



# Optimizing Shoot Formation in *Gentiana kurroo* Royle for Gentiopicroside Production

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## Abstract

The roots and shoots of *Gentiana kurroo* Royle are rich sources of gentiopicroside (GPD). The plant is used traditionally for curing many metabolic diseases. The exploitation of *G. kurroo* in its native habitat has placed the plant on the critically endangered list of plants in India. One of the ways of creating an alternative source of *G. kurroo* is through in vitro propagation. Although a number of in vitro propagation methods for *G. kurroo* exist, there are no studies that have optimized methods for rapid in vitro shoot production and the production of GPD. The objective of this study was to develop an effective in vitro shoot multiplication system of *G. kurroo*. Furthermore, the influence of solid and liquid induction media were investigated. Shoots were regenerated from embryogenic callus and transferred to solid and liquid Murashige and Skoog (MS) and Gamborg (B<sub>5</sub>) media fortified with various concentrations of BA containing different auxins. It was observed that the liquid medium produced a higher number of shoots than the solid media. MS supplemented with BA (2 mg/L) and IAA (0.5 mg/L) produced ~5.58 shoots per explant on the solid medium, while ~16 shoots per explant was obtained in the liquid medium. High-Performance Liquid Chromatography (HPLC) analysis of in vitro shoots grown in the liquid medium produced 9.13 mg/g dry weight (DW) of GPD which is seven-fold higher than that of naturally growing plant shoots. The in vitro protocol for *G. kurroo* developed in this study may be used for industrial production of GPD.

**Keywords** *Gentiana kurroo* · Somatic embryo · Multiple shoots · Liquid culture · Gentiopicroside

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## Introduction

*Gentiana kurroo* Royle, a perennial herb of the family Gentianaceae, is endemic to the Himalayas and is one of the many critically endangered species of India (Wani et al. 2013). The rooting system of *G. kurroo* contain iridoid glycosides such as gentiopicroside, gentiamarin,

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amaroswerin, and the alkaloid gentianin (Baba and Malik 2014). The leaves also contain the iridoid glycoside (2, 3-dihydroxybezoyloxy)-7-ketologanin (Kumar et al. 2003). The plant is included in Siddha and Ayurveda medicinal systems in India and its rhizomes and roots are used to treat a variety of health problems including bronchial asthma, dyspepsia, flatulence, colic, anorexia, helminthiasis, inflammations, amenorrhea, dysmenorrhea, hemorrhoids, constipation, leukoderma, leprosy and urinary infections (Sharma et al. 1993).

The commercial demand for GPD is increasing. In vitro studies show that GPD has anti-inflammatory (Mubashir et al. 2014; Zhang et al. 2019) and antidiabetic activity (Yang et al. 2018). In vivo studies show that GPD has analgesic (Chen et al. 2008), antinociceptive (Liu et al. 2016), antibacterial and free radical scavenging activities (Kumarasamy et al. 2003) and it is used to treat acute jaundice and chronic active hepatitis (Deng et al. 2013). Extensive collection of *G. kurroo* from its natural habitat is posing a severe threat to its wild populations. The narrow distribution, poor seedling establishment and recalcitrance to domestication has led to the depletion of the species in India (Behera and Raina 2011). To conserve its natural populations from being over exploited for its wide medicinal uses, in vitro propagation methods of the plant have been established (Sharma et al. 1993, 2014; Fiuk and Rybczyński 2007, 2008a, b).

It is important to further improve the existing tissue culture protocols for commercial scale production of the plant. The in vitro mass multiplication of plantlets in a liquid culture system is able to minimize production costs and is an imperative step to automate the shoot multiplication technique (Sandal et al. 2001; Pati et al. 2011). The added advantage of the liquid culture method is the uniform media distribution, renewal of culture media without changing the culture vessel and simplicity in cleaning containers (Pati et al. 2011). Although in vitro morphogenesis of *G. kurroo* was reported previously from different explants (Sharma et al. 1993, 2014; Fiuk and Rybczyński 2007, 2008a) there are no reports on the production of large number of multiple shoots in a liquid medium. Moreover, there are no reports demonstrating the production of GPD in in vitro shoot cultures of *G. kurroo*.

