



# Induction and submerged cultivation of *Valeriana jatamansi* adventitious root cultures for production of valerenic acids and its derivatives

Ashok Gehlot<sup>1</sup> · Neha Chaudhary<sup>1,4</sup> · Jyoti Devi<sup>1,2</sup> · Robin Joshi<sup>1</sup> · Dinesh Kumar<sup>2,3</sup> · Shashi Bhushan<sup>1,2</sup>

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## Abstract

In vitro adventitious roots were induced from leaves of *Valeriana jatamansi* to assess their potential as a sustainable alternative to extract pharmaceutically important phytoconstituents. Among the different media used, a significantly ( $p \leq 0.05$ ) high root induction (90%) was achieved on Schenk and Hildebrandt (SH) medium fortified with 9.84  $\mu\text{M}$  indole-3-butyric acid (IBA). In addition, various process parameters i.e. IBA concentration, sucrose and medium strength were also optimized under submerged cultivation. The maximum fresh root biomass ( $144.09 \pm 11.36$  g/L) with a high relative growth rate ( $2.01 \pm 0.04$ ) and growth index (13.41) was achieved in half-strength SH medium having 2% sucrose and 4.92  $\mu\text{M}$  IBA. Further, a significantly high yield of total valerenic acid derivatives [1525.14  $\mu\text{g/g}$  dry weight (DW)] was recorded in adventitious roots as compared to donor plant parts. Individually, valerenic acid (506.27  $\mu\text{g/g}$  DW) was accumulated higher in plant rhizomes, while acetoxyvalerenic (534.91  $\mu\text{g/g}$  DW) and hydroxyl valerenic acid (919.57  $\mu\text{g/g}$  DW) in adventitious roots. Interestingly, hydroxy valerenic acid was unmeasurable in donor plant parts. The phenolic compounds were also found maximum in adventitious roots (451.85  $\mu\text{g/g}$  DW) with the dominance of pharmaceutically important kaempferol and rutin. A substantial increase in phytochemicals was evident at subsequent culture stages with shortened in vitro cultivation cycle (2 months) than field-grown plants (24 months). Moreover, adventitious roots also accumulated 0.059% essential oil with patchouli alcohol (24%) as a key constituent. Conclusively, an enriched metabolic profile and substantially shorter growth cycle under submerged cultivation undoubtedly demonstrated the potential of induced *V. jatamansi* adventitious roots as a feasible source of phytoconstituents.

## Key message

In vitro adventitious roots induced leaf of *Valeriana jatamansi* showed improved metabolic profile and shorter cultivation cycle, thereby exhibiting potential as a sustainable alternative for extraction of industrially important phytoconstituents.

**Keywords** Adventitious roots · Medium strength · Tissue culture · Valerenic acid · Pharmaceutical

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✉ Shashi Bhushan  
sbhushan@ihbt.res.in

<sup>1</sup> Division of Biotechnology, CSIR—Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh 176061, India

<sup>2</sup> Academy of Scientific and Innovative Research, Ghaziabad, Uttar Pradesh 201002, India

<sup>3</sup> Division of Chemical Technology, CSIR—Institute of Himalayan Bioresource Technology, Palampur 176061, Himachal Pradesh, India

<sup>4</sup> Present Address: Department of Biological Sciences and Engineering, Netaji Subhas University of Technology, New Delhi 110078, India

## Abbreviations

AVA	Acetoxyvalerenic acid
DW	Dry weight
GC–MS	Gas chromatography-mass spectrometry
GI	Growth index
HVA	Hydroxyvalerenic acid
IBA	Indole-3-butyric acid
IVA	Isovalerenic acid
Min	Minute
RGR	Relative growth rate
SH	Schenk and Hildebrandt
UPLC	Ultra performance liquid chromatography
VA	Valerenic acid
µg	Microgram
µM	Micromolar
g	Gram
%	Percent
w/v	Weight/volume
mg	Milligram
mL	Millilitre

## Introduction

*Valeriana jatamansi* Jones (Synonym *V. wallichii*) is a vulnerable Himalayan medicinal and aromatic plant. It belongs to Valerianaceae family and grows between 2000 and 3500 m elevation. In Asia, this species is extensively used as a substitute for European *V. officinalis* in several traditional as well as modern system of medicine, especially in sleep supplements (Mathela et al. 2005). Nearly 200 species are reported from Europe, Asia and North America hemisphere with dominance of *V. officinalis*, *V. jatamansi*, *V. quadrangularis*, *V. hardwickii*, *V. wallichii*, *V. edulis* and *V. glechomifolia* (Nandhini et al. 2018). In botanical nomenclature, earlier there was ambiguity between *V. jatamansi* and *Nardostachys jatamansi*, however, published reports further revealed that both plant species are altogether different (Dhiman and Bhattacharya 2020). Valeriana is the most frequently used herb alone or in combination to treat anxiety and insomnia around the globe and is ranked among the top 10 therapeutics sold in the United States (Lanje et al. 2020). The rhizome extract contains sesquiterpenes which primarily includes valerenic acid (VA), isovalerenic acid (IVA), acetoxy valerenic acid (AVA) and hydroxy valerenic acid (HVA), and is primarily known for their tranquilizer activities (Bos et al. 1996; Jugran et al. 2019). The pharmacological effects of its extract are generally attributed to VA and its derivatives that act on GABA receptors of the central nervous system (Becker et al. 2014; Yuan et al. 2004). In addition, its rhizomes also contain essential oil (0.05–1.66%) having an array of applications in aromatic and pharmaceutical industries (Singh et al. 2010). A wide

range of products are presently available on market shelves such as ‘VitaGreen’ (Brain tonic), ‘Mushkbala Valerian root oil’ (Perfume, Spa, Beauty), ‘Calm Bliss’ (Good sleep) etc.

In India, the estimated annual trade of dried *V. jatamansi* roots has increased by more than tenfold since 2001–2002 to about 2000 metric ton in 2019 (Purohit et al. 2015; NMPB 2019). However, a huge gap exists between demand and supply of *V. jatamansi* material. At present, over 70% demand of herbal-based industries is mainly met by uprooting of plants from wild. This destructive means of collection and limited cultivation has put *V. jatamansi* under the category of vulnerable Himalayan plant species (Ved et al. 2017; Dhiman et al. 2020). Hence, there is necessity to develop alternate cultivation technologies to get desired phytochemicals, and plant cell and tissue cultures seem to be a feasible and sustainable option. In this regard, several reports are available that demonstrated the potential of plant cell and organ cultures for the production of secondary metabolites at an industrial scale (Eibl et al. 2018; Khan et al. 2021). In the past few years, the use of differentiated in vitro adventitious roots advocated to be an efficient and stable production system (Murthy et al. 2016). Unlike hairy roots, these can be induced naturally without genetic transformation from non-root plant parts such as leaves, stem and hypocotyl (Devi et al. 2021). These roots are easy to grow under liquid condition and have a fast proliferation rate with the capability of synthesizing specific bioactive compounds (Rahmat and Kang 2019). A number of studies have been reported at the pilot scale, especially in medicinal plants like *Panax ginseng*, *Hypericum perforatum*, *Echinacea purpurea* and *Polygonum multiflorum* (Devi et al. 2021). Similar to other tissue culture systems, their success is also influenced by various in vitro factors such as explant type, medium, light, temperature, and phytohormones, especially auxin and needs to be optimized firstly at lab scale. In the case of *V. jatamansi*, few reports are available on in vitro induction of shoot cultures (Kaur et al. 1999), indirect organogenesis, hairy root induction and their phytochemical assessment (Banerjee et al. 1998; Das et al. 2013; Chen et al. 2014; Pandey et al. 2020). However, no information was found in the literature with respect to adventitious root induction from *V. jatamansi* and their utilization for the extraction of phytoconstituents.

