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The effects of plant growth regulators on *in vitro* seedling development in *Verbascum bugulifolium* and thidiazuron-induced changes in phenolic substance contents in its callus tissues

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ABSTRACT:
Verbascum bugulifolium is a rare and potentially multi-purpose plant species with a narrow natural distribution limited to Southeastern Bulgaria and Northwestern Türkiye. This study examined the effects of several plant growth regulators on seed germination, organ development, and callogenesis in *V. bugulifolium* to identify an efficient *in vitro* culture protocol which would aid in conservation efforts and estimate its medicinal potential. Gibberellic acid (GA_3) treatment at 0.5 mg L^{-1} promoted seed germination and organ development. The efficacy of GA_3 treatment in enhancing seed germination and callogenesis increased when combined with thidiazuron (TDZ) at 2.0 mg L^{-1}. The GA_3 treatment also increased hypocotyl lengths when combined with other PGRs, except when combined with kinetin (KIN). Leaf production increased in response to GA_3 treatment alone or in combination with 6-benzylaminopurine (BAP) and TDZ. High concentrations of auxin treatments limited leaf production. Treatment with 1-naphthaleneacetic acid (NAA) at 0.25 mg L^{-1} increased root production while significantly decreasing its elongation. All cytokinin and auxin treatments except indole-3-butyric acid (IBA) at 0.25 and 0.5 mg L^{-1} significantly reduced root elongation. GA_3 treatments and the combination of GA_3 with IBA at 0.5 mg L^{-1} increased root lengths. TDZ was the most effective callus-inducing growth regulator, and its effect increased when combined with GA_3. The calli yielded promising amounts of total phenolic, flavonoid, and tannin contents, which varied in response to different TDZ concentrations. The protocol described here may be used to promote enhanced seedling growth and callogenesis in the species.

Keywords: flavonoid, organ development, phytohormones, Riva mullein, sustainable biodiversity, tannin

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INTRODUCTION

Verbascum bugulifolium Lam. (Riva mullein) is a rare perennial plant species belonging to the Scrophulariaceae (figwort) family. It is native to the Euro-Siberian phytogeographic area (Cabi *et al.* 2022) and is naturally found between northwest Türkiye and southeast Bulgaria (POWO 2024). The species primarily prefers areas with a mild climate and regular rainfall throughout the year, and it is found in forest clearings, dry heaths and on acidic rocks (Cabi *et al.* 2022). It is classified as an endangered (EN) species in Bulgaria and has been included in the Red List of Bulgarian vascular plants (Petrova & Vladimirov 2009). The threat category of the species has been reassessed and suggested as EN according to a study based on its populations in Türkiye (Cabi *et al.* 2019). Populations of the species are limited to a narrow geographical area, indicating their sensitivity to habitat and environmental changes. Thus,

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efficient conservation strategies should be developed to preserve the natural populations of the species and to sustain plant biodiversity.

Verbascum species have significant ethnobotanical value. They are used to treat a wide range of diseases, such as haemorrhoids, gastric ulcers, asthma, rheumatism, exanthema, otitis, and various infectious parasitic diseases (Blanco-Salas *et al.* 2021). In addition to its limited natural distribution and conservation status, *V. bugulifolium* has also been reported to have pharmaceutical value originating from its anti-inflammatory, antioxidant, and antibacterial properties (Gökmen *et al.* 2021). Thus, the species has the potential to be used as a medicinal plant. The main medicinal compounds with antioxidant properties in plants are phenolic substances, which include phenolic acids, flavonoids, and tannins among their main subgroups. Various plant organs from the roots to the flowers, include these secondary metabolites (Sun & Shahrajabian 2023). The economic potential of the plant is not limited only to its medicinal properties. Compared with other *Verbascum* species, *V. bugulifolium* also has aesthetic flowers which bloom in various combinations of colours, such as yellow, orange, brown, green, and blue. Therefore, the species also has the potential for use as an ornamental plant. All these properties suggest the multi-purpose use of *V. bugulifolium*.

In vitro plant propagation involves the use of tissue culture techniques to sustainably produce new plants from seeds or available explants. This technique allows rapid plant propagation, which can then be used to produce plant material for medicinal purposes, trigger the synthesis of active compounds in plant tissues (Rather *et al.* 2022), or protect plant species from extinction following successful acclimatisation processes (Mežaka *et al.* 2023). One of the main factors which affects the success of plant organ development in *in vitro* cultures is the use of culture medium supplements such as plant growth regulators (PGRs). Thus, numerous previous studies have aimed to increase the efficacy of culture media by testing the effects of PGR concentrations (Redhwan *et al.* 2023) and their combinations (Lijalem & Feyissa 2020) on seed germination and organ development in threatened plant species to ensure an efficient *in vitro* production protocol for conservation studies (Fallah *et al.* 2019).

Evaluating culture medium components to support the *in vitro* propagation and conservation efforts of *V. bugulifolium* would require a thorough investigation and comparison of the effects of different PGRs and their concentrations on organ development, seed germination, and callogenesis. Callus tissues can be utilised in various applications in plant biotechnology, including mass propagation and the sustainable production of secondary metabolites (Efferth 2019). Therefore, investigating the preliminary callus-inductive potentials of the PGRs tested on the species would benefit further studies. Also, an investigation on the determination of phenolic substance contents in the species' callus tissues would provide insights into the pharmaceutical potential of the plant. To the best of our knowledge, no such study has been conducted on *V. bugulifolium*. On the other hand, *in vitro*-grown Riva mulleins should be returned to their natural environment to contribute to biodiversity conservation studies. Thus, our objectives were to assess the impact of PGRs on *in vitro* seed germination and organ development in *V. bugulifolium* and to evaluate the phenolic substance content in its callus tissues to support future propagation studies on the sustainable use of the species for conservation and pharmaceutical purposes. In this regard, our study represents a pioneering effort on the species.

MATERIAL AND METHODS

Seed source and disinfection method. Seeds of *Verbascum bugulifolium* Lam. were collected from the Çınarlıdere Recreation Area in the Derince District of Kocaeli Province in Türkiye with the official permission of the General Directorate of Nature Protection and National Parks of the Ministry of Agriculture and Forestry of Türkiye. The capsules containing seeds were maintained in a dry place at room temperature until the start of the study. The seeds were placed in a filter paper pouch and surface sterilised by successively treating them with 70% (*v*:*v*) ethanol for 1 min and 1% (*v*:*v*) sodium hypochlorite for 8 min. A few drops of Tween-20 (Duchefa Biochemie) were added to the latter treatment. The pouches containing the seeds were then rinsed three times with sterilised distilled water, and the seeds were used for inoculations.

Culture medium preparation, *in vitro* seed germination, organ development, and callogenesis. The disinfected seeds were cultured on Murashige and Skoog's (MS; Murashige & Skoog 1962) medium. All the media variants were supplemented with 30 g L^{-1} sucrose and solidified using 3.5 g L^{-1} phytagel (Sigma-Aldrich). The pH of the medium was adjusted to 5.7. The media were distributed into the culture vessels (Magenta™, GA-7) at a volume of 30 mL following sterilisation at 121°C under pressure of 118 kPa for 20 min.

