Induction and Submerged Cultivation of *Valeriana Jatamansi* Adventitious Root Cultures for Production of Valerenic Acid and its Derivatives

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Abstract

In vitro adventitious roots were induced from *Valeriana jatamansi* to assess their quality as an alternative ingredient for extraction of secondary metabolites to meet the demand of phytopharmaceutical industries. A significantly ($p \leq 0.05$) high root induction (90 %) was achieved on Schenk and Hildebrandt medium fortified with 9.84 µM indole-3-butyric acid. A maximum root biomass (144.09 ± 11.36 g/L) with high relative growth rate (2.01 ± 0.04) and growth index (13.41) was achieved in submerged cultivation. The total valerenic acid derivatives (1525.14 µg/g DW) and acetoxy valerenic acid (534.91µg/g DW) were significantly high in induced adventitious roots, with notable quantity of hydroxyl valerenic acid (919.57 µg/g DW) that otherwise not quantifiable in parent plant parts. In addition, 0.059% essential oil yield consisting of 24.00% patchouli alcohol was also obtained from induced adventitious roots. The phenolic acid derivatives were also significantly higher in adventitious roots (451.58 µg/g DW) as compared to rhizome (187.79 µg/g DW) and leaves (263.68 µg/g DW) of the parent plant. Notably, a substantial increase in phytochemicals was evident at subsequent culture stages with significantly reduced *in vitro* cultivation cycle (2 months) as compared to field grown plants (24 months). Conclusively, a comparable metabolic profile of *in vitro* induced *V. jatamansi* adventitious roots and considerably shorter growth cycle clearly determines its potential as a feasible source of phytoconstituents.

Introduction

Valeriana jatamansi (Synonyms *Valeriana wallichii*) is an important medicinal and aromatic plant of Valerianaceae family grows between 1000–3000m elevation in Himalayan region. It is known by various vernacular names such as Indian Valerian, Muskbala, Sugandhbala (Hindi) and Tagar (Sanskrit) in India (Ved et al. 2003). *V. jatamansi* is an vulnerable plant species used in number of herbal medicine as a substitute for European *V. officinalis* (Mathela et al. 2005). As a consequence, Indian government included *V. jatamansi* as a prioritized plant species under cultivation programme of National Medicinal Plants Board (NMPB). The rhizome extract is known for its tranquilizer activities, besides antimicrobial, anti-inflammatory, insecticidal, antiviral, anti-leishmanial and antioxidant properties (Jugran et al. 2019). It is the most commonly used herbal supplement in combination with other herbs and ranked among top 10 therapeutics sold in United States (Lanje et al. 2020). In 2020, Valerian is ranked among top ten herbal medicines used in phytopharmaceutical industry around the globe (Link 1) as compared to 19th position during 2018 (Link 2). There are number of bioactive compounds reported from *V. jatamansi* including sesquiterpenes that consists of valerenic acid, acetoxy valerenic acid and hydroxyl valerenic acid (Bos et al. 1996; Singh et al. 2006). Valerenic acid is known to have spasmolytic and muscle relaxant properties, while isovalerenic acid contribute for its aroma (Murti et al. 2011). The hydroxy valerenic acid acts on GABA receptor of the central nervous system and is a major inhibitory neurotransmitter that helps in muscle relaxation (Trauner et al. 2008; Neuhaus et al. 2008; Felgentreff et al. 2012). In addition, the essential oil extracted from *V. jatamansi* roots, which varies from 0.05% -1.66% (Singh et al. 2010). The yield of essential oil influenced by method and duration of distillation, species and growing habitat (Verma et al. 2013). The major component of essential oil are monoterpenes and sesquiterpenes (Jugran et al. 2019). An array of valerian products (root extracts, dried roots, powdered roots, essential oils and tinctures) alone or in combination with other plant ingredients are commercially available in market 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 was about 1000–2000 MT in 2019,[3] which increased by more than 10 fold from 111.5 tonnes in 2001–2002 (Purohit et al. 2015). This huge jump in demand of raw ingredient by local healer, trader and exporter led to overexploitation of *V. jatamansi* from natural habitats and thus, adversely affecting the demand-supply chain of quality raw ingredient on sustainable basis. In addition, the low supply-high demand also led to adulteration in raw ingredients, thus affecting the human health at large (Dhiman et al. 2020).

In this regard, a number of biotechnological strategies are available and can be explored to produce bioactive ingredients. Among different plant cell and organ cultures, *in vitro* adventitious roots revealed to be an efficient system for the production of phytoconstituents at industrial scale (Murthy et al. 2016; Rahmat and Kang 2019). Generally, the adventitious roots formed by above ground plant parts such as leaf, stem, hypocotyl and non-pericycle tissues of the primary roots under adverse environmental condition (Verstraeten et al. 2014). There formation is an intricate process consists of three interdependent phases (induction, initiation and expression) and known to be influenced by plant hormones, especially auxin (Kevers et al. 1997). Primarily, the particular cells of an explant or wounded tissue undergo fate transition, divide and form root primordia that enlarge through cortex to get expressed as adventitious roots (Lischweski et al. 2015). Under *in vitro* conditions, the establishment of adventitious root cultures influenced by number of factors such as substrate, light, temperature, humidity and phytohormones (Murthy et al. 2008). These roots are easy to grow and have fast proliferation rate with capability of synthesizing specific bioactive compounds (Rahmat and Kang, 2019). A number of studies in medicinal plants like *Hypericum perforatum* (Cui et al. 2011), *Panax vietnamensis* (Tam et al. 2015), *Gynostemma pentaphyllum* (Khai and Minh 2018), *Oplopanax elatus* (Han et al. 2019) and *Polygonum multiflorum* (Ho et al. 2019) have demonstrated adventitious roots as a stable production system for array of secondary metabolites. Considering the vulnerability of *V. jatamansi* in Himalayas and consumer driven demand for naturals by herbal industries, the present study was carried out to assess the possibility of *in vitro* induced adventitious roots as an alternate source to extract bioactives and essential oil on sustainable basis.

Materials And Methods

Plant Materials

V. jatamansi (approximately 2 years old) plants were collected from Chamba District, Himachal Pradesh of north-west Himalayan region during May, 2018 and maintained under poly-house conditions at CSIR-IHBT. The leaves of *V. jatamansi* was used as explant for *in vitro* induction of adventitious root.

Explants preparation and surface sterilization

Explants were washed with autoclaved distilled water having 2–3 drops of Tween-20. Thereafter, the leaf explants were treated with Bavistin (0.1 % w/v) and Streptomycin (0.1 % w/v) for 10 min. to prevent fungal and microbial contamination. The explants were rinsed two-three times with autoclaved distilled water.

Finally, surface sterilized with mercuric chloride (0.1 % w/v) for 2 min. under laminar flow cabinet and washed thoroughly with distilled water for three-four times. Under aseptic condition, explants were placed on autoclaved blotting paper to remove free water before inoculation.

Media preparation and culture conditions

For optimization of adventitious root initiation process, sterilized leaf explants were inoculated on hormone free Murashige and Skoog (MS, 1962), Schenk and Hildebrandt (SH, 1972) and Gamborg (B5, 1968) medium. The medium was fortified with different concentrations (0.49, 2.46, 4.92, 9.84 & 19.69 µM) of indole-3-butyric acid (IBA) and sucrose (3 %) for induction of adventitious root and their multiplication in liquid medium. In addition, different strength of SH medium (1/4X, 1/2X, 3/4X & 1X) and sucrose concentrations (1.0, 2.0, 3.0, 4.0 & 5.0%) were also optimized for efficient multiplication. Medium pH was set at 5.8 ± 0.2 with sodium hydroxide (1N) or hydrochloric acid (1N) and autoclaved at 121°C and 15psi for 15 min. The explant inoculation and further sub-culturing was done aseptically under laminar airflow hood. The cultures were kept in tissue culture rooms on racks under dark condition at controlled temperature (25 ± 2°C). The multiplication of *in vitro* induced adventitious roots was done in liquid SH medium having IBA (4.92 µM) with 1.0 % inoculum density. The root cultures were kept in incubator shaker (INNOVA 5000) at 70 rpm and 25 ± 2°C temperature under dark condition.

Determination of adventitious root biomass and growth parameters

The adventitious roots removed from semi-solid medium with the help of forceps after 8 weeks of cultivation and fresh weight was measured. In case of liquid cultures, roots were drained on sieve (1.0 mm) under aseptic condition and then rinsing once with autoclaved distilled water. Adventitious roots were dried on sterile filter paper to remove free surface water, after that fresh weight was measured. Finally, roots were dried in hot air oven at 40º C, until constant dry weight reached. The relative growth rate (RGR) and growth index (GI) was measured as per method reported by Ho et al. (2017) on fresh weight basis using following equations:

i. $RGR = (\ln W2 - \ln W1)/CP$,
ii. $GI = W2\ (g) - W1\ (g) / W1\ (g)$

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

Preparation of sample and determination of valerenic acid derivatives

The parent plant rhizome, leaves and *in vitro* induced adventitious roots from different culture stages were dried and powdered for phytochemical analysis. From each sample, 200 mg dried powder was extracted with 3.0 mL of methanol (HPLC grade) using sonication method for 60 min. and thereafter centrifuged at 2000 rpm for 10 min. The left-over residual 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 Puradisc syringe filter (0.2 µm) and stored at 4 ºC. Final concentration of extract in respective samples was made to 40.0 mg/mL. The standards of valerenic acid (VA), acetoxyvalerenic acid (AVA) and hydroxyvalerenic acid (HVA) were procured from Sigma India and stock solutions (1 mg/mL) made in HPLC grade methanol to prepare calibration curve for comparative evaluation. The valerenic acid derivatives were quantified using Acquity Ultra Performance Liquid Chromatography (UPLC) - e photodiode array detector (Waters, India). System have two mobile phases: (A) phosphoric acid (0.1 %) in water and (B) acetonitrile. All the samples were injected at 5 µL concentration. The absorbance was measured at 280 nm and quantification performed using standard curve of VA, AVA and HVA. Each sample was analysed in triplicate.