The aim of this study was to multiply shoots from *G. kurroo* in vitro and measure the content of GPD in the resulting shoots. A previous study assessed GPD content only in the roots and rhizomes of the wild *G. kurroo* (Wani et al. 2013). The objectives of this study were (i)

to optimize production of adventitious shoots in *G. kurroo*, and (ii) to measure the concentration of GPD in these shoots.

## Materials and Methods

### Plant Material and Apical Meristem Culture

Two months old plants of *G. kurroo* were obtained from Dr. Y. S Parmar, University of Horticulture and Forestry, Solan, Himachal Pradesh, India. The apical meristem was separated with a sterile blade and disinfected with 1% sodium hypochlorite and then 0.1% mercury chloride under aseptic conditions. The surface sterilized explants were rinsed 4–5 times in sterile distilled water and blotted on sterile filter paper to remove excess water. The explants were seeded on MS basal medium (Murashige and Skoog 1962) enriched with 0.5 and 1 mg/L 2, 4-D and Kn 1 mg/L (Kotvi et al. 2016) and cultures were maintained under 16 h photoperiod at  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 24 °C. All the plant growth regulators (PGRs) were purchased from Sigma-Aldrich (Mumbai, India), while basal salts were purchased from Himedia (Mumbai, India).

### Embryogenic Culture and Shoot Establishment

The actively growing embryogenic callus cultures derived from the apical meristem were transferred to the liquid MS medium fortified with 2 mg/L IBA and 1 mg/L IAA and placed on a Gyrotory shaker at 100 rpm at 24 °C. After two months, embryogenic liquid cultures with different stages of differentiated somatic embryos were carefully transferred to fresh semisolid MS and B<sub>5</sub> media (Gamborg et al. 1968) for further regeneration and development of shoot cultures.

Regenerated shoots from the somatic embryos were cut into two nodal length pieces along with the auxiliary shoot buds and transferred on MS medium supplemented with various concentrations of BA (0.5 to 2.0 mg/L) along with IAA (0.2 to 1.0 mg/L), NAA (0.5 mg/L) and Kn (0.5 to 2.0 mg/L) for adventitious shoot induction. All the cultures were maintained under a 16 h photoperiod of  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 24 °C. A minimum of 12 replicates were used in all the combinations with 3 explants in each replication. A PGR free medium was used as a control. The PGR combination that produced the highest number of auxiliary shoots on semisolid (1.7 g/L phytigel) media was selected for further studies in liquid culture medium.

## Optimization of Liquid Volume for Shoots Culture

Different volumes of MS medium fortified with BA 2 mg/L and IAA 0.5 mg/L were used to optimize the growth of axillary shoots. Approximately 5 to 6 cm long shoots were inoculated into 250 ml wide mouth Erlenmeyer flasks containing different volumes of medium in the range of 10 mL to 40 mL. (Table 3) The flasks were kept on a Gyrotory shaker at 90 rpm under  $40 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 23 °C for 35 days. The media volume was chosen based on the number of new adventitious shoot formation with no hyperhydricity effect. The volume of the media producing the highest shoot production was selected for GPD production and quantification.

## Sample Preparation and Extraction of Gentiopicroside

Exactly 100 mg well dried fine powder of *G. kurroo* shoots from different sources (naturally growing, green house saplings and in vitro) was added separately to 5 mL volumetric flasks. Approximately, 3–4 mL of HPLC grade methanol (Merck) was added and the tubes were sonicated for 15 min using Cole-Parmer (Mumbai, India) ultrasonic bath and incubated in a boiling water bath at 75°C for 15 min. The samples were further sonicated for 10 min. The final volumes were adjusted to 5 mL. Exactly, 1 mL of the sample was centrifuged in 2 mL Eppendorf vial at 15,000 rpm for 15 min; the supernatant was transferred to a new vial and centrifuged again at the same speed. The resulting supernatant was directly used for HPLC analysis to quantify the GPD.