In the first phase of the experiments, the seeds were then cultured on media supplemented with thidiazuron (TDZ; Duchefa Biochemie), kinetin (KIN; Sigma-Aldrich), or 6-benzylaminopurine (BAP; Caisson Labs) as cytokinins and 1-naphthaleneacetic acid (NAA; Sigma-Aldrich), indole-3-butyric acid (IBA; Sigma-Aldrich), or 3-indole-acetic acid (IAA; Sigma-Aldrich) as auxins at 0.25, 0.5, 1.0, and 2.0 mg L^{-1} concentrations to examine the impact of commonly-used PGRs on the germination of seeds and the development of organs. In the second phase of the experiments, the effects of gibberellic acid (GA_3; Merck-Schuchardt) on the same parameters were tested at 0.5 and 1.0 mg L^{-1} concentrations. The treatments with the best germination results in the first and second phases were combined and tested on the same parameters, and their effects were compared to those of the PGRs tested in the first phase. The calli formed around the hypocotyls during organ development were collected, stored at -18°C prior to use, and employed in the biochemical assays.

Parameters measured and culture conditions. At the end of the incubation period of 30 days, the germination rate (%), the number of leaves, the length of the hypocotyls, and the number and length of the roots were measured and/or calculated. The callus induction percentage of the PGR treatments was calculated, and the observations on callus morphology were recorded at the end of the same incubation period. The cultures were incubated at 23 ± 1°C under a photon flux density of 60 µmol m^{-2} s^{-1} provided by fluorescent lamps with a photoperiod of 16/8 h light/dark.

Acclimatisation of the plantlets. At the end of the incubation period, the caps of the culture vessels were replaced with filtered caps for a week. The *in vitro*-regenerated plants were removed from the culture vessels after 1 week and cleaned thoroughly with tap water to remove the medium residue. The well-rooted seedlings were then transferred into paper cups filled with a peat soil and perlite (3:1, *v*:*v*) mixture and the cups were covered with transparent bags to maintain the humidity for 4 weeks. The temperature of the acclimatisation room was set to 25 ± 1°C, and the same illumination conditions used for the *in vitro* cultures were applied. The relative humidity of the acclimatisation room was 40%. The plants were irrigated with tap water once every three days. The transparent bags were removed at the end of the period, and the acclimatised

plants were kept in the same previous environmental conditions until they were transferred to their natural habitat.

The chemicals used in the biochemical assays and extract preparation from the callus tissues. The callus tissues formed from the hypocotyls of the seedlings grown in the medium which gave the best callus induction ratio in the first phase were collected, freeze-dried, and powdered. The extracts were prepared according to the method proposed by Kiran Acemi *et al.* (2020) with slight modifications. Briefly, a 150 mg callus sample was extracted in 3 mL 80% (*v*:*v*) aqueous methanol using an ultrasonic bath (ISOLAB, 300 W, 50 kHz) for 60 min at 60°C. Following a 10 min centrifugation at 4400 × g, the supernatants were collected and directly employed in the preliminary analyses to determine the phenolic compounds, and flavonoid, condensed tannin contents.

Estimation of the total phenolic, flavonoid, and condensed tannin contents. The total phenolic contents in the TDZ-induced calli (TIC) were determined according to the method described by Singleton *et al.* (1999) with modifications. A 50 µL aliquot of the extract was combined with 250 uL of the Folin-Ciocalteu reagent (PanReac AppliChem) and 3 mL dH_2O. After incubating them for 1 min, 750 µl of 20% (*w*:*v*) Na_2CO_3 (Merck) solution was added. The solution was vortexed and incubated at room temperature for 30 min. The solution was then diluted to a final volume of 5 mL with dH_2O. The absorbance of the solution was recorded at 750 nm using a spectrophotometer (T60U, PG Instruments). The standard curve was prepared using 0.1–0.5 mg mL^{-1} gallic acid (AlfaAesar). The results were expressed as mg gallic acid equivalent g^{-1} callus dry weight (mg GAE g^{-1} callus DW).

The total flavonoid content was determined using the method proposed by Dewanto *et al.* (2002). A 500 µL aliquot of the extract combined with 75 µL of 5% (*w*:*v*) $NaNO_2$ (Merck) was incubated for 5 min at room temperature. Then, 150 µL of 10% (*w*:*v*) $AlCl_3 \bullet 6H_2O$ (PanReac AppliChem) was added to the solution. After 5 min of incubation, 0.5 mL of 1M NaOH (Merck) was added, and the solution was vortexed. The volume of the final solution was adjusted to 2.5 mL with dH_2O. The absorbance of the solution was directly recorded at 405 nm using a spectrophotometer. The standard curve was prepared using 0.2-1.0 mg mL^{-1} quercetin (TCI). The results were expressed as mg quercetin equivalent g^{-1} callus dry weight (mg QE g^{-1} callus DW).

The condensed tannin content was determined using the method described by Fadda & Mulas (2010). A 0.4 mL aliquot of the extract (diluted to 1 mg mL^{-1}) was combined with 0.2 mL of 96% (*v*:*v*) ethanol and 0.4 mL of the vanillin solution (1% vanillin in 70% sulphuric acid). The final solution was incubated at room temperature for 30 min. The absorbance was measured at 500 nm using a spectrophotometer. The standard curve was prepared using 1–100 µg mL^{-1} catechin (TCI). The results were expressed as mg catechin equivalent g^{-1} callus dry weight (mg CE g^{-1} callus DW).

Statistical analyses and data visualisation. Each *in vitro* experiment was repeated five times, and each culture vessel represented a single replicate. Nine seeds were cultured in each culture vessel, and 45 seeds were used per treatment. The acclimatization success of the seedlings was calculated based on 90 randomly selected plants sown into the soil. The biochemical assays were performed in triplicate. The results were displayed as the mean ± standard deviation. The callus induction rates were calculated as the percentage of the number of callus-forming seedlings relative to the total number of seedlings. IBM SPSS Statistics 22 software was used to conduct Duncan's multiple range test (MRT) at a significance level of $p < 0.05$ following the variance analysis.

The results of the seed germination and morphometric parameters were normalised to the [0, 1] interval, and the data were processed using the Interactive Clustered Heat Map Builder (Ryan *et al.* 2020) to conduct hierarchical cluster analysis (HCA) and visualise the similarities and differences between the treatments based on their results. The HCA was based on the Euclidean distance and the complete-linkage clustering method.

RESULTS

The effects of the PGRs on seed germination. The seeds began to germinate on the sixth day of culture in all the medium variants. In the first phase of the study, where the effects of commonly used PGRs were tested, the germination frequency of the control group was 75.53 ± 3.87%. The lowest NAA concentration increased the germination rate to 88.89 ± 3.85%, which was the highest rate in the first phase of the study. IBA treatment at 1.0 mg L^{-1} and BAP treatment at 0.5 mg L^{-1} reduced the germination rate to 46.67 ± 11.55% and 48.89 ± 3.85%, respectively. These were the lowest germination rates (Fig. 1). KIN treatment at 2.0 mg L^{-1} induced effects which were statistically similar to the control. Also, KIN at 1.0 mg L^{-1} and TDZ at 0.5 and 2.0 mg L^{-1} produced statistically similar outcomes. In the second phase of the study, the GA_3 treatments produced better results than most of the auxin and cytokinin treatments tested in the first phase. In the medium with GA_3 at 0.5 mg L^{-1}, 93.33 ± 6.65% of the seeds germinated. GA_3 treatment at 0.5 mg L^{-1} in combination with TDZ at 2.0 mg L^{-1} gave the highest germination frequency (95.33 ± 8.08%), representing the highest rate for both phases of the study. Subsequently, GA_3 treatment at 0.5 mg L^{-1} combined with BAP at 1.0 mg L^{-1} resulted in a germination rate of 86.67 ± 6.67% (Fig. 1). In contrast, GA_3 at 0.5 mg L^{-1} treatment in combination with IAA and NAA at 0.25 mg L^{-1} reduced the germination rate, yielding statistically similar lowest ratios (62.22 ± 3.85% and 62.22 ± 10.18%, respectively).

