

Elucidating the role of different culture medium on in vitro plant regeneration of *Valeriana jatamansi* Jones ex Roxb. a high value medicinal plant of the temperate Himalayas

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Abstract

Valeriana jatamansi (Tagar) is a threatened Himalayan medicinal plant facing population decline due to habitat degradation and overexploitation. This study aimed to develop an efficient micropropagation protocol and evaluate biochemical attributes under *in vitro* and field conditions. A completely randomized design (CRD) was used to compare Murashige and Skoog (MS) and Gamborg's B5 media, with data analyzed using two-way ANOVA and DMRT, revealing significant effects ($p \leq 0.01$) of basal media and plant growth regulators (PGRs). Gamborg's B5 medium showed superior performance, with maximum shoot proliferation (3 ± 0.81 shoots per explant) and leaf number (11.33 ± 4.71) at 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA. Root induction was optimal on MS medium with IBA, producing the highest root number (35.66 ± 9.8) at 4.0 mg L^{-1} and maximum root length ($4.33 \pm 0.94 \text{ cm}$) at 3.0 mg L^{-1} . Field-grown plants exhibited higher carotenoids, soluble sugars, starch, and antioxidant activity, whereas *in vitro* cultures showed higher amino acid content ($5.44 \pm 0.08 \text{ mg g}^{-1}$ FW). These findings indicate that B5 medium is superior for *in vitro* regeneration, providing a reliable protocol for large-scale propagation and conservation of *V. jatamansi*.

Key message

Optimized micropropagation of threatened Himalayan via Gamborg's B5 medium achieves superior shoot proliferation; biochemical profiling reveals distinct vs. field metabolite profiles for effective conservation.

Introduction

Valeriana is a genus with about 200 distributed worldwide and these plants are used to treat various ailments on the place where the plant is endemic. *Valeriana* species have been used for generations. These species are often utilized in traditional medicine systems, particularly in areas where they are naturally found. The use of *Valeriana* species dates back centuries, with historical records documenting their medicinal applications in multiple cultural contexts. The most important commercial species are *V. officinalis*, *V. wallichii*, *V. fauriei* and *V. edulis*. (Houghton, 1997). Indian valerian *Valeriana jatamansi* Jones ex Roxb. also known as *Valeriana wallichii* DC. belongs to the Valerianaceae family and is an important medicinal and aromatic plant in the temperate Himalayan area. Indian valerian is commonly referred to as Tagar or Sugandhbala in Sanskrit, and Mushkbala in Hindi. It thrives in subtropical to temperate regions, growing naturally at altitudes ranging from 1500 to 3000 meters. Natural populations of the species are found mostly as undercover on moist slopes along the streams (Gautam et al., 2021). The plant can also be found in the eastern Himalayan region (Raina & Negi, 2015). *V. jatamansi* roots are used as one of the ingredients in the preparation of snake bites and it is also used for liver, kidney and spleen disorder. *V. jatamansi* is also used in various industries such as cosmetics and perfumery (Charmakar et al., 2021). Important constituents from rhizomes and roots of *V. jatamansi* used by different industries are (Wills et al., 2000) bakkenoloids type sesquiterpenoids, essential oils and phenolics (Gautam et al., 2021). Overexploitation of the plant for industrial and pharmaceutical purposes

has led to a decline in its wild populations. If the current rate of exploitation continues unchecked, the plant faces the risk of becoming extinct in the near future (Nawchoo et al., 2012). *V. jatamansi* has been assessed as a Vulnerable (VU) species under the IUCN criteria due to overharvesting, habitat degradation, and restricted distribution in the Himalayan region (Gowthami et al., 2021). Sustainable alternatives to wild collection are urgently needed to ensure the continued availability of this valuable species for industrial and pharmacological applications.

In recent years, plant tissue culture has gained substantial importance in the propagation and preservation of medicinal and aromatic plants (MAPs), many of which are facing population decline due to anthropogenic pressures such as habitat fragmentation, overharvesting, climate change, and unregulated trade. The sustainable cultivation of these plants through plant tissue culture significantly reduces the dependency on wild populations, thereby alleviating pressure on natural ecosystems and contributing to biodiversity conservation. (Cuce & Inceer 2024), (Cuce et al., 2022), (Inceer et al., 2022), (Cüce & Karaismailoğlu, 2023), (Cuce et al., 2025). In addition to propagation, plant tissue culture also serves as a platform for secondary metabolite enhancement through advanced biotechnological interventions such as elicitation, precursor feeding, and genetic transformation. These strategies have been successfully employed to boost the production of bioactive compounds in several commercially important plant species (Murthy et al., 2014). Such approaches are essential for developing plant lines with improved compatibility and productivity for industrial applications, particularly in terms of biomass yield and the accumulation of pharmacologically active constituents. (Fazili et al., 2022), However, the success of such *in vitro* applications is strongly influenced by the composition of the culture medium, which governs cell differentiation, organogenesis, and metabolite biosynthesis (Neumann et al., 2005). Among the various commonly used basal media, Murashige and Skoog (MS) medium, developed in 1962, is widely used for general-purpose *in vitro* culture due to its high nutrient and salt content (Murashige & Skoog 1962). Nevertheless, plant species exhibit varied responses to the same culture medium, and the optimization of medium composition including macro and micronutrients, vitamins, carbon sources, and growth regulators is crucial for enhancing growth and productivity *in vitro* (Pierik 1997) (George et al., 2007). Gamborg's B5 medium, formulated in 1968 for the culture of soybean suspension cells, has been shown to be effective for certain plant species, especially for those sensitive to the high nitrogen content of MS medium. Comparative evaluations have highlighted Gamborg's B5 medium's superior performance in promoting organogenesis and root induction in selected medicinal plants, suggesting its broader applicability across different plant species. (Gamborg et al., 1968) (Hussain et al., 2012) (Verma et al., 2015), (Jacob et al., 2005).

In the current investigation, *V. jatamansi* an important high-altitude medicinal herb endemic to the Himalayan region and valued for its essential oil and valepotriates was cultured under *in vitro* conditions using Murashige and Skoog (MS) medium and Gamborg's B5 medium supplemented with varying concentrations and combinations of auxins and cytokinins. The objective was to determine the optimal medium for achieving high-frequency shoot proliferation, leaf development, and rooting. The experimental results revealed that Gamborg's B5 medium demonstrated significantly higher efficacy compared to MS medium in promoting all observed growth parameters. This study is the first

documented report highlighting the superior response of *V. jatamansi* to B5 medium, which not only contributes to the understanding of species-specific media requirements but also opens new research areas for more effective tissue culture protocols for this medicinally important species. The successful micropropagation of *V. jatamansi* using Gamborg's B5 medium offers a reliable way to produce plants in large numbers, which can help both pharmaceutical industries and conservation efforts. Finally, the study strengthens the case for further exploration of alternative and species-specific media formulations for other endangered or commercially valuable medicinal plants.

