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BIOACTIVE/MEDICINAL COMPOUNDS

Optimisation of gentiopicroside production in *Gentiana kurroo* Royle from adventitious root cultures in a liquid culture system

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Abstract

Gentiana kurroo Royle is a critically endangered medicinal herb of the Indian Himalaya. It has bioactive seco-iridoid glycosides, notably gentiopicroside, in the rhizome and roots. In this study, we report on the *in vitro* production of gentiopicroside (GPD) from adventitious (AD) roots induced directly from the leaf, nodal, and suspension cell cultures of *G. kurroo*. Murashige and Skoog (MS) media fortified with Indole-3-butyric acid (IBA) 2 mg L^{-1} + Naphthaleneacetic acid (NAA) 0.5 mg L^{-1} and IBA 2 mg L^{-1} + Indole-3-acetic acid (IAA) 1 mg L^{-1} produced the maximum number of roots. Suspension cell cultures derived AD roots showed a 1.41-fold higher biomass production than adventitious roots induced from leaf and nodal explants. Among the various concentrations of MS media salts evaluated, half-strength MS suspension media favored the higher biomass and GPD accumulation. The maximum accumulation of GPD (2.58 mg g^{-1} dry weight (DW)) and AD root biomass (18.96 g L^{-1} DW) was observed on the 48th day. Furthermore, GPD produced from AD root cultures were separated by using TLC, characterized using Nuclear Magnetic Resonance (NMR) and High-Resolution Liquid Chromatograph-Mass Spectrometer (HR LC-MS) and quantified with High-Performance Liquid Chromatography (HPLC).

Keywords *Gentiana kurroo* · Cell suspension culture · Adventitious root culture · Gentiopicroside · NMR · HPLC · HR LC-MS

Introduction

Gentiana kurroo Royle, commonly known as the Indian or Himalayan gentian, is included in the Indian pharmaceutical codex (Behera and Raina 2012; Skinder et al. 2017). It grows in high altitude regions of Western and Northwestern Himalayas (Sharma et al. 2014). The roots of *G. kurroo* are widely used to treat liver and stomach inflammation in India (Ando et al. 2007). In Europe and Asia, gentian root extracts are used as an anti-inflammatory, analgesic, anti-rheumatic, antipyretic, antidiuretic, and hypoglycemic agent (Sezik et al. 2005; Niiho et al. 2006; Chen et al. 2009; Wani et al. 2013). Annually, about 6000 tons of gentian plants are harvested for its roots (Ando et al. 2007). The wild population of *G. kurroo* is limited due to unregulated over exploitation and has become red-listed as critically endangered by the International Union for Conservation of Nature (IUCN) in India (Ved et al. 2015). Hence, this plant has been banned from being exported by the Ministry of Commerce, Government of India vide Notification no. 2 (RE-98) 1997–2002 dated April 13, 1998, (Hayta et al. 2011). Chemical analysis of *Gentian*a spp. root extracts showed that gentiopicroside (GPD), swertiamarin, and loganic acid are the major bioactive compounds in the species *G. lutea* and *G. kurroo* (Ando et al. 2007; Wani et al. 2013). The presence of these three bioactive compounds (gentiopicroside, swertiamarin, and loganic acid) was used for the quality assessment of gentian roots for medicinal purposes (Huang et al. 2014). Thus, an alternative source is needed to meet the ever-growing demand for Gentian roots.

The *in vitro* culture system is an alternative way for the large-scale production of economically valuable secondary metabolites in a sustainable manner (Siva et al. 2012). Plant tissue culture has been shown to produce a higher level of secondary metabolites that are more stable (Silja and Satheeshkumar 2015). Previously, several reports on the induction of hairy root in *Gentiana* species have been accomplished using *Agrobacterium*-mediated transformation and regeneration (Nakatsuka et al. 2006). Few researchers have

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attempted to produce GPD using hairy root cultures of *Gentiana* species (Huang et al. 2014). The content of GPD in hairy root cultures was investigated in *G. macrophylla* by Zhang et al. (2010), *G. cruciata* L. (Hayta et al. 2011), and *G. scabra* (Huang et al. 2014). Since secondary metabolite production using genetically transformed root cultures were given less attention by pharmaceutical industries, adventitious root cultures were alternatively used to scale up the biomass and production of useful compounds (Silja and Satheeshkumar 2015).

Previous findings have suggested that adventitious root formation can be activated by adding an appropriate quantity of exogenous auxins such as IAA, IBA, or NAA to the culture medium (Park et al. 2017). This study presents for the first time the induction of adventitious roots from leaf, nodes, and suspension cell cultures of *G. kurroo*. The objectives of this study were (i) to optimize an *in vitro* system for the production of roots in *G. kurroo*, and (ii) to quantify the GPD content.

Materials and methods

Plant material and direct adventitious root formation Leaves and nodes of *G. kurroo* were collected from greenhouse-grown seedlings, washed with 1% sodium hypochlorite and 0.1% mercury chloride for surface sterilization. The plant material was cut into 1 cm length pieces. At least 12 pieces of the leaves and nodes were transferred to semi-solid MS basal medium (Himedia, Mumbai, India) supplemented with 30 g L^{-1} sucrose and 1.7 g L^{-1} phytagel (Sigma-Aldrich, Mumbai, India) and fortified with three types of auxins (IAA, IBA, and NAA) singly or in combination of varying concentrations for direct adventitious root organogenesis. Medium with no auxins was used as a control. The cultures were maintained at 25 ± 2°C under 16 h photoperiod. The adventitious roots that developed directly from the leaf and nodal region were transferred onto solid medium with the same Plant Growth Regulators (PGRs) singly or in combination for two subcultures at 45-day intervals. These were then cultured in a suspension medium to ascertain the level of root biomass production. The PGR combination that produced the highest biomass was used for GPD production. All the PGRs were purchased from Sigma-Aldrich.

Adventitious root formation from cell suspension Friable callus developed in MS medium supplemented with 1 mg/L 2, 4-D and 0.5 mg L^{-1} Kn (kinetin) from *in vitro* shoot derived leaves (Fiuk and Rybczyński 2008). To prepare the cell

Table 1. Adventitious root induction using leaf and nodal explants of *G. kurroo* on solidified MS media