The effects of the PGRs on organ development. In the hypocotyl development, the treatments resulted in diverse concentration-dependent effects. The mean hypocotyl length was 0.18 ± 0.05 cm in the control, while a slight increase (0.25 ± 0.02 cm) was recorded in the presence of IBA at 0.5 mg L^{-1} in the medium. NAA and IBA treatments at 1.0 mg L^{-1} reduced the mean hypocotyl

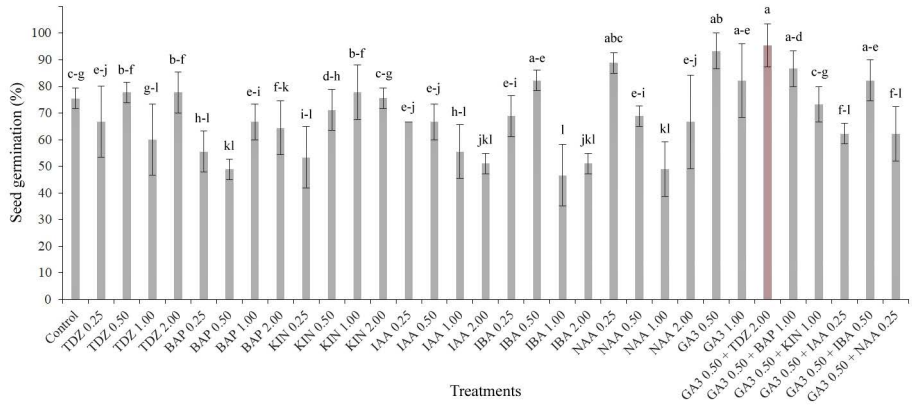

Fig. 1. The effects of the PGR treatments on the *in vitro* germination of *Verbascum bugulifolium* seeds. Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. The numbers following the PGR abbreviations indicate their concentrations as mg L^{-1} in the culture medium.

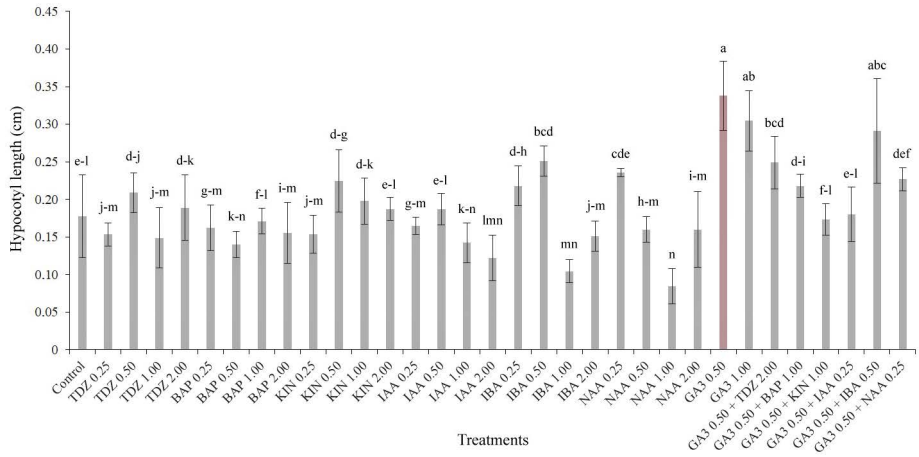

Fig. 2. The effects of the PGR treatments on *in vitro* hypocotyl elongation in *Verbascum bugulifolium*. Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. The numbers following the PGR abbreviations indicate their concentrations as mg L^{-1} in the culture medium.

Fig. 3. The effects of the PGRs on the germination of *Verbascum bugulifolium* seeds and the *in vitro* development of its organs. The panels represent general views of *in vitro* seedlings (A) from the control (B), from the medium with 2.0 mg L^{-1} TDZ (C), 2.0 mg L^{-1} BAP (D), 1.0 mg L^{-1} KIN (E), 0.25 mg L^{-1} IAA (F), 2.0 mg L^{-1} IBA (G), and 0.25 mg L^{-1} NAA (H). The panels from I to L represent the general views of seedlings from the medium with 0.5 mg L^{-1} GA_3 (I) and its combination with TDZ at 2.0 mg L^{-1} (J), BAP at 1.0 mg L^{-1} (K), and IBA at 0.5 mg L^{-1} (L). The last two panels represent the plants acclimatised (M) and transferred to nature (N). The size, brightness and sharpness of the photographs were adjusted when required. The scale bars correspond to 1 cm in the panels.

Table 1. The changes in the callus induction ratio and callus morphology around hypocotyls depending on the plant growth regulator treatment during *in vitro* seedling development in *Verbascum bugulifolium*

Plant growth regulators	Concentrations (mg L^{-1})	Callus induction ratio (%)	Callus morphology
Control	0.0	0.00	n/a
TDZ	0.25	42.20	C / G-Lg
	0.5	42.20	C / Lg
	1.0	33.30	C-F / Lg
	2.0	51.10	C-F / G-Lg-W
BAP	0.25	11.10	C-F / Lg
	0.5	20.00	C-F / G-Lg
	1.0	24.40	C / G-Lg
	2.0	13.30	C / G-Lg
KIN	0.25	0.00	n/a
	0.5	0.00	n/a
	1.0	0.00	n/a
	2.0	0.00	n/a
IBA	0.25	0.00	n/a
	0.5	0.00	n/a
	1.0	0.00	n/a
	2.0	0.00	n/a
IAA	0.25	11.10	F / Cw
	0.5	26.60	F / Cw
	1.0	17.70	F / Cw
	2.0	13.30	F / Cw
NAA	0.25	20.00	F / Cw-W
	0.5	35.50	F / W-Cw
	1.0	28.80	F / W-Cw
	2.0	20.00	F / W-Cw
GA_3	0.5	0.00	n/a
	1.0	0.00	n/a
GA_3 + TDZ	0.5 + 2.0	100	C-F / G-Lg-W
GA_3 + BAP	0.5 + 1.0	91.30	C-F / G-Lg
GA_3 + KIN	0.5 + 1.0	52.17	F / Lg-G
GA_3 + IBA	0.5 + 0.25	0.00	n/a
GA_3 + IAA	0.5 + 0.5	20.00	F / Cw
GA_3 + NAA	0.5 + 0.25	60.00	F / W-Cw

The callus morphology indicators were given as callus texture and color. F: Fragile, C: Compact, G: Green, Lg: Light green, W: White, Cw: Creamy-white, n/a: not available. The first letter for the color indicator indicates the dominant callus color in the cultures.

length to 0.08 ± 0.03 cm and 0.10 ± 0.01 cm, respectively (Fig. 2). The GA_3 treatments promoted hypocotyl elongation. The plants cultured on media containing GA_3 at 0.5 mg L^{-1} alone produced the longest mean hypocotyl length in the study (0.34 ± 0.05 cm). On the other hand, the combination of GA_3 with KIN and IAA reduced the hypocotyl lengths to 0.17 ± 0.02 cm and 0.18 ± 0.04 cm, respectively. The latter combination provided statistically similar results to the control (Fig. 2). The general appearance of the seedlings and the impact of the PGRs on organ development are shown in Fig. 3. Furthermore, the TDZ and BAP treatments revealed abnormal growth around the hypocotyls, form-

ing compact, green-coloured calli. In addition, the NAA treatments formed fragile white-coloured calli around the roots. The effects of the PGRs on callus production during the seedling development are shown in Table 1. Among the individual PGR treatments, higher callus induction ratios were observed for the medium enriched with TDZ concentrations. The medium containing 2.0 mg L^{-1} TDZ induced the highest callogenesis response (51.10%), whereas the KIN, IBA and GA_3 treatments did not induce any response. The combination of TDZ with GA_3 elevated the callogenesis response to 100% in the seedlings.