Materials and methods

Plant material and explant inoculation

Three- to four-year-old plants of *V. jatamansi* were procured from the Pothibasa field station (2200 m above sea level) of the High-Altitude Plant Physiology Research Centre, Hemvati Nandan Bahuguna Garhwal University, Srinagar, Uttarakhand. The plants were maintained in the glasshouse of the HAPPRC main research complex (550 m above sea level) at Srinagar, Uttarakhand. Healthy nodal segments of *V. jatamansi* were selected as explants (Fig. 5B). Nodal segments were excised using sterilized scissors under aseptic conditions. The explants were initially washed under running tap water using fine mesh nets to prevent material loss and to remove surface debris. They were then trimmed to uniform sizes and rinsed thoroughly 3 to 4 times with tap water, followed by washing with double-distilled water. Surface sterilization was done by treating with surfactant Tween-20 for 2 to 4 minutes. For antifungal treatment, the explants were cleaned with 0.1–0.5% Bavistin solution using a soft brush and subsequently soaked for 30 to 40 min. Following fungicide treatment, explants were thoroughly rinsed 5 to 6 times with double-distilled water to remove any residual chemicals. Nodal segments of *V. jatamansi* were treated with 0.5% (w/v) mercuric chloride (HgCl_2) for 5 minutes to establish an optimal surface sterilization protocol for *in vitro* culture initiation.

Culture conditions

Two basal media were used in this study: Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) and Gamborg's B5 medium (Gamborg et al., 1968), both supplemented with 3% (w/v) sucrose. Plant growth regulators (PGRs) were incorporated in different concentrations and combinations, namely T0: control (no PGR), T1: BAP 0.5 mg L^{-1} , T2: BAP 1.0 mg L^{-1} , T3: BAP 1.5 mg L^{-1} , T4: BAP 2.0 mg L^{-1} , T1A1: BAP 0.5 mg L^{-1} + IAA 0.125 mg L^{-1} , T2A2: BAP 1.0 mg L^{-1} + IAA 0.25 mg L^{-1} , T3A3: BAP 1.5 mg L^{-1} + IAA 0.375 mg L^{-1} , and T4A4: BAP 2.0 mg L^{-1} + IAA 0.5 mg L^{-1} . For rooting, regenerated shoots were transferred to MS medium supplemented with auxins: T0: control, T1: NAA 0.2 mg L^{-1} , T2: IBA 0.2 mg L^{-1} , T3: IAA 0.2 mg L^{-1} , T4: IBA 2.0 mg L^{-1} , T5: IBA 3.0 mg L^{-1} , and T6: IBA 4.0 mg L^{-1} . The pH of the media was adjusted to 5.8 and sterilized by autoclaving at 121°C and 105 kPa for 15 min and solidified with 0.7% (w/v) plant tissue culture grade agar. Cultures were maintained in a growth chamber at $24 \pm 2^\circ\text{C}$, 50–60% relative humidity, under a 16/8 h light/dark photoperiod with a photosynthetic

photon flux density of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool daylight fluorescent lamps. The PGRs were added to the growth medium after adjusting the pH and before adding the gelling agent. Cultures were maintained in 250 mL Erlenmeyer flasks sealed with sterile cotton plugs wrapped in absorbent fabric. Rooted plantlets were removed from the culture vessels, washed, and transferred to pots containing sand, soil, perlite, cocopeat, and vermiculite in a 1:1:1 (v/v) ratio for hardening under greenhouse conditions (27°C, 90% humidity). All media components, PGRs, and chemicals were procured from HiMedia Pvt. Ltd., India.

Chlorophyll and Carotenoid Estimation

For pigment extraction, 50 mg of fresh leaf tissue from both *in vitro*-grown and field-grown plants of *V. jatamansi* was homogenized in 5 mL of absolute acetone and incubated in the dark to prevent pigment degradation. The homogenate was centrifuged at 3,000 rpm for 15 min, and the resulting supernatant was collected and diluted with an additional 10 mL of acetone. The absorbance of the extract was recorded at 480, 645, and 663 nm using a UV-VIS spectrophotometer. Chlorophyll a, chlorophyll b, and total carotenoid contents were quantified following the method of Arnon (1949), using the following equations:

$$\text{Chlorophyll a (mg L}^{-1}\text{)} = 12.7 (A_{663}) - 2.69 (A_{645})$$

$$\text{Chlorophyll b (mg L}^{-1}\text{)} = 22.9 (A_{645}) - 4.68 (A_{663})$$

$$\text{Total chlorophyll (mg L}^{-1}\text{)} = 20.2 (A_{645}) + 8.02 (A_{663})$$

Total Carbohydrate Estimation

The anthrone method was employed to estimate the total soluble sugar and starch contents in leaf tissues of *V. jatamansi*. Freshly harvested leaves (200 mg) from *in vitro* grown and field grown plants were extracted with 5 mL of 80% boiling ethanol and centrifuged at 3,000 rpm for 10 minutes. The resulting supernatant was used for the estimation of soluble sugars, while the pellet was reserved for starch quantification. Sucrose was used as the standard reference for both estimations.

For total soluble sugar estimation, appropriate volumes of the ethanol supernatant were diluted to 1 mL with distilled water. To this, 4 mL of cold anthrone reagent (prepared by dissolving 200 mg of anthrone in 100 mL of 95% sulfuric acid) was added. The mixture was cyclomixed for 2–3 minutes and the absorbance was measured at 620 nm using a UV-VIS double beam spectrophotometer. All estimations were carried out in triplicates

For starch estimation, the ethanol-insoluble pellet was resuspended in 5 mL of 52% perchloric acid and centrifuged at 3,000 rpm for 12 minutes. The supernatant was then diluted to 1 mL with distilled water,

and 4 mL of anthrone reagent was added. After thorough mixing, absorbance was recorded at 620 nm. A standard curve was prepared using known concentrations of sucrose for quantification.

Estimation of Reducing Power Assay

2.5 ml of methanol plant extract (1mg/ml in methanol) was mixed with 2.5 ml of sodium phosphate buffer (0.2M; pH 6.6) and 2.5 ml of potassium ferricyanide (1%) in a test tube. The test tubes were then covered by silver foil and placed in water bath at 50°C for 20 mins. The test tubes were then removed from the water bath and allowed to cool down in room temperature and then 2.5 ml of trichloro-acetic acid (TCA 10%) was added. The mixture was then centrifuged at 3000 rpm for 10 minutes. Different concentrations of 25µl to 125 µl supernatant, 1.25 ml distilled water and 250 µl ferric chloride (0.1%) was added to the test tubes. The test tubes mixture was mixed well and absorbance was taken at 700 nm using spectrophotometer.

Estimation of total free amino acids

Total amino acid (TAA) content was estimated using the ninhydrin method (**Moore et al., 1954**). An acetate buffer (pH 5.0) was prepared by dissolving 1.64 g of sodium acetate in 100 mL of distilled water. Ninhydrin reagent was prepared by dissolving 750 mg of ninhydrin in 26.25 mL of 80% ethanol and 11.25 mL of acetate buffer. Ethanolic extract (50 mg) was dissolved in 5 mL of ethanol, centrifuged at 4°C, 3000 rpm for 10 minutes, and the supernatant diluted to prepare 1 mL test solutions. To each, 2 mL of ninhydrin reagent was added, vortexed, and incubated at 100°C for 20–30 minutes. After cooling, absorbance was measured at 570 nm. TAA was calculated as mg/g fresh weight using a glycine standard curve.

