substance contents and antioxidant capacity

In the aerial parts (shoots and leaves) generated during the multiplication phase, TDZ induced the highest TPC numerically, followed by 2-iP and BAP, while the control showed the lowest TPC value. TDZ and 2-iP treatments induced statistically similar TPC values. However, TDZ treatment reduced TFC content compared with the control. BAP treatment induced the highest TFC content, followed by 2-iP treatment. A similar trend was also observed for TTC, except for the control and TDZ treatments. All cytokinin treatments increased TTC, with BAP being the most effective treatment, followed by 2-iP (Table 4).

In the phosphomolybdenum assay using 1000 µg mL^{-1} extract, total antioxidant capacity also differed significantly among treatments. The BAP treatment produced the highest antioxidant capacity, followed by 2-iP and the control, whereas TDZ showed the lowest value (Table 4).

Discussion

The effectiveness of PGRs in in vitro propagation of ornamental plants varies significantly by species, genotype, and PGR type (Darqui et al. 2017). They can also modulate the biosynthesis and accumulation of secondary metabolites, including phenolic compounds, flavonoids, and tannins (Jamwal et al. 2018).

In vitro seed germination and early seedling development

In the present study, the type and concentration of the applied cytokinin affected the germination and early seedling development of *D. barbatus*. The in vitro germination response of *D. barbatus* varied among cytokinin