The seedlings grown in the control group produced 3.96 ± 0.14 leaves per plant. Among the cytokinin treatments, TDZ at 0.5 mg L^{-1} significantly increased the mean leaf number to 4.27 ± 0.07, yielding the highest leaf production. In contrast, the media containing high concentrations of auxins resulted in the lowest leaf numbers. Notably, the NAA at 1.0 mg L^{-1} medium produced the fewest leaves (1.36 ± 0.15). Also, NAA and IAA at 2.0 mg L^{-1} and IBA at 1.0 mg L^{-1} concentrations produced low and statistically similar leaf numbers. However, the lowest concentration of NAA induced the highest leaf numbers among the other auxins tested, which was statistically similar to the control (Fig. 4). The results indicated that adding GA_3 to the culture medium increased leaf production, with GA_3 at 0.5 mg L^{-1} yielding the maximum number of leaves per plant (4.51 ± 0.20). Additionally, GA_3 and cytokinin combinations also triggered leaf production. For instance, TDZ at 2.0 mg L^{-1} in combination with GA_3 at 0.5 mg L^{-1} and BAP at 1.0 mg L^{-1} in combination with GA_3 at 0.5 mg L^{-1} in the culture medium induced 4.40 ± 0.59 and 4.40 ± 0.60 leaves per plant, respectively. These combinations of GA_3 with cytokinins yielded statistically similar results (Fig. 4). In contrast, the combination of GA_3 with auxins decreased the number of leaves compared to the control and the media with GA_3 alone. GA_3 at 0.5 mg L^{-1} combined with 0.25 mg L^{-1} IAA reduced the leaf count to 2.93 ± 0.07 per plant. Visual observations indicated that GA_3 treatments alone or combined with the other PGRs promoted petiole elongation. Also, chlorosis symptoms were observable in some plants at the end of the incubation period, regardless of the PGR treatments.

The auxin treatments significantly triggered the rhizogenic response. NAA treatment at 0.25 mg L^{-1} increased root production, yielding the highest root number (1.11 ± 0.10), while the control medium induced only 0.82 ± 0.04 roots per plant. The other concentrations of NAA treatments dramatically reduced the root number in a concentration-dependent manner (Fig. 5). KIN treatments at 1.0 mg L^{-1} and 2.0 mg L^{-1} improved the rhizogenic response, yielding 0.91 ± 0.04 and 0.89 ± 0.15 roots per plant, respectively, whereas other cytokinin treatments reduced root production compared to the control. GA_3 treatments alone at 0.5 mg L^{-1} and 1.0 mg L^{-1} resulted in close but relatively higher mean root numbers (0.93 ± 0.07 and 0.87 ± 0.12, respectively) than the control. Also, 0.5 mg L^{-1} GA_3 combined with 0.5 mg L^{-1} IBA yielded statistically similar root production results to those of 0.5 mg L^{-1} IBA and 0.25 mg L^{-1} IAA treatments. However, all the GA_3 and PGR combinations showed a lower rooting response compared to the control. Notably, the direct root production induced by the NAA treatment decreased when combined with GA_3.

The root elongation was stimulated only by IBA, GA_3 and their combination at specific concentrations. IBA treatments at 0.25 mg L^{-1} and 0.5 mg L^{-1} achieved the highest mean root lengths (3.89 ± 0.37 cm and 4.11 ± 0.33 cm, respectively) among the cytokinins and auxins tested. Besides these IBA treatments, the control group also induced a significantly higher mean root length (3.71 ± 0.43 cm) than all the other cytokinin and auxin treatments. BAP and NAA treatments at 1.0 mg L^{-1} resulted in the minimum mean root lengths of 0.04 ± 0.00 and 0.05 ± 0.00 cm, respectively. Most TDZ, BAP, and NAA treatments induced statistically similar results (Fig. 6). The GA_3 treatment at 0.5 mg L^{-1} produced the best root elongation result (4.74 ± 0.71 cm).

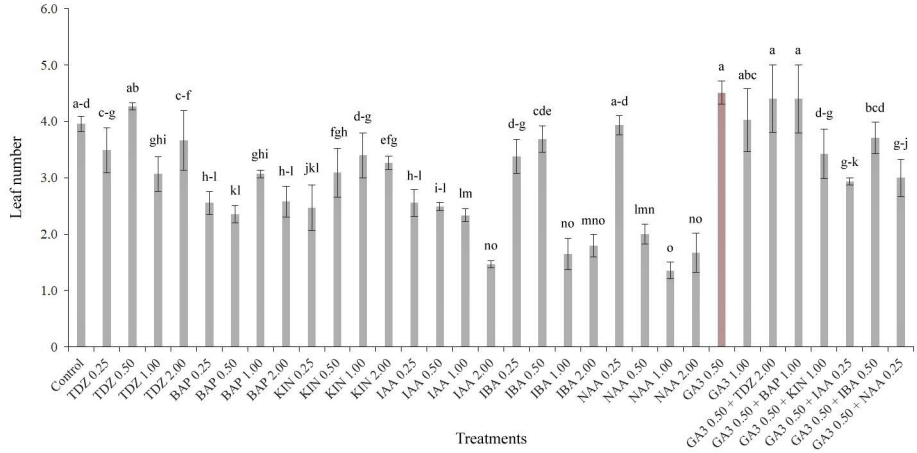

Fig. 4. The effects of the PGR treatments on *in vitro* leaf production in *Verbascum bugulifolium*. Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. The numbers following the PGR abbreviations indicate their concentrations as mg L^{-1} in the culture medium.

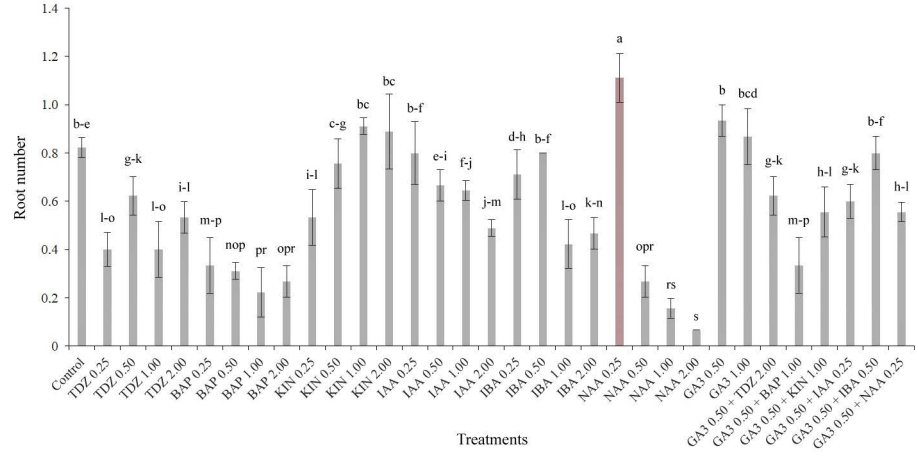

Fig. 5. The effects of the PGR treatments on *in vitro* root production in *Verbascum bugulifolium*. Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. The numbers following the PGR abbreviations indicate their concentrations as mg L^{-1} in the culture medium.