Statistical analysis

The experiment was executed following a completely randomized design (CRD), to evaluate the effects of different plant growth regulators (PGRs) on the direct organogenesis of *Valeriana jatamansi* using two different culture media (MS and B5). For shoot proliferation study three nodal explants were transferred in one 250ml erlenmeyer flask and three flasks were used for each treatment. a factorial two-way analysis of variance (ANOVA) was used to evaluate the effects of two independent factors, namely types of growth medium and concentrations of PGRs on the *in vitro* regeneration capacity. Results were evaluated as mean $\pm$ SD. Significant differences for multiple comparisons were determined using Duncan's multiple range test by SPSS 26.0, Correlation and box plot analysis was also done using R software version 2025.09.2.

Results and Discussions

Direct Organogenesis and Shoot Proliferation

Shoot regeneration in *Valeriana jatamansi* was significantly influenced by both basal medium composition and plant growth regulator (PGR) treatments (Fig. 3). Two-way ANOVA confirmed that the main effects of medium and PGRs were highly significant for shoot number ($p \leq 0.01$) and shoot length ($p \leq 0.001$ for medium), while their interaction effects varied by parameter (Table 1). Box plot simulation showed higher growth potential across all shoot growth parameters (Fig. 1).

Table 1. Interaction effects of basal medium composition and plant growth regulator (PGR) concentrations on the *in vitro* morphogenic response of *Valeriana jatamansi*.

Experimental parameters	Variance source of two-way ANOVA				Interaction P × M	
	Growth medium (M)		PGR treatments (P)			
	<i>F</i>	<i>P-value</i>	<i>F</i>	<i>P-value</i>	<i>F</i>	<i>P-value</i>
No. of shoots	11.560**	0.002	3.19**	0.008	1.21 NS	0.321 NS
Shoot length	43.416***	0.000	4.37**	0.001	2.33**	0.039 NS
No. of leaves	9.58**	0.004	1.92 NS	0.086	1.16 NS	0.346 NS
NS non-significant * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$						

Shoot Number

The interaction between growth medium and PGR treatments for shoot number was non-significant ($F = 1.21$; $p = 0.321$), indicating that the influence of the basal medium remained consistent across different hormonal regimes (Table 1). Consequently, the main effects were analyzed independently. When data were pooled across all PGR levels, Gamborg’s B5 medium produced a significantly higher mean shoot number (1.96 ± 0.97 per explant) compared to MS medium (1.33 ± 0.55 per explant) (Table 2). Among the PGR treatments, the highest shoot proliferation (2.33 ± 1.03 shoots per explant) was achieved using either 1.5 mg L^{-1} BAP alone or the combination of 2.0 mg L^{-1} BAP with 0.5 mg L^{-1} IAA (Table 2) (Fig. 5C). Mean separation using Duncan’s multiple range test confirmed that these treatments were statistically superior to the control (1.00 ± 0.00) (Fig. 5E).

Table 2

Main effects of basal media and plant growth regulators on the quantitative growth characteristics of *Valeriana jatamansi* under *in vitro* conditions.

Factor			Shoot number/explant	Shoot length/explant	Leaf number/explant
Growth Medium					
MS			1.33 ± 0.55	1.69 ± 0.61	4.48 ± 2.81
Gamborg's B5			1.96 ± 0.97	2.89 ± 1.07	7.11 ± 3.84
PGR Treatments (mg L ⁻¹)					
Control	BAP	IAA			
0.0			1.00 ± 0.00 ^a	1.00 ± 0.00 ^a	2.00 ± 0.00 ^a
	0.5	0.0	1.16 ± 0.40 ^a	2.4 ± 0.87 ^b	5.16 ± 3.12 ^{ab}
	1.0	0.0	1.5 ± 0.54 ^{ab}	3.11 ± 1.75 ^b	6.66 ± 4.13 ^b
	1.5	0.0	2.33 ± 1.03 ^b	2.41 ± 0.80 ^b	5.5 ± 3.33 ^{ab}
	2.0	0.0	1.66 ± 0.81 ^{ab}	2.33 ± 1.40 ^b	5.5 ± 4.18 ^{ab}
	0.5	0.125	1.16 ± 0.40 ^a	2.08 ± 0.82 ^{ab}	6 ± 2.75 ^{ab}
	1.0	0.5	2 ± 1.09 ^{ab}	2.63 ± 0.47 ^b	7.16 ± 3.81 ^b
	1.5	2.0	1.66 ± 0.81 ^{ab}	2.16 ± 0.51 ^{ab}	5.66 ± 2.33 ^{ab}
	2.0	0.5	2.33 ± 1.03 ^b	2.5 ± 1.04 ^b	8.5 ± 4.88 ^b
Values represent mean ± SD. Means followed by distinct letters within a column indicate significant differences (DMRT, $p \leq 0.05$). Data were recorded 5 weeks after the culture and represent a total of three replicates of 10 plants per treatment BAP 6-Benzylaminopurine, Indole-3-Acetic Acid.					

Shoot Length

In contrast to shoot number, shoot elongation exhibited a significant interaction between the basal medium and PGR treatments ($F = 2.33$; $p = 0.039$), indicating that the response to hormonal supplementation depended on the specific nutrient composition of the basal medium (Table 1). The longest shoots (4.33 ± 1.25 cm) were recorded on B5 medium supplemented with 1.0 mg L^{-1} BAP, followed by B5 medium containing 2.0 mg L^{-1} BAP (3.50 ± 0.00 cm). Across all PGR concentrations, shoots regenerated on B5 medium (2.89 ± 1.07 cm) were consistently longer than those on MS medium (1.69 ± 0.61 cm) (Table 2). Correlation analysis further supported these findings, revealing a strong positive association between BAP concentration and shoot proliferation in Gamborg's B5 medium ($r = 0.86$), (Fig. 2A), whereas a weaker correlation was observed in MS medium ($r = 0.41$), (Fig. 2B).

Leaf Number

The interaction effect for leaf number was non-significant ($F = 1.16$; $p = 0.346$), (Table 1). When pooled across PGR treatments, Gamborg's B5 medium supported significantly higher leaf production (7.11 ± 3.84 leaves per explant) compared to MS medium (4.48 ± 2.81 leaves per explant) (Table 2). Among the hormonal treatments, the combination of 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} IAA yielded the maximum leaf count (8.5 ± 4.88), (Fig. 5C) which was significantly different from the control according to the DMRT (Table 2). A strong positive correlation was observed between leaf number and shoot length in both MS ($r = 0.81$) and Gamborg's B5 ($r = 0.79$) media, (Fig. 2), indicating that enhanced shoot elongation is closely linked to increased leaf development under *in vitro* conditions.

The superior performance of Gamborg's B5 medium suggests that *Valeriana jatamansi* exhibits a preferential response to the nutrient composition of Gamborg's formulation. Gamborg's B5 medium contains relatively higher levels of potassium nitrate and additional nitrogen sources, including ammonium sulfate, which are known to enhance nitrogen assimilation, protein synthesis, and overall morphogenic competence in plant tissue cultures (Aziz et al., 2010; Yin et al., 2017; Bari et al., 2022). Furthermore, the elevated thiamine content of Gamborg's B5 medium may support enzymatic activity and metabolic processes essential for active cell division and tissue differentiation (Goyer et al., 2010; Fitzpatrick et al., 2020; Vollmer et al., 2023). Comparable medium-specific responses have been reported in *Bergenia ciliata*, underscoring the importance of species-specific optimization of basal salt composition for efficient *in vitro* regeneration of threatened Himalayan medicinal plants (Kurmi et al., 2020)

Rooting and acclimatization.