Test sample	Growth regulators (mg/L)			Leaf			Node		
S.No.	IBA	IAA	NAA	No. of root formed	Root length (cm)	% of response	No. of root formed	Root length (cm)	% of response
C	_	_	-	-	-	-	-	-	-
1	0.2	-	-	5.50 ± 1.75^{abcde}	2.35 ± 0.58^{abcd}	33.33	-	-	-
2	0.5	-	-	8.60 ± 1.20^{cdef}	3.18 ± 0.68^{bcde}	41.67	-	-	-
3	1	-	-	8.57 ± 1.39^{cdef}	3.21 ± 0.19^{bcde}	58.33	1.33 ± 0.25^{a}	0.67 ± 0.11^{abc}	25.0
4	1.5	-	-	8.71 ± 0.89^{cdef}	3.52 ± 0.18^{cde}	58.33	1.67 ± 0.37^{a}	1.10 ± 0.21^{abcde}	25.0
5	2	-	-	9.00 ± 0.51^{efg}	3.21 ± 0.74^{bcde}	66.67	2.25 ± 0.85^{ab}	0.73 ± 0.25^{abcd}	33.3
6	0.5	0.5	-	8.00 ± 1.32^{defg}	3.06 ± 0.31^{bcde}	41.67	-	-	-
7	1	0.5	-	13.50 ± 1.48^{fgh}	3.37 ± 0.13^{cde}	83.33	3.00 ± 0.57^{ab}	1.25 ± 0.30^{abcde}	25.0
8	2	0.5	-	16.63 ± 1.29^{hi}	3.61 ± 0.13^{cde}	66.67	3.50 ± 0.64^{ab}	0.40 ± 0.10^{cde}	33.3
9	0.5	1	-	9.25 ± 1.10^{defg}	2.43 ± 0.26^{abcde}	33.33	1.50 ± 0.50^{a}	0.40 ± 0.17^{a}	16.7
10	1	1	-	8.67 ± 1.12^{cdef}	2.77 ± 0.19^{abcde}	50.00	2.33 ± 0.47^{ab}	0.85 ± 0.17^{abcde}	25.0
11	2	1	-	13.33 ± 1.52^{fgh}	3.50 ± 0.20^{cde}	50.00	2.80 ± 0.80^{ab}	1.44 ± 0.19^{e}	41.7
12	0.5	-	0.5	6.75 ± 1.11^{abcde}	2.75 ± 0.42^{abcde}	33.33	1.50 ± 0.64^{ab}	1.15 ± 0.34^{bcde}	16.7
13	1	-	0.5	9.71 ± 1.42^{efg}	2.67 ± 0.14^{abcde}	58.33	4.25 ± 0.70^{b}	1.48 ± 0.18^{de}	66.7
14	2	-	0.5	20.22 ± 1.44^{i}	3.97 ± 0.19^{e}	75.00	3.20 ± 0.66^{ab}	1.02 ± 0.10^{abcde}	41.7
15	0.5	-	1	8.20 ± 1.24^{bcdef}	2.82 ± 0.14^{abcde}	41.67	2.50 ± 0.50^{ab}	0.65 ± 0.15^{abc}	16.7
16	1	-	1	10.00 ± 1.37^{efg}	2.73 ± 0.17^{abcde}	50.00	1.33 ± 0.25^{a}	0.62 ± 0.08^{abc}	25.0
16	2	-	1	14.13 ± 1.32^{gh}	3.74 ± 0.24^{de}	66.67	2.17 ± 0.30^{ab}	0.88 ± 0.10^{abcde}	50.0
17	-	-	0.2	2.50 ± 0.50^{a}	1.95 ± 0.35^{abc}	16.67	-	-	-
18	-	-	0.5	3.50 ± 0.67^{abc}	2.13 ± 0.37^{abc}	33.33	-	-	-
19	-	-	1	3.00 ± 1.00^{ab}	1.65 ± 0.39^{ab}	16.67	1.33 ± 0.17^{a}	0.57 ± 0.20^{abc}	25.0
20	-	-	1.5	3.67 ± 1.20^{abc}	1.35 ± 0.25^{a}	25.00	2.00 ± 0.41^{ab}	0.50 ± 0.10^{ab}	16.7
21	-	-	2	3.50 ± 1.20^{abc}	2.65 ± 0.45^{abcde}	16.67	1.50 ± 0.20^{a}	0.76 ± 0.16^{abcde}	16.7
22	-	0.5	1	2.67 ± 0.60^{a}	2.55 ± 0.51^{abcde}	25.00	-	-	-
23	-	0.5	1.5	2.50 ± 0.88^{a}	3.85 ± 0.25^{de}	8.33	2.33 ± 0.27^{ab}	0.80 ± 0.12^{abcde}	25.0
24	-	0.5	2	4.33 ± 0.48^{abcd}	3.43 ± 0.85^{abc}	25.00	2.67 ± 0.33^{ab}	0.70 ± 0.11^{abc}	25.0

The values represent the mean of 12 experimental data. Mean ± SE; means within each *column* followed by the same *letter* are not significantly different ($P > 0.05$; Duncan's multiple range test), *C* control (media without PGRs)

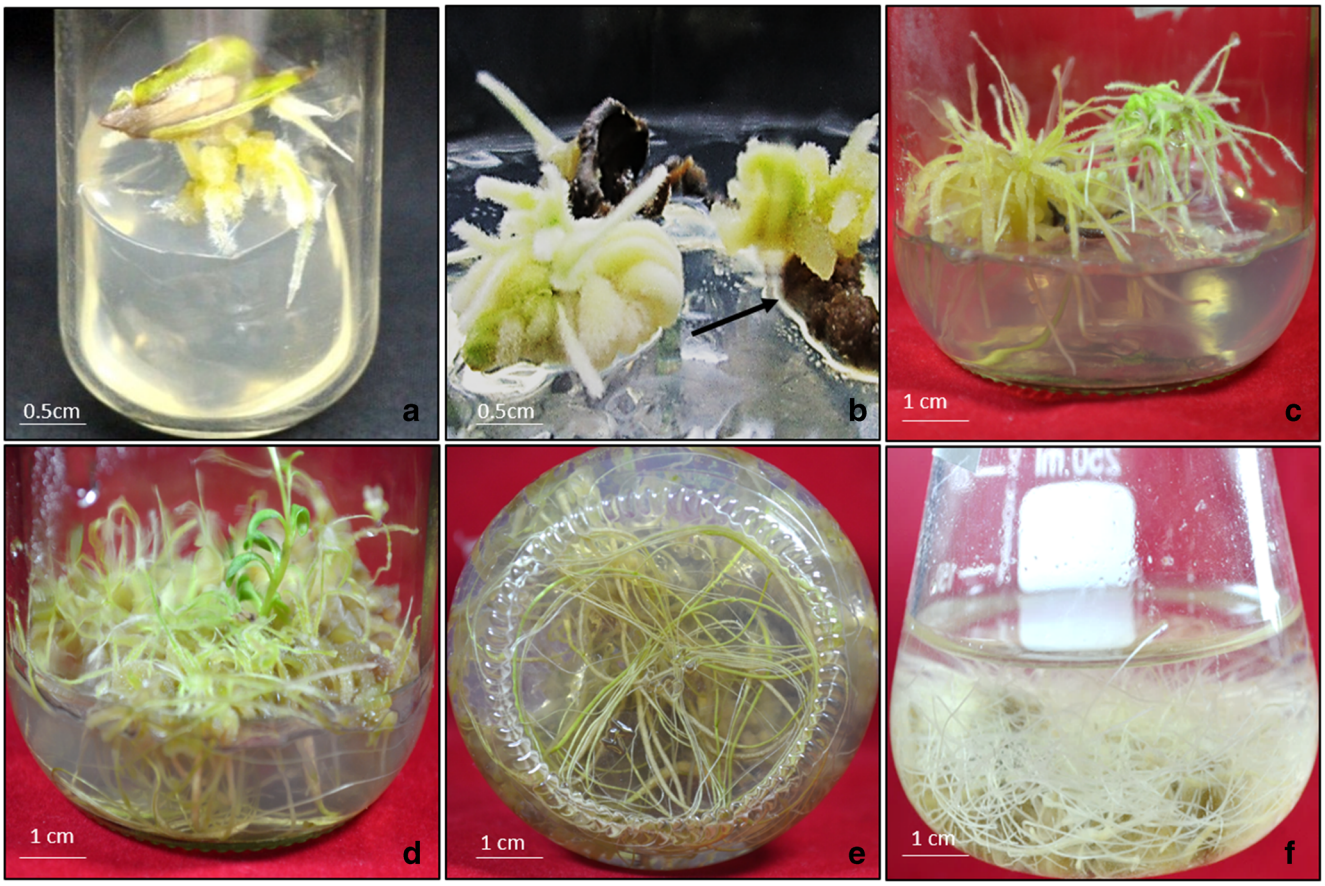

Figure 1 Adventitious (AD) root formation on solid medium and scale-up in suspension medium: (*a*) AD root formation from leaf explant; (*b*) AD root formation from nodal explant; (*c*, *d*, and *e*) Different stages of AD root elongation; (*f*) AD root growing in suspension medium.