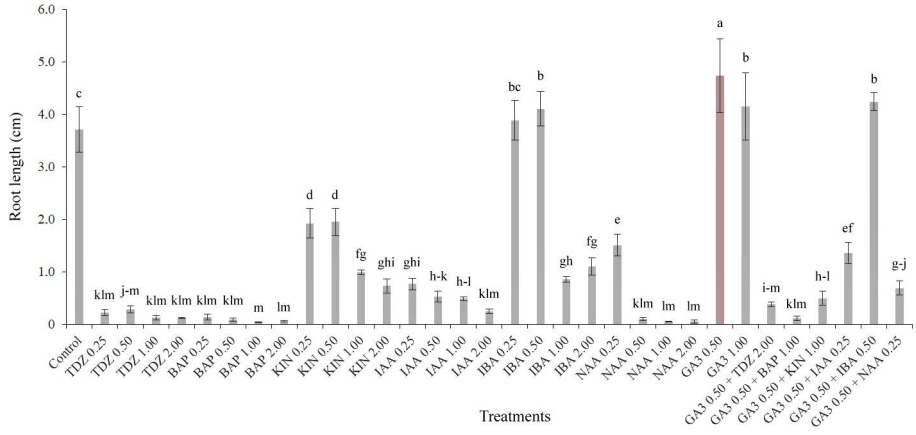

Fig. 6. The effects of the PGR treatments on *in vitro* root elongation in *Verbascum bugulifolium*. Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. The numbers following the PGR abbreviations indicate their concentrations as mg L^{-1} in the culture medium.

Additionally, the combination of GA_3 with IBA treatments both at 0.5 mg L^{-1} provided a higher (4.24 ± 0.17 cm) mean root length than that of the control and GA_3 treatment alone at 1.0 mg L^{-1} (4.15 ± 0.65 cm). However, all the other treatments with GA_3 and PGR combinations resulted in lower root lengths than the control and the other treatments. GA_3 treatments, IBA at 0.5 mg L^{-1}, and its combination with GA_3 at the same concentration yielded statistically similar results (Fig. 6).

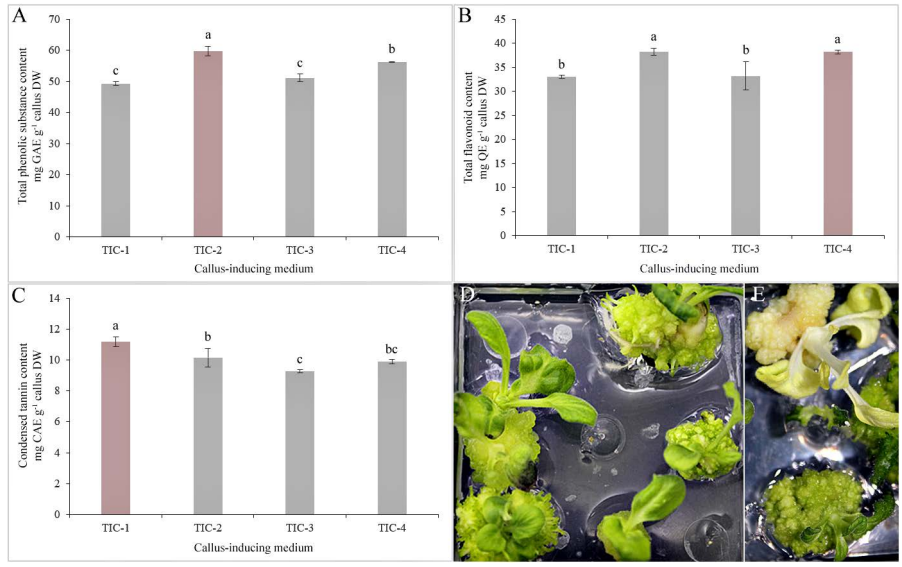

Fig. 7. The effects of the TDZ treatments on the total phenolic (A), total flavonoid (B), and condensed tannin contents (C) in calli tissues developed from *Verbascum bugulifolium* seedlings during their growth on the culture medium with 0.25 mg L^{-1} (D) and 2.0 mg L^{-1} TDZ (E). Data represent mean ± standard deviation. The means with the same letters are not significantly different according to Duncan's MRT ($p < 0.05$). The red-coloured bar indicates the maximum value for the parameter. TIC: TDZ-induced calli from the medium with 0.25 (1), 0.5 (2), 1.0 (3), and 2.0 mg L^{-1} (4) TDZ.

Acclimatisation success. *In vitro*-raised plants with healthy leaves without any symptoms of chlorosis and well-developed organs were considered to be successfully acclimatised (Fig. 3). The acclimatisation success was calculated at 71.11%. After the first stage of acclimatisation, where the culture vessels were replaced with filtered caps for a week, mild chlorosis symptoms were observed in the interveinal tissue of some seedlings' leaves.

The total phenolic, flavonoid, and condensed tannin contents in TDZ-induced callus tissues. The highest total phenolic content (59.70 ± 1.60 mg GAE g^{-1} DW) was measured in the calli induced by the medium enriched with TDZ at 0.5 mg L^{-1} (TIC-2), whereas the medium enriched with 0.25 mg L^{-1} TDZ (TIC-1) induced the lowest content (49.27 ± 0.70 mg GAE g^{-1} DW). Statistically similar total phenolic contents were recorded for both TIC-1 and TIC-3. The calli cultured in the TDZ at 2.0 mg L^{-1} (TIC-4) medium exhibited the highest (38.18 ± 0.41 mg QE g^{-1} DW) total flavonoid content, while no statistically significant differences were observed in the calli from the 0.5 mg L^{-1} TDZ (TIC-2) medium. The condensed tannin content increased up to 11.17 ± 0.31 mg CAE g^{-1} DW in the calli cultured in the medium with 0.25 mg L^{-1} TDZ (TIC-1), whereas the lowest level (9.27 ± 0.12 mg CAE g^{-1} DW) was recorded for the medium with 1.0 mg L^{-1} TDZ (TIC-3). The total phenolic, flavonoid, and condensed tannin contents for all the TIC treatments and the physical appearance of the calli are shown in Fig. 7.

Hierarchical cluster analysis of the normalised data. The analysis produced two distinct primary clusters, each with two major sub-clusters. Treatment with GA_3 at 0.5 mg L^{-1} yielded the highest developmental results, whereas NAA at 1.0 mg L^{-1} resulted in the lowest growth values based on the total evaluation of all the parameters measured. All Kin treatments except the lowest concentration were found within the same subcluster. Similarly, the total effects of both GA_3 treatments, IBA treatment at 0.5 mg L^{-1} and its combination with GA_3 at 0.5 mg L^{-1} were also grouped within the same subcluster. These treatments produced the most favourable results based on an overall developmental performance (Fig. 8).