Distillation and phytochemical analysis of essential oil

Freshly harvested *V. jatamansi* plant parts (rhizome and leaves) and *in vitro* grown adventitious roots were distilled in Clevenger apparatus. Chopped samples were washed with distilled water and air-dried on sterile filter paper. Thereafter, 1 Kg (FW) of each sample was added to 5.0 L round bottom flask and hydro-distilled (400 mL water) for 4 hours. The extracted essential oil filtered and stored under dark in sealed vials at 4°C.

Gas Chromatography–Mass Spectrometry (GC-MS) analysis of essential oil extracted from all the samples was performed on Shimadzu QP2010 series fitted with AOC-500 auto-sampler and SH-RXI-55ILMS capillary column (30 m x 0.25 mm i.d., film thickness 0.25 µm). Helium (99.99 % pure) was used as carrier gas with 1.05 mL/min flow rate, linear velocity 37.60 cm/s, pressure 65.30 kPa, split ratio 1: 10, mass scan 40–800 amu at a sampling rate of 1.0 scan/s, scan speed: 1666 u/s and interval: 0.5 s. The oven temperature was programmed as mentioned for GC analysis. Electron impact ionization at 70 eV with 0.9 kV detector voltage was used. 10 µL oil samples were mixed with 2 mL DCM (GC grade) and 2 µL of this solution was injected. Ion source temperature was 200°C, interface temperature was 240°C and injector temperature was maintained at 250°C. The constituents were identified with the help of relative retention indices and by comparison with known mass spectral data, National Institute of Standards and Technology (NIST) libraries. A mixture of *n*-alkanes (C_8-C_{24}) was used as reference for the calculation of relative retention indices (RRI) in temperature-programmed run. Moreover, decane was also used as an internal standard. The analysis of every sample was performed in triplicate.

Phenolic acids derivatives characterization

Plant part (rhizome and leaves) and adventitious root samples (100 mg each) were analysed for determination of phenolic acids derivatives i.e. gallic acid, p-coumaric acid, rutin, ferulic acid, cinnamic acid and kaempferol (Sigma) using Ultra Performance Liquid Chromatography - e photodiode array detector (UPLC-PDA, Waters, India) system. All the samples were dried, powdered and extracted in 70 % Methanol using sonication methods for 10 min. followed by centrifugation at 8000 rpm for 10 min. This process was repeated three times upto 5 ml of solvent. The samples were filtered through 0.22 µm syringe filter and transferred into vials for further analysis. In the UPLC system, the analytical column used was Waters HSS-T3 C18 column (2.1mm 100 mm, 5 mm, 1.8 µm). Detection wavelength was set at 270 nm. The gradient elution system was used, mobile phase A contain 0.1% formic acid in water, mobile phase B was 0.1 % formic acid in acetonitrile (ACN). The gradient started from 0 min. at 10% B; then from 0–1 min, 10% B; 1–8 min, linear gradient from 10% B to 45% B; 8–9 min, 95% B; 9–10 min, 95% B, then again mobile phase ran on initial conditions, 10–11 min, 10% B, 11–13 min, 10% B. Elution was performed at a solvent flow rate of 0.25 mL/min. The entire sample was analysed in triplicate.

Analysis of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) activity

The free radical scavenging activity of the samples (as described preparation of sample for determination of valerenic acid derivatives) were analyzed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH, Sigma) method (Singh et al. 2016; Bhardwaj et al. 2018). 0.2 mL methanolic extracts were mixed with 3 mL of 100 µM DPPH solution and incubated at room temperature (25 ± 2°C) for 30 min under dark condition (A_E). 0.2 mL of methanol instead of above sample extracts mixed with 3 mL DPPH (100 µM) solution was taken as Blank (A_B). Finally, absorbance was measured at 517nm using UV-Visible Spectrophotometer. DPPH activity was measured as:

% Inhibition (DPPH activity) = Absorbance of blank (A_B) – Absorbance of sample (A_E)/Absorbance of blank (A_B) x 100

Design of experiment and data analysis

The resultant data obtained from different experiments were analysed using one-way analysis of variance using statistical software (SPSS version14) for all parameters i.e. induction percent, number and length of adventitious roots, fresh-weight, dry-weight, RGR and GI. The design of experiments used in the study was completely randomized design. The experimental data for adventitious roots induction and multiplication was recorded after 4 weeks and 8 weeks, respectively. The main effects of treatment (factor) 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 for all the treatments.

Results And Discussion

The ever-rising demand for natural products and continuously diminishing plant population in natural habitat necessitates to develop alternative method to generate herbal ingredients of industrial importance. Hence, *in vitro* adventitious roots of *V. jatamansi* are worked out in this study as a substitute to obtain valerenic acid derivatives, essential oil and other derivatives.

Induction of adventitious root cultures

Murashige and Skoog (1962) Schenk and Hildebrandt (1972) and Gamborg (1968) medium were tried to induce *in vitro* adventitious roots from leaf explant of *V. jatamansi*. For this purpose, leaves were surface sterilized and cut into 4–5 mm sections and inoculated on different hormone free media under aseptic conditions.

The selection of explant was based on earlier reports that showed leaf explant as a best source for initiation of adventitious roots cultures in *P. vietnamensis* (Trinh et al. 2012; Tam et al. 2015; Linh et al. 2019) *C. tenuiflora* (Gómez-Aguirre et al. 2015) and *P. multiflorum* (Ho et al. 2019). Among different media tried, the induction of adventitious root was only observed in hormone free SH medium after four weeks of inoculation. This selective response of adventitious root induction might be due to variation in macronutrient, micronutrient and vitamins (myo-inositol) composition of respective basal media (SH, B5 and MS medium).

For instance, SH media have low ammonium: nitrate ratio (1:9) as compared to MS medium (1:2), which probably be helping in organogenesis. SH media also have 10 times higher myo-inositol (1000 mg/L) concertation than B5 and MS medium. It is pertinent to mention that myo-inositol known to accelerate cell division rather than increasing the cell size (Staudt 1984), which may be one of the reason for induction of adventitious root in SH medium. Similar observation was reported for adventitious root growth in five species of *Scutellaria* genus in SH medium fortified with IBA (1.0 mg/L) as compared to B5 and MS medium (Barska et al. 2011).

In general, adventitious root formation is a complex process and tightly regulated by various phytohormones. However, auxin is a key hormone for induction and regulation of adventitious root development. There are two types of auxins i.e. synthetic (NAA [1-naphthaleneacetic acid]) and natural analogue (IAA [indole-3-acetic acid] and IBA), which are routinely used in plant tissue culture (Bartel et al. 2001; Piotrowska-Niczyporuk et al. 2014). Therefore, the further improvement in adventitious root induction was attempted by using natural auxin analogue 'IBA'. The basal SH medium was fortified with various concentration of IBA (0.49, 2.46, 4.92, 9.84 & 19.69 µM). The induction of adventitious roots was evident as hair-like outgrowth from cut ends of the leaves within 8 days of inoculation, as compared to 28 days in hormone free medium. Furthermore, induction of roots was also observed from the margins of the leaf sections at several points during extended period up to four weeks of incubation. Experimental data releveled a significantly ($p \leq 0.05$) high percentage of adventitious root induction (90%) as well as their number (5.72 ± 0.18) and length (1.73 ± 0.06 cm) in medium fortified with 9.84 µM IBA (Table 1). However, the rooting potential found to be decreased beyond this concentration of IBA. It was noticed that above this IBA concertation i.e. 9.84 µM IBA, leaves showed more callusing than adventitious root formation. It can be deduced from the results that SH medium with 9.84 µM IBA fortification is efficient for inducing adventitious roots from *V. jatamansi* leaf explant at reasonably shorter time as compared to other combinations.

Table 1
Differential response of adventitious root induction with varying concentrations IBA growing on SH semi-solid medium with 3% sucrose after 4 weeks of incubation.

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 within the column followed by different letters significantly different at $p \leq 0.05$ level as determined using Duncan's multiple range test. A value represents mean ± standard error.

In accordance to present study, IBA has been demonstrated to be an efficient hormone for induction and multiplication of adventitious roots in number of plants (Chan et al. 2004; Agullo-Anton et al. 2011; Ghimire et al. 2018). In general, critical auxin concentration generates several signals which influence the induction as well as other phases of adventitious root development. Auxin generally promote the cell division in the initiation phase and leads to the formation of root primordia. The level of auxin in the explant is regulated by oxidation, amino acid conjugation and glycosylation (Jackson et al. 2002; Staswick 2009).