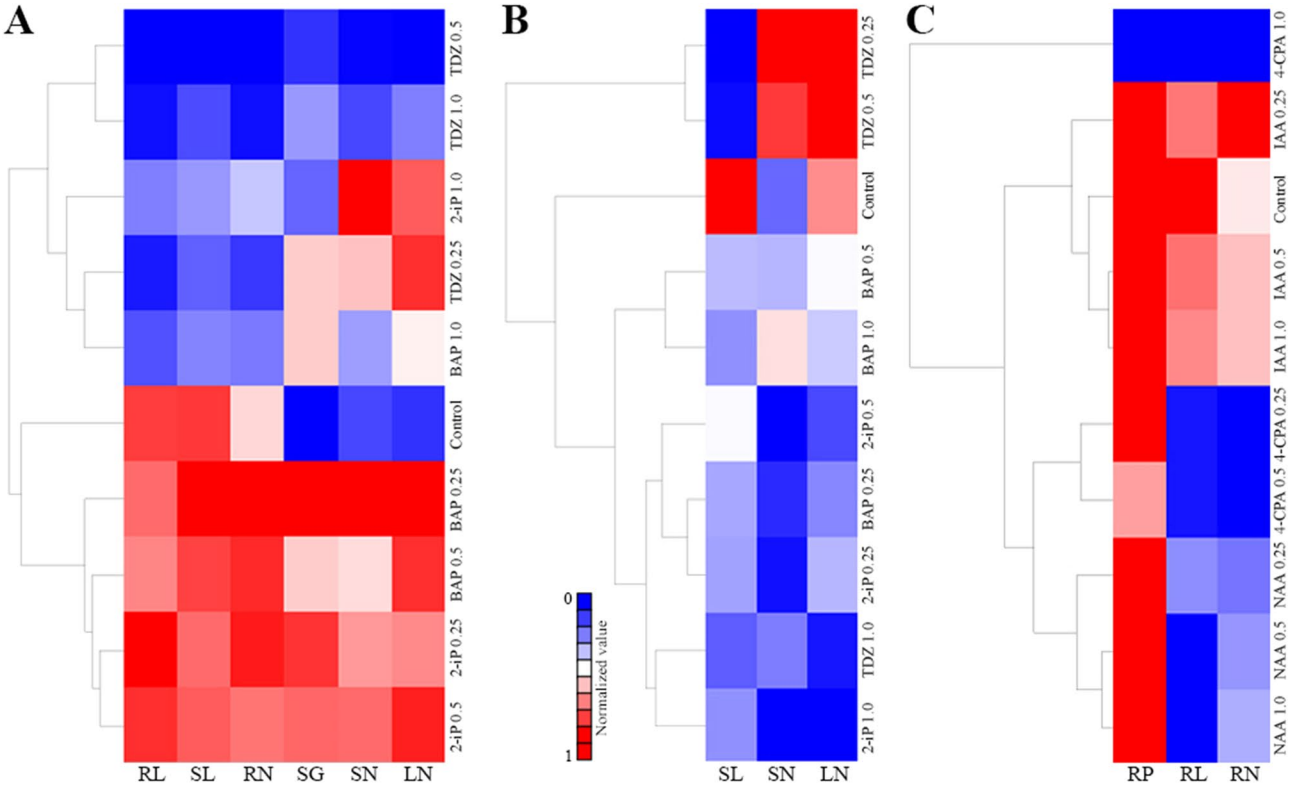


Fig. 2 Hierarchical clustered heat maps showing the effects of plant growth regulator treatments on (**A**) seed germination and seedling development, (**B**) axillary shoot multiplication, and (**C**) rooting of *Dianthus barbatus* shoots. SG: seed germination percentage, SN: shoot number, SL: shoot length, RN: root number, RL: root length, LN: leaf number, RP: rooting percentage

Table 4 Effects of cytokinin treatments on total phenolic, flavonoid, and condensed tannin contents in the aerial parts of *Dianthus barbatus* after the shoot multiplication phase

Treatment (mg L^{-1})	Total phenolic content (mg GAE g^{-1} DW)	Total flavonoid content (mg QE g^{-1} DW)	Condensed tannin content (mg CE g^{-1} DW)	Total antioxidant capacity (µg AAE mL^{-1} extract)
Control	16.09 ± 0.21 b	5.90 ± 0.03 c	2.63 ± 0.01 d	1.33 ± 0.01 c
TDZ 0.25	26.43 ± 0.95 a	4.11 ± 0.03 d	2.74 ± 0.01 c	1.27 ± 0.01 d
BAP 0.25	20.88 ± 3.42 ab	8.08 ± 0.05 a	4.18 ± 0.01 a	1.61 ± 0.00 a
2-iP 0.25	23.75 ± 3.00 a	7.32 ± 0.03 b	3.79 ± 0.01 b	1.43 ± 0.00 b

In vitro-derived aerial parts were used in the experiments. Data are expressed as mean ± standard deviation. Different letters within the same column indicate significant differences among treatments according to Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$)

treatments, with the highest germination percentage (66.7%) at 0.25 mg L^{-1} BAP, followed by 0.25 mg L^{-1} 2-iP and TDZ. This response was comparable to or higher than those reported for several other *Dianthus* species, including *D. giganteus* ssp. *croaticus* (42%) and *D. ciliatus* ssp. *dalmaticus* (70%) cultured on MS medium supplemented with 1 mg L^{-1} gibberellic acid (GA_3) (Radojević et al. 2010), and *D. henteri*, which showed 100% germination under light and 50% under dark conditions on MS medium containing 1 mg L^{-1} GA_3 (Cristea et al. 2010).

Cytokinin treatments applied during seed germination also affected subsequent shoot development in *D. barbatus*. In the present study, BAP, particularly at 0.25 and 0.5 mg L^{-1}, markedly promoted shoot proliferation, whereas TDZ treatments resulted in the lowest shoot numbers. In contrast, shoot elongation was greatest in the hormone-free control and BAP at low concentrations, while TDZ treatments markedly reduced shoot length. The contrasting responses of shoot number and shoot length suggest that cytokinin-induced shoot proliferation may be accompanied by reduced shoot elongation, particularly under TDZ treatments (Çelikel et al. 2021). A similar concentration-dependent response was observed in root development, with the low and moderate 2-iP concentrations (0.25 and 0.5 mg L^{-1}) resulting in numerically greater root production and root elongation in the seedlings. Similar concentration-dependent effects of cytokinins on shoot development have been reported in *Dianthus* species, although the magnitude and direction of these responses vary according to species, cytokinin type, and concentration (Farkas et al. 2025).

Axillary shoot multiplication and morphogenetic responses

Consistent with the responses observed during seed germination, shoot multiplication of axillary shoots in *D. barbatus* was strongly influenced by cytokinin type and concentration. Radojević et al. (2010) reported that nodal segments provided a better multiplication index than apical segments in *Dianthus* species. Among the cytokinins used in this study, the shoot multiplication data indicated that TDZ, a phenylurea-derived cytokinin, exhibits high morphogenetic activity on *D. barbatus* even at low concentrations. Similarly, Abu-Qaoud (2013) reported that TDZ is a highly effective cytokinin for shoot regeneration in carnation (*D. caryophyllus*) cultivars. Our finding also agrees with recent reviews of *D. caryophyllus* micropropagation, which indicate that TDZ is frequently among the most effective cytokinins for inducing multiple shoot formation, although the optimum concentration varies with genotype and explant type (Sirohi et al. 2025). Similar to our findings, TDZ markedly increased shoot proliferation in *D. petraeus*, although prolonged exposure resulted in hyperhydricity, emphasizing the importance of subsequent transfer to a regulator-free medium (Tsoktouridis et al. 2013). This success of TDZ can be attributed to its ability to shift the endogenous hormone balance in favor of cytokinins and to increase the expression of genes that stimulate cell division (Pai and Desai 2018). However, the exact biosynthetic and molecular mechanism behind the results remains to be investigated in *D. barbatus*.