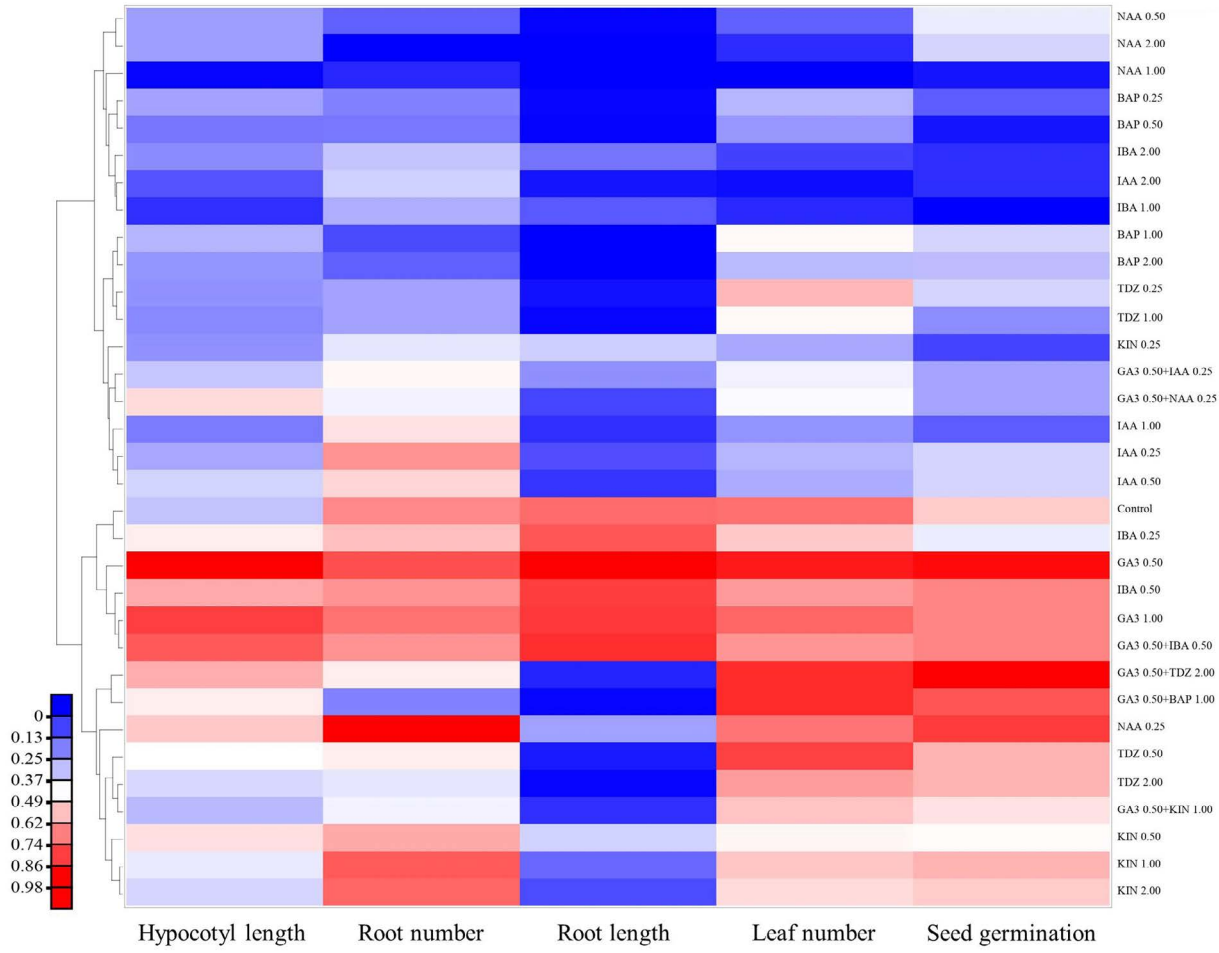

Fig. 8. A comparison of the effects of the PGR treatments on seed germination and organ development in *in vitro*-grown *Verbascum bugulifolium* seedlings through a hierarchical clustering heatmap

DISCUSSION

In vitro techniques have been used in the production of *Verbascum* species due to their biologically active molecules or as part of conservation strategies. To date, *in vitro* propagation techniques have been studied on *V. thapsus* (Turker *et al.* 2001), *V. speciosum* (Karamian & Ghasemlou 2012), *V. davidoffii* (Stanilova *et al.* 2016), *V. eriophorum* (Yordanova *et al.* 2016), *V. letourneuxii* (Farid *et al.* 2020), *V. phrygium* (Akin 2021), and *V. scamandri* (Cambaz & Çördük 2023). However, no such study has been carried out on the rare *V. bugulifolium* either for the conservation of the species or the *in vitro* production of its biologically active molecules. A deeper understanding of PGRs' effects on *in vitro* seed germination and organ development in plant cultures contributes both to developing an efficient propagation method for conservation purposes and establishing *in vitro* cultures to produce biomass for sustainable metabolite identification. In this context, the current study describes an *in vitro* culture method for *V. bugulifolium* by analysing the effects of PGRs on seed germination, organ development and callogenesis. It also elucidates phenolic accumulation levels in TDZ-induced calli during seedling growth.

The effects of PGRs on seed germination. The results suggest a concentration- and combination-dependent variation in seed germination ratios in response to PGR treatments. The minimum concentration of NAA treatment yielded the most promising germination result among the individual PGR treatments. The success of NAA in seed germination might be attributed to

the enhanced antioxidant capacity in seeds (Xing *et al.* 2023). However, germination occurred as radicle emergence from the seeds in the NAA-fortified media, and organ development remained insufficient at increased NAA levels. Interestingly, combining the most effective NAA treatment with GA_3 decreased the germination rate, suggesting a possible antagonistic interaction between these PGRs and seed germination in *V. bugulifolium*. This finding also affected other organ developmental parameters. However, in terms of the percentage of seeds which germinated and turned into plants, the best result was obtained from the second phase of the study on media containing GA_3 and its combination with TDZ. In contrast, individual PGR treatments in the first phase of the study produced lower germination ratios than the GA_3 + TDZ treatment. These results suggest a synergistic effect of GA_3 in combination with TDZ and BAP when used at appropriate concentrations. Similar results were also reported by Ahmad *et al.* (2021), who found that the germination percentage improved in *Pterocarpus marsupium* when GA_3 and TDZ were combined in the culture medium. The seed germination-triggering effect of TDZ when applied at optimal concentrations has also been reported in *Pacteilis radiata* (Kim et al. 2019). Additionally, the success of GA_3 in promoting seed germination has been attributed to the weakened seed dormancy promoted by increased lipid degradation, which provides the solutes necessary for seed germination (Evensen & Loy 1978; Mimi *et al.* 2023). Therefore, a similar physiological mechanism might be the reason behind the increased seed germination in *V. bugulifolium*. In a study on another *Verbascum* species, Hilooğlu *et al.* (2018) found that GA_3 treatments at 100 and 200 µM increased seed germination to 39% and 54.5% in the *V. calycosum* endemic to Türkiye. Our study also showed that GA_3 treatment is more effective in breaking seed dormancy than the other PGRs tested in Riva mullein when used at 0.5 mg L^{-1} in the culture medium. In *Mandragora autumnalis*, the researchers found that adding GA_3 to the culture medium effectively promotes seed germination. This study also found that pre-cold treatment of seeds and the removal of their coats can improve germination (Al-Ahmad 2020). Our study indicated that no such pre-treatments or scarification treatments are required to enhance the germination of *V. bugulifolium* seeds.