Considering the vulnerability of *V. jatamansi* in the Himalayas, limited cultivation and rising consumer-driven demand for naturals, the present study was carried out to assess the possibility of leaf-induced adventitious roots as an alternate source of phytochemicals. In addition, an efficient protocol is also established by optimizing variables like type of media, auxin concentration, media strength and sucrose level under submerged condition. The developed process seems to be a feasible and stable method for the production of *V. jatamansi* based phytoconstituents.

## Materials and methods

### Plant materials and explant preparation

*V. jatamansi* ( $\approx$  2 years old) plants were collected from Holi, Chamba District, Himachal Pradesh (32°33'19.12" N latitude, 76°07'35.29" E longitude & 1867.00 m altitude) and maintained under poly-house conditions at CSIR-IHBT campus (32°06'29" N Latitude, Longitude 76°33'35" E & 1472 m Altitude) having 20–30 °C temperature and 40–50% relative humidity. Leaves from these plants were taken as explants to induce adventitious roots. Explants were surface sterilized by washing with water having Tween-20 (2–3 drops) and treated with 0.1% (w/v) Bavistin and Streptomycin for 10 min. These explants were then further sterilized with mercuric chloride (0.1% w/v) for 2 min under laminar flow and subsequently washed with autoclaved water. Free water was removed prior to inoculation by placing the explants on autoclaved blotting paper.

### Media preparation, culture conditions and inoculation of explants

Different sterilized media i.e. MS (Murashige and Skoog 1962), SH (Schenk and Hildebrandt 1972) and B5 (Gamborg et al. 1968) medium were used in this study to induce adventitious roots from leaf explant of *V. jatamansi*. The adventitious root induction was only achieved in SH medium. Therefore, further independent experiments to improve and maximize the growth of adventitious roots were conducted in SH medium fortified with different concentrations (0.49, 2.46, 4.92, 9.84, and 19.69  $\mu$ M) of indole-3-butyric acid (IBA), medium strength (1/4 $\times$ , 1/2 $\times$ , 3/4 $\times$ , and 1 $\times$ ) and sucrose concentration (1.0, 2.0, 3.0, 4.0, and 5.0%). All the experiments of root proliferation and multiplication under submerged conditions were carried out in 250 mL conical flask containing 50 mL liquid SH medium, which was fortified with 4.92  $\mu$ M IBA and inoculated at 1.0% inoculum density. The cultures were kept in an incubator shaker (INNOVA 5000) at 70 rpm under the dark condition at a fixed temperature (25  $\pm$  2 °C).

The sterile leaf explants of *V. jatamansi* were cut into 4–5 mm sections and inoculated in autoclaved petri-plates 90  $\times$  15 mm on semi-solid hormone-free MS, SH and B5 medium for induction of adventitious roots. The cultures were kept in tissue culture racks at 25  $\pm$  2 °C temperature under dark condition.

### Determination of root biomass and growth parameters

The adventitious roots were harvested after 8 weeks of cultivation, free medium removed by keeping on sterile filter paper and fresh weight recorded as gram (g). The relative growth rate (RGR) and growth index (GI) was calculated using following equations (Ho et al. 2017):

$$\text{RGR} = \frac{(\ln W_2 - \ln W_1)}{\text{CP}}, \quad (1)$$

$$\text{GI} = \frac{W_2(\text{g}) - W_1(\text{g})}{W_1(\text{g})} \quad (2)$$

where ln: natural log, W1: initial fresh weight, W2: final fresh weight and CP: culture period.

### Quantification of phytoconstituents

The donor plant parts (rhizome and leaves) and in vitro raised adventitious roots obtained after 8 weeks of cultivation in optimized media (half-strength SH medium supplemented with 2% sucrose and 4.92  $\mu$ M IBA) were dried and powdered for further analysis. The samples (200 mg) were sonicated for 60 min with 3.0 mL of High-performance liquid chromatography (HPLC) grade methanol and thereafter centrifuged at 2000 rpm for 10 min to extract phytochemicals. The left-over residue of each sample was again extracted with 2 mL of solvent and supernatant pooled together in 5.0 mL sample collecting vials. Finally, respective supernatants were filtered with a Puradisc syringe filter (0.2  $\mu$ m) and stored at 4 °C. The standards of VA, AVA and HVA were procured from Sigma India and stock solutions (1 mg/mL) made in HPLC grade methanol to prepare the calibration curve. The VA and its derivatives were quantified at 280 nm using Acquity Ultra Performance Liquid Chromatography—e $\lambda$  photodiode array detector (UPLC-PDA, Waters). All the samples were injected at 5  $\mu$ L concentration. Moreover, the quantification of valerenic acid and its derivatives was also done during different culture stages i.e. adventitious roots induced on SH media fortified with 9.84  $\mu$ M IBA (induction stage, P0), proliferation (first subculture) on semi—solid SH media with 4.92  $\mu$ M IBA (proliferation stage, P1) and submerged cultivation in liquid SH media having 4.92  $\mu$ M IBA (multiplication stage, P2) on production.

In addition, the phenolic derivatives (gallic acid, p-coumaric acid, rutin, ferulic acid, cinnamic acid and kaempferol) were quantified in respective samples (100 mg each plant root, leaf, and adventitious roots) using UPLC-PDA system fitted with HSS-T3 C18 column (2.1 mm 100 mm, 5 mm, and 1.8  $\mu$ m) at 270 nm. All the samples were extracted

in 70% HPLC grade methanol for 10 min by sonication, followed by 10 min centrifugation at 8000 rpm. The samples were filtered through 0.22 µm syringe filter and transferred to vials for further analysis.

### Extraction and analysis of essential oil

Freshly harvested *V. jatamansi* plant parts (rhizome and leaves) and in vitro adventitious roots were chopped, washed with distilled water and air-dried on sterile filter paper. The samples (1 kg each fresh weight) were put in 5.0 L round bottom flask and hydro-distilled (400 mL water) for 4 h in Clevenger apparatus. The extracted oil was filtered and stored under dark in sealed vials at 4 °C. The components of extracted essential oil were quantified by Gas Chromatography–Mass Spectrometry (GC–MS), Shimadzu QP2010 series fitted with AOC-500 auto-sampler and SH-RXI-55ILMS capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). The constituents were identified by comparing their relative retention indices (RRI) with known mass spectral data available at National Institute of Standards and Technology libraries. A mixture of *n*-alkanes (C<sub>8</sub>–C<sub>24</sub>) was used as a reference for the calculation of RRI in temperature-programmed run. Moreover, decane was also used as an internal standard.

### Design of experiment and data analysis

The experiments were conducted under a completely randomized block design with four replicates per treatment. The experimental data for induction and multiplication was recorded after 4 and 8 weeks, respectively. The results obtained from different independent experiments i.e. adventitious root induction in different media as well as optimization of IBA concentration, medium strength and sucrose concentration in SH media performed in triplicate were analysed by one-way analysis of variance (ANOVA) using SPSS (version14) software. The main effects and their interactions with dependent factor were studied for the test of significance ( $p \leq 0.05$ ) and compared using Duncan Multiple Range Test. The degree of variations was represented as mean and standard error.