In *Panax ginseng*, IBA was reported to have better adventitious root induction efficiency as compared to NAA in SH medium (Kim et al. 2003). Similarly, Jayakodi et al. (2014) and Linh et al. (2019) also reported highest adventitious root induction on SH medium containing IBA from leaf explant of *P. ginseng* and *P. vietnamensis*, respectively. In case of *V. jatamansi*, supplementation of MS medium with BAP (6 - benzylaminopurine) and NAA (2.0 mg/L) found to promote rhizogenesis from leaves of micro propagated plants (Saini et al. 2018).

The effect of auxin on adventitious root induction was further confirmed by fortification of basal SH, MS and B5 media with optimized concentration of IBA (9.84 µM). Surprisingly, adventitious roots were induced from inoculated leaf explants in all the media. However, significantly high ($p \leq 0.05$) rhizogenic potentials in terms of induction (90.0%) as well as number (5.44 ± 0.32) and length (1.47 ± 0.09 cm) of adventitious roots was obtained in SH medium as compared to MS and B5 (Fig. 1). Thus, the earlier results hold true that the SH medium with 9.84 µM IBA concentration was optimum for induction of adventitious roots. These roots were repeatedly sub-cultured on the above optimized medium for further multiplication and maintenance of mother stock. Consistent with observation of present investigation, Kim et al. (2003) described the use of SH media fortified with IBA for inducing and yielding high adventitious roots biomass in Korean ginseng as compared to NAA. However, the concentration of IBA (24.6 µM) used in this study was 2.5 times higher as compared to present work. In addition, Nhut et al. (2012) also induced adventitious roots in *Vietnamese ginseng (Panax vietnamensis)* on SH and MS media. Similar to our results, authors observed highest root proliferation on SH medium, however, with NAA fortification. It is hypothesized by the authors that ammonium and nitrate ratio as well vitamins (thiamine-HCL and myo-inositol) concentration of SH media might have played a significant role in high rate of adventitious root formation. Vitamin B or thiamine-HCL known to promote better root growth, thus helps in better nutrient absorption. Whereas, myo-inositol take part in the auxin (IAA) transport and play a significant role in plant cell growth and development (Loewus and Murthy 2000).

Effect of IBA on multiplication of adventitious roots in submerged cultivation

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 augmented with various IBA concentrations. The growth of adventitious roots was determined based on biomass yield and relative growth rate (RGR) after 8 weeks of cultivation. Experimental results showed a significant ($p \leq 0.05$) effect of IBA concentration on multiplication of adventitious roots in liquid medium. A significantly high root biomass yield (123.39 ± 7.11 g/L FW) and relative growth rate (RGR) (1.95 ± 0.03) was recorded in SH liquid medium at 4.92 µM IBA concentration after 8 weeks of cultivation (Table 2). In addition, the growth index (11.34 ± 0.71) (GI) was also found highest in same medium (Fig. 2a). However, the IBA concertation beyond 4.92 µM was not able to support further growth of adventitious roots. Furthermore, these results suggest that the multiplication of adventitious root in submerged condition was obtained in just half IBA concentration to that of induction medium (9.48 µM).

Table 2
Effect of different concentrations of IBA on biomass production in SH liquid medium after 8 weeks of incubation.

IBA (µM)	Fresh weight (g/L)	Dry weight (g/L)	Relative growth rate
Control	21.03 ± 1.18a	2.56 ± 0.26a	1.19 ± 0.02a
0.49	61.96 ± 4.84b	3.74 ± 0.26b	1.64 ± 0.04b
2.46	84.44 ± 5.79c	5.49 ± 0.31c	1.78 ± 0.03c
4.92	123.39 ± 7.11e	7.44 ± 0.44d	1.95 ± 0.03d
9.84	107.47 ± 7.29d	6.90 ± 0.43d	1.88 ± 0.04d
19.69	67.89 ± 4.79b	4.99 ± 0.32c	1.69 ± 0.03b

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

Contrary to present results, Kannan et al. (2020) observed higher adventitious roots induction potential (i.e. 134.33 root number per explant) from leaf explants of *Morinda coreia* on ½ strength MS media augmented with 1.0 mg/L IBA, whereas, maximum proliferation efficiency of induced roots (i.e. 1.568 g/L FW, 5.082 growth ratio and 0.163 g/L DW) were attained on liquid MS medium enriched with same IBA concentration. Similarly, Wu et al. (2006) also reported induction of *Echinacea 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 negative impact on root biomass growth beyond the 2.0 mg/L IBA. From the above results it can be deduced that 4.92 µM IBA in SH media is optimum for maximum adventitious root biomass accumulation under *in vitro* condition.

Effect of medium strength on multiplication of adventitious roots in submerged cultivation

In general, medium type and their elemental composition found to influence growth as well as overall productivity of *in vitro* plant cultures. Different strength of liquid SH medium (1/4X, 1/2X, 3/4X & 1X) supplemented with best responsive IBA concentration (i.e. 4.92 µM) were investigated to maximize the yield of root biomass. A significantly ($p \leq 0.05$) high roots biomass (126.40 ± 23.90 g/L) and relative growth rate (1.86 ± 0.09) was recorded in half strength (1/2X) SH medium (Table 3) as compared to other medium strength tested in the study. GI was also highest in half strength (1/2X) SH medium (Fig. 2b). SH medium having 1/4x concentration exhibited slowest growth as evident from the root biomass yield. From the above results, it can be deduced that optimal concentration of macro and micronutrients in media seems to be the determining factor for *in vitro* induced adventitious root growth.

Table 3
Differential response of adventitious root multiplication on varying strength of SH liquid medium supplemented with IBA (4.92 µM) after 8 weeks of incubation.

Strength of SH media	Fresh weight (g/L)	Dry weight (gm/L)	Relative growth rate
1/4	97.87 ± 16.12ab	7.74 ± 1.14ab	1.77 ± 0.08ab
1/2	126.40 ± 23.90c	9.76 ± 1.64b	1.86 ± 0.09b
3/4	63.20 ± 13.11a	5.17 ± 1.00a	1.57 ± 0.08a
1/0	106.40 ± 15.04ab	9.71 ± 1.58b	1.83 ± 0.07b
Values within the column followed by different letters significantly different at $p \leq 0.05$ level as determined using Duncan's multiple range test. A value represents mean ± standard error.			

Corroborating to our results, adventitious root induced from *Echinacea angustifolia* showed maximum biomass yield in half strength MS medium supplemented with IBA (2.0 mg/L) as compared to 0.25, 0.75, 1.00, 1.50 and 2.00 strength (Wu et al. 2006). Similarly, half strength of MS medium fortified with IBA (3.0 mg/L) also found optimum for multiplication of adventitious roots induced from rhizome of *P. hexandrum* (Rajesh et al. 2014). Whereas, 1/4x strength of MS nutrients in combination with IBA (1.0 mg/L) resulted in maximum biomass accumulation in *Periploca sepium* adventitious roots (Zhang et al. 2011). It is well known that strength of the medium plays crucial role in growth and development of plant cells/tissues during cultivation under *in vitro* condition. The low salt concentration in the medium also increase the availability of nutrient ions (George et al. 2008).

Influence of various concentrations of sucrose on multiplication of adventitious roots in submerged cultivation

In plant cell and tissue cultures, sucrose is the principal carbohydrate used as energy source for growth and development. It is catabolized into glucose and fructose and it's absorption rate varies with type of cultures (George et al. 2008). In the present work, diverse sucrose (1 to 5 % w/v) concentration in optimized media i.e. ½ strength SH media having 4.92 µM IBA were tried in submerged cultivation to improve further *V. jatamansi* adventitious roots multiplication. Among different concentrations of sucrose tested, a significantly high ($p \leq 0.05$) root biomass (144.09 ± 11.36 g/L FW) and relative growth rate (2.01 ± 0.04) was obtained in medium augmented with 2% (w/v) sucrose after cultivation of 8 weeks (Table 4). Also, same media showed highest growth index (13.41 ± 1.14) as compared to other concentration (Fig. 2c). However, the root biomass yield on dry weight basis depicted dissimilar pattern with subsequent increase in sucrose concentration (4–5%). It may be due to formation of more extracellular polysaccharides with respect to the higher concentration of sucrose in the medium (Saiman et al. 2012).

Table 4
Effect of different concentrations of sucrose in ½ strength SH liquid medium supplemented with IBA (4.92 µM) on biomass production after 8 weeks of incubation.

Sucrose (%)	Fresh weight (gm/L)	Dry weight (gm/L)	Relative growth rate
1.0	100.30 ± 9.81b	6.63 ± 0.69a	1.84 ± 0.05b
2.0	144.09 ± 11.36c	8.91 ± 0.69b	2.01 ± 0.04d
3.0	136.81 ± 14.46c	9.59 ± 0.68b	1.98 ± 0.05bc
4.0	100.57 ± 8.65b	12.31 ± 0.56c	1.85 ± 0.04b
5.0	58.47 ± 9.69a	8.28 ± 0.88ab	1.58 ± 0.06a
Values within the column followed by different letters significantly different at $p \leq 0.05$ level as determined using Duncan's multiple range test. A value represents mean ± standard error.			