The effects of PGRs on hypocotyl elongation. Phytohormones regulate the process of hypocotyl elongation, which facilitates the shoot's emergence above the soil's surface. Hypocotyl elongation is influenced by a number of external variables, including temperature, light intensity, and water availability (Wang & Shang 2020), as well as endogenous interactions between numerous PGRs (Lorrai *et al.* 2018). Along with environmental stimuli such as light and temperature, the transport and metabolism of auxins also play an integral role in hypocotyl development in plants (Zheng *et al.* 2016). In our experiments, the environmental factors were stable for each treatment during the incubation period. Thus, the results were affected only in response to the PGR treatments. Our experiments demonstrated that the hypocotyl elongation-enhancing effects of auxins were found when tested at low concentrations (0.5 mg L^{-1} for IAA and IBA, and 0.25 mg L^{-1} for NAA). Also, cytokinins KIN and TDZ (at 0.5 mg L^{-1}) noticeably triggered hypocotyl elongation. In this context, Pasternak & Steinmacher (2024) noted that the action or effect of cytokinin is dependent on the ability of the plant cell to produce endogenous auxin, while exogenous cytokinin induces callus, shoot, or root formation from shoot-related explants in the majority of dicotyledons. Also, in addition to its cytokinin-like properties, TDZ may also be regarded as a stressor in plant tissue culture (Nautiyal *et al.* 2023). This data also supports TDZ's ability to stimulate callus formation around *V. bugulifolium* hypocotyls. Our results also suggest the possible influence of low-concentration exogenous auxin and cytokinin

treatments and an interaction among PGRs on the regulation of hypocotyl development in *V. bugulifolium*. Considering that hypocotyl elongation results from cell elongation (Lee *et al.* 2022), our findings indicate that GA_3 treatment significantly improves hypocotyl cell elongation in *V. bugulifolium*. However, its effect decreased when combined with other PGRs, suggesting a possible crosstalk between these PGRs in hypocotyl development.

The effects of the PGRs on leaf production. The aerial parts of *Verbascum* species are medicinally valuable and rich in biologically active compounds such as iridoids, phenolic acids, flavonoids, and saponins (Donn *et al.* 2023). It is mainly the leaves and flowers which have been historically used to treat various diseases, making them beneficial in traditional medicine (Tatli & Akdemir 2006). Therefore, the number of leaves is crucial for obtaining enough biomass to extract valuable bioactive compounds. In the first phase of our study, treatment with TDZ at 0.5 mg L^{-1} resulted in more favourable leaf production than other individual cytokinin and auxin treatments. In the second phase of the study, GA_3 alone and its combination with TDZ and BAP triggered leaf production. These findings are similar to those of previous studies, which reported the effectiveness of TDZ and BAP treatments in promoting leaf production in plants such as *Pancratium maritimum* (Redhwan *et al.* 2023) and *Trigonella foenum-graecum* (Mala *et al.* 2021), respectively. In contrast, another study found that *Strelitzia reginae* produces fewer leaves when GA_3 is present in the culture medium (Paiva *et al.* 2023). Also, in *Cyclamen persicum*, Oh & Kim (2014) found that GA_3 treatments supported the elongation of the leaf petioles. In our study, we also observed that the GA_3 treatments affected the elongation of the leaf petioles in *V. bugulifolium*. Consequently, we propose that the most effective *in vitro* leaf production treatments for *V. bugulifolium* are GA_3 at 0.5 mg L^{-1} or its combination with TDZ at 2.0 mg L^{-1} and BAP at 1.0 mg L^{-1}. Noticeably, the leaves of some seedlings showed chlorosis symptoms. This observation may be attributed to the species' possible sensitivity to the macro- and/or micro-element composition of the culture medium. Additionally, it could be a result of ethylene accumulation in *in vitro* cultures (Park *et al.* 2016). However, these hypotheses warrant further studies.

The effects of the PGRs on root development. Auxins are crucial in controlling many aspects of plant development, particularly in the root system architecture, where they promote root elongation and lateral root formation (Alarcón *et al.* 2019). The auxin type in our study significantly affected root development in the first phase. The NAA treatment at minimum concentrations induced root induction, whereas only IBA at low concentrations triggered root elongation in the seedlings. The concentration-dependent root inductive effect of NAA treatment may be related to its ability to decrease IAA-oxidase activity, which catalyses the degradation of IAA, and to stimulate antioxidant enzyme activities, both of which are associated with enhanced rooting ability (Yan *et al.* 2014). Likewise, NAA was found to be the most effective treatment for increasing root numbers in grapevine cultures (Kim *et al.* 2023). Although the NAA treatments increased the root numbers in our study, the roots were hairy and exhibited stunted growth. Endogenous auxin concentration may be alleviated by the exogenous application of PGRs such as NAA used in our study, a synthetic auxin analogue which functions similarly to natural auxin IAA, but is more stable (Pasternak & Steinmacher 2024). In addition, KIN treatments at 1.0 and 2.0 mg L^{-1} on *V. bugulifolium* yielded promising results in root production, but dramatically reduced root length. In *Arabidopsis thaliana* the researchers found that cytokinin (*trans*-Zeatin) treatment accelerates the shift from elongation to differentiation and stimulates growth cessation

in the distal elongation zone in roots (Liu *et al*. 2022). Thus, the root length restriction in *V. bugulifolium* after cytokinin treatments may be attributed to a similar mechanism, which should be investigated in further experiments. In root elongation in *V. bugulifolium*, IBA proved to be the most effective among the tested auxins. IBA has been shown to stimulate an auxin response when applied to plants. This response is associated with the conversion of IBA to IAA by a set of peroxisomal enzymes rather than the plants' direct perception of IBA (reviewed in Casanova-Sáez *et al.* 2021). However, the inhibition of root elongation in response to IAA treatments indicates that IBA may promote elongation through another indirect mechanism in *V. bugulifolium*.

The quality of *in vitro* root development plays a crucial role as it directly impacts the ability of plants to absorb nutrients and water from the soil when acclimatised and planted in their natural environment (Redhwan *et al.* 2023). In *V. thapsus*, NAA and 2,4-D treatments were found to stimulate rooting frequency and high root numbers were reported in the presence of NAA at 5.37 μM (1.0 mg L^{-1}) in the culture medium (Turker *et al.* 2001). Yordanova *et al.* (2016) also reported similar results in *V. eriophorum*, where treatment with 0.5 mg L^{-1} IBA had the most potent effect on root elongation. In the second phase of our study, although the GA_3 treatments did not lead to a statistically significant increase in root production, they yielded superior root elongation results compared to IBA, which was the most effective treatment for the same parameter in the first phase. Therefore, the results of the second phase of our study suggest a synergistic effect of IBA in combination with GA_3 in promoting root production and elongation in *V. bugulifolium*. Our results also indicate the complexity of root development in *V. bugulifolium*, often driven by multiple endogenous factors. In addition, our study also revealed that the use of TDZ, BAP, and NAA promotes the formation of callus tissues in *V. bugulifolium*, which is beneficial for the sustainable use of the species for pharmaceutical studies and is necessary to establish cell cultures.