## Results and discussion

### Induction and proliferation of adventitious roots

#### Effect of medium on induction of adventitious root

Hormone free MS, SH and B5 semi-solid media were used to induce in vitro adventitious roots from leaf explant of *V. jatamansi*. Among different media tried, the induction of

root was only observed in hormone free SH medium after 4 weeks of inoculation. It might be due to the nutritional composition of SH media that have low ammonium:nitrate ratio (1:9) as compared to MS (1:2) and 10 times higher myo-inositol (1000 mg/L) concentration than both B5 and MS medium. It is pertinent to mention here that ammonium:nitrate ratio is important for organogenesis, whereas myo-inositol known to accelerate cell division rather than increasing the cell size (Staudt 1984), and plays a significant role in plant cell growth and development (Loewus and Murthy 2000).

#### Effect of auxin (IBA) on adventitious root induction

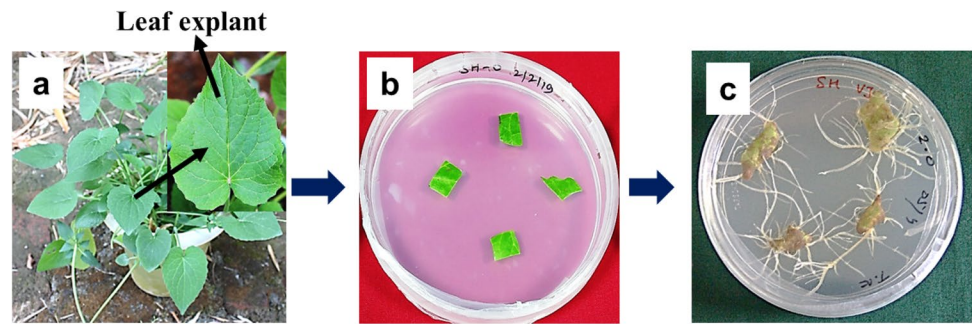
Further improvement in adventitious root induction was done by fortifying optimized SH medium with different 'IBA' concentration. The induction of adventitious roots was observed from the cut ends of leaves within 8 days of inoculation, as compared to 28 days in hormone free medium. A significantly ( $p \leq 0.05$ ) high percentage of adventitious root induction (90%) as well as number ( $5.72 \pm 0.18$ ) and length ( $1.73 \pm 0.06$  cm) was recorded in medium fortified with 9.84 µM IBA (Table 1; Fig. 1). However, the rooting potential was found to be decreased beyond this IBA concentration. It is probably due to more callusing that has been observed in the inoculated plant tissues. In accordance to present the study, SH medium enriched with IBA (24.6 µM) too has shown high adventitious root induction efficiency in *P. ginseng* as compared to NAA (1-naphthaleneacetic acid) (Kim et al. 2003). Similarly, high adventitious root induction was also reported on SH medium containing IBA (7.0 mg/L) from leaf explant of *P. vietnamensis* (Linh et al. 2019). In contrast, Saini et al. (2018) reported induction of adventitious root from leaves of micropropagated *V. jatamansi* plants in MS medium with BAP (6-benzylaminopurine) and NAA (2.0 mg/L).

**Table 1** Adventitious root induction response at different IBA concentrations

IBA (µM)	Induction %	No. of adventitious root (NoAR)	Length of adventitious root (cm) (LoAR)
Control	10.00 ± 10.00a	1.50 ± 0.29a	0.15 ± 0.03a
0.49	30.00 ± 19.14ab	2.33 ± 0.21b	0.38 ± 0.02b
2.46	40.00 ± 24.49ab	2.50 ± 0.19b	0.46 ± 0.05b
4.92	65.00 ± 23.62ab	3.38 ± 0.14c	0.70 ± 0.06c
9.84	90.00 ± 10.00b	5.72 ± 0.18e	1.73 ± 0.06e
19.69	80.00 ± 20.00b	4.69 ± 0.18d	1.16 ± 0.06d

Values (mean ± standard error) within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test (DMRT)

**Fig. 1** Induction of adventitious root from *V. jatamansi* leaves. **a** Parent plant, **b** Leaf section inoculated on semisolid SH medium and **c** Induction of adventitious roots from explant



The effect of auxin on adventitious root induction was further confirmed by fortification of basal SH, MS and B5 media with the optimized concentration of IBA (9.84  $\mu\text{M}$ ). Remarkably, adventitious roots were induced in all the media from inoculated leaf explants. However, significantly high ( $p \leq 0.05$ ) rhizogenic induction potentials (90%) as well as number ( $5.44 \pm 0.32$ ) and length ( $1.47 \pm 0.09$  cm) of roots was obtained in SH medium as compared to MS and B5 (Fig. 2). Thus, the earlier results hold true and the SH medium with 9.84  $\mu\text{M}$  IBA concentration was found optimum for induction of adventitious roots in a shorter time. These roots were repeatedly sub-cultured and maintain as mother stock for further experiments.

## Submerged cultivation

### Effect of IBA on growth of adventitious roots

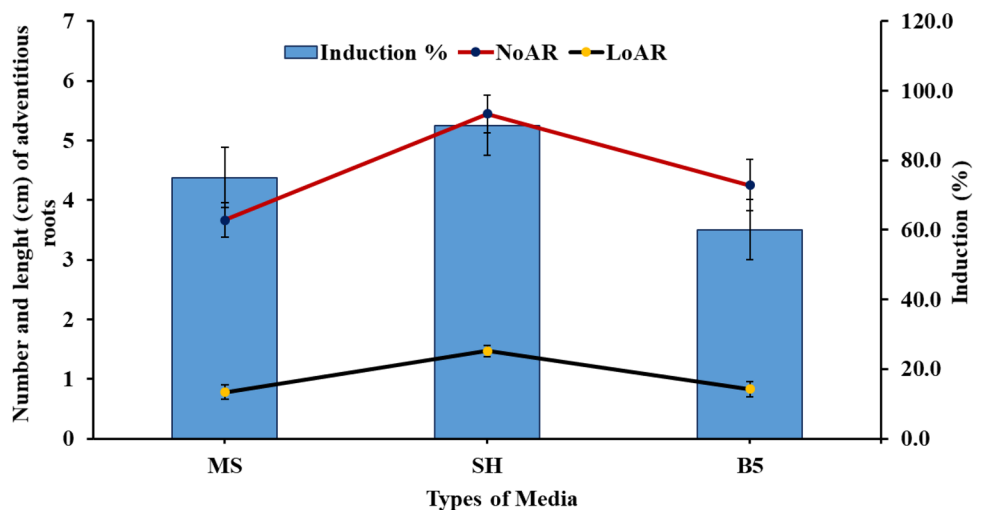
In order to evaluate the capability of adventitious roots for large-scale cultivation, in vitro induced roots were inoculated (1.0% inoculum density) in SH liquid medium having various IBA concentrations. A significantly ( $p \leq 0.05$ ) high root biomass yield ( $123.39 \pm 7.11$  g/L FW) and RGR ( $1.95 \pm 0.03$ ) was recorded at 4.92  $\mu\text{M}$  IBA after 8 weeks

of cultivation (Table 2). In addition, the growth index ( $11.34 \pm 0.71$ ) (GI) was also found highest in the same medium. However, IBA concentration beyond 4.92  $\mu\text{M}$  was not able to support further growth of adventitious roots. It is evident from the results that just half IBA concentration is sufficient during submerged cultivation of adventitious root than required for optimum induction (9.48  $\mu\text{M}$ ). Contrary to present results, Kannan et al. (2020) observed maximum proliferation efficiency of induced roots (1.568 g/L FW) on liquid MS medium enriched with the same IBA concentration (1.0 mg/L) as optimized for induction from leaf explants of *M. coreia*. Similarly, Wu et al. (2006) also reported induction of *E. angustifoli* adventitious roots from root explants on half strength liquid MS media supplemented with IBA (1.0 mg/L), however, growth of induced root was maximum (11.8 g/L FW and 6.0 growth rate) in same strength liquid media fortified with 2.0 mg/L IBA. They also observed a negative impact on root biomass growth beyond 2.0 mg/L IBA.