Similar to the present observations, Murthy and Praveen (2013) reported maximum accumulation of *W. somnifera* adventitious root biomass (113.58 g/L FW and 8.70 growth ratio) on half strength liquid MS media having 2% sucrose after four weeks of culture period. They also observed negative effect of increased sucrose concentration in media (3–8%) on accumulation of adventitious root biomass. In another study, 2% sucrose (w/v) concentration in MS medium was found optimum for highest yield of *Gynura procumbens* adventitious root biomass i.e. 13.8 ± 1.60 g FW per flask after four weeks of cultivation. Further, they also observed decrease in root biomass with further increase in sucrose concentration (7–10% w/v) in the media (Saiman et al. 2012).

It may be inferred from the results that 2 % sucrose in the ½ strength SH medium is optimum for the growth of *V. Jatamansi* adventitious root cultures under *in vitro* condition.

Determination of valerenic acid and its derivatives using Ultra Performance Liquid Chromatography analysis

Valerenic acid (VA) and its derivatives acetoxyvalerenic acid (AVA) and hydroxyvalerenic (HVA) are main active components of the herb *V. jatamansi*. In present study, the parent plant material (rhizome and leaves) and *in vitro* induced adventitious root samples were analysed for the identification and quantification of VA, AVA and HVA acid using UPLC-PDA. The yield of total valerenic acid derivatives (1525.14 ± 68.85 µg/g DW) was significantly ($p \leq 0.05$) high in adventitious roots in comparison to parent plant parts i.e. rhizome (624.78 ± 13.67 µg/g DW) and leaves (200.17 ± 4.27 µg/g DW). However, valerenic acid (506.27 ± 10.34 µg/g DW) in rhizome was found higher as compared to leaves of the parent plant (79.23 ± 4.56 µg/g DW) as well as *in vitro* induced adventitious roots (70.66 ± 0.36 µg/g DW). Whereas, AVA content was significantly ($p \leq 0.05$) high in adventitious root samples (534.91 ± 39.57 µg/g DW) than parent plant parts i.e. rhizome (118.51 ± 4.16 µg/g DW) and leaves (120.94 ± 7.48 µg/g DW). Similarly, Tousi et al. (2010) recorded higher fraction of VA (0.38 %), AVA (0.55%) and HVA (0.44%) in *Valeriana officinalis* adventitious roots induced from petiole explant on MS medium supplemented with IAA.

Interestingly, *in vitro* adventitious roots showed presence of HVA at significantly high amount (919.57 ± 28.85 µg/g DW), which otherwise not quantifiable in leaves as well as rhizome parts of the parent plants (Table 5 and Fig. 3). It is also pertinent to mention here that the comparative evaluation performed was between two-month (eight weeks) old *in vitro* adventitious roots versus rhizomes and leaves of approximately 2-year old plants grown under conventional agricultural cultivation condition. Thus, results of present study clearly suggesting the potential of *V. jatamansi* adventitious roots as a good alternative source of valerenic acid and its derivatives. In addition, *in vitro* induced adventitious roots could be a novel source of hydroxyvalerenic acid that was unquantifiable in parent plant parts.

Table 5
Quantitative analysis of valerenic acid, acetoxy valerenic acid and hydroxyl valerenic acid from plant part and adventitious root samples of *V. jatamansi* using Ultra Performance Liquid Chromatography (UPLC-PDA) system.

Sample	Metabolite content (µg/gm DW)			
	VA	AVA	HVA	Total valerenic acid derivatives yield
Rhizome	506.27 ± 10.34b	118.51 ± 4.16a	0.00 ± 0.00*a	624.78 ± 13.67b
Leaves	79.23 ± 4.56a	120.94 ± 7.48a	0.00 ± 0.00*a	200.17 ± 4.27a
Adventitious roots	70.66 ± 0.36a	534.91 ± 39.57b	919.57 ± 28.85b	1525.14 ± 55.20c
*NQ: Not quantifiable. Values within the column followed by different letters significantly different at $p \leq 0.05$ level as determined using Duncan's multiple range test. A value represents mean ± standard error.				
VA: Valerenic acid; AVA: Acetoxyvalerenic acid; AVA: Hydroxyvalerenic acid.				

Earlier, Singh et al. (2006) quantified valerenic acid in rhizome of *V. officinalis* and *V. jatamansi* plants by High performance thin layer chromatography (HPTLC). They found relatively higher valerenic acid content (0.42%) in *V. officinalis* than *V. jatamansi* (0.12% valerenic acid). Similarly, Srivastava et al. (2010) reported presence of valerenic acid (0.11%) in *V. jatamansi* rhizomes using HPTLC methods. Furthermore, Batra et al. (2016)found relatively low valerenic acid content (0.001%) in rhizomes of *V. jatamansi*. There is very limited information available on tissue culture raised *V. jatamansi* plants or tissues.

Recently, Partap et al. (2020) studied the effect of methyl jasmonate and yeast extract on accumulation of valerenic acid derivative from leaves and roots of nursery-grown, aeroponic and pot cultivated *V. jatamansi* plants. They reported maximum amount of VA (4.19 mg/g) in roots of plants grown under pot

cultivation followed by nursery grown (3.18 mg/g) and aeroponic cultivation (1.78 mg/g). AVA content was found higher (2.38 mg/g) in roots grown under aeroponic condition.

Determination of essential oil, patchouli alcohol and other derivatives using Gas Chromatographic – Mass Spectroscopic (GC – MS) analysis

Valeriana jatamansi plant parts (rhizome & leaves) and adventitious root (1000g each, FW) were used for the extraction of essential oil using hydro-distillation method in a Clevenger apparatus. The plant rhizome yielded relatively higher essential oil content (0.4% v/w) as compared to *in vitro* induced adventitious root (0.059% v/w). However, trace amount of essential oil was obtained from the leaves of parent plants.

Earlier, Singh et al. (2013) reported a yield of 0.1–0.5% essential oil from two-year-old *V. jatamansi* plants fresh rhizomes cultivated in western Himalayas. However, comparatively high essential oil content (0.60–1.66%) was reported in thirteen *V. jatamansi* chemotypes collected from various agro-climatic zones of Himachal Pradesh, India (Singh et al. 2013, 2015). Previously, Sati et al. (2005) reported extraction of essential oil (0.03%) through steam distillation method from *Valeriana wallichii* leaves collected from Western Himalaya. Also, Coassini and Moneghini (1989) reported 0.1–0.45% essential oil yield from fresh leaves collected from three populations of *Valeriana officinalis*.

In present study, adventitious root induced from leaves yielded 0.059% v/w essential oil, which is reasonably low as compared to rhizomes harvested from field grown parent plants. However, it is worth mentioning that *in vitro* cultivation period of adventitious root is significantly low (2 months) as compared to field conditions (2 years).

Furthermore, the essential oil extracted from different samples was analysed by GC-MS to characterize its individual constituents. In this regard, a total of thirty-one phytochemical constituents were characterized and identified (Table 6, Fig. 4). These constituents represent 98.15%, 79.11% and 96.56% of essential oil obtained from rhizome, leaves and adventitious root samples, respectively. Overall, the GC-MS analysis exhibited presence of nine common constituents i.e. patchouli alcohol, α – patchoulene, β – patchoulene, seychellene, cis-β- guaiene, trans-β – guaiene, α – selinene, kessane and veridiflorol in essential oil extracted from parent plant parts (rhizome and leaves) and adventitious root samples. However, the percentagewise contribution (Fig. 5) of individual constituent in essential oil was patchouli alcohol > seychellene > trans-β – guaiene > veridiflorol > α- patchoulene of rhizome, patchouli alcohol > n - valeric acid > α-trans-bergamotene > seychellene > veridiflorol of leaves and patchouli alcohol > trans-β – guaiene > seychellene > α - gurjunene > α- guaiene of *in vitro* induced adventitious roots, respectively.

Table 6
Extraction and analysis of essential oil, patchouli alcohol and other derivatives using Gas Chromatographic – Mass Spectroscopic (GC – MS) system.

S.No.	Phytochemical constituents	Retention time (RT)	Retention index (RI)	Plant sample		Adventitious roots
				Rhizome	Leaves	
1.	n - Valeric acid	5.771	953	-	15.78	-
2.	Hexenyl isovalerate	15.197	1236	-	1.79	-
3.	Hexyl Isovalerate	15.425	1242	-	0.93	-
4.	β - Patchoulene	20.539	1386	1.72	0.98	0.42
5.	β - Elemene	20.68	1390	0.76	-	2.12
6.	Pentanoic acid	20.778	1393	-	1.06	-
7.	α-trans-Bergamotene	22.124	1434	-	8.31	4.08
8.	α - Guaiene	22.229	1438	2.49	-	6.19
9.	Aromadendrene	22.492	1446	1.89	1.02	-
10.	Seychellene	22.708	1452	6.34	4.43	9.52
11.	α - Gurjunene	22.751	1454	-	-	6.31
12.	α - Humulene	22.914	1458	1.19	-	3.63
13.	α - Patchoulene	23.116	1464	2.54	1.19	3.98
14.	Curcumene	23.671	1481	-	2.59	-
15.	α - Selinene	22.242	1497	0.86	-	-
16.	Caryophyllene	23.983	1490	-	-	0.41
17.	α - Bisabolene	-	1493	-	-	0.2
18.	Chamigrene	24.204	1496	-	-	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	-	5.26
24.	Isopatchoulane	27.104	1589	1.67	-	-
25.	β - Gurjunene	27.328	1596	-	1.9	1.3
26.	Veridiflorolol	27.359	1603	0.67	-	-
27.	Geranyl isovalerate	27.494	1601	-	2.93	-
28.	Longipinanol	27.686	1608	-	1.34	-
29.	Humulene epoxide II	27.815	1612	1.17	-	-
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 %
*TA: Trace amount.						