suspension exactly, 1.5 g of friable callus was added to the 250 mL conical flask containing 80 mL of liquid MS media fortified with 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kn and kept under 16 h photoperiod in 25 ± 2°C. The suspension culture was maintained at 90 rpm in orbital shaker under illumination (40 μmol m^{-2} s^{-1}). These suspension cell cultures were further sub-cultured on the different types of auxins of varying concentrations and in combination for adventitious root organogenesis in suspension media. The cultures were incubated on an orbital shaker at 70 rpm, 85% relative humidity, under 25 ± 2°C in darkness. The biomass of the roots was obtained and GPD content was evaluated using HPLC, LC-MS, and NMR. The biomass and GPD content of AD roots obtained by direct organogenesis and those obtained from cell suspensions were compared.

Comparison of biomass To select the best method for upscaling AD root biomass, 1 g of AD roots developed directly either from the leaf or nodal region and suspension cells were inoculated into a 250 ml Erlenmeyer flask containing 75 mL of liquid MS medium and 2 mg L^{-1} IBA, 0.5 mg L^{-1} IAA and 30 g L^{-1} sucrose. All the culture flasks were incubated at room temperature at 90 rpm in an ORBITEK Pilot Shaker (Scigenics Biotech, Chennai, India) under 16 h photoperiod (40 μmol m^{-2} s^{-1}) at 25 ± 2°C. To estimate the accumulation of biomass, AD roots were harvested after 45 d of inoculation, placed on Whatman blotting paper to remove the moisture and the fresh weight (FW) was calculated. The fresh AD roots were dried in a hot-oven at 50°C for 2 d and the dry weight (DW) was measured. The GPD concentration was estimated using HPLC as described below. The highest biomass producing AD root system was adopted for further scale-up of biomass and GPD production.

Extract preparation Adventitious roots grown in the solid media and suspension media were dried and ground into a fine powder separately. Exactly, 100 mg powder was placed in 5 mL volumetric flasks and 3–4 mL of HPLC grade methanol was added to the flasks. The flasks were sonicated for 15 min and incubated in a boiling water bath at 75°C for 15 min. in a Cole-Pharmer ultrasonic bath (Mumbai, India). The flask was sonicated again for 10 min. The final volume was made up to 5 mL using HPLC grade methanol. Exactly, 1 mL was transferred to an Eppendorf tube and centrifuged twice at 15,000 rpm for 15 min. The supernatant was used for separation, characterization and quantification of GPD.

Table 2. Adventitious root induction using suspension cell cultures of *G. kurroo* in suspension medium

Test sample	IBA (mg/L)	IAA (mg/L)	NAA (mg/L)	% of response	Root length (cm)	Root biomass (gm/ flask)
C	-	-	-	-	-	-
1	0.5	-	-	50.00	1.13 ± 0.22^{ab}	5.30 ± 0.34^{ab}
2	1	-	-	58.33	0.93 ± 0.18^{ab}	4.77 ± 0.40^{a}
3	1.5	-	-	75.00	1.10 ± 0.32^{ab}	6.24 ± 0.68^{bcd}
4	2	-	-	75.00	1.77 ± 0.49^{ab}	7.19 ± 0.38^{de}
5	0.5	0.5	-	50.00	1.40 ± 0.48^{ab}	6.70 ± 0.39^{cde}
6	1	0.5	-	91.67	2.26 ± 0.51^{b}	10.11 ± 0.19^{fgh}
7	2	0.5	-	75.00	1.33 ± 0.42^{ab}	10.49 ± 0.44^{gh}
8	0.5	1	-	33.33	1.22 ± 0.58^{ab}	9.08 ± 0.19^{f}
9	1	1	-	50.00	1.02 ± 0.21^{ab}	6.39 ± 0.47^{bcde}
10	2	1	-	100.0	1.94 ± 0.39^{ab}	11.18 ± 0.31^{h}
11	0.5	-	0.5		-	-
12	1	-	0.5	33.33	0.58 ± 0.045^{a}	9.89 ± 0.31^{fg}
13	2	-	0.5	50.00	1.12 ± 0.20^{ab}	8.46 ± 0.87^{fg}
14	0.5	-	1	16.67	1.20 ± 0.80^{ab}	$9..19 \pm 0.30^{f}$
15	1	-	1		-	-
16	2	-	1	25.00	0.57 ± 0.06^{a}	7.62 ± 0.86^{e}
17	-	-	0.2		-	-
18	-	-	0.5		-	-
19	-	-	1		-	-
20	-	0.2	-		-	-
21	-	0.5	-	16.67	0.66 ± 0.11^{a}	5.76 ± 0.29^{abc}
22	-	1	-	50.00	1.19 ± 0.37^{ab}	6.51 ± 0.30^{bcde}

The values represent the mean of 12 experimental data. Mean ± SE; means within each *column* followed by the same *letter* are not significantly different ($P > 0.05$; Duncan's multiple range test); *C* control (media without PGRs)

Separation of GPD using TLC About 5 μL of the root extract from the suspension media was placed on TLC aluminum plates coated with silica gel (Merck, Mumbai, India). Separation of the extract was carried out using chloroform and methanol (80: 20 v/v). A GPD standard (Sigma-Aldrich) was used for the identification of the compound. The TLC plates were observed under UV illuminator (Cole-Parmer). For purification of GPD, 20 × 30 cm long TLC glass plates were prepared manually using silica-gel (Himedia). About 300 μL of the root extract was spotted on the plates and separation was carried out using chloroform and methanol (80: 20 v/v). The appropriate band was identified under an UV illuminator (Cole-Parmer) using a GPD standard (Sigma-Aldrich). The appropriate spot containing GPD was scraped out and loaded onto a 50 mL separation burette and GPD was eluted with chloroform and methanol (85: 15 v/v). The eluted GPD was again confirmed with analytical HPLC for purity.

Quantification of GPD using HPLC The quantitative analysis was carried out as per Tanaka et al. (2014) with few modifications. GPD quantification was performed using analytical HPLC (JASCO liquid chromatography, PU-2080 Plus, Tokyo, Japan) equipped with an auto sampler (AS-2055) and variable wavelength detector. The separation was carried out on a Thermo Scientific (Chennai, India) column packed with RP C8 (particle size 5 μm, 4.6 mm×150 mm) using water: methanol (60:40, v/v) mobile phase at a flow rate of 1 mL min^{-1} and UV detection at 272 nm. The Chrompass software was used for data collection and integration. Peak identification and peak area calculation were done by comparing with the calibration curve prepared using 1 mg mL^{-1} (Ho et al. 2017) stock standard GPD solution (data not shown).