### Effect of medium strength

Medium type and their elemental composition play a crucial role in the growth as well as overall productivity of

**Fig. 2** Effect of different media on induction of adventitious roots. Data represented in bar chart showed mean  $\pm$  SE of five replicates and significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test (DMRT). MS Murashige and Skoog, SH Schenk and Hildebrandt, B5 Gamborg, NoAR Number of adventitious roots, LoAR Length of adventitious roots (cm)



**Table 2** Effect of IBA, media strength and sucrose concentration on adventitious root biomass production

Growth parameter	IBA ( $\mu\text{M}$ )		Strength of SH media							
	Control	0.49	2.46	4.92	9.84	19.69	1/4X	1/2X	3/4X	1X
Fresh weight (g/L)	21.03 $\pm$ 1.18a	61.96 $\pm$ 4.84b	84.44 $\pm$ 5.79c	123.39 $\pm$ 7.11e	107.47 $\pm$ 7.29d	67.89 $\pm$ 4.79b	97.87 $\pm$ 16.12ab	126.40 $\pm$ 23.90c	63.20 $\pm$ 13.11a	106.40 $\pm$ 15.04ab
Relative growth rate	1.19 $\pm$ 0.02a	1.64 $\pm$ 0.04b	1.78 $\pm$ 0.03c	1.95 $\pm$ 0.03d	1.88 $\pm$ 0.04d	1.69 $\pm$ 0.03b	1.77 $\pm$ 0.08ab	1.86 $\pm$ 0.09b	1.57 $\pm$ 0.08a	1.83 $\pm$ 0.07b
Growth index	1.22 $\pm$ 0.02a	1.62 $\pm$ 0.05b	1.76 $\pm$ 0.04c	1.95 $\pm$ 0.03d	1.87 $\pm$ 0.04d	1.72 $\pm$ 0.03c	1.47 $\pm$ 0.08ab	1.56 $\pm$ 0.09b	1.26 $\pm$ 0.08a	1.53 $\pm$ 0.07b
Growth parameter	Sucrose (%)									
	1.0	2.0	3.0	4.0	5.0					
Fresh weight (g/L)	100.30 $\pm$ 9.81b	144.09 $\pm$ 11.36c	136.81 $\pm$ 14.46c	100.57 $\pm$ 8.65b	58.47 $\pm$ 9.69a					
Relative growth rate	1.84 $\pm$ 0.05b	2.01 $\pm$ 0.04d	1.98 $\pm$ 0.05bc	1.85 $\pm$ 0.04b	1.58 $\pm$ 0.06a					
Growth index	1.54 $\pm$ 0.05b	1.71 $\pm$ 0.04d	1.68 $\pm$ 0.05 cd	1.55 $\pm$ 0.04bc	1.28 $\pm$ 0.06a					

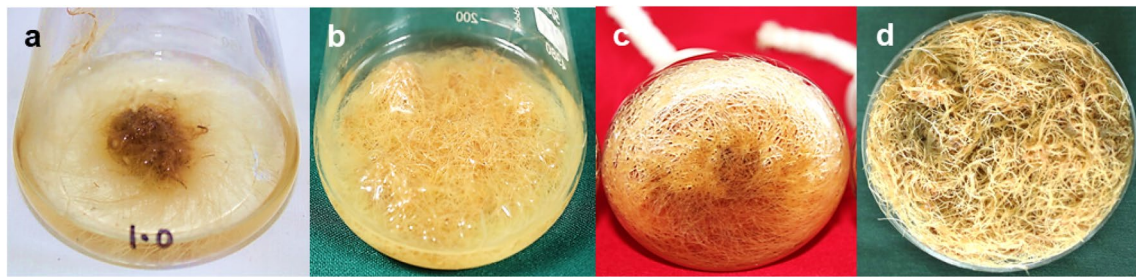
Values (mean  $\pm$  standard error) within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test (DMRT)

in vitro plant tissue cultures. In this experiment, different strengths of liquid SH medium (1/4X, 1/2X, 3/4X, and 1X) supplemented with the best responsive IBA concentration (4.92  $\mu\text{M}$ ) were investigated to maximize the biomass yield. A significantly ( $p \leq 0.05$ ) high roots biomass (126.40  $\pm$  23.90 g/L) and RGR (1.86  $\pm$  0.09) was recorded in 1/2  $\times$  strength SH medium (Table 2). GI was also highest in 1/2  $\times$  strength SH medium. In contrast, 1/4  $\times$  strength SH medium exhibited lowest growth. Corroborating to our results, adventitious root induced from *E. angustifolia* showed maximum biomass yield in half strength MS medium supplemented with IBA (Wu et al. 2006). Similarly, half strength of MS medium fortified with IBA (3.0 mg/L) was also found optimum for multiplication of adventitious roots induced from rhizome of *P. hexandrum* (Rajesh et al. 2014). It can be inferred that low salt concentration favouring the growth of root tissues, as is also known to increase the availability of nutrient ions in the medium (George et al. 2008). It will also help in reducing the overall cost of the process.

### Influence of sucrose concentrations

Sucrose is the principal energy source catabolized easily by plant tissues into glucose and fructose. In the present work, 1/2  $\times$  strength SH media having 4.92  $\mu\text{M}$  IBA was fortified with 1–5% (w/v) sucrose concentration to further improve roots biomass yield under submerged cultivation. The medium augmented with 2% sucrose showed significantly high root biomass (144.09  $\pm$  11.36 g/L FW), RGR (2.01  $\pm$  0.04) and GI (13.41  $\pm$  1.14) (Table 2). Thereafter, a continuous decrease was noticed in the biomass yield with a further increase in sucrose concentration (3–5%). This subsequent decrease in root biomass can be attributed to relatively higher osmotic pressure (Cui et al. 2010). Similar to these observations, Murthy and Praveen (2013) reported maximum accumulation of *Withania somnifera* adventitious root biomass (113.58 g/L FW) on half strength liquid MS media having 2% sucrose after 4 weeks of culture period. They also observed a negative effect on the accumulation of root biomass of increased sucrose concentration (3–8%). In another study, MS medium having 2% sucrose was also yielded highest adventitious root biomass (13.8  $\pm$  1.60 g FW per flask) in *Gynura procumbens* after 4 weeks of cultivation (Saiman et al. 2012).

It can be summarized from the above discussed data that half strength SH medium supplemented with 2% sucrose and 4.92  $\mu\text{M}$  IBA is optimum to obtain maximum adventitious root yield under submerged cultivation (Fig. 3). The complete in vitro process of *V. jatamansi* adventitious root cultivation have 2-month cycle period, which is considerably very low than 2 years under field condition. Overall, the whole process can be divided into three stages i.e.