In accordance with present investigation, patchouli alcohol (48.47–65.04%) was reported to be the major constituent of essential oil extracted from rhizomes of *V. jatamansi* chemotypes obtained from Western Himalayas (Singh et al. 2013). Recently, a comprehensive study was performed to assess the effect of phenological stages and altitude on various constituents of essential oil extracted from *V. jatamansi* rhizomes cultivated in North-West Himalayas (Jugran et al. 2020). A quite significant variation in various constituents was reported with respect to phenological stage as well as altitude, however, patchouli alcohol (36.52–52.68%) was found to be the major component followed by β-patchoulene (1.14–2.70%), α-guaiene (0.28–5.08%), δ – guaiene (0.86–9.49%) and seychellene (1.06–4.56%) including various other minor molecules.

Contrary to present results, Das et al. (2011) reported maaliol (26.1%) as a major component followed by patchouli alcohol (9.3%) in fresh rhizomes of *V. jatamansi* collected from North-East Himalayas. This variation in the constituents of *V. jatamansi* essential oil can be attributed to the age of plants, time of

harvest and geographical differences. However, it is quite clear from the results of present investigation as well as above discussion that patchouli alcohol is one of the major volatile component of *V. jatamansi* essential oil.

Determination of phenolic acids derivatives using Ultra Performance Liquid Chromatography analysis

Phenolic acids derivatives i.e. gallic acid, p-coumaric acid, rutin, ferulic acid, cinnamic acid and kaempferol were determined from plant rhizome, leaves and adventitious root using Ultra Performance Liquid Chromatography (UPLC-PDA) system. In vitro raised adventitious roots reported significantly higher phenolic acid derivatives (451.58 µg/g) as rhizome (187.79 µg/g) and leaves (263.68 µg/g) parts of the plant. In particular, adventitious root also showed significantly higher content of gallic acid (34.24 ± 1.20 µg/g), p-coumaric acid (162.46 ± 8.68 µg/g), rutin (217.86 ± 0.32 µg/g), ferulic acid (6.65 ± 0.12 µg/g) and kaempferol (22.82 ± 8.36 µg/g) as compared to parent plant part rhizome gallic acid (33.14 ± 2.90 µg/g), rutin (138.32 ± 1.91µg/g) and ferulic acid (5.39 ± 0.11 µg/g), whereas plant leaves showed gallic acid (33.41 ± 4.04 µg/g), p-coumaric acid (161.90 ± 8.09 µg/g), rutin (49.78 ± 0.13 µg/g), ferulic acid (0.72 ± 0.12 µg/g) and kaempferol (17.87 ± 1.85 µg/g). it was also observed that rhizome part does not showed detectable amount of p-coumaric acid and kaempferol, whereas leaves does not showed presence of cinnamic acid (Table 7; Fig. 6).

Table 7
Determination of phenolic acids derivatives and DPPH activity in plant parts and adventitious roots.

Plant sample	Phenolic acid derivatives (µg/g)							DPPH activity (%)
	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	85.78 ± 1.62b
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	61.98 ± 0.97a
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	87.09 ± 0.63b

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

In addition, rhizome part recorded higher amount of cinnamic acid (10.95 ± 4.16 µg/g) and compared to adventitious root (7.82 ± 5.77 µg/g) (Table 7). Theses bioactive compounds have antioxidant, antimaicrobial, anticancer, anticholesterolemic acid and adventitious root culture could be alternate route for their biosysnthesis and production. In earlier studies, Bhatt et al. (2012) determined phenolic compounds i.e. gallic acid, hydroxybenzoic acid, caffeic acid, catechin, chlorogenic acid p-coumaric acid in different parts of planted and wild cultivated *V. jatamansi* plants. They reported significantly higher content of caffeic acid (158.56 mg/100 g) and hydroxybenzoic acid (390.58 mg/100 g DW) in planted condition, whereas, wild cultivated *V. jatamansi* plants recorded higher content of gallic acid (8.70 mg/100 g), coumeric acid (2.89 mg/100 g), chlorogenic acid (5.52 mg/100 g) and catechin (229.59 mg/100 g). In addition, aerial portion of wild cultivated *V. jatamansi* plants revealled higher content of gallic acid, p-coumaric acid, chlorogenic acid and catechins. However, caffeic acid and hydroxybenzoic acid content was found higher in aerial portion of planted *V. jatamansi* plants. In another study, Jugran et al. (2020) observed significant variations in different phenolic i.e. gallic acid, p-coumaric acid, chlorogenic acid, catechin, caffeic acid and hydroxy-benzoic acid in pre-flowering, flowering and post-flowering stages of V. jatamansi plant population wrt their occurance in high, intermediate and low altitude. Gallic acid content was found to be highest (9.39 mg/100 g DW) in pre - flowering stage of plant population of low altitude region. p-Coumaric acid content was maximum (24.34 mg/100 g DW) at high altitude during pre-flowering stage of the plant. In case of chlorogenic acid, post-flowering stage showed highest (7.45 mg/100 g DW) content at high altitude. Catechin content was maximum (6.03 mg/100 g DW) in post-flowering stage of plant population at high altitude conditions. Caffeic acid and hydroxy-benzoic acid was both detected highest (2.61 & 6.24 mg/100 g DW) in pre-flowering stage of plant population collected from intermediate altitudinal region. Presence of above bioactives compound in adventitious root culture showed an alternate source for their production on sustainable basis for medicinal uses.

Analysis of DPPH (2, 2-diphenyl-1-picrylhydrazyl) activity

Comparative analysis of antioxidant activity using DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay was done using methanolic extracts of rhizome, leaves and adventitious roots samples. The data revealed that adventitious root sample (87.09 %) showed higher free radical scavenging activity as compared to rhizome (85.78 %) and leaves (61.98 %) samples (Table 7). However, leaf induced adventitious root shows higher DPPH activity with leaf samples of parent plant. DPPH generally used to evaluate the free radical-scavenging activity of natural antioxidants from the plant and this free radical activity trigger production of marker compounds and act as major factor in biological damages during different stages of culture (Boo et al. 2018). However, DPPH activity in the plant also reflect the stress levels confronted by the tissues. Adventitious root of *H. perforatum* showed a steady increase in the free radical scavenging activity (Cui et al. 2011) and are specific to plants. The results of present investigation are in agreement with report of Boo et al. (2018), where a proportional increase (10 mg mL-1) in concentration of free radical scavenging activity was found higher than 80% in adventitious root culture of *Platycodon grandiflorum*.

Effect of culture stages on production of valerenic acid derivatives

In general, *in vitro* plant cell and tissue culture shows variability in growth as well as metabolite production at various culture stages. Therefore, production of valerenic acid derivatives at different culture stages i.e. a) induced from leaf explants on semi - solid SH media + 9.84 µM IBA (P_0), b) multiplied on semi - solid SH media + 4.92 µM IBA (P_1) and c) submerged cultivation in liquid SH media + 4.92 µM IBA (P_2) of *V. jatamansi* adventitious root development was also studied in present investigation. The results showed presence of VA, AVA and HVA in all the stages of adventitious root formation and multiplication (Fig. 7).

Total valerenic acid derivatives yield were significantly ($p \leq 0.05$) increased (302.28–1625.98 µg/g DW) from culture stage 1 to stage 3. In case of individual metabolite, there was significant ($p \leq 0.05$) enhancement in AVA (92.51–620.72 µg/g DW) and HVA (140.42–933.95 µg/g DW) content with respect to different culture stages (Fig. 7). From the trend, it can be deduced that in culture stage 1 the priority of the tissue is specifically towards its growth and development, whereas during stage 2 and 3 the high metabolic yield indicates the shift or activation of secondary metabolism. Also, there might be a possibility that submerged cultivation create more stressing environment, thus enhancing biosynthesis of valerenic acid derivatives, especially in stage 3 cultures.

Similar information on secondary metabolite production in relation to *in vitro* plant tissue culture stages/passage is very limited. Hagimori et al. (1980) quantified digoxin content in passage one and second of callus induced from seedlings and leaf disk of *Digitalis* species inoculated on different media under light and dark condition. The induced callus showed diverse tendency of metabolic profile in first and second passage of culture. Callus induced from seedling showed high digoxin content in first passage. Whereas, in second passage digitoxin content was not detected. However, leaf disc induced callus exhibited similar metabolite content in first passage as in inoculum. In contrast, callus proliferated during second passage revealed varying digoxin content in different *Digitalis* species. In another study on effect of passage on *in vitro* shoot multiplication of *Cassia angustifolia*, Siddique et al. (2015) studied the impact of TDZ on shoot cultures up to 5th passages of sub-culture in MS medium. They observed continuous increase in shoot number (12.0 ± 0.9) and length (6.9 ± 0.29 cm) up to fifth passages on MS medium fortified with 5.0 µM TDZ. In the current research work, the aim was to determined metabolite content in adventitious roots of *V. jatamansi* at different passage times.