Confirmation of GPD using LC-MS and NMR The purified GPD was confirmed with HPLC analysis and subjected to NMR analysis. The ^{1}H-NMR spectra were measured as described by Tanaka et al. (2014) using the VarianVNMRS 500 MHz NMR (Palo Alto, CA). The HR-LCMS analysis was carried out using1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs (Agilent Technologies, CA) as previously described

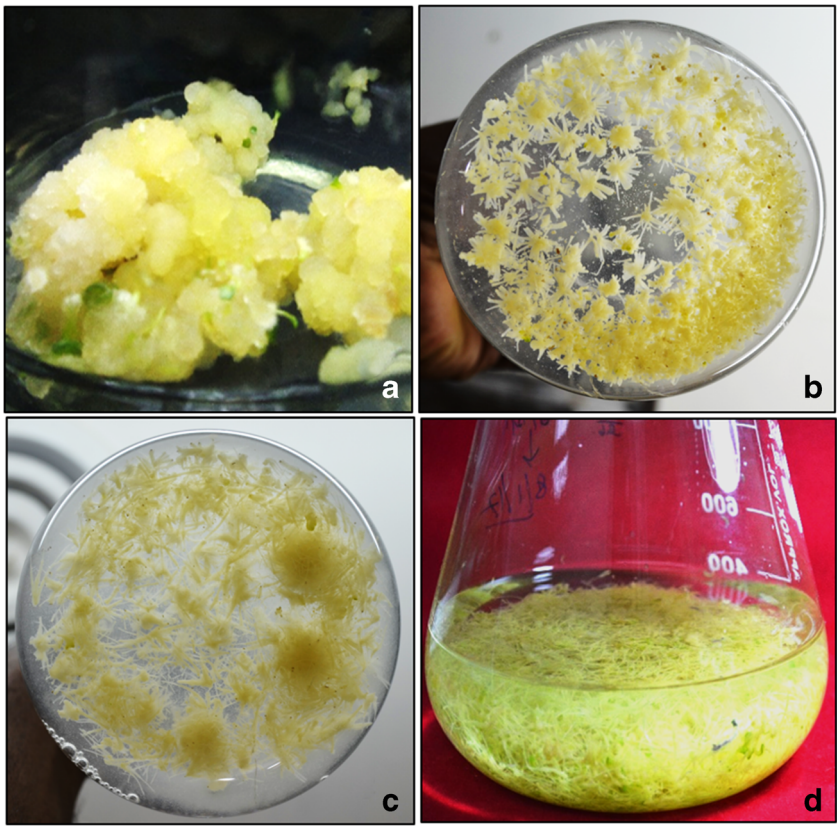

Figure 2. Different stages of adventitious root formation on suspension medium: (*a*) friable callus formation on solid medium; (*b*) initial stages of AD root formation from suspended cells; (*c* and *d*) AD root elongation.

(Chen et al. 2009). An Agilent MassHunter METLIN library was used for mass spectrum analysis.

Optimization of salt strength and growth kinetics The influence of salt concentration on the biomass and GPD production was assessed. Adventitious roots were inoculated on MS basal medium supplemented with 2 mg L^{-1} IBA and 0.5 mg L^{-1} IAA but varying salt concentrations (1/4x, 3/4x, 1/2x, and 2x). Normal MS basal medium without PGRs was used as a control. After 40 days, fresh roots were harvested, the biomass of the roots was estimated, and the concentration of GPD was analyzed. The optimal period for adventitious root formation was done by harvesting the roots at 8-d intervals for up to 48 d.

Statistical analysis All root induction and growth treatments were comprised of 12 duplicates and the experiment was repeated twice. One-way analysis of variance (ANOVA) was employed for data analysis using the SPSS 16.0 statistical

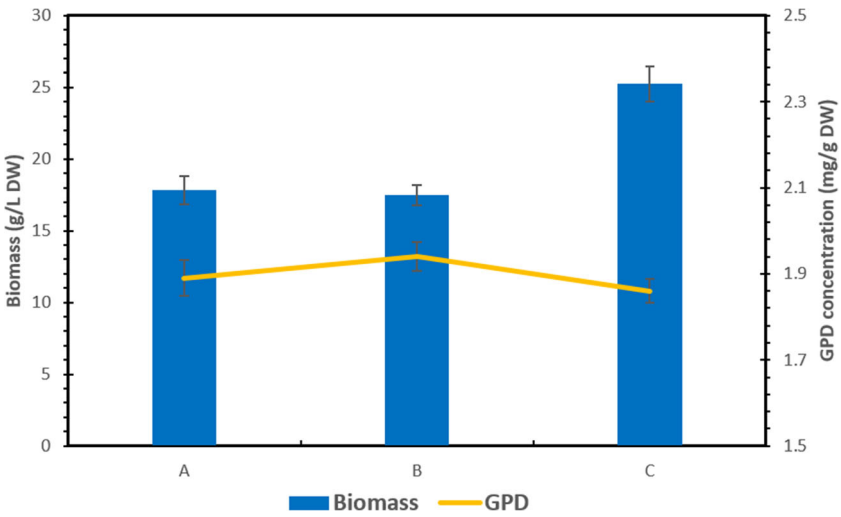

Figure 3. Comparison of different AD root Biomass and GPD content: (*a*) leaf-derived AD root on solid media, (*b*) leaf-derived AD root in liquid media; (*c*) suspended cells derived AD roots in liquid media.