In direct organogenesis, rooting was induced in Gamborg's B5 medium with treatments of BAP ($0.5\text{--}2 \text{ mg L}^{-1}$) and BAP combined with IAA ($0.5 \text{ mg L}^{-1} + \text{IAA } 0.125 \text{ mg L}^{-1}$, $1 \text{ mg L}^{-1} + \text{IAA } 0.25 \text{ mg L}^{-1}$), (Fig. 5F) while MS medium failed to induce rooting. These findings align with earlier studies demonstrating the role of nitrogen sources in improving root architecture in various species. For instance, ammonium sulfate has been linked to enhanced lateral root growth, while potassium nitrate supports nutrient uptake and antioxidant defences, improving plant resilience in challenging environments (Rafi et al., 2018; Wang et al., 2016). This suggests that Gamborg's B5 medium's formulation is well-suited to promoting effective rooting in tissue culture systems.

Shoot-induced plantlets without roots were sub-cultured in MS medium supplemented with auxins (NAA, IBA, IAA) for root induction. No rooting occurred in the control or treatments with NAA (0.2 mg L^{-1}), IBA (0.2 mg L^{-1}), or IAA (0.2 mg L^{-1}). The maximum number of roots (35.66 ± 9.8 roots per explant) was obtained with IBA at a concentration of 4 mg L^{-1} , followed by progressively lower root numbers at 3 mg L^{-1} and 2 mg L^{-1} concentrations of IBA, respectively. Maximum root length ($3.66 \pm 0.94 \text{ cm}$) and the least root induction time (21.66 ± 2.36 days) were achieved with IBA (3 mg L^{-1} and 4 mg L^{-1} , respectively) (Fig. 6) (Table 3). These results suggest that IBA is more effective than NAA and IAA for

adventitious root induction in *Valeriana jatamansi*, which may be attributed to its conversion into active auxin (IAA) and its role in promoting root initiation processes (Fattorini et al., 2017; Frick & Strader, 2018). Plantlets with well-developed roots were transferred to potting mixture containing cocopeat + perlite (1:1) in small clay pots under controlled condition. (27°C and 90% relative humidity) (Fig. 6E). The plants achieved a height of 3–4 cm after four weeks of transplantation. After raising under controlled conditions plants were transferred to large pots containing soil, sand and farmyard manure (1:1:1) and were maintained in green house.

Table 3
Influence of exogenous auxin supplementation on the in vitro rhizogenic response and root architecture of *Valeriana jatamansi*.

Treatment	No. of days for root emergence	Rooting (%)	No of roots (Per explant)	Root length (cm)
Control	0 ± 0 ^a	0%	0 ± 0 ^a	0 ± 0 ^a
MS + NAA 0.2 L ⁻¹	0 ± 0 ^a	0%	0 ± 0 ^a	0 ± 0 ^a
MS + IBA 0.2 L ⁻¹	0 ± 0 ^a	0%	0 ± 0 ^a	0 ± 0 ^a
MS + IAA 0.2 L ⁻¹	0 ± 0 ^a	0%	0 ± 0 ^a	0 ± 0 ^a
MS + IBA 2 L ⁻¹	24 ± 4.32 ^b	100%	19.33 ± 7.02 ^b	3.83 ± 1.17 ^b
MS + IBA 3 L ⁻¹	25 ± 4.08 ^b	100%	32.33 ± 5.73 ^c	4.33 ± 0.94 ^b
MS + IBA 4 L ⁻¹	21.66 ± 2.36 ^b	100%	35.66 ± 9.8 ^c	3.66 ± 0.94 ^b
Values represent mean ± SD. Means followed by distinct letters within a column indicate significant differences (DMRT, p ≤ 0.05). Data were recorded 5 weeks after the culture and represent a total of three replicates of 10 plants per treatment BAP 6-Benzylaminopurine, Indole-3-Acetic Acid.				

Phytochemical analysis

Pigment Analysis

Pigment analysis revealed that field-grown plants contained significantly higher levels of chlorophyll a (2.80 ± 0.01 mg/g FW), chlorophyll b (4.41 ± 0.009 mg/g FW), (Fig. 4A), and total carotenoids (336 ± 22.86 mg/g FW), (Fig. 4B), compared to *in vitro*-grown plants. In contrast, *in vitro*-grown plants exhibited lower pigment concentrations, with chlorophyll a at 1.773 ± 0.004 mg/g FW, chlorophyll b at 2.932 ± 0.01 mg/g FW, and carotenoids at 167.2 ± 1.30 mg/g FW, (Table 4). Similar results could be found in studies conducted on various phytochemical comparison of field grown and *in vitro* plants. (Talreja, 2011; Khan et al., 2021; Malik et al., 2020). This suggests that field conditions favor the synthesis of both chlorophyll a, chlorophyll b and carotenoids, possibly due to enhanced light exposure and nutrient availability.

Table 4
Comparative evaluation of photosynthetic pigments and primary metabolite profiles in *in vitro* propagated and field-grown *Valeriana jatamansi* Jones.

Chlorophyll a	Field grown plants	<i>In vitro</i> plants
	2.80 ± 0.01 mg/g FW	1.773 ± 0.004 mg/g FW
Chlorophyll b	4.41 ± 0.009 mg/g FW	2.932 ± 0.01 mg/g FW
Carotenoids	336 ± 22.86 mg/g FW	167.2 ± 1.30 mg/g FW
Total soluble sugar	31.25 ± 1.8 mg/g FW	14.5 ± 0.7 mg/g FW
Total soluble starch	28.125 ± 1.6 mg/g FW	13.05 ± 0.6 mg/g FW
Total amino acids	5.17 ± 0.18 mg/g FW	5.44 ± 0.08 mg/g FW
FW: Fresh weight		

Total Carbohydrate estimation

Total soluble sugar was found in higher amount in field grown plants (31.25 ± 1.8 mg/g FW) as compared to *in vitro* plants (14.5 ± 0.7 mg/g FW), (Fig. 4C), (Table 4). Total soluble starch was found in higher quantity in the field grown plants (28.125 ± 1.6 mg/g FW) in comparison with *in vitro* grown plants (13.05 ± 0.6 mg/g FW), (Fig. 4D), (Table 4). Similar results could be found in studies conducted on various phytochemical comparison of field grown and *in vitro* plants. (Talreja, 2011; Khan et al., 2021; Malik et al., 2020). This suggests that field conditions favour the synthesis of these compounds, possibly due to enhanced light exposure and nutrient availability in field condition.

Total amino acid

The total amino acids were present in higher amount in the plants grown *in vitro* (5.44 ± 0.08 mg/g FW), as compared to plants in cultivation (5.17 ± 0.18 mg/g FW), (Fig. 4E), (Table 4). The higher accumulation of amino acids in *in vitro*-grown plants compared to field-grown plants can be attributed to the controlled nutrient conditions in the *in vitro* environment. In contrast to field conditions, which present a complex and variable nutrient landscape, *in vitro* conditions provide a consistent supply of nutrients that can enhance amino acid uptake and accumulation. Moreover, the controlled environment of *in vitro* culture may induce specific stress responses that lead to increased amino acid levels, similar to how certain amino acids are involved in plant adaptation to stress. These findings align with the concept that controlled conditions can amplify the effects of biostimulants, leading to higher concentrations of bioactive compounds in plant tissues. (Lardos et al., 2024).