The effects of TDZ concentration on the total phenolic, flavonoid, and condensed tannin contents. TDZ is also known as an elicitor in tissue cultures since it has been found to enhance secondary metabolite production in plants (Turkyilmaz Unal 2018). Our data reflected that the concentration-dependent changes in the total phenolic content due to TDZ treatments in *V. bugulifolium* were in accordance with the developmental parameters, indicating a direct interaction between the level of tissue/organ development and phenolic content. In *Plectranthus amboinicus*, methanolic extracts of *in vitro*-grown plants in TDZ-enriched medium exhibited higher total phenolic (81.23 ± 0.72 mg GAE g^{-1} DW), flavonoid (42.77 ± 0.43 mg QE g^{-1} DW), and tannin (55.68 ± 0.48 mg tannic acid equivalent g^{-1} DW) contents than *ex vitro*-grown individuals (Faisal *et al.* 2023). However, the methanolic extracts of callus tissues induced in the presence of BAP at 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA from *in vitro*-grown *Vaccinium corymbosum* leaves contained significantly lower total phenolic and flavonoid contents than extracts from both untreated field and *in vitro*-grown leaves (Kolarević *et al.* 2021). In *Zingiber officinale* callus, the chloroform/methanol (1:1, *v:v*) extracts had 33.6 ± 0.07 mg GAE g^{-1} DW phenolic content, while yeast treatment as an elicitor to callus increased the value to 45.91 ± 1.8 mg GAE g^{-1} DW. Flavonoids, on the other hand, were undetectable in the callus, and occurred only in the rhizomes at 40.25 ± 0.21 mg QE g^{-1} DW (Ali *et al.* 2018). Water extracts of *Lavandula officinalis* callus formed on medium with BAP and NAA at 0.5 mg L^{-1} were reported to yield the highest total phenolic content (35.74 ± 0.48 mg GAE g^{-1} DW), while medium with 0.5 mg L^{-1} BAP and 1.0 mg L^{-1} 2,4-D induced the highest (32.42 ± 0.46 mg QE g^{-1} DW) total flavonoid content (Buran & Topdemir 2022). In *Limonium delicatulum*, lower amounts of flavonoid (9.23

± 0.60 mg CAE g^{-1} DW) but higher tannin (17.6 ± 7.67 mg CAE g^{-1} DW) contents were observed in methanolic extracts from the shoots of the plants in the vegetative stage than the TIC of *V. bugulifolium*. Higher tannin contents up to 48.38 ± 0.75 mg CAE g^{-1} DW from the same species were reported in the flowering stage (Medini *et al.* 2014). Besides inter-species variation, the contrasting content levels among the afore-mentioned medicinal species and the TIC of *V. bugulifolium* may be attributed to differences in extraction methods and the use of different equivalents in calculations. All the samples in the cited reports were extracted either by placing the samples in mechanical stirrers or shakers or in stationary extraction solvents for specific periods ranging from 1 to 72 h. It is important to note that while the extraction method we used in our study was rapid, efficient, and cost-effective, other methods, such as Soxhlet extraction, may be more efficient at fully extracting and showing the bioactive compound contents.

CONCLUSION

In this study, the impacts of PGRs on seed germination and organ development in *Verbascum bugulifolium* were assessed in a concentration- and combination-dependent manner in order to support conservation and secondary metabolite production efforts. A preliminary screening of antioxidant compounds in its callus tissues was also carried out. In conclusion, this study's findings revealed that GA_3 treatments, IBA treatment at 0.5 mg L^{-1}, and the combination of both treatments at 0.5 mg L^{-1} induced similar and the most potent developmental results, while GA_3 at 0.5 mg L^{-1} combined with TDZ at 2.0 mg L^{-1} induced the highest stimulating effect on seed germination and callogenesis. However, GA_3 treatment at 0.5 mg L^{-1} proved optimal for seedling growth without forming callus tissue around the seedling. In addition, auxin treatments such as NAA and IBA at low concentrations exerted stimulatory effects on root production and elongation, respectively. GA_3 treatments in combination with TDZ or BAP were found to have the potential for callus production, which we recommend to facilitate mass propagation and large-scale biomass production in *V. bugulifolium* since the species has high regeneration potential. A medium optimisation study is also recommended since chlorosis symptoms were visible in some individuals at the end of the incubation periods. TDZ treatment concentration was found to be a decisive factor in the production of phenolics, flavonoids, and tannins in the calli of *V. bugulifolium*. TDZ treatment at 0.5 mg L^{-1} can effectively trigger total phenolic content and flavonoid accumulation, while treatment at 0.25 mg L^{-1} is advantageous for the accumulation of tannins. The method developed in our study may be used in the establishment of tissue culture systems and the sustainable *in vitro* production of *V. bugulifolium* seedlings, without the need to harvest wild adults, for various purposes ranging, from conservation to pharmaceutical research.

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COMPREHENSIVE PLANT BIOLOGY

REZIME

Efekti regulatora rasta biljaka na *in vitro* razvoj klijanaca *Verbascum bugulifolium* i promene sadržaja fenolnih supstanci u tkivima kalusa izazvane tidiazuronom

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Verbascum bugulifolium je retka i potencijalno višenamenska biljna vrsta sa uskom distribucijom ograničenom od jugoistočne Bugarske do severozapadne Turske. Ova studija je ispitivala efekte nekoliko regulatora rasta biljaka na klijanje semena, razvoj organa i kalogenezu kod *V. bugulifolium* da bi se identifikovao efikasan protokol kulture *in vitro* koji bi pomogao u naporima za očuvanje vrste i procenile njegove lekovite vrednosti. Tretman giberelinskom kiselinom od 0,5 mg L^{-1} je podstakao klijanje semena i razvoj organa. Efikasnost tretmana GA3 u poboljšanju klijanja semena i kalogeneze se povećala kada se kombinuje sa tidiazuronom (TDZ) u dozi od 2,0 mg L^{-1}. Tretman GA3 je takođe povećao dužinu hipokotila kada se kombinuje sa drugim PGR, osim kada se kombinuje sa kinetinom (KIN). Proizvodnja listova se povećala kao odgovor na samo tretman GA3 ili u kombinaciji sa 6-benzilaminopurinom (BAP) i TDZ. Visoke koncentracije tretmana auksinom ograničavaju proizvodnju listova. Tretman 1-naftalensirćetnom kiselinom (NAA) pri 0,25 mg L^{-1} povećao je proizvodnju korena uz značajno smanjenje njegovog izduženja. Svi tretmani citokininom i auksinom osim indol-3-maslačne kiseline (IBA) u dozi od 0,25 i 0,5 mg L^{-1} značajno su smanjili izduživanje korena. Tretmani GA3 i kombinacija GA3 sa IBA od 0,5 mg L^{-1} povećali su dužinu korena. TDZse pokazao kao regulator koji najefikasnije povećava rast kalusa, a njegov efekat se povećava kada se kombinuje sa GA3. Kod kalusa je detektovan prinos značajnih koncentracija ukupnih fenola, flavonoida i tanina, koji su izmenjeni kao odgovor na koncentracije TDZ. Protokol koji je ovde opisan može se koristiti za pojačan rast klijanaca i kalogenezu vrste.

Ključne reči: flavonoid, razvoj organa, fitohormoni, divizma Riva, održivi biodiverzitet, tanin