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 fifth sub-culture, after that is decrease trend. The erysodin and erysovin, content was only quantified in the seventh sub-culture of cotyledons induced callus culture. In addition, Coppede et al. (2014) quantified higher amount of Quinonemethide triterpenoid (QMTs) i.e. maytenin (1,147.90 µg/g) and 22b-hydroxymaytenin (1,032.89 µg/g) in leaf induced callus of *Maytenus ilicifolia* after twelve days of inoculation. However, during subsequent subculturing from sixteen to forty-eight days, it showed decreases pattern of QMTs.

Also, Le et al. (2019) reported a consistent increase in ginsenosides i.e., protopanaxadiol and protopanaxatriol during long (twenty-year old) and short-term (one-year old) cultivation of *Panax ginseng* adventitious roots. The accumulation of secondary metabolites under *in vitro* condition significantly affected by numbers of factors i.e. explants, selection of genotypes, friability, somaclonal variations, repetitive subculturing for a longer time, morphological variations, DNA methylation and genetic stability (Bourgaud et al. 2001; Fu et al. 2012; Coppede et al. 2014). The above facts clearly stated the significant role of culture stages on accumulation of metabolite content under *in vitro* condition.

Overall process of induction, multiplication and submerged cultivation

The complete process of *V. jatamansi* adventitious root culture cultivation can be divided into three stages; namely, adventitious root induction, multiplication and submerged cultivation for scale up production (Fig. 8). The detailed bioprocess can be summarized under following heads:

Adventitious root induction: In this stage, adventitious roots were induced from leaf explants on optimized SH media fortified with 9.84 µM IBA within eight days of inoculation under aseptic condition.

Multiplication of adventitious roots: After four weeks of induction, induced roots were further amplified on semi-solid ½ strength SH media having 4.92 µM IBA and 2.0 % sucrose.

Submerged cultivation of adventitious roots: Considering the development of alternative route for production of valerenic acid derivatives, large-scale multiplication of adventitious roots was done through submerged cultivation in optimized ½ strength liquid SH media enriched with 4.92 µM IBA and 2.0 % sucrose for two months (eight weeks) under *in vitro* condition.

Considering the developed *in vitro* protocol, the complete process took two months (eight weeks) after induction of adventitious roots from leaf explants of *V. jatamansi*. Whereas, the generation of commercially valued rhizomes or root biomass through conventional means generally takes 2 years after transplanting in field condition (Singh et al. 2010). In addition, the conventional cultivation gets jeopardised by slow germination, poor viability and long dormancy of seeds as well as limited planting material through vegetative propagation (Rana et al. 2004). These issues not only affecting the availability of quality raw material to herbal industries on sustainable basis, but also affecting the plant population in its natural habitat. The feasibility of submerged cultivation bioprocess as compared to conventional cultivation can be assessed on the basis that *in vitro* adventitious roots can yield over two times higher essential oil (Table 8).

Table 8
Comparative analysis of essential oil and total valerenic acid derivative yield in conventional and submerged cultivation.

Type of cultivation	Cycle (Month)	Essential oil yield (%)	Total valerenic acid derivative (µg/g)	Yield/two year	
				Essential oil (%)	Total valerenic acid derivative (µg/g)
Conventional*	24	0.500	624.78	0.500	624.78**
In vitro adventitious root (submerged cultivation)	02	0.059	1525.14 (one cycle)	0.708*** (6x2 year = 12 cycle)	18301.68*** (6x2 year = 12 cycle)
*Singh et al., 2010.					
** quantified during present investigation					
***estimated figures					

Conclusion

In vitro adventitious roots were successfully induced from leaf explant of *Valeriana jatamansi* on SH medium having IBA (9.84 µM), whereas maximum multiplication and submerged cultivation was achieved in half strength SH media supplemented with 4.92 µM IBA and 2.0 % sucrose. The significantly high yield of total valerenic acid derivatives, with notable content of AVA and HVA in induced adventitious roots as compared to parent plant parts makes them a viable proposition as an alternate source. Furthermore, presence of hydroxyvalerenic acid in induced adventitious roots (otherwise unquantifiable in parent plant parts) advocates use of this technology for biosynthesis of novel compounds without transformation of host plants. A low essential oil yield of *in vitro* adventitious roots as compared to rhizomes of parent plants needs to be investigated. In addition, it also extracted significant content of phenolic acids. However, a significantly shorter *in vitro* cultivation cycle (2 month) than conventional means (2 years) could be a crucial factor to support the feasibility of developed process at industrial scale. Conclusively, the results of present study are clearly exhibiting the potential of *in vitro* induced adventitious roots as an sustainable source to meet industrial demand around the year.

Declarations

Author contributions

AG Methodology, Validation, Formal analysis, Data Curation, Investigation, 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.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interest.

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Figures

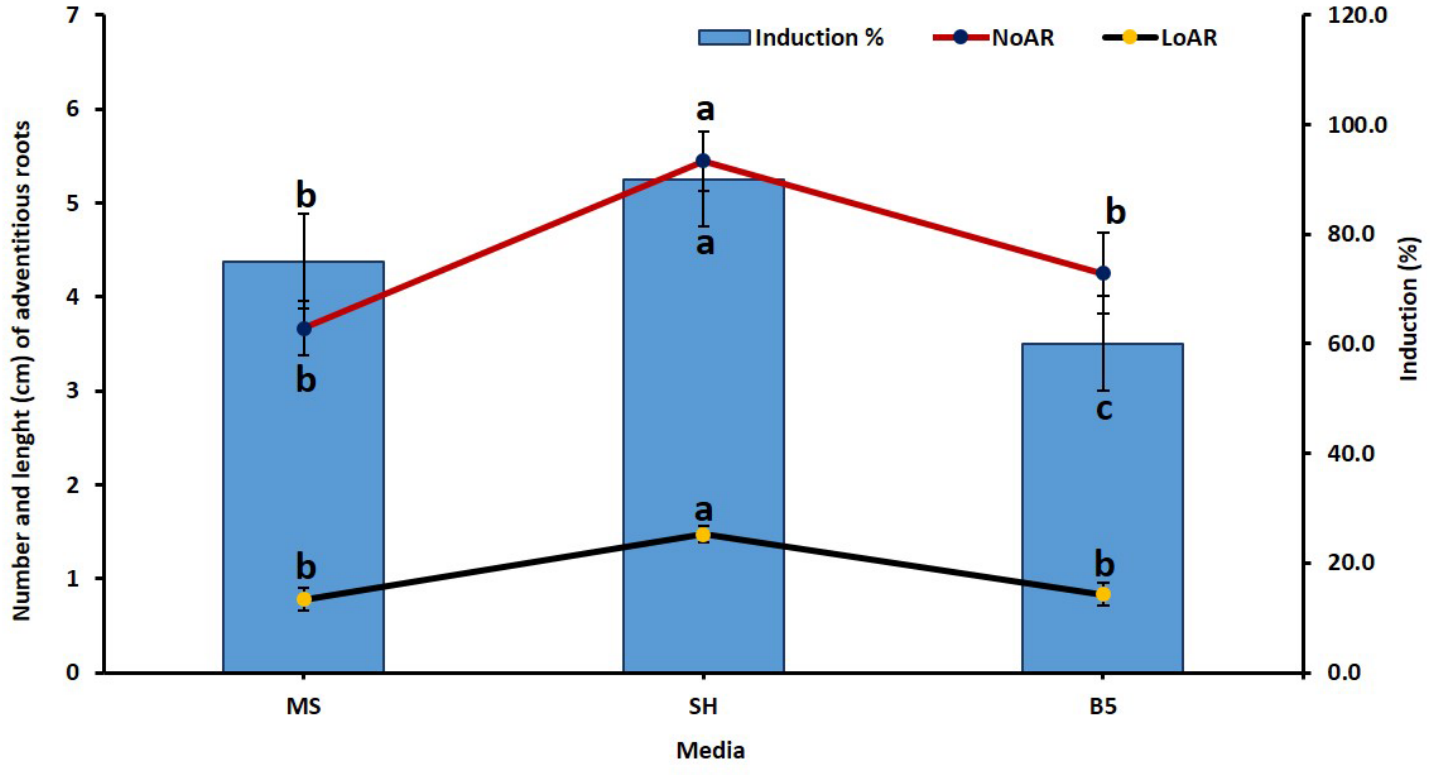

Figure 1

Effect of different media (MS, SH & B5) enriched with IBA 9.84 µM on induction of adventitious root culture of V. jatamansi. Data represented in bar chart showed mean ± SE of five replicates and significantly different at p ≤0.05 level as determined using Duncan's multiple range test (DMRT). Note: NoAR, Number of adventitious roots; LoAR, Length of adventitious roots (cm).