Total reducing power assay

When compared with standard of ascorbic acid with methanolic extracts of *in vitro* grown plants and field grown plants, field grown plants showed highest reducing power 0.334 as compared to *in vitro* grown plants that is 0.111 (Fig. 4F), (Table 4). A higher absorbance of the reaction mixture corresponds to an increased reducing power. Several similar studies have shown that field-grown plants typically

demonstrate a higher reducing power compared to *in vitro* cultured plants. (Hakkim et al., 2007; **Khorasani et al., 2015**; Kutty et al., 2014). Field-grown plants are subjected to various environmental stressors such as extreme temperatures, drought, and pathogen exposure, which can induce the production of higher levels of antioxidants as a defence mechanism (Bansal et al., 2024). These natural conditions stimulate the synthesis of phenolic compounds and other antioxidant metabolites, contributing to a greater reducing power compared to plants grown in controlled, sterile *in vitro* environments (**Thiruvengadam et al., 2015**).

Conclusion

This study successfully established a comprehensive and reproducible micropropagation protocol for *V. jatamansi*, a high-value medicinal plant endemic to the Himalayas, whose natural populations are critically threatened by overexploitation and a lack of organized cultivation. The study aimed to address this urgent need for conservation and sustainable propagation strategies by comparing the efficacy of two widely used culture media, Murashige and Skoog (MS) and Gamborg's B5, for direct organogenesis. The findings unequivocally demonstrate that the Gamborg's B5 medium significantly outperforms the MS medium in key growth parameters, offering a more effective solution for the *in vitro* propagation of this species. The primary focus of this investigation was to determine the optimal medium for achieving high-frequency shoot proliferation, leaf development, and rooting. The results showed that B5 medium fortified with BAP (2 mg L^{-1}) and IAA (0.5 mg L^{-1}) was the most effective for shoot regeneration, producing a maximum of 3 ± 0.816 shoots per plant, which was a notably superior response compared to the best MS medium treatment (BAP 1.5 mg L^{-1}), which yielded 2.33 ± 0.471 shoots per plant. This finding aligns with the observation that Gamborg's B5 medium's distinct nutrient profile, including higher levels of potassium nitrate and ammonium sulfate, enhances nutrient uptake and protein synthesis, which are critical for tissue development. Furthermore, the maximum shoot length ($4.33 \pm 1.247 \text{ cm}$) and the highest number of leaves (11.33 ± 4.714 leaves per plant) were achieved on B5 medium, confirming its overall superior performance for the aerial parts of the plant. Beyond shoot induction, this study also provided a detailed analysis of rooting and acclimatization. The results indicate that rooting was observed exclusively in B5 medium during the direct organogenesis phase. However, root development was also observed in MS medium when shoot induced plantlets were subcultured in MS medium supplemented with auxins. The maximum number of roots (35.66 ± 9.8) and the longest root length ($4.33 \pm 0.94 \text{ cm}$) were recorded in MS medium supplemented with IBA at 4 mg L^{-1} and 3 mg L^{-1} , respectively. This two-step protocol initial shoot proliferation on B5 medium followed by rooting on MS medium provides a comprehensive and highly efficient strategy for mass multiplication.

The study further evaluated the biochemical attributes of *in vitro*-grown plants in comparison to field-grown counterparts. Phytochemical analysis confirmed that field conditions promote the synthesis of a number of key compounds. Field-grown plants had significantly higher levels of chlorophyll a ($2.80 \pm 0.01 \text{ mg/g FW}$), chlorophyll b ($4.41 \pm 0.009 \text{ mg/g FW}$), total carotenoids ($336 \pm 22.86 \text{ mg/g FW}$), total soluble sugars ($31.25 \pm 1.8 \text{ mg/g FW}$), and total starch ($28.125 \pm 1.6 \text{ mg/g FW}$). Moreover, field-grown plants

showed superior antioxidant potential, with a higher reducing power (absorbance of 0.334) compared to *in vitro*-grown plants (absorbance of 0.111). Conversely, *in vitro*-grown plants exhibited a higher total amino acid content (5.44 ± 0.08 mg/g FW) compared to field-grown plants (5.17 ± 0.18 mg/g FW). These differences are likely due to environmental stressors in the field that stimulate the production of secondary metabolites as a defence mechanism, while the controlled *in vitro* environment may enhance the accumulation of primary metabolites like amino acids.

In conclusion, this research provides an efficient species-specific tissue culture protocol that offers a sustainable and scalable method for the mass propagation of *Valeriana jatamansi*. By enabling the production of genetically uniform, high-quality plantlets, this approach can alleviate the pressure on wild populations and support the establishment of commercial cultivation, thereby ensuring the long-term availability of this valuable plant for pharmaceutical, cosmetic, and perfumery industries. This study establishes the importance of optimizing media formulations for specific plant species and highlights the potential of plant tissue culture as a critical tool for both conservation and the sustainable production of threatened medicinal plants.

Abbreviations

ANOVA

Analysis of Variance

B5

Gamborg's Medium

BAP

Benzyl acetyl pyruvate

Cm

Centimetre

°C

Degree Celsius

DMRT

Duncan's Multiple Range Test

FW

Fresh weight

h

Hour(s)

HgCl₂

Mercuric Chloride

IAA

Indole-3-acetic acid

IBA

Indole-3-butyric acid

kPa

kilopascal
mg
Milli gram
Mg/g
milligrams per gram
ml
Millilitre
MS
Murashige and Skoog's (1962) medium
NAA
Naphthalene Acetic Acid
nm
nanometer
pH
Potential of hydrogen
PGRs
Plant growth regulators
rpm
Revolution per minute
SD
Standard deviation
UV
Ultra violet
UV-VIS
Ultraviolet-Visible
 μ l
microlitres
 μ mol
micromole
v/v
Volume by volume
w/v
Weight by volume

Declarations

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author, [Geken Riba], upon reasonable request.

Funding Declaration

The authors declare that no specific funding was received for this research from any funding agency in the public, commercial, or not-for-profit sectors.

Ethics Declaration

Not applicable.

Author Contribution

G.R conceptualized and designed the study, performed the plant collection and laboratory experiments, analyzed the data, and drafted the manuscript. B.P provided institutional supervision and critical insights throughout the research process. R.R.K contributed to the experiment conceptualization phase and provided technical assistance during the experimental work. D.J assisted in writing and was responsible for formatting the manuscript. All authors have read and approved the final version of the manuscript.