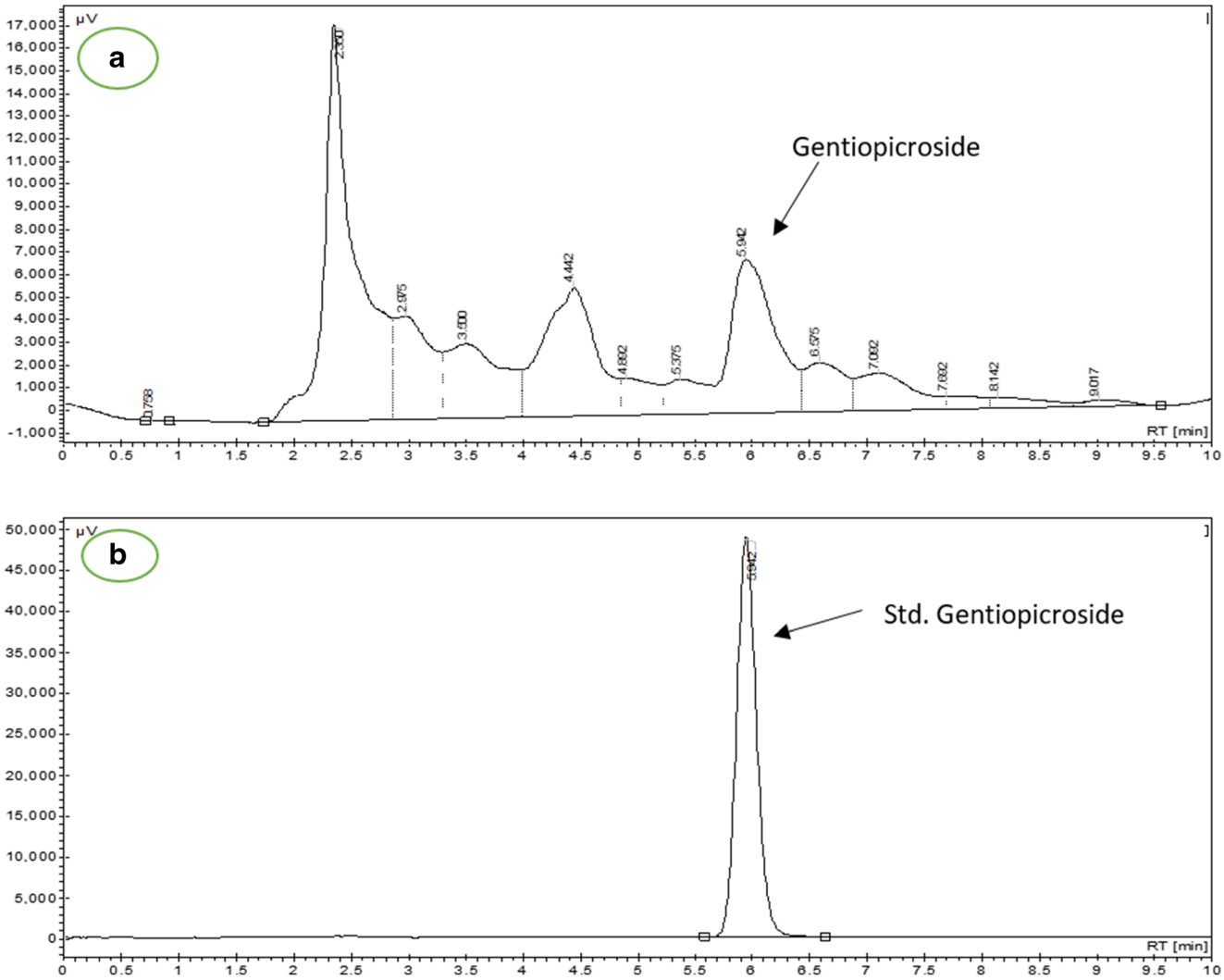

Figure 4. Quantification of GPD using HPLC: *a* HPLC profile of methanolic extracts of *in vitro* AD root, *b* standard GPD chromatogram.

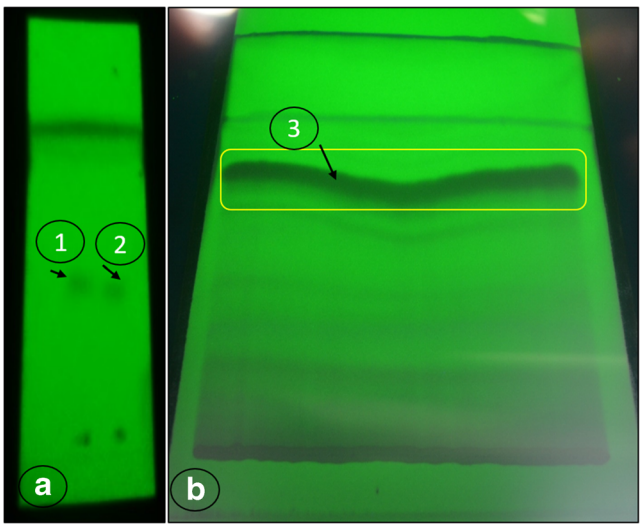

Figure 5. Separation of gentiopicroside (GPD) from crud extract using TLC method: (*a*) separation of GPD along with standard (1, standard GPD; 2, crude extract); (*b*) separation of GPD in large TLC plates (3-GPD band).

package. Means were compared using Duncan's multiple range test (DMTRT) at a significance of $p = 0.05$

Results and discussion

Adventitious root induction on solid medium Among various auxins used individually, 2 mg L^{-1} of IBA produced 9.00 ± 0.51 roots with an average root length of 3.21± 0.74 in leaf explant after 6 weeks. When used in combination, 2 mg L^{-1} IBA with 0.5 mg L^{-1} NAA produced the maximum number of roots (20.22 ± 1.42) with a mean length of 3.97 ± 0.19 cm from the leaf explant after 4 weeks of culture. A similar response was observed in leaf explants from *Withania somnifera* (Wadegaonkar et al. 2006) and *Plumbago rosea* (Silja and Satheeshkumar 2015) where the maximum adventitious root formation was achieved using MS medium fortified with IAA (1 mg L^{-1}) in combination with NAA (1.5 mg L^{-1}). Another study showed

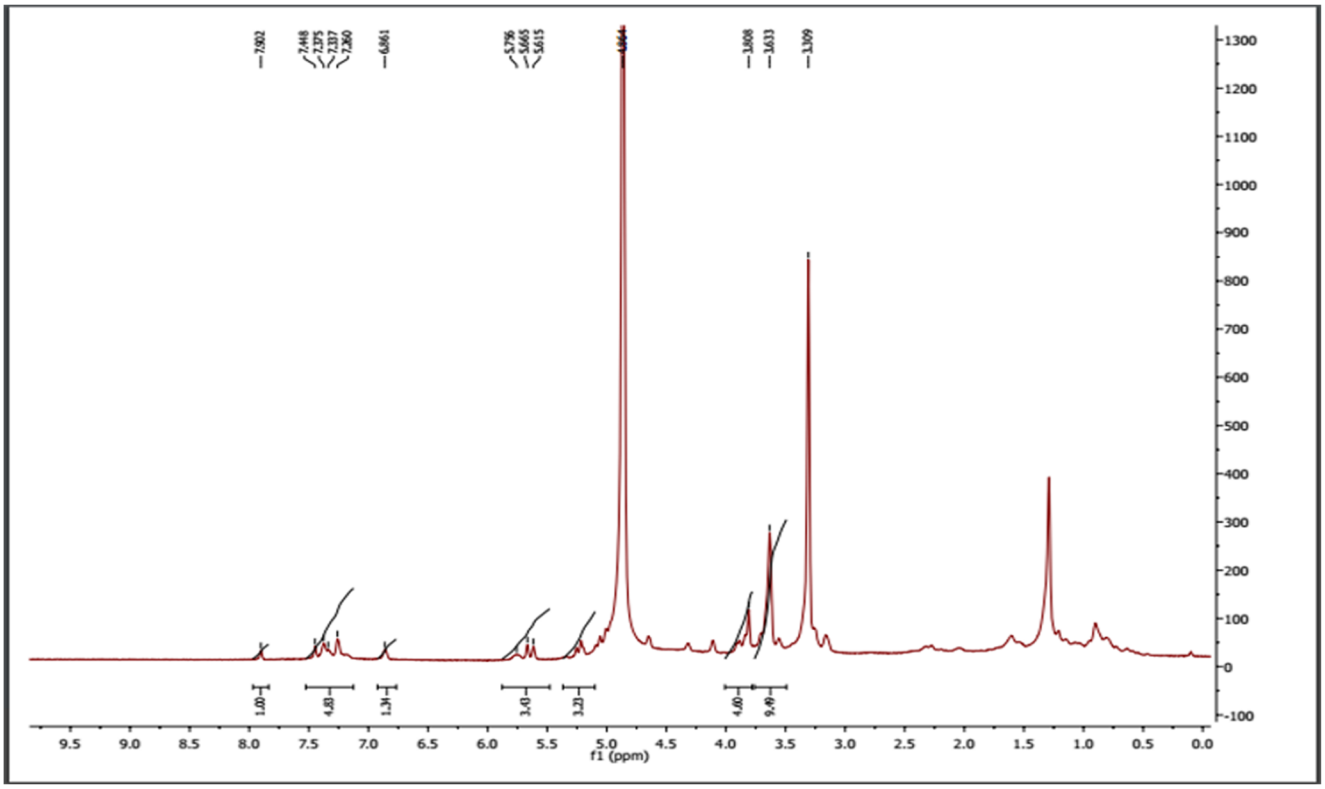

Figure 6. ^{1}H NMR spectrum of gentiopicroside purified from TLC plate.