**Fig. 3** Growth of in vitro induced adventitious roots at different interval under submerged cultivation in shake flask cultures. **a** 2-week old, **b** 8-week old, **c** 8-week old (bottom side view) and **d** fresh roots harvested after completion of cultivation

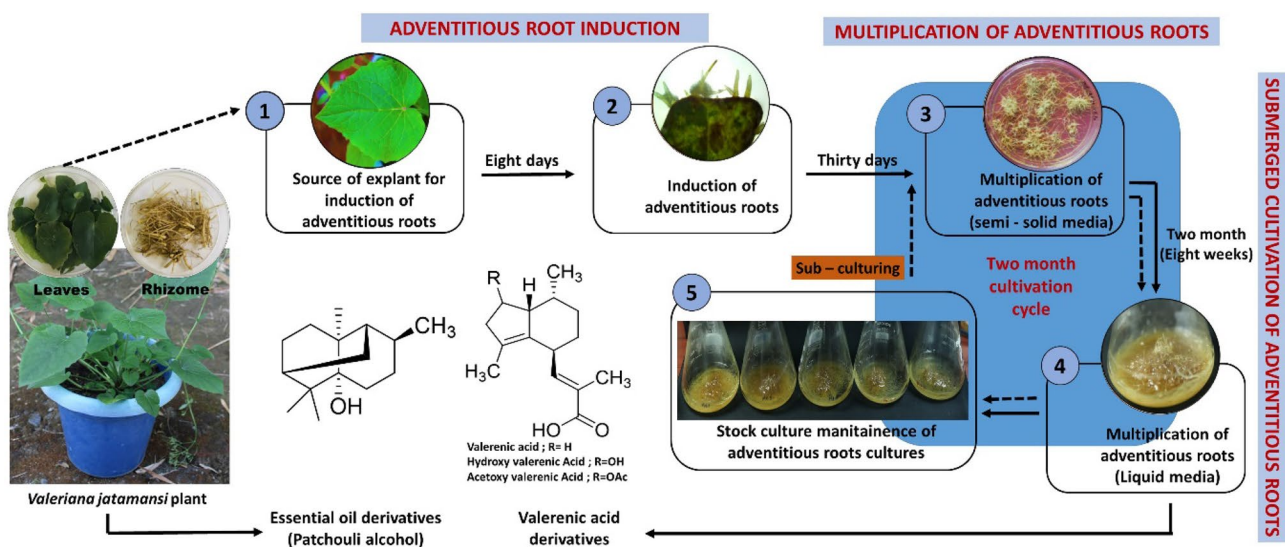
adventitious root induction, multiplication and submerged cultivation as described in Fig. 4.

### Quantitative analysis of phytoconstituents

#### Valerenic acid and its derivatives

Comparative evaluation of donor plant parts and in vitro induced adventitious root was done to assess marker phytoconstituents. Among various samples, the VA contents were significantly high in plant rhizomes ( $506.27 \pm 10.34 \mu\text{g/g DW}$ ) followed by leaves, whereas AVA was dominant in induced roots (Table 3; Fig. 5). Unexpectedly, HVA was not quantifiable in donor plant parts, however, present in good quantity ( $919.57 \pm 28.85 \mu\text{g/g DW}$ ) in adventitious root (Table 3) as evident from the comparative UPLC spectra of donor plant parts [rhizome (Fig. 5d) and leaves (Fig. 5e)] and in vitro induced root samples (Fig. 5f). Considerably high accumulation of HVA in induced adventitious roots had also resulted in maximum total VA derivatives yield

i.e. 59.01 and 86.91% higher than rhizome and leaves of donor plant. Here, it is also pertinent to mention that the comparative evaluation performed was between 2-month (8 weeks) old in vitro adventitious roots versus rhizomes and leaves harvested from approximately 2-year old field grown plants. The information available on *V. jatamansi* cell and tissue culture is limited, especially under submerged cultivations. However, few reports on *V. officinalis* are available that demonstrated the effect of different explants on root induction and phytochemical production. Tousi et al. (2010) assessed the phytochemical potential of adventitious roots induced from different explants of in vitro grown *V. officinalis* plants, Authors reported the highest VA (0.38%), AVA (0.55%) and HVA (0.44%) from petiole-induced roots on MS medium supplemented with IAA. Similarly, Ghaderi and Jafari (2014) demonstrated that the tissue culture-raised *V. officinalis* plants are capable of producing higher content of valtrate and valerenic acid. In studies performed on field grown plants, Singh et al. (2006) found relatively higher VA content (0.42%) in *V. officinalis* than *V. jatamansi* (0.12%)



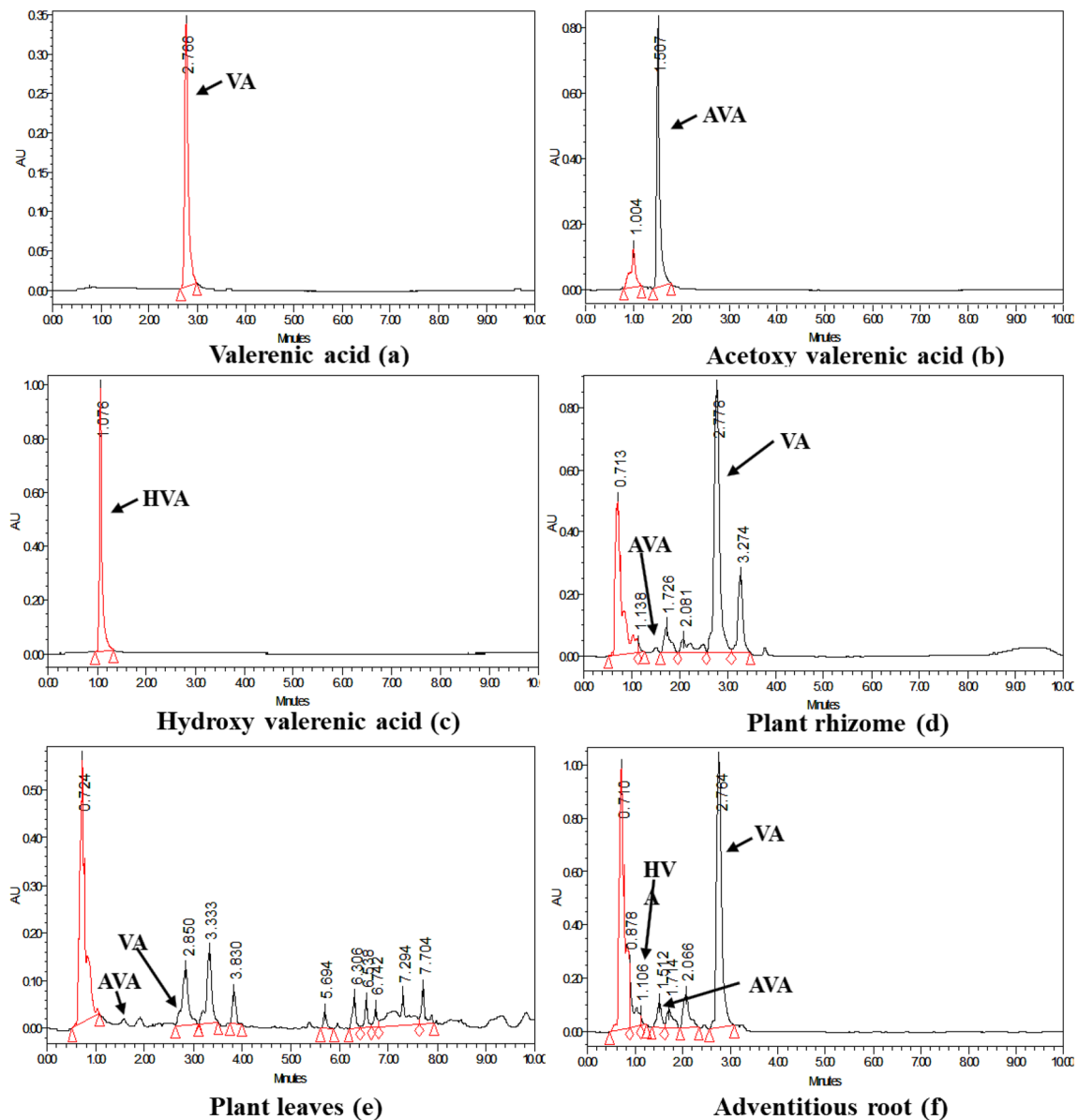
**Fig. 4** Overall process for production of phytoconstituents using leaf-induced in vitro adventitious root cultures