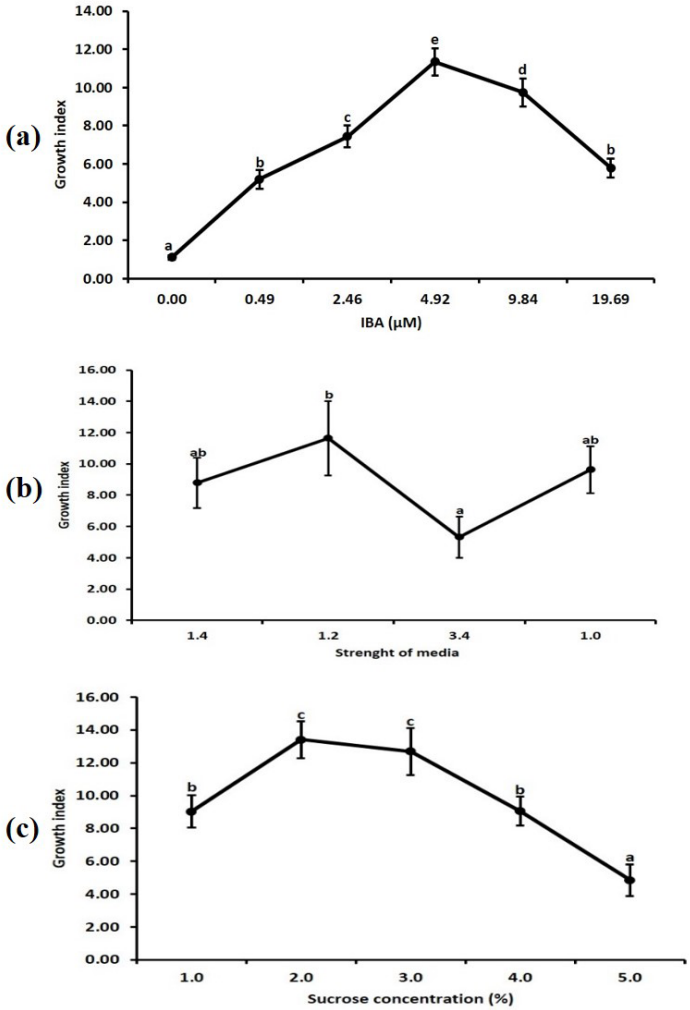

Figure 2

Effect of different growth parameters of adventitious root culture (a) IBA concentration in SH media, (b) Strength of SH media & (c) sucrose concentration on biomass accumulation (growth index) of adventitious root culture of V. jatamansi. Data represented in bar chart showed mean ± SE of five replicates and significantly different at p ≤0.05 level as determined using Duncan's multiple range test (DMRT).

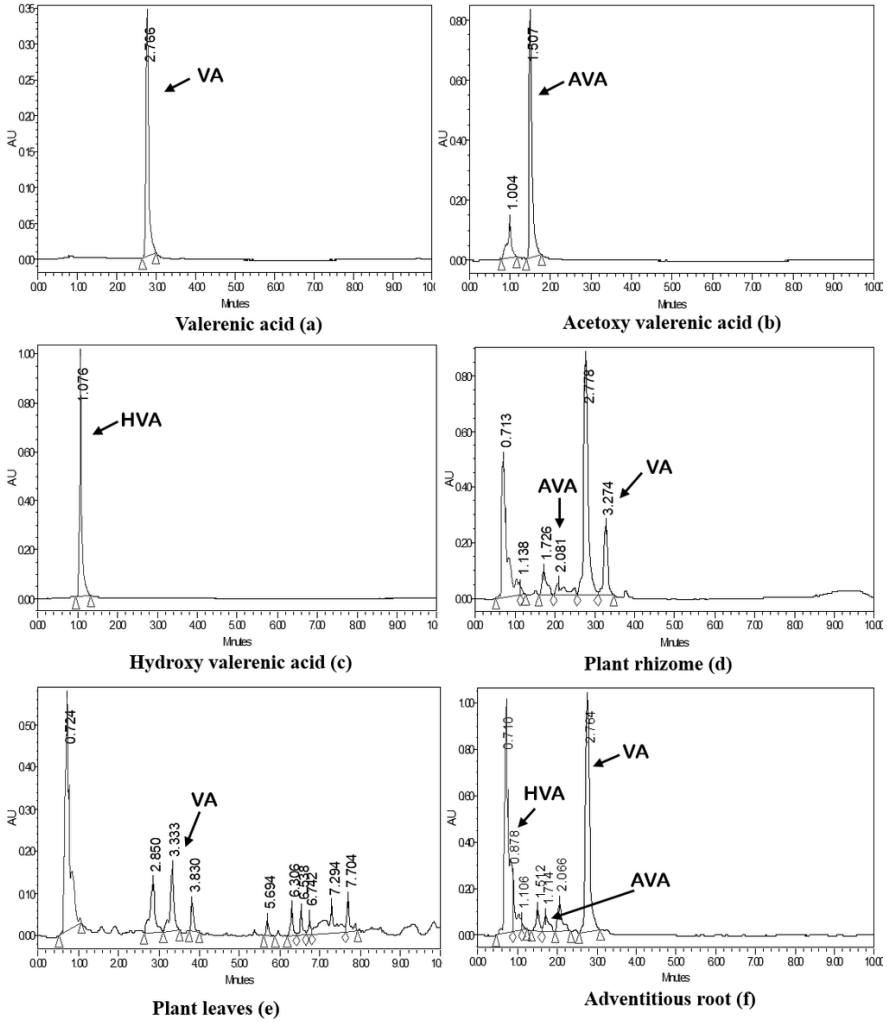

Figure 3

Determination of valerenic acid (VA), acetoxy valerenic acid (AVA) and hydroxyl valerenic acid (HVA) in plant and in vitro adventitious root samples of V. jatamansi using Ultra Performance Liquid Chromatography (UPLC-PDA) system. (a, b & c) standard mix of valerenic acid, acetoxy valerenic acid, hydroxyl valerenic acid, (d) plant rhizome, (e) plant leaves and (f) adventitious roots.

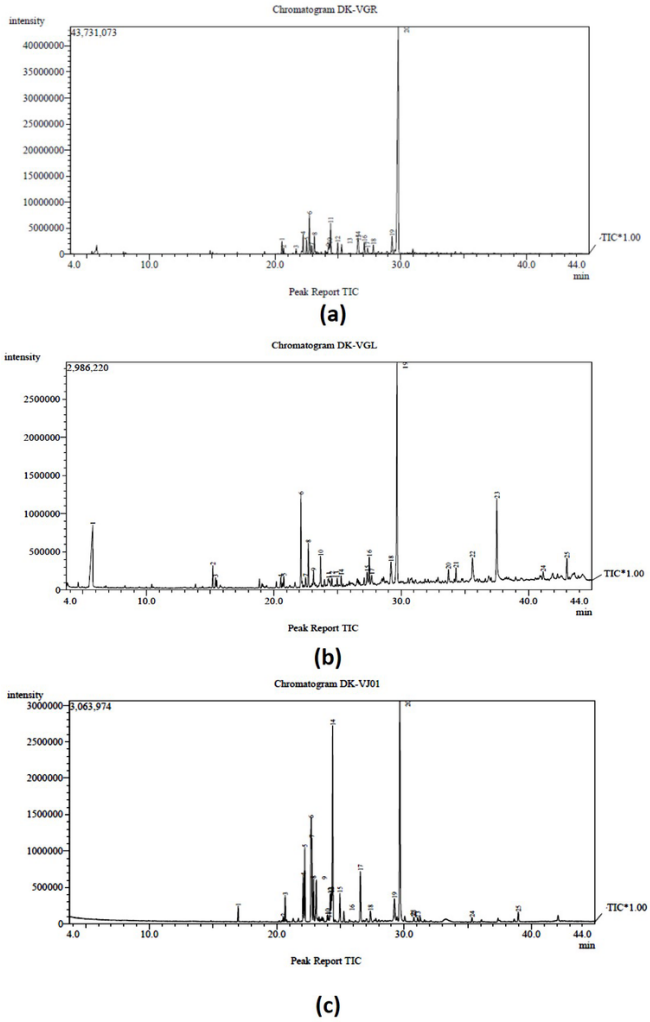

Figure 4

Determination of essential oil derivatives using Gas Chromatographic – Mass Spectroscopic (GC – MS) system from parent plant part (rhizome & leaves) and adventitious root of V. jatamansi. (a) Plant rhizome (b) plant leaves and (c) adventitious roots.

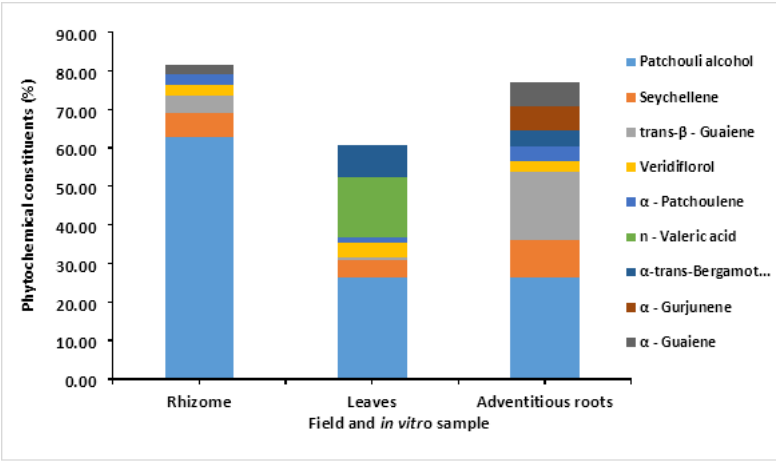

Figure 5

Gas Chromatographic – Mass Spectroscopic (GC – MS) analysis of major constituents present in essential oil extracted from rhizome, leaves and adventitious roots of V. jatamansi.