that 0.5 mg L^{-1} IBA alone is capable of inducing the maximum number of roots (17.50 roots) in leaf explant of *W. somnifera* (Praveen and Murthy 2010). In *Castilleja tenuiflora*, rhizogenesis was achieved on B5 media supplemented with only NAA (10 μM L^{-1}) and 8% sucrose in leaf explants (Gómez-Aguirre et al. 2012). In the present study, nodal explants of *G. kurroo* produced an average 4.25 ± 0.70 roots with a mean length of 1.48 ± 0.18 cm after 6 weeks on MS medium supplemented with 1 mg L^{-1} IBA with 0.5 mg L^{-1} IAA (Table 1, Fig. 1*a–f*). Khanam et al. (2018) reported that when 0.5 μM L^{-1} IBA was used solely with 4% sucrose, a mean of 2. 60 ± 0.16 roots was produced in nodal segments of *Allamanda cathartica.* This study showed that the formation of adventitious roots under *in vitro* condition varies on the basis of exogenous addition of different auxins, explant type, and plant species.

Adventitious root induction on liquid medium The initial suspension cultures in MS medium augmented with 2 mg L^{-1} IBA + 1 mg L^{-1} IAA yielded a fresh weight of 11.18 g/flask of roots. It was possible to measure the root length with subsequent subcultures and was found to reached a maximum of 1.94 cm (Table 2, Fig. 2). The roots formed from the suspension cells yielded the maximum biomass. The increased number of AD root formation in suspension cells may be due to the switch of a larger number of cells to root primordia. This could have been activated by submergence-mediated ethylene production as reported earlier (Steffens and Rasmussen 2016).

Similarly, suspension cell cultures of *Curcuma amada* in MS medium supplemented with 0.3 mg g L^{-1} IBA alone produced AD root (Soundar Raju et al. 2015). In *W. somnifera*, it was observed that there was a higher frequency of AD root induction in half-strength MS liquid medium supplemented with 0.5 mg L^{-1} IBA + 0.25 mg L^{-1} IAA from suspension cell culture after 4 weeks of incubation (Thilip et al. 2015). These results suggested that suspended cells are the more promising explants for the production of AD root in *G. kurroo.*

Comparison of biomass and gentiopicroside content The highest yield of roots was obtained from the suspension cultures compared to those obtained from the leaf and nodal explant. The dry weight of roots obtained from the suspension cultures was 20.34 g L^{-1}, from the leaf 10.39 g L^{-1} and nodal regions was 7.24 g L^{-1} (Fig. 3). This is the first report which showed that AD roots can be obtained directly from leaf explants on solid medium and from cell suspension cultures of *G. kurroo*. This is also the first study that produced GPD from AD roots in *G. kurroo* without any genetic modification. In the past, a few attempts were made to produce GPD in transformed root lines in the genus *Gentiana*. For example, genetic transformation of *G. scabra* with *Agrobacterium rhizogenes* resulted in the formation of

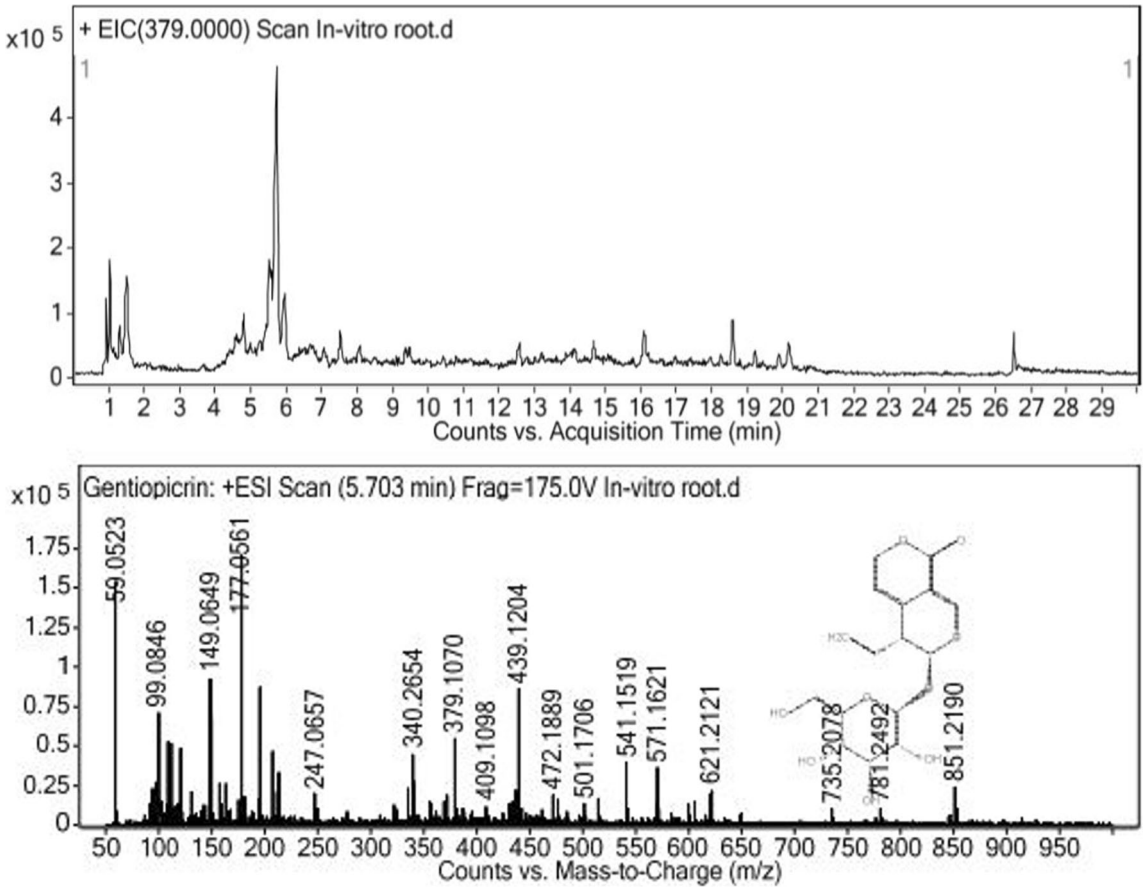

Figure 7. HR-LC MS profile: (*a*) HR-LC MS chromatogram of adventitious root methanolic extract; (*b*) mass spectrum showing GPD.

226 mg L^{-1} DW of hairy roots which were able to produce GPD (55 mg g^{-1} DW) and swertiamarin (Huang et al. 2014). Similarly, Zhang et al. (2010) produced hairy roots in *G. macrophylla* with a fresh weight of 7.1 g/150 mL and 0.11 mg g^{-1} DW of GPD. In comparison with above two studies, our study produced a much higher root mass and greater amount of GPD. HPLC based quantification of GPD revealed that the naturally grown roots contain a higher amount of GPD (41.9 ± 0.84 mg g^{-1} DW) compared to AD roots established in this study (1.94 mg g^{-1} DW). There was not much differences in GPD content among the three types of AD roots (leaf-derived AD root on solid media, leaf-derived AD root in liquid media, and suspended cells derived AD roots) used in this study, whereas there was a difference in root biomass among the three types of explants (leaf, node, and suspended cells obtained from *in vitro* leaf-derived callus) with the greatest biomass produced in the suspension cells derived root cultures (Fig. 3). Though the GPD content is comparatively low in the AD root, it is possible to produce a higher biomass through *in vitro* culture and increase the production of this valuable compound. Therefore, the AD roots derived from suspended cells were used for the production of GPD in shake flask cultures.