**Table 3** Quantitative analysis of marker compounds present in *V. jatamansi* plant parts and in vitro induced adventitious roots

Sample (s)	Metabolite content ( $\mu\text{g}/\text{gm DW}$ )			
	VA	AVA	HVA	Total valerenic acid derivatives yield
Rhizome	$506.27 \pm 10.34\text{b}$	$118.51 \pm 4.16\text{a}$	$0.00 \pm 0.00^*\text{a}$	$624.78 \pm 13.67\text{b}$
Leaves	$79.23 \pm 4.56\text{a}$	$120.94 \pm 7.48\text{a}$	$0.00 \pm 0.00^*\text{a}$	$200.17 \pm 4.27\text{a}$
Adventitious roots	$70.66 \pm 0.36\text{a}$	$534.91 \pm 39.57\text{b}$	$919.57 \pm 28.85\text{b}$	$1525.14 \pm 55.20\text{c}$

VA valerenic acid, AVA acetoxyvalerenic acid, HVA Hydroxyvalerenic acid

\*NQ: Not quantifiable. Values (mean  $\pm$  standard error) within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test (DMRT)



**Fig. 5** Identification of marker compounds in different *V. jatamansi* samples. **a** VA Valerenic acid, **b** AVA acetoxyvalerenic acid, **c** HVA hydroxyvalerenic acid, **d** plant rhizome, **e** plant leaves and **f** in vitro induced adventitious roots



rhizomes. Similarly, Srivastava et al. (2010) reported higher valerenic acid content (0.31%) in *Nardostachys jatamansi* as compared to *V. jatamansi* (0.11%). Recently, Partap et al. (2020) studied the effect of methyl jasmonate and yeast extract on accumulation of VA derivatives in leaves and roots of *V. jatamansi*. They reported maximum amount of VA (4.19 mg/g DW) in plants roots grown under pot followed by the nursery and aeroponic cultivation. Thus, it can be concluded that in vitro adventitious roots could be a potential alternative for field grown plants to extract phytoconstituents.

#### Determination of phenolic acids derivatives

In this study, phenolic compounds were quantified to comprehend any notable change in their profile. The data exhibited a significantly high amount of total phenolic acid derivatives (451.58 µg/g DW) in adventitious roots than rhizome (187.79 µg/g DW) and leaves (263.68 µg/g DW) of donor plants (Table 4). Strikingly, rutin (217.86 ± 0.32 µg/g DW) and kaempferol (22.82 ± 8.36 µg/g DW) were significantly high in induced roots as compared to donor plant part (Table 4; Fig. 6). These compounds are strong antioxidants and predominantly used to develop phytopharmaceuticals for inflammation related diseases (Choy et al. 2019). Thus, induced roots can be considered as a good source for these bioactives in addition to other phenolic compounds. Earlier, gallic acid and p-coumaric acid were reported as dominating phenolic compounds in aerial and root of *V. jatamansi* plants with total yield varying from 8.76 to 13.16 mg GAE/g DW (Jugran et al. 2020). Likewise, Bhatt et al. (2012) reported comparable phenolic profile of both aerial and root *V. jatamansi* plant parts collected from the wild. A small number of flavonoid glycosides including kaempferol and rutin are also isolated from underground parts of *V. jatamansi* plant (Jugran et al. 2019).

#### Effect of culture stages on production of valerenic acid derivatives

Generally, plant tissues show variability with respect to their growth as well as phytochemicals production at various developmental stages and environment. Consequently, the VA derivatives were quantified in adventitious root harvested from different culture stages i.e. (a) induction stage (P0), (b) proliferation stage (P1) and (c) multiplication stages (P2). The results showed the presence of VA, AVA and HVA in all the stages of root formation and multiplication (Fig. 7). However, a significant ( $p \leq 0.05$ ) increase was noticed in total VA derivatives (302.28–1625.98 µg/g DW) from culture stage P0 to stage P2. It seems that P0 stage primarily focused on growth and development, whereas tissue at the culture stage P1 and P2 displayed the shift towards secondary metabolism probably due to change in the cultivation environment. Comparable information on secondary metabolite production with respect to culture stages/passage is limited, however, few reports available are available. In *Digitalis lanata* calli, Hagimori et al. (1980) observed a decrease in digoxin and digitoxin contents in second as compared to the first passage. Similarly, Garica-Mateos et al. (2005) reported significant accumulation of alkaloids i.e. alpha (43.26–45.58%) and beta (49.75–52.44%) erythroidines up to the fifth sub-culture and found a subsequent decrease thereafter. In general, more efforts are needed to establish the relationship between culture passage and overall productivity for developing commercially feasible bioprocess.

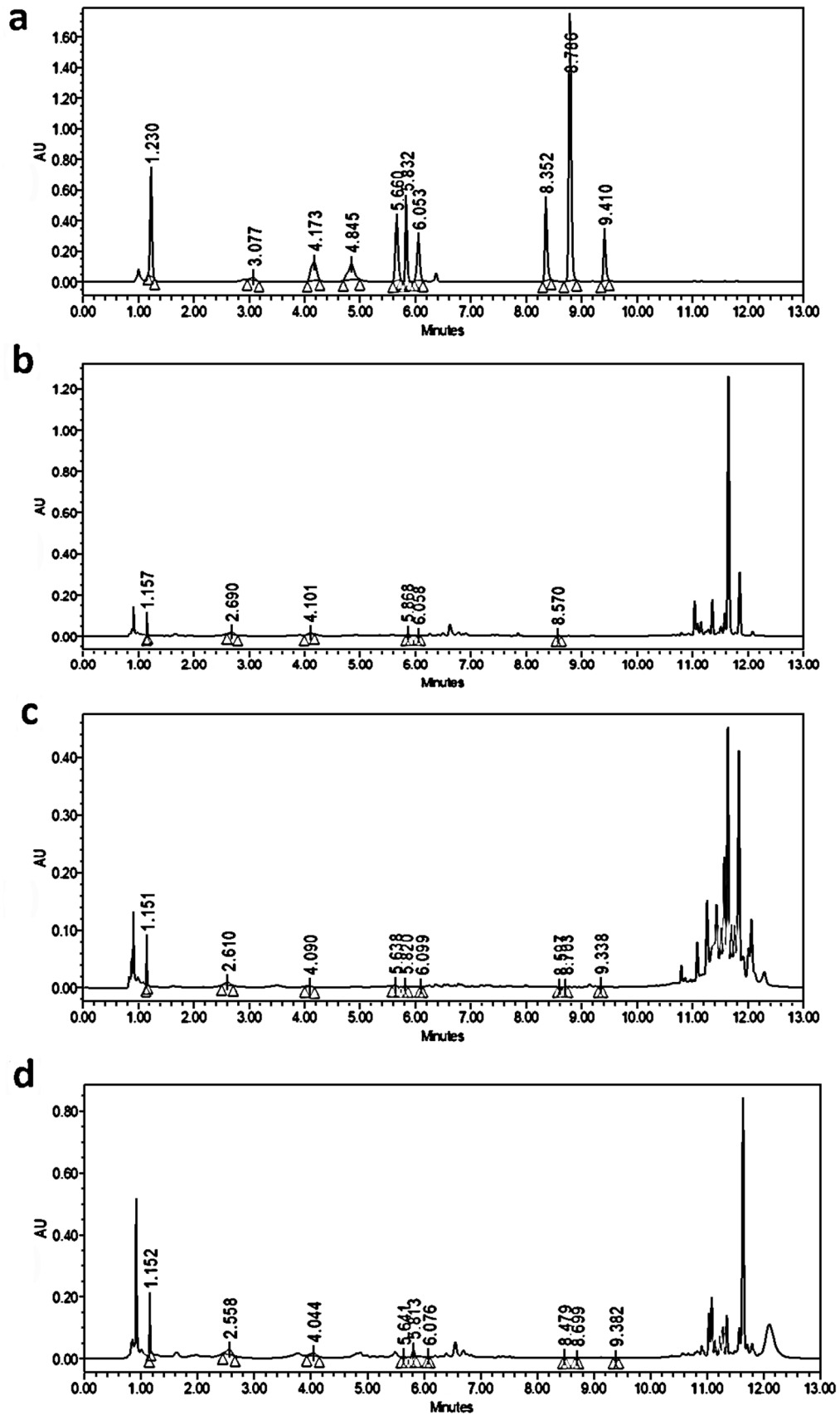
#### Extraction and characterization of essential oil