Pareek et al. (2004) reported that BAP combined with NAA promoted efficient shoot regeneration in several *Dianthus* species, whereas our results indicate that TDZ alone at a considerably lower concentration produced greater shoot multiplication. Marković et al. (2013) reported that adding a BAP and NAA combination at 1.0 mg L^{-1} and 0.1 mg L^{-1}, respectively, to the medium significantly increased shoot production in nodal cuttings of *D. deltoides*, whereas Khatun et al. (2013) obtained the highest shoot regeneration from nodal explants of *D. caryophyllus* using 1.0 mg L^{-1} BAP. Likewise, Bazanis and Papafotiou (2024) reported that 0.1 mg L^{-1} BAP was the most effective treatment for shoot multiplication in *D. cruentus*. On the other hand, Farkas et al. (2025) reported that cytokinin responses varied among *Dianthus* species, with 2-iP showing favorable effects on shoot quality during the elongation stage. In contrast, 2-iP treatments produced the lowest shoot numbers in *D. barbatus* in the present study, indicating a lower shoot multiplication efficiency than TDZ and BAP. These findings indicate that the optimal PGR regime is species-dependent within the genus *Dianthus* and depends on endogenous hormone balance, explant origin, and culture conditions.

In our study, the control group produced the longest shoots, while all cytokinin treatments reduced shoot elongation. The fact that shoots remained short despite a marked increase in shoot number following TDZ treatments indicates that exogenous cytokinins promote adventitious meristem formation and shoot proliferation rather than shoot elongation in *D. barbatus*. This finding is consistent with previous findings that high cytokinin activity promotes multiple shoot formation while limiting shoot elongation (Kumari et al. 2018). Recent work in *Dianthus* species has also demonstrated considerable variation in shoot number and shoot length in response to different cytokinins, highlighting the species- and cytokinin-dependent nature of these responses (Farkas et al. 2025). The treatments of 0.25 and 0.5 mg L^{-1} TDZ also increased leaf production. However, 1.0 mg L^{-1} TDZ significantly reduced leaf development. This suggests that low and moderate cytokinin activity promotes production of new leaf primordia, but excessive TDZ concentrations may disrupt the balance of organogenesis in *D. barbatus*.

In vitro rooting and morphogenetic responses

Considering the rooting results, the high rooting observed on hormone-free medium suggests that endogenous auxin levels may have been sufficient. In *D. ingoldbyi*, *D. juniperinus* subsp. *bauhinorum* and *D. fruticosus* subsp. *occidentalis*, cuttings were treated with indole-3-butyric acid (IBA), and rooting rates were reported as 100%, 57.14%, and 28.57%, respectively. The authors also reported that untreated cuttings did not root (Sarropoulou and Maloupa 2022). In *D. caryophyllus* cuttings, NAA was reported to promote early rooting and increase root number and length, outperforming IAA and IBA treatments (Kumar et al. 2014). These findings suggest species-specific rooting needs in *Dianthus* species. Our results show that a low concentration of IAA increased root length, indicating that this natural auxin promotes cell elongation more efficiently than the other auxins tested on *D. barbatus*. Compared to the control, NAA treatments reduced root production, while 4-CPA dramatically suppressed both root number and length. Although 4-CPA was included to compare the effects of chemically distinct auxin classes on in vitro rooting responses in *D. barbatus*, the poor rooting response indicates that not all compounds with auxin activity promote adventitious root formation, emphasizing the importance of auxin structure in regulating rhizogenic competence in *Dianthus* species.