## Quantification of Gentiopicroside

HPLC was used to separate GPD on a JASCO system (PU-2080 Plus, Japan) equipped with an auto sample injector (AS-2055 plus Japan). An isocratic elution was carried out using methanol and 0.5% acetic acid in deionized water (40:60, v/v) in a Thermo scientific (Chennai, India) reverse phase C18 (particle size 5  $\mu\text{m}$ , 4.6 mm  $\times$  150 mm) column. The flow rate was set to 1 mL/min. The UV absorption maximum for GPD was determined using a JASCO Photodiode-Array Detector with authentic GPD (Sigma-Aldrich, Mumbai, India). The UV absorption maximum was 272 nm for GPD and this spectrum was further set for analytical HPLC system.

## Statistical Analysis

The data were subjected to one-way ANOVA using SPSS software version 16.0. Mean values were compared by Duncan's Multiple Range Test and reported as mean  $\pm$  standard error (SE). A probability of  $P \leq 0.05$  was considered to be significant.

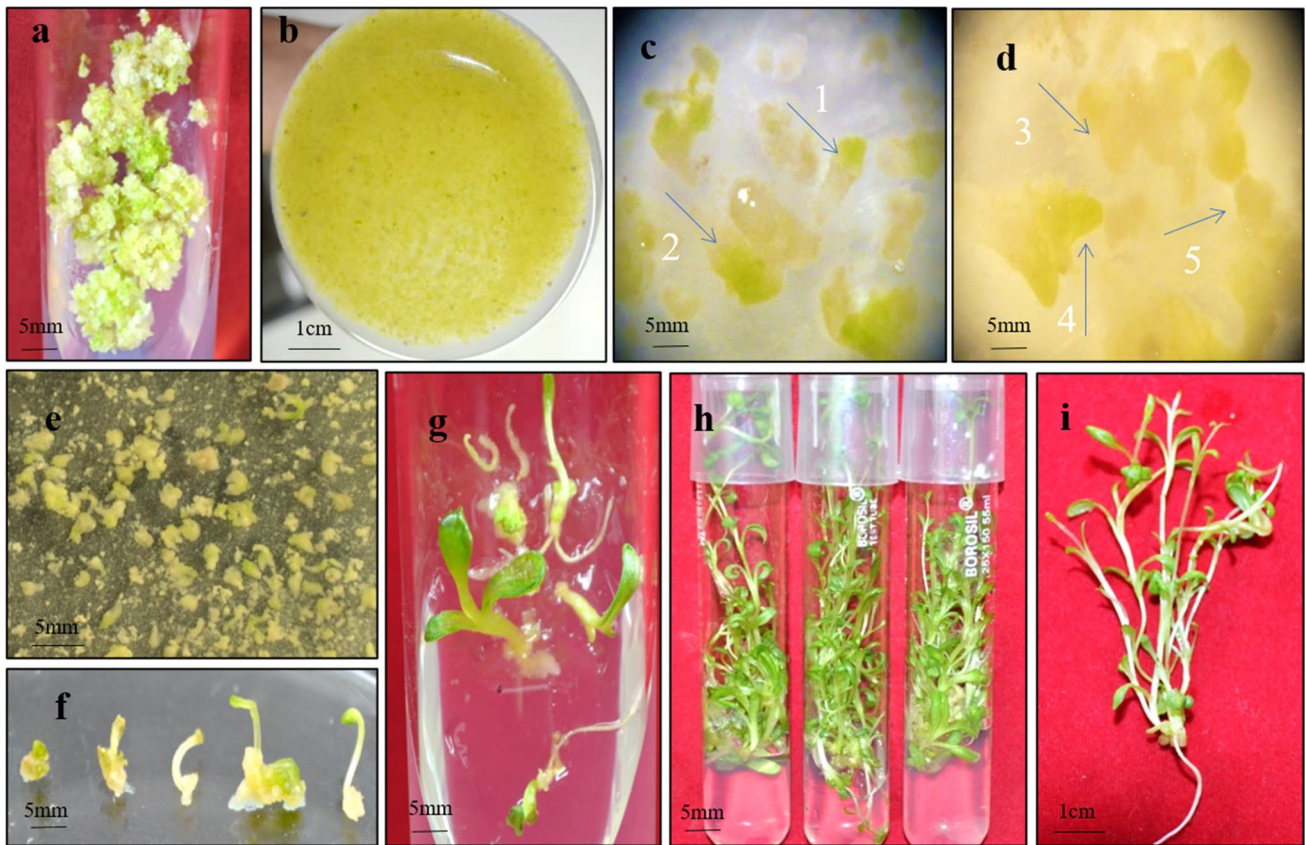
## Results

### Somatic Embryo Formation in Liquid Medium and Shoot Multiplication

No somatic embryos were observed in the embryogenic callus cultures that were maintained on MS solid medium fortified with cytokines (Kn 0.5 mg/L) and auxins (2,4-D 1 mg/L) even after four subcultures (Fig. 1a). Therefore, the embryogenic callus cultures were transferred to MS liquid medium fortified with 2 mg/L IBA and 1 mg/L IAA. This media combination produced the differentiated embryos after 30 days of culture (Fig. 1b). The torpedo, globular, heart and cotyledon stages of somatic embryos (Fig. 1c–f) were then transferred carefully onto semisolid B<sub>5</sub> and MS medium fortified with and without phytohormones for shoot establishment. The embryos that were cultured on PGR free media did not develop any shoots. A greater number of somatic embryos regenerated into shoots on the semisolid B<sub>5</sub> medium containing 1 mg/L BAP + 0.5 mg/L Kn with an average of 4.58 plantlets per culture tube after 5 weeks compared to other PGR combinations tested (Fig. 1g; Table 1). The shoots