The plant rhizome had higher essential oil (0.4% v/w) yield, with trace amount in leaves harvested from donor plants (Table 5). Whereas, induced adventitious root yielded 0.059% v/w essential oil. Understandably, it is significantly low than plant roots, however, results are quite encouraging considering the shorter in vitro cultivation cycle. Furthermore, a total of thirty-one phytochemical constituents

**Table 4** Quantification of phenolic compounds yield in *V. jatamansi* plant parts and in vitro induced adventitious roots

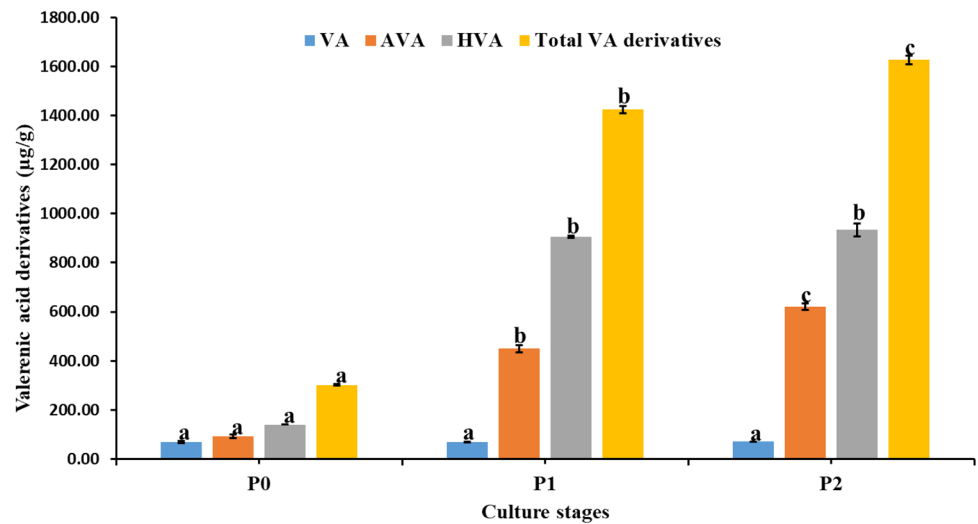
Sample (s)	Phenolic acid derivatives (µg/g DW)						
	Gallic acid	p-coumaric acid	Rutin	Ferulic acid	Cinnamic acid	Kaempferol	Total Phenolic acid derivatives
Rhizome	33.14 ± 2.90a	0.00 ± 0.00a	138.32 ± 1.91b	5.39 ± 0.11b	10.95 ± 4.16c	0.00 ± 0.00a	187.79 ± 1.81a
Leaves	33.41 ± 4.04b	161.90 ± 8.09b	49.78 ± 0.13a	0.72 ± 0.12a	0.00 ± 0.00a	17.87 ± 1.85b	263.68 ± 0.28b
Adventitious roots	34.24 ± 1.20c	162.46 ± 8.68c	217.86 ± 0.32c	6.65 ± 0.12c	7.82 ± 5.77b	22.82 ± 8.36c	451.85 ± 0.85c

Values (mean ± standard error) within the column followed by different letters significantly different at  $p \leq 0.05$  level as determined using Duncan's multiple range test (DMRT)



**Fig. 6** UPLC chromatograms depicting phenolic compounds profile. **a** Standard mix of phenolic compounds, **b** plant rhizome, **c** plant leaves and **d** in vitro induced adventitious roots

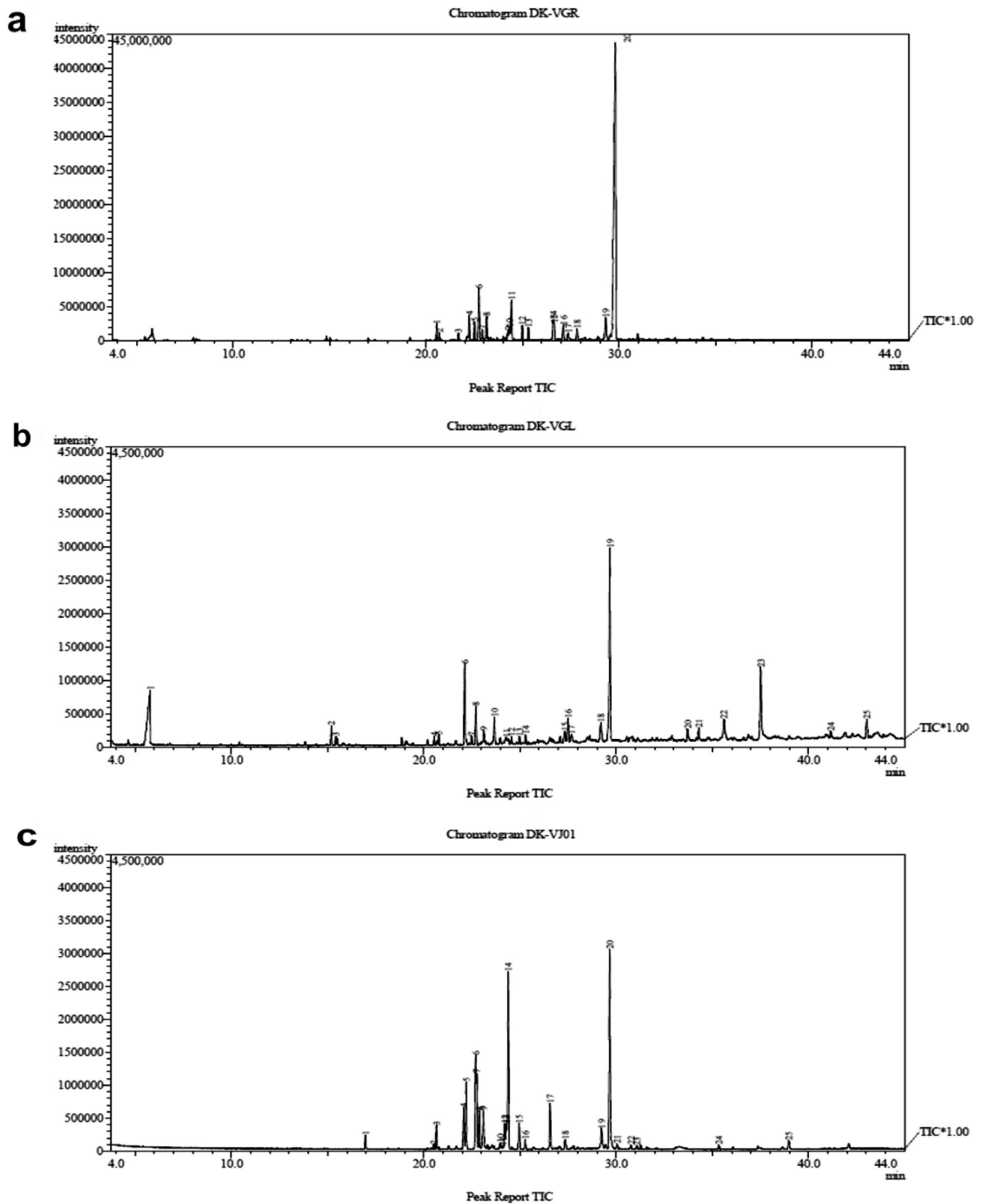
**Fig. 7** Production of valerenic acid and its derivatives at different culture stages i.e. induction stage (P0); proliferation stage (first subculture) (P1) and multiplication stage (P2). VA Valerenic acid, AVA acetoxyl-valerenic acid, HVA hydroxyvalerenic acid