Cytokinin-mediated changes in phenolic substance contents and antioxidant capacity

In the aerial parts generated during the multiplication phase in *D. barbatus*, cytokinin treatment significantly influenced TPC. This pronounced response indicates that the type of cytokinin can substantially influence phenolic accumulation in *D. barbatus*. Consistent with the phytochemical potential observed in the present study, Celik et al. (2024) reported considerable variation in phenolic composition among seven endemic *Dianthus* species from Türkiye, with *D. balansae* showing the highest TPC. Similarly, Aliyazicioglu et al. (2017) reported substantial polyphenolic content in *D. carmelitarum*. Although these studies differed in species, plant material, and extraction procedures, they collectively support *Dianthus* species' capacity to accumulate phenolic compounds. The pronounced increase in TFC under cytokinin treatments also suggests that cytokinins favored the accumulation of phenolic-derived metabolites in *D. barbatus*. Wang et al. (2024) reported substantial tissue-dependent variation in phenolic and flavonoid contents in *D. caryophyllus*, with flower tissues showing the highest levels. Galeotti et al. (2008) also demonstrated considerable tissue-dependent variation in flavonoid composition in *D. caryophyllus*, identifying vicenin-2 and kaempferol glycosides among its major flavonoids. Researchers have previously detected tannins in *Dianthus* species, including *D. crinitus* and *D. basuticus*, and associated them with biological activities (Lamula and Ashafa 2014; Ahmad et al. 2024). In *D. caryophyllus*, roots and flowers showed higher radical scavenging activity than shoots and leaves, suggesting flowers and roots contained potent antioxidants (Wang et al. 2024). In *D. superbus*, the researchers reported that different extraction methods can affect the antioxidant activities of the extracts (Gou et al. 2011). Beyond species differences, antioxidant capacity varies by organ, extraction method, and solvent. Thus, our results showed that aboveground parts of cytokinin-treated *D. barbatus* also differ in antioxidant capacities. The variation in phenolic compound contents and antioxidant capacity among cytokinin-treated *D. barbatus* shoots likely reflects cytokinin-mediated, structure-dependent regulation of phenylpropanoid metabolism. To build on these findings, future studies should investigate the underlying mechanisms, validate antioxidant potential using free radical scavenging assays, and utilize single-donor clonal lines to minimize genetic variance associated with seed-derived explants, since the mixed seeds employed in our study may have high genetic variability, and individual explants may respond differently to PGRs and exhibit varying metabolite accumulation.

Conclusion

This study established an efficient in vitro culture system for *D. barbatus* encompassing seed germination, axillary shoot multiplication, rooting, and acclimatization. Cytokinin responses were strongly stage-dependent, with BAP favoring seedling development and TDZ promoting

shoot proliferation, whereas hormone-free conditions or IAA were more favorable for aspects of root development. Cytokinin treatments also markedly influenced the phytochemical profile of the regenerated plants. These findings demonstrate that cytokinin treatment can differentially regulate plant development and phytochemical accumulation in *D. barbatus*, providing a basis for its efficient in vitro culture and further evaluation of its bioactive potential. Biochemical measurements in our study alone are insufficient to determine the specific biological activity or therapeutic efficacy of *D. barbatus*. Thus, further compound-specific and activity-based investigations are necessary.

Abbreviations

2-iP	6-(γ,γ-dimethylallylamino)purine riboside
4-CPA	4-chlorophenoxyacetic acid
AAE	Ascorbic acid equivalent
BAP	6-benzylaminopurine
CE	Catechin equivalent
DW	Dry weight
GA_3	Gibberellic acid
GAE	Gallic acid equivalent
IAA	Indole-3-acetic acid
MS	Murashige and Skoog medium
NAA	α-naphthaleneacetic acid
PGR	Plant growth regulator
PPM	Plant preservative mixture
QE	Quercetin equivalent
TAC	Total antioxidant capacity
TDZ	Thidiazuron
TFC	Total flavonoid content
TPC	Total phenolic content
TTC	Total condensed tannin content

Acknowledgements
Not applicable.

Author contributions
G. K., A. R., and A. A. contributed to the conception of the study. A. A. designed the experiments. G. K., A. R., and Ay. A. performed the experiments. G. K. and A. R. analyzed the data, performed statistical comparisons, and prepared the figures. A. A., G. K., and A. R. wrote and edited the manuscript. All authors commented on and contributed to the revision of the manuscript. All authors read and approved the final manuscript.

Funding
No funding was received for conducting this study.

Data availability
All data generated or analyzed during this study are included in this published article and/or available from the corresponding author.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 17 September 2026 / Accepted: 3 November 2026
Published online: 10 November 2026

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