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Figures

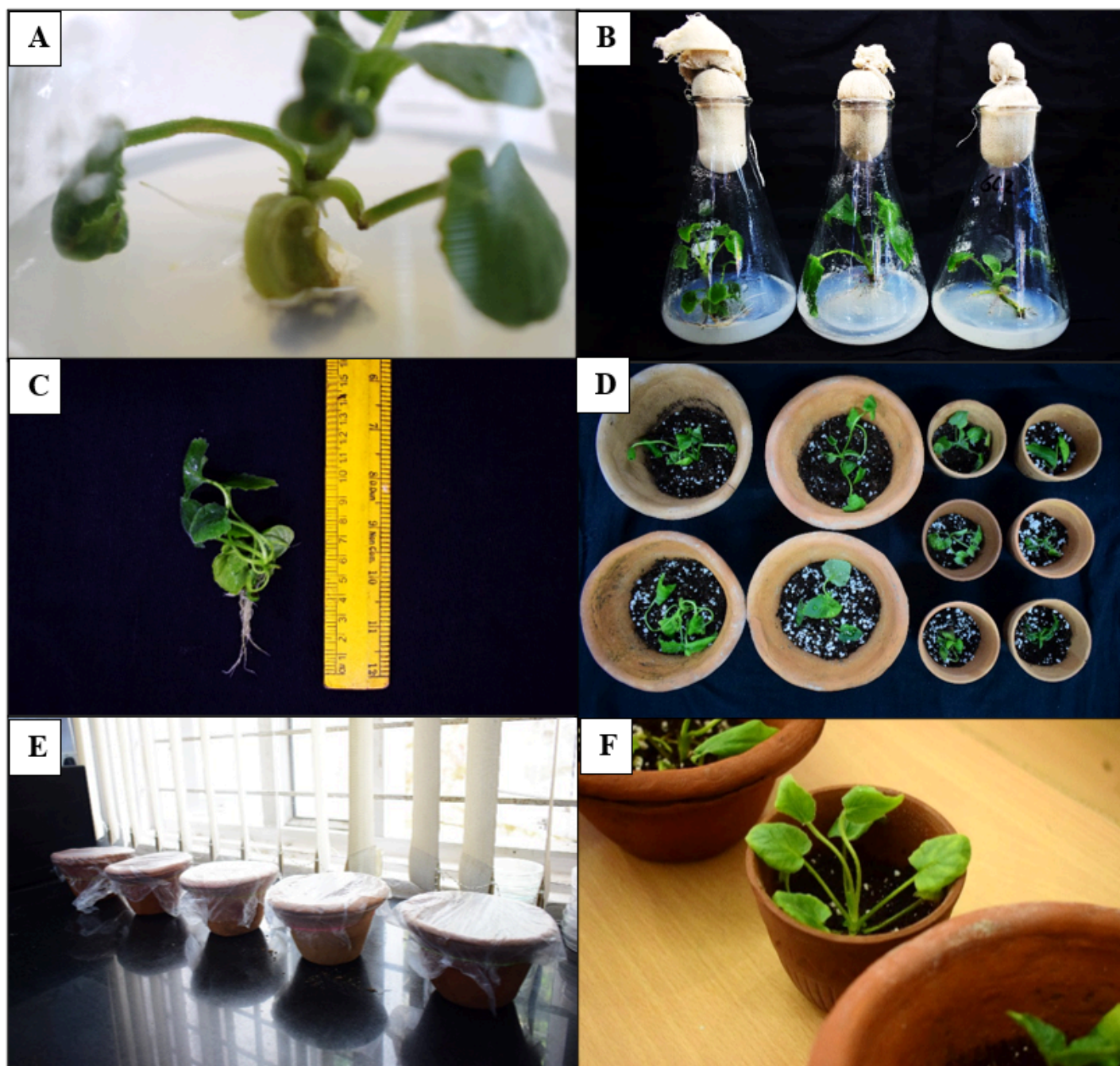


Figure 1

Box plot representation of shoot growth parameters (Number of leaves, number of shoots, and shoot length) of *Valeriana jatamansi* cultured on Murashige and Skoog (MS) and Gamborg B5 media under *in vitro* conditions.

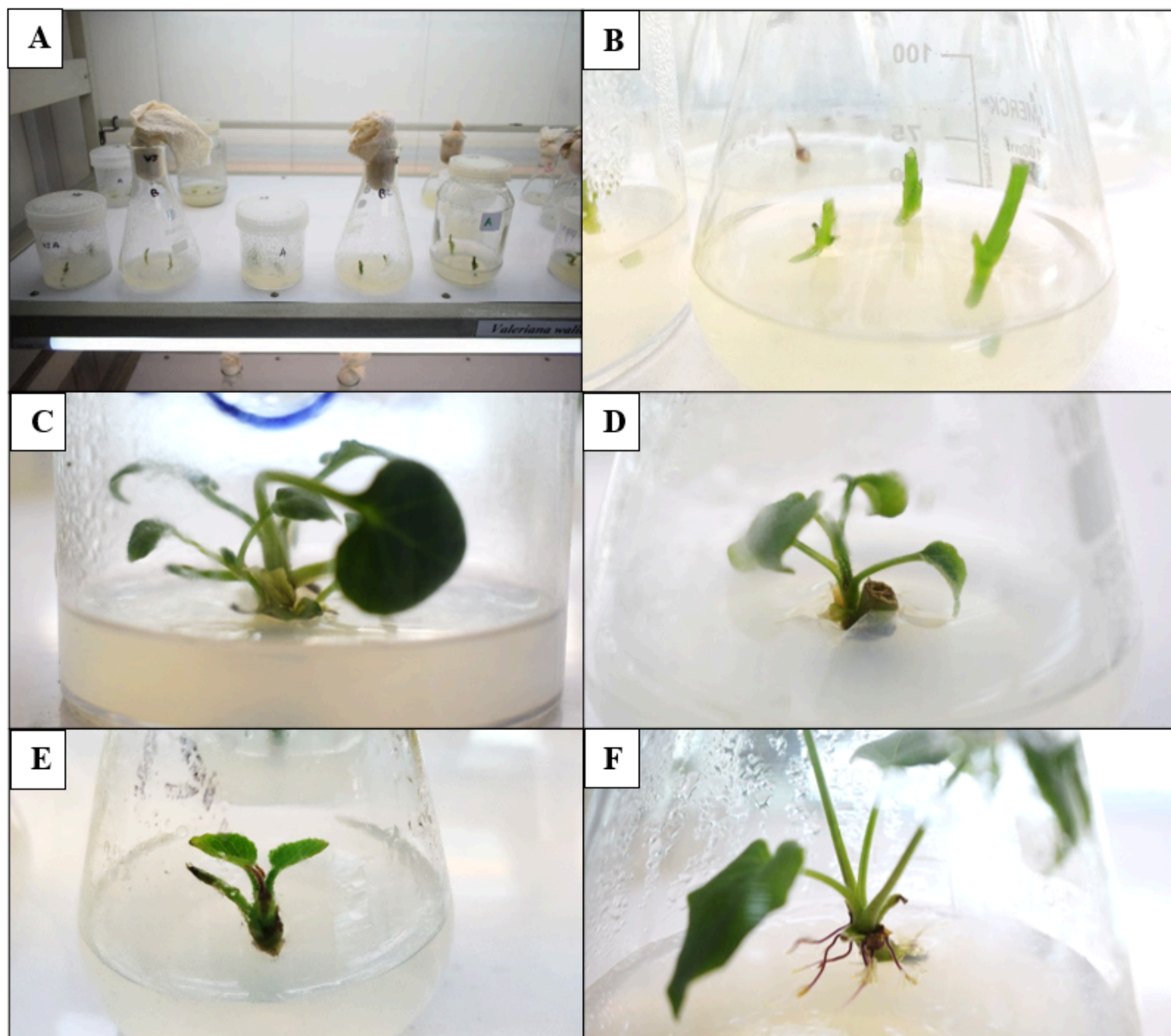


Figure 2

Correlation heatmap illustrating the relationships between shoot growth parameters of *Valeriana jatamansi* and plant growth regulators under *in vitro* conditions on (A) Murashige and Skoog (MS) medium and (B) Gamborg B5 medium.