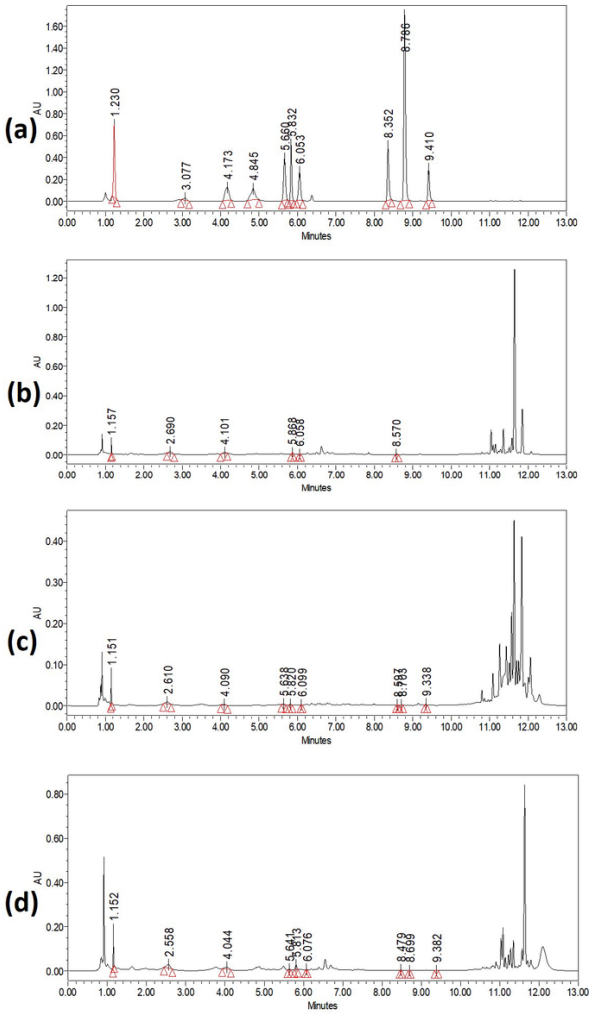

Figure 6

Determination of phenolic acids derivatives using Ultra Performance Liquid Chromatography (UPLC-PDA) system. (a) Standard mix of phenolic compounds (b) plant rhizome, (c) plant leaves and (d) adventitious roots.

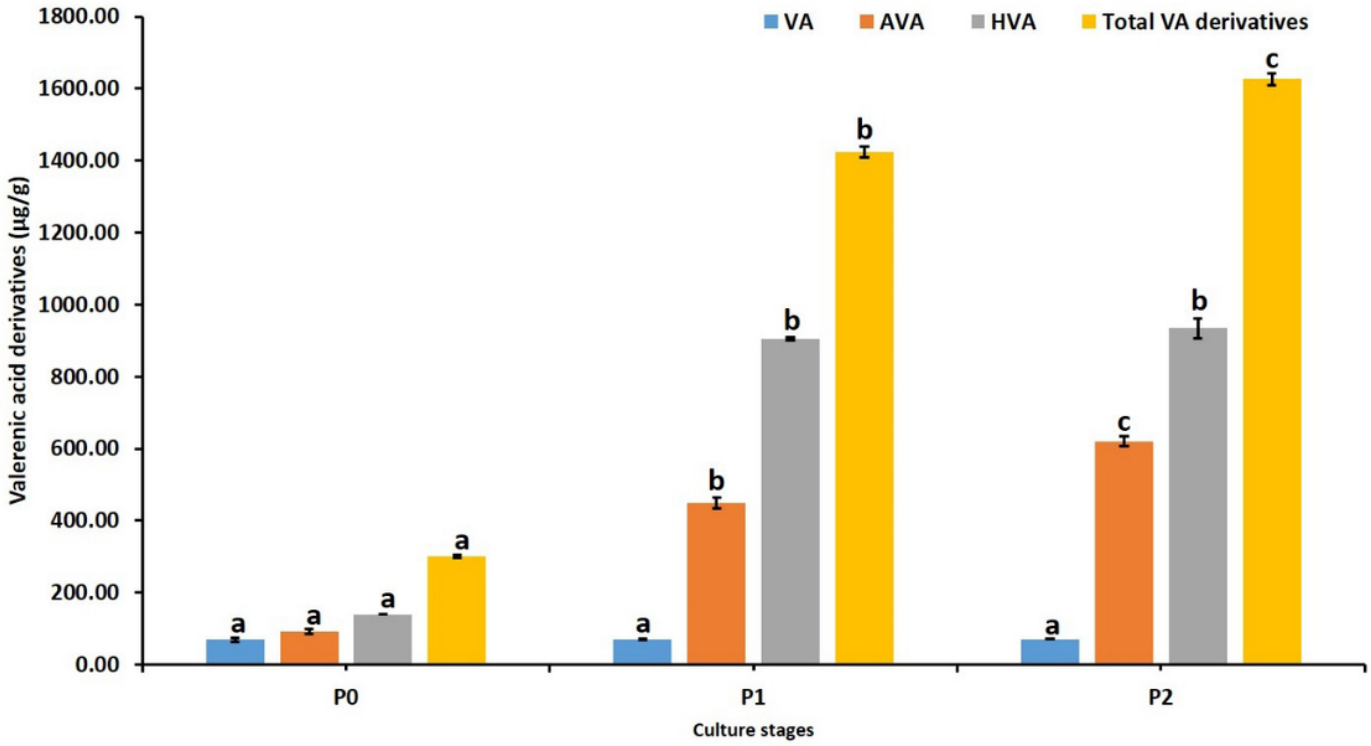

Figure 7

Effect of culture stage on production of valerenic acid derivatives from in vitro adventitious root culture of V. jatamansi. P0) Adventitious induced from leaf explants on semi - solid SH media + 9.84 µM IBA, P1) Adventitious multiplied on semi - solid SH media + 4.92 µM IBA & P2) Submerged cultivation in liquid SH media + 4.92 µM IBA.

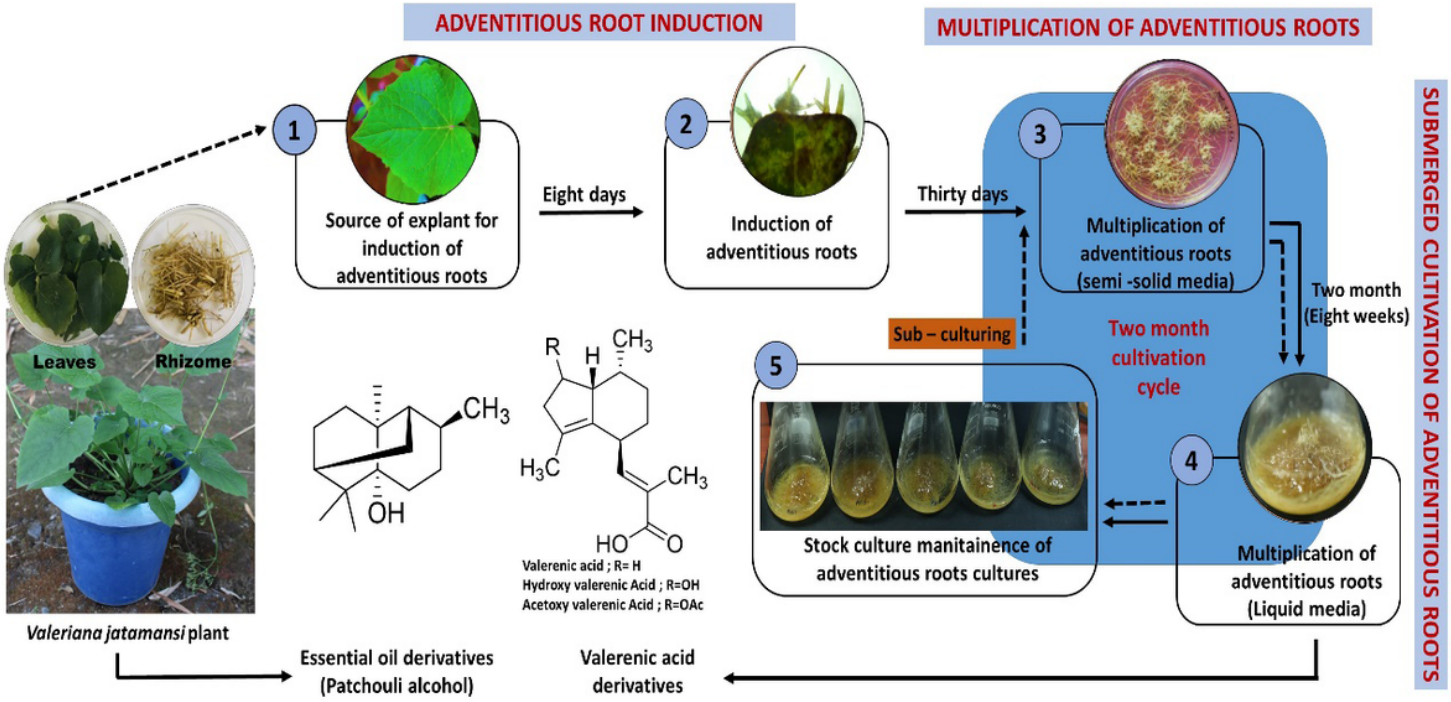

Figure 8

Growth cycle of in vitro induced adventitious root culture of V. jatamansi.