**Table 5** Phytoconstituents of essential oil extracted from *V. jatamansi* plant parts and in vitro induced adventitious roots

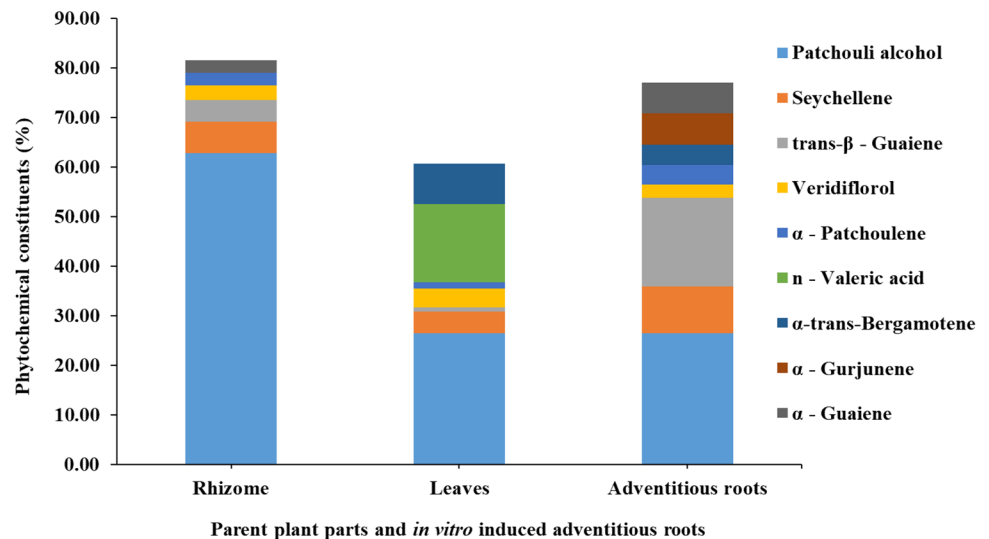
S. No.	Constituents (%)	Retention time (RT)	Retention index (RI)	Plant sample		Adventitious roots
				Rhizome	Leaves	
1.	n-Valeric acid	5.771	953	NQ	15.78	NQ
2.	Hexenyl isovalerate	15.197	1236	NQ	1.79	NQ
3.	Hexyl isovalerate	15.425	1242	NQ	0.93	NQ
4.	β-Patchoulene	20.539	1386	1.72	0.98	0.42
5.	β-Elemene	20.68	1390	0.76	NQ	2.12
6.	Pentanoic acid	20.778	1393	NQ	1.06	NQ
7.	α-trans-Bergamotene	22.124	1434	NQ	8.31	4.08
8.	α-Guaiene	22.229	1438	2.49	NQ	6.19
9.	Aromadendrene	22.492	1446	1.89	1.02	NQ
10.	Seychellene	22.708	1452	6.34	4.43	9.52
11.	α-Gurjunene	22.751	1454	NQ	NQ	6.31
12.	α-Humulene	22.914	1458	1.19	NQ	3.63
13.	α-Patchoulene	23.116	1464	2.54	1.19	3.98
14.	Curcumene	23.671	1481	NQ	2.59	NQ
15.	α-Selinene	22.242	1497	0.86	NQ	NQ
16.	Caryophyllene	23.983	1490	NQ	NQ	0.41
17.	α-Bisabolene	–	1493	NQ	NQ	0.2
18.	Chamigrene	24.204	1496	NQ	NQ	2.82
19.	cis-β-Guaiene	24.291	1499	1.41	1.96	2.47
20.	trans-β-Guaiene	24.554	1507	4.42	0.82	17.82
21.	α-Selinene	24.975	1521	1.56	0.83	2.5
22.	Kessane	25.288	1532	1.32	1.01	0.85
23.	Epiglobulol	26.587	1573	2.42	NQ	5.26
24.	Isopatchoulane	27.104	1589	1.67	NQ	NQ
25.	β-Gurjunene	27.328	1596	NQ	1.9	1.3
26.	Veridiflorol	27.359	1603	0.67	NQ	NQ
27.	Geranyl isovalerate	27.494	1601	NQ	2.93	NQ
28.	Longipinanol	27.686	1608	NQ	1.34	NQ
29.	Humulene epoxide II	27.815	1612	1.17	NQ	NQ
30.	Veridiflorol	29.196	1659	2.96	3.81	2.68
31.	Patchouli alcohol	29.671	1675	62.76	26.43	24.00
32.	Essential oil content (% FW)	–	–	0.400	TA	0.059

NQ not quantifiable, TA trace amount



**Fig. 8** GC–MS chromatograms exhibiting metabolic profile of *V. jatamansi* essential oil extracted from **a** plant rhizome, **b** plant leaves and **c** in vitro induced adventitious roots

**Fig. 9** Major compounds present in essential oil extracted from different *V. jatamansi* samples



were measured in respective essential oil fractions with a comparative chromatographic profile given in Fig. 8. These constituents represent 98.15%, 79.11% and 96.56% of essential oil obtained from rhizome, leaves and adventitious root samples, respectively. The GC–MS analysis exhibited the presence of nine common constituents with the dominance of patchouli alcohol (Fig. 9). Earlier also, patchouli alcohol (35–65%) was reported to be the major constituent of essential oil extracted from of *V. jatamansi* rhizomes (Singh et al. 2013; Raina and Negi 2015; Jugran et al. 2020).

## Conclusion

An efficient in vitro protocol has been developed for the induction of adventitious roots from leaf explant of *Valeriana jatamansi* on SH medium. Thereafter, these adventitious roots were successfully cultivated under submerged condition using standardized half strength SH media supplemented with 4.92 μM IBA and 2.0% sucrose. Induced adventitious roots had a high total valerenic acid derivatives yield with the notable presence of HVA. Further, phytochemical evaluation of these roots also exhibited considerable accumulation of phenolic constituents, including pharmaceutically important rutin and kaempferol. The adventitious roots are also a good source of essential oil with the dominance of patchouli alcohol. The developed bioprocess has a significantly shorter submerged cultivation cycle (2 months) than field grown plants (2 years) that could be a crucial factor to gauge feasibility of the developed process at an industrial scale. Conclusively, the results of the present study are clearly demonstrating the potential of in vitro induced adventitious roots as an alternate source for the production of phytoconstituents including essential oil on a sustainable basis.

**Author contributions** AG Methodology, Validation, Formal analysis, Data Curation, Writing—Review & Editing, NC Methodology, Formal analysis, Data Curation, JD Methodology, Formal analysis, Writing—Original Draft, RJ Formal analysis, Validation, DK Formal analysis, Validation, Writing—Review & Editing; SB Conceptualization, Supervision, Writing—Review & Editing, Project administration, Funding acquisition.

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**Data availability** The data generated during research work are available from the corresponding author on reasonable request.

**Code availability** Not applicable.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** Not Applicable.

**Consent for publication** Authors declare consent for publication.

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