that were formed from the somatic embryos was further used for optimization of adventitious shoot formation on solid medium. Among the various PGRs tested for multiple shoot induction, MS medium supplemented with 2 mg/L BA and 0.5 mg/L IAA produced an average of 5.58 auxiliary shoots per explant after 30 days of culture (Fig. 1h, i; Table 2). This PGR combination was further used for optimization liquid shoot culture system.

### Shoot Culture Establishment in Liquid Medium

The in vitro shoots obtained from somatic embryo regeneration was inoculated into different concentrations of liquid MS medium (10, 15, 20, 25, 30, 35 and 40 mL) supplemented with BA 2 mg/L and IAA 0.5 mg/L in 250 mL conical flask for axillary shoot proliferation. Among the different volumes of liquid media used, 15 mL of medium produced the highest number ( $16.30 \pm 0.49$ ) of adventitious shoots per explant with highest shoots length ( $6.35 \pm 0.34$  cm) (Fig. 2). Though, the flasks with 20 mL of medium also produced nearly a similar ( $15.66 \pm 0.61$ ) number of shoots, the shoots failed to grow more than  $3.61 \pm 0.25$  cm in length. Both (15 mL and 20 mL) medium showed 100% culture survival at the end of 35 days after inoculation (Table 3), while shoots in 20 mL of medium were very fragile and pale green in color compared to the shoots grown in 15 mL of medium. The number of multiple shoot formation per



**Fig. 1** In vitro shoot regeneration and multiple shoot induction of *G. kurroo*: **a** Embryogenic callus (MS+0.5 mg/L Kn and 1 mg/L 2, 4-D); **b** Somatic embryo establishment in suspension culture on MS+2 mg/L IBA and 1 mg/L IAA); **c–f** Different stages of Somatic embryos found in suspension culture (1 & 2 -torpedo, 3-heart, 4-

cotyledon, 5- globular stages. **g** Regeneration of somatic embryos in to shoots on B5+1 mg/L BAP+0.5 mg/L Kn); **h** Multiple shoots induction in solid media (MS+BA 2 mg/L and IAA 0.5 mg/L); **i** A single shoot showing multiple shoot formation

explant in liquid medium was 2.96-fold higher than that of the solid media (Fig. 3).