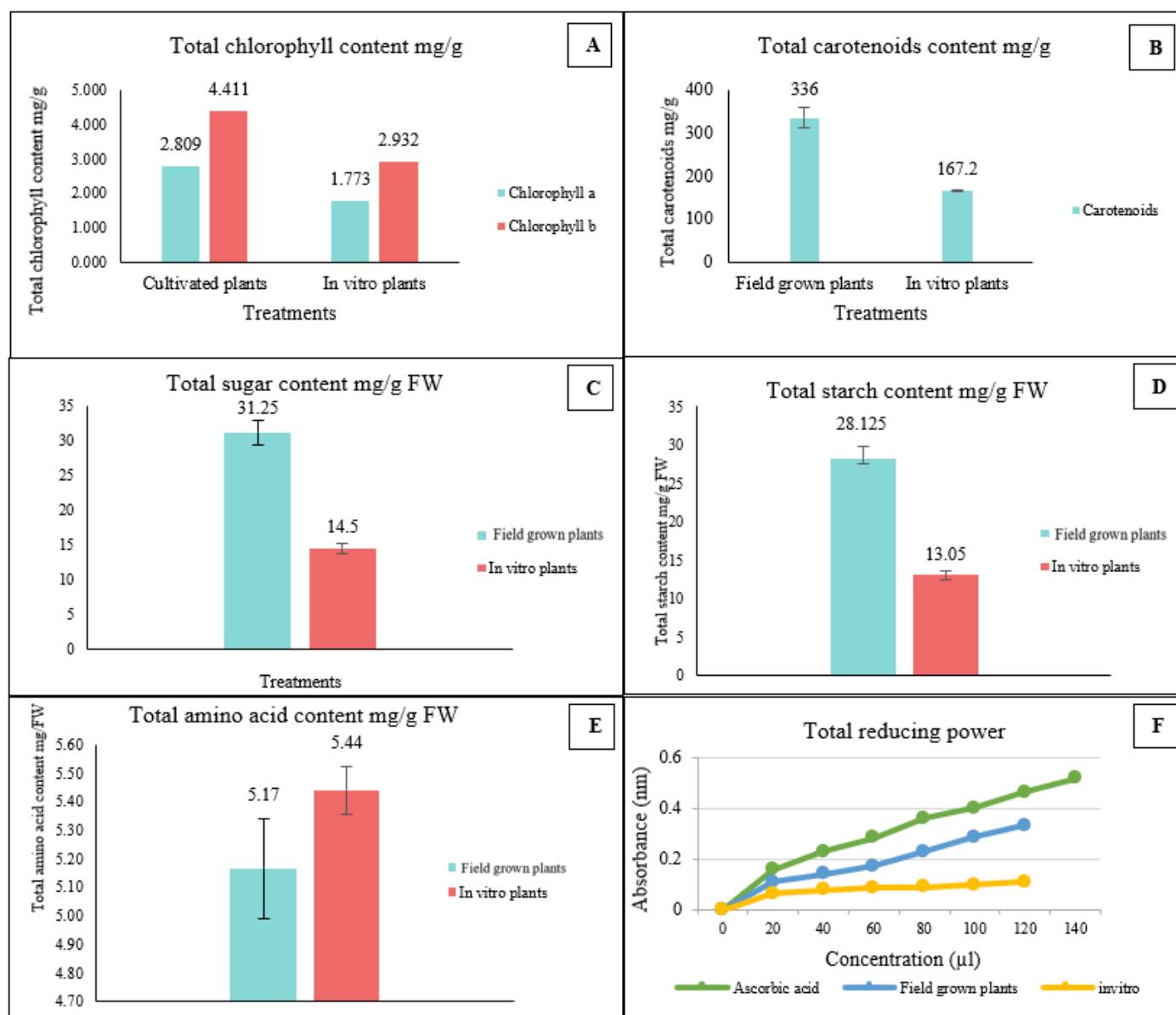


Figure 3

Synergistic effects of growth media and plant growth regulators on shoot growth parameters of *Valeriana jatamansi* under *in vitro* conditions.

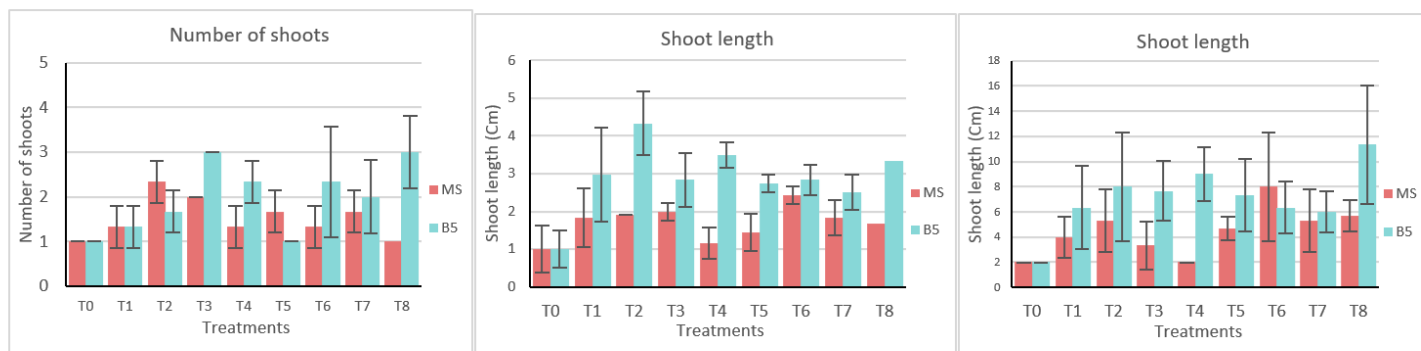


Figure 4

Phytochemical analysis of field grown and *in vitro* grown *Valeriana jatamansi*. (A) Total chlorophyll content, (B) Total Caretonoids content, (C) Total sugar content Mg/g FW, (D) Total starch mg/g FW, (E) Total amino acid content mg/g FW, (F) Comparison of total reducing power in *in vitro* grown and field grown *Valeriana jatamansi*.