Characterization and quantification of gentiopicroside Quantitative analysis of GPD showed that 2.58 mg g^{-1} DW was produced in the roots from the suspension cultures after 48 days (Fig. 4). The GPD was purified using TLC (Fig. 5) and then analyzed with ^{1}H NMR for further confirmation (Fig. 6). The NMR signals obtained at 5 ppm matches those of previous analysis carried out in *G. radix and G. scabrae radix* (Tanaka et al. 2014). The molecular mass of the GPD was further confirmed with HR LC-MS analysis (Fig. 7*a* and *b*). The mass spectra of GPD obtained in this study were similar to that of *G. macrophylla* (Chen et al. 2009).

Optimization of salt strength and growth kinetics This study assessed the influence of MS basal media on AD root biomass and production of GPD. Previous studies have shown that biomass accumulation of *in vitro* root cultures is influenced by salt strength of the culture medium (Hayta et al. 2011; Murthy et al. 2016). The interactions between the nutritious salts in the culture medium increase the accessibility of ions to the roots and thus enhance growth of AD roots and secondary metabolites synthesis (Soundar Raju et al. 2015). Therefore, in this study, the optimum salt concentration for root production from the suspension cells was investigated. Initially, 20 mg

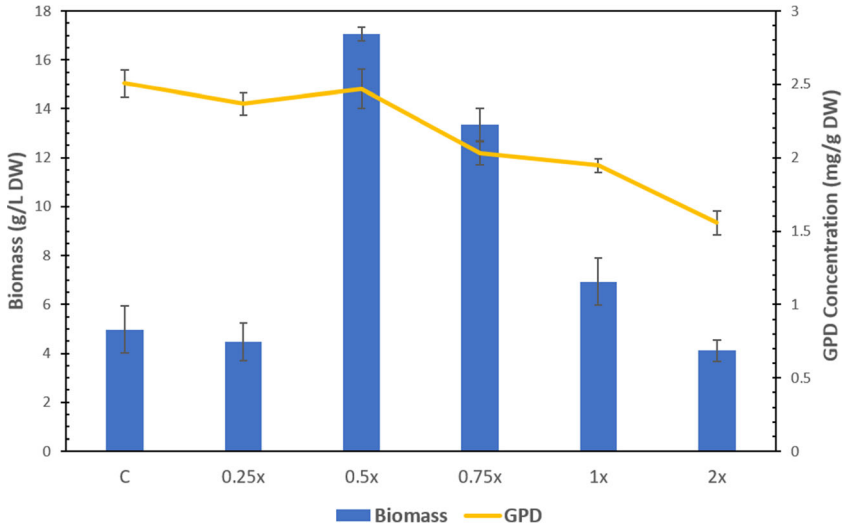

Figure 8. Effect of MS salt concentration on biomass and GPD.

L^{-1} of each AD root lines was inoculated onto varying salt concentrations (1/4x, 1/2x, 3/4x and 2x) of suspension MS medium enriched with 2 mg L^{-1} IBA and 0.5 mg L^{-1} IAA. The roots were harvested after 48 days and analyzed for fresh weight, dry weight, and GPD content.

Among the different salt concentrations used in this study, half-strength MS medium produced a maximum fresh root biomass of 120.8 g/L and 2.50 mg/g DW GPD (Fig. 8). This finding confirms the influence of salt strength on root biomass and GPD accumulation in *G. kurroo.* This is similar to that reported by Rajesh et al. (2014) in *Podophyllum hexandrum* and *Curcuma amada* (Soundar Raju et al. 2015). Similarly, the hairy roots developed from *G. macrophylla* showed the highest biomass accumulation in ½ strength MS medium (Tiwari et al. 2007).

In order to ascertain the time required for maximum yield of roots and GPD production, the suspension cultures in ½ MS medium were examined at 8-d intervals. The highest accumulation of biomass and GPD was achieved on the 48th day (Fig. 9). There was no increase in biomass after 48 d of culture. In another study, maximum biomass was reached at 35 d and the highest accumulation of GPD observed on 28 d in hairy root cultures of *G. scabra* (Huang et al. 2014). Similarly, in *G. macrophylla*, hairy root cultures showed maximum biomass production after 35 d (Tiwari et al. 2007). These studies implicate that biomass accumulation may vary depending on the root system and plant species in the genus *Gentian*.

Conclusion

This study showed that it was possible to develop AD roots in *G. kurroo* using leaves, nodal segments, and suspension cultures under *in vitro* conditions. It also showed that the roots from the suspension cultures had a higher biomass and a substantial amount of GPD. The presence of GPD was confirmed using HPLC, NMR, and HR-LCMS. The method developed in this study could be an alternative way of producing a substantial amount of GPD and could be scaled up to meet the industrial demand of GPD. In addition, an *in vitro* method of

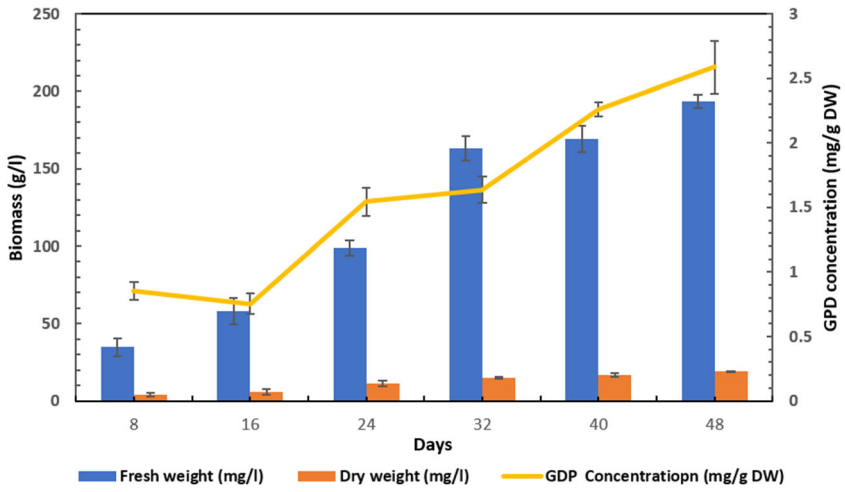

Figure 9. Growth kinetics of suspension derived AD roots.

producing GPD will reduce exploitation of this plant in the wild population and prevent its extinction.