### Gentiopicroside Production by Shoot Cultures

The relative content of GPD in the *G. kurroo* shoots grown in the wild, potted plants grown in green house and in vitro shoots grown on solid and liquid medium was quantified using HPLC. An equal amount (100 mg) of dry powder of the wild, potted plants and in vitro shoots was used for extraction and quantification of GPD. The representative HPLC chromatogram of *G. kurroo* shoots from different sources and standard GPD was presented in overlay mode for comparison in Fig. 4. The GPD was identified in the crude sample based on the authentic GPD retention time. The quantitative analysis showed that in vitro shoots grown in liquid medium contained 9.13 mg/g DW of GPD which relatively higher than field-grown (1.2 mg/g DW) and greenhouse-grown shoots (2.03 mg/g DW). In addition, difference in GPD content was observed in the shoots cultured on solid

and liquid media (Fig. 5). Thus, the liquid shoot culture of *G. kurroo* produced relatively higher amount of GPD than the other samples.

### Discussion

In the present study, we report for the first time a protocol for high somatic embryo regeneration and in vitro mass multiplication of *G. kurroo* using a liquid agitated culture system without any hyperhydricity effect.

A successful in vitro propagation system of critically endangered medicinal plants is one way of protecting the plants from being over exploited from their natural habitats. In addition, it enables the large-scale production of biomass from which secondary metabolites to meet industrial needs can be obtained (Ramachandra Rao and Ravishankar 2002; Satdive et al. 2003). In this study, apical meristems derived calli were used to develop well differentiated embryos in MS liquid medium supplemented with IBA 2 mg/L and 0.5 mg/L

**Table 1** Effect of different plant growth regulators on somatic embryo regeneration

Basal salt	Plant growth regulators (mg/L)			Culture observation parameters	
	BAP	IAA	KN	Average no. of somatic embryo regenerated	Frequency of regeneration (%)
<b>MS medium</b>					
	1.0	0.2	–	1.4 ± 0.5 <sup>a</sup>	41.7
	1.0	0.5	–	1.2 ± 0.4 <sup>a</sup>	75.0
	1.0	1.0	–	1.8 ± 0.84 <sup>abc</sup>	41.7
	1.0	1.5	–	2.0 ± 0.82 <sup>abcd</sup>	58.3
	2.0	0.2	–	1.9 ± 0.78 <sup>abcd</sup>	75.0
	2.0	0.5	–	1.2 ± 0.45 <sup>a</sup>	41.7
	2.0	1.0	–	1.3 ± 0.46 <sup>a</sup>	66.7
	2.0	1.5	–	1.3 ± 0.58 <sup>a</sup>	33.3
	1.0	–	0.2	2.0 ± 1.1 <sup>abcd</sup>	91.7
	1.0	–	0.5	2.1 ± 1.04 <sup>abcde</sup>	91.7
	1.0	–	1.0	2.1 ± 1.38 <sup>abcde</sup>	100.0
	–	–	–	1.2 ± 0.4 <sup>a</sup>	50.0
<b>B<sub>5</sub> Medium</b>					
	1.0	0.2	–	1.4 ± 0.70 <sup>a</sup>	83.3
	1.0	0.5	–	3.4 ± 1.16 <sup>f</sup>	100.0
	1.0	1.0	–	1.7 ± 0.79 <sup>ab</sup>	83.3
	1.0	1.5	–	2.8 ± 0.97 <sup>bcd</sup>	100.0
	2.0	0.2	–	3.3 ± 0.87 <sup>f</sup>	100.0
	2.0	0.5	–	2.1 ± 1.04 <sup>abcde</sup>	100.0
	2.0	1.0	–	2.9 ± 1.16 <sup>def</sup>	100.0
	2.0	1.5	–	3.1 ± 0.74 <sup>ef</sup>	91.7
	1.0	–	0.2	3.1 ± 1.27 <sup>cdef</sup>	100.0
	1.0	–	0.5	4.6 ± 0.98 <sup>g</sup>	100.0
	1.0	–	1.0	3.6 ± 0.67 <sup>f</sup>	91.7
	–	–	–	1.2 ± 0.45 <sup>a</sup>	41.7

The values are presented as mean ± SE; n = 12

Means within each column followed by the same letter are not significantly different ( $P < 0.05$ ; Duncan's multiple range test)