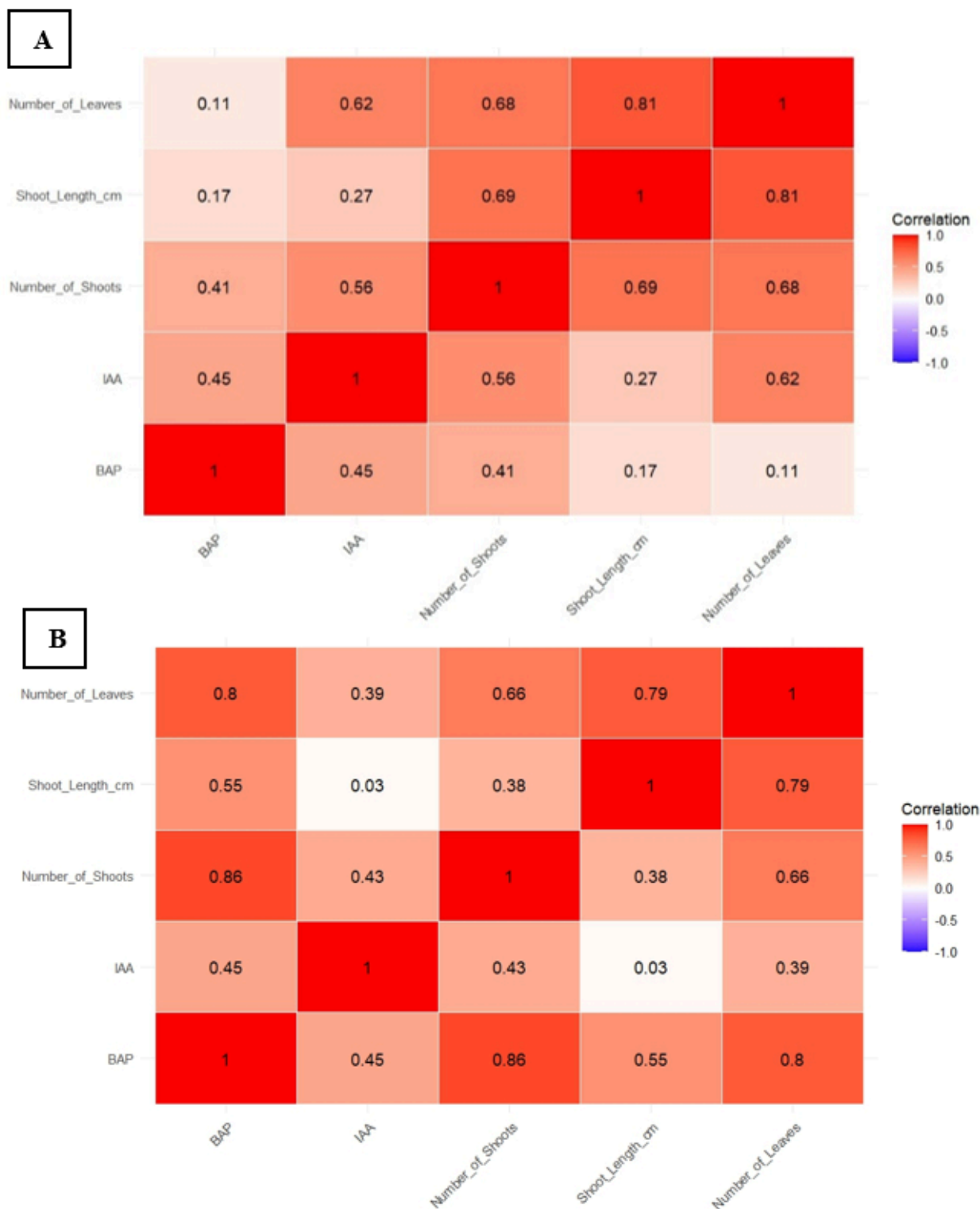


Figure 5

Direct organogenesis of *Valeriana jatamansi* in MS and B5 medium. Inoculated nodal segments under growth chamber conditions; (B) Nodal segments cultured on Murashige and Skoog (MS) medium; (C) Direct organogenesis on Gamborg B5 medium supplemented with BAP (2 mg L⁻¹) and IAA (0.5 mg L⁻¹) after 5 weeks of inoculation; (D) Direct organogenesis on MS medium supplemented with BAP (1.5 mg L⁻¹) after 5 weeks of inoculation; (E) Control (MS medium without plant growth regulators); (F) Root

induction on Gamborg B5 medium supplemented with BAP (2 mg L^{-1}) and IAA (0.5 mg L^{-1}) after 5 weeks of inoculation.

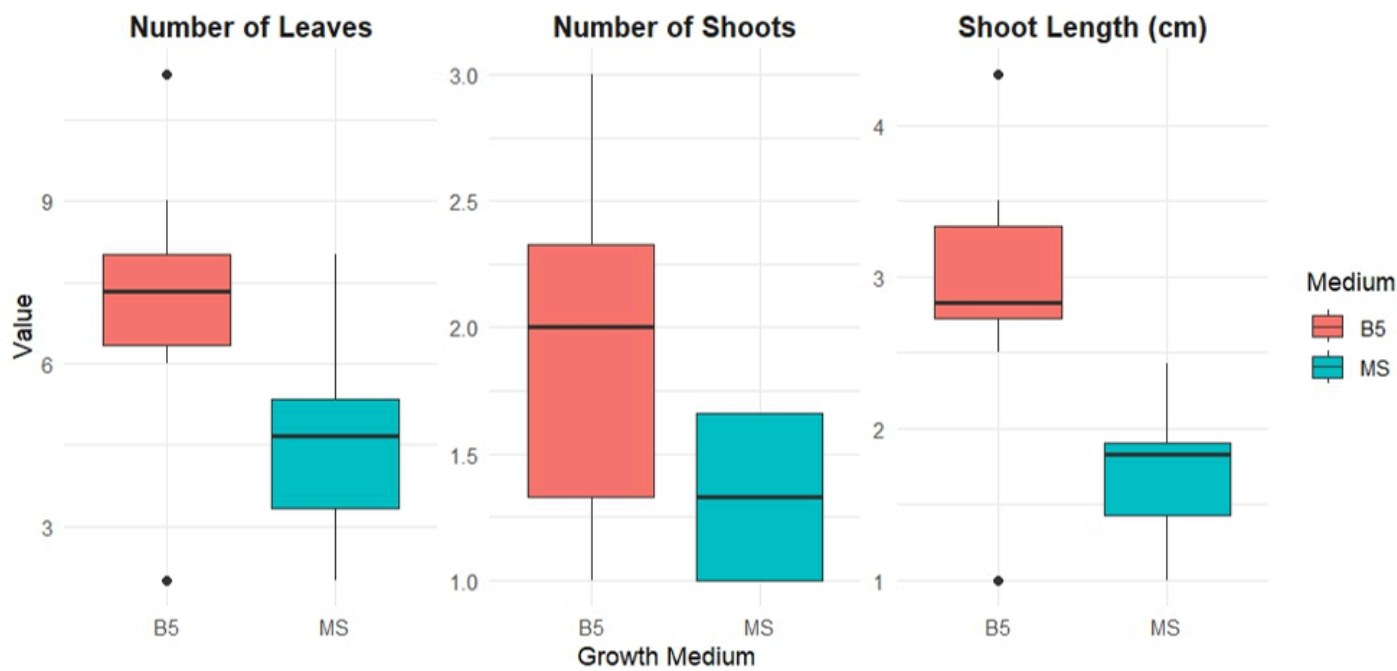


Figure 6

Root induction and acclimatization of *Valeriana jatamansi*. (A) Root induction on Murashige and Skoog (MS) medium supplemented with IBA (3 mg L^{-1}) after 20 days of subculture; (B) Rooted plantlets on MS medium supplemented with IBA (4 mg L^{-1}) ready for acclimatization; (C) Well-developed rooted plantlet on MS medium supplemented with IBA (4 mg L^{-1}); (D) Transfer of plantlets to clay pots; (E) Pots covered with polythene sheets to maintain high humidity; (F) Plants after 5 weeks of acclimatization.