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References

Ando H, Hirai Y, Fujii M, Hori Y, Fukumura M, Niiho Y, Nakajima Y, Shibata T, Toriizuka K, Ida Y (2007) The chemical constituents of fresh Gentian Root. J Nat Med 61:269–279. https://doi.org/10.1007/s11418-007-0143-x

Behera MC, Raina R (2012) Gentiana kurroo Royle – A critically endangered bitter herb. Int J Medi Arom Plants 2(1):22–29

Chen L-Y, Chen Q-L, Xu D, Hao JG, Schläppi M, Xu ZQ (2009) Changes of gentiopicroside synthesis during somatic embryogenesis in *Gentiana macrophylla*. Planta Med 75:1618–1624. https://doi.org/10.1055/s-0029-1185808

Fiuk A, Rybczyński JJ (2008) Morphogenic capability of *Gentiana kurroo* Royle seedling and leaf explants. Acta Physiol Plant 30:157–166. https://doi.org/10.1007/s11738-007-0104-8

Gómez-Aguirre YA, Zamilpa A, González-Cortazar M, Trejo-Tapia G (2012) Adventitious root cultures of *Castilleja tenuiflora* Benth. as a source of phenylethanoid glycosides. Ind Crops Prod 36:188–195. https://doi.org/10.1016/j.indcrop.2011.09.005

Hayta S, Gurel A, Akgun I, Altan F, Ganzera M, Tanyolac B, Bedir E (2011) Induction of *Gentiana cruciata* hairy roots and their secondary metabolites. Biologia (Bratisl) 66(4):618–625. https://doi.org/10.2478/s11756-011-0076-4

Ho T-T, Lee K-J, Lee J-D, Bhushan S, Paek KY, Park SY (2017) Adventitious root culture of *Polygonum multiflorum* for phenolic compounds and its pilot-scale production in 500 L-tank. Plant Cell Tissue Organ Cult PCTOC 130:167–181. https://doi.org/10.1007/s11240-017-1212-9

Huang S-H, Vishwakarma R, Lee T-T, Chan HS, Tsay HS (2014) Establishment of hairy root lines and analysis of iridoids and secoiridoids in the medicinal plant *Gentiana scabra*. Bot Stud 55:17. https://doi.org/10.1186/1999-3110-55-17

Khanam MN, Anis M, Ahmad S (2018) Establishment of adventitious root cultures of *Allamanda cathartica* L. for the production of iridoid glycosides and its identification using HPTLC MS. Ind Crops Prod 125:198–206. https://doi.org/10.1016/j.indcrop.2018.08.044

Murthy HN, Dandin VS, Paek K-Y (2016) Tools for biotechnological production of useful phytochemicals from adventitious root cultures. Phytochem Rev 15(1):129–145

Nakatsuka T, Nishihara M, Mishiba K, Hirano H, Yamamura S (2006) Two different transposable elements inserted in flavonoid 3′,5′-hydroxylase gene contribute to pink flower coloration in *Gentiana scabra*. Mol Genet Genomics 275:231–241. https://doi.org/10.1007/s00438-005-0083-7

Niiho Y, Yamazaki T, Nakajima Y, Yamamoto T, Ando H, Hirai Y, Toriizuka K, Ida Y (2006) Gastroprotective effects of bitter principles isolated from Gentian root and Swertia herb on experimentally-induced gastric lesions in rats. J Nat Med 60:82–88. https://doi.org/10.1007/s11418-005-0014-2

Park S-H, Elhiti M, Wang H, Xu A, Brown D, Wang A (2017) Adventitious root formation of in vitro peach shoots is regulated by auxin and ethylene. Sci Hortic 226:250–260. https://doi.org/10.1016/j.scienta.2017.08.053

Praveen N, Murthy HN (2010) Production of withanolide-A from adventitious root cultures of *Withania somnifera*. Acta Physiol Plant 32:1017–1022. https://doi.org/10.1007/s11738-010-0489-7

Rajesh M, Sivanandhan G, Arun M, Vasudevan V, Theboral J, Girija S, Manickavasagam M, Selvaraj N, Ganapathi A (2014) Factors influencing podophyllotoxin production in adventitious root culture of *Podophyllum hexandrum* Royle. Acta Physiol Plant 36:1009–1021. https://doi.org/10.1007/s11738-013-1479-3

Sezik E, Aslan M, Yesilada E, Ito S (2005) Hypoglycaemic activity of *Gentiana olivieri* and isolation of the active constituent through bioassay- directed fractionation techniques. Life Sci 76:1223–1238. https://doi.org/10.1016/j.lfs.2004.07.024

Sharma A, Kaur R, Sharma N (2014) In vitro morphogenic response of different explants of Gentiana kurroo Royle from Western Himalayas—an endangered medicinal plant. Physiol Mol Biol Plants 20(2):249–256

Silja PK, Satheeshkumar K (2015) Establishment of adventitious root cultures from leaf explants of *Plumbago rosea* and enhanced plumbagin production through elicitation. Ind Crops Prod 76:479–486. https://doi.org/10.1016/j.indcrop.2015.07.021

Siva R, Mayes S, Behera SK, Rajasekaran C (2012) Anthraquinones dye production using root cultures of *Oldenlandia umbellata* L. Ind Crops Prod 37:415–419. https://doi.org/10.1016/j.indcrop.2011.12.027

Skinder B, Ganai B, Wani A (2017) Scientific study of Gentiana kurroo Royle. Medicines 4(4):74

Soundar Raju C, Varutharaju K, Thilip C, Aslam A, Shajahan A (2015) Rhizogenesis in cell suspension culture from mango ginger: a source of isosorbide and n-Hexadecanoic acid. Advances in Botany 2015:1–7

Steffens B, Rasmussen A (2016) The physiology of adventitious roots. Plant Physiology 170:603–617. https://doi.org/10.1104/pp.15.01360

Tanaka R, Hasebe Y, Nagatsu A (2014) Application of quantitative 1H-NMR method to determination of gentiopicroside in *Gentianae radix* and Gentianae scabrae radix. J Nat Med 68:630–635. https://doi.org/10.1007/s11418-014-0833-0

Thilip C, Raju CS, Varutharaju K, Aslam A, Shajahan A (2015) Establishment of adventitious root culture from cell suspensions of *Withania somnifera* (L.) Dunal: an in vitro approach for production of withanolides. Int J Pharma Bio Sci 6(1):1030–1037

Tiwari RK, Trivedi M, Guang ZC, Guo GQ, Zheng GC (2007) Genetic transformation of *Gentiana macrophylla* with *Agrobacterium rhizogenes*: growth and production of secoiridoid glucoside gentiopicroside in transformed hairy root cultures. Plant Cell Rep 26:199–210. https://doi.org/10.1007/s00299-006-0236-0

Ved D, Saha D, Ravikumar K, Haridasan K (2015) Gentiana kurroo. The IUCN red list of threatened species 2015:e.T50126594A50131345. https://doi.org/10.2305/IUCN.UK.2015

Wadegaonkar PA, Bhagwat KA, Rai MK (2006) Direct rhizogenesis and establishment of fast growing normal root organ culture of *Withania*

somnifera Dunal. Plant Cell Tissue Organ Cult 84:223–225. https://doi.org/10.1007/s11240-005-9011-0

Wani BA, Ramamoorthy D, Rather MA, Arumugam N, Qazi AK, Majeed R, Hamid A, Ganie SA, Ganai BA, Anand R, Gupta AP (2013) Induction of apoptosis in human pancreatic MiaPaCa-2 cells through the loss of mitochondrial membrane potential (ΔΨm) by *Gentiana kurroo* root extract and LC-ESI-MS analysis of its principal constituents. Phytomedicine 20:723–733. https://doi.org/10.1016/j.phymed.2013.01.011

Zhang HL, Xue SH, Pu F, Tiwari RK, Wang XY (2010) Establishment of hairy root lines and analysis of gentiopicroside in the medicinal plant *Gentiana macrophylla*. Russ J Plant Physiol 57:110–117. https://doi.org/10.1134/S1021443710010152