IAA. Previously somatic embryos were produced from cotyledons (Fiuk and Rybczyński 2007) and hypocotyl derived protoplast cultures (Fiuk and Rybczyński 2008b) of *G. kurroo*. The somatic embryogenesis was also achieved in other *Gentiana* species such as *G. cruciata*, *G. pannonica*, and *G. tibetica* on solid MS medium fortified with 2,4-D (Fiuk and Rybczyński 2008c). In this study, well differentiated somatic embryo formation was observed in liquid medium (Fig. 1c–f). The advantage of a liquid medium for tissue culture is that there is a continuous nutrient source for the somatic embryos and the method is cost effective since no solid medium is required (Egertsdotter et al. 2019).

The well-developed somatic embryos (Fig. 1f) were used for shoot regeneration on different semisolid medium. The highest somatic embryo regeneration was achieved on B<sub>5</sub>

basal salt medium fortified with 1 mg/L BAP + 0.5 mg/L Kn. However, the regenerated shoots did not produce any axillary shoots even after two sub cultures in this medium. Therefore, this study assessed the effect of various combinations of auxins and cytokinins for higher shoot multiplication on a semisolid medium. A previous study reported that MS medium supplemented with 8.9 μM BA and 1.1 μM NAA produced optimal axillary shoot induction with 3.5 ± 0.7 shoots per nodal explants of *G. kurroo* (Sharma et al. 1993, 2014). In our study, 5.58 auxiliary shoots were produced per explant on MS medium supplemented with 2 mg/L BA and 0.5 mg/L IAA with an average shoot length of 7.25 cm. All the media combinations enriched with BA showed axillary shoot formation. Interestingly, medium enriched with BA with any one of the auxins (IAA, NAA) produced a relatively higher number of shoots in comparison with medium containing only cytokinins (Table 2). This pattern of synergistic effect of cytokinin with auxin for auxiliary shoot formation was also observed in *Mucuna pruriens* (Faisal et al. 2006), *Balanites aegyptiaca* (Siddique and Anis 2009), *Salix tetrasperma* Roxb (Khan et al. 2011), *Jatropha curcas* L. (Sahoo et al. 2012), and *Vitex trifolia* (Ahmad et al. 2013). On the contrary, in *Psoralea corylifolia* L root segments produced multiple shoots in a medium fortified with only cytokinin (Jani et al. 2015).

To ascertain the effect of type of media on shoot proliferation in *G. kurroo*, plantlets derived from somatic embryos were cultured in liquid and solid (2 g/L phytigel) MS media for a period of 40 days. It was shown that 15 mL of MS medium supplemented with 2 mg/L BA and 0.5 mg/L IAA in 250 mL conical flasks produced 16.8 axillary shoots per explant. This is 3.1-fold higher than that produced in the solid media (which reduced 38-fold of the total cost needed for per shoot). Similar results of higher axillary shoot proliferation in liquid media were observed in many other plants such as *Camellia sinensis* (Sandal et al. 2001), *Ananas comosus* (Firoozabady and Gutterson 2003), *Drosera aglica*, *Drosera binata* (Kawiak et al. 2003), *Centaurium erythraea* (Piatczak et al. 2005) and *Knautia sarajevensis* (Karalija et al. 2017). This may be due to the increased surface exposure of shoots to liquid medium (Piatczak et al. 2005). The increased number of axillary shoot formation in a liquid medium may be due to the elimination of apical dominance during the continuous orbital movement of the culture vessel (Takayama and Akita 2005).

Elimination of a solidifying agent in plant tissue culture has added advantages as it reduces the culture costs substantially. For example, complete elimination of the solidifying agent in shoot multiplication in *Catharanthus roseus* reduced the cost 5.2-fold (Pati et al. 2011). Similarly, a cost reduction of 5.83% was achieved in the shoot multiplication of *Camellia sinensis* in a liquid culture system (Sandal et al. 2001). Another significant finding of this

**Table 2** Induction of multiple shoots using different auxins and cytokinins on solid MS medium

Growth regulators (mg/L)				Observation		
BAP	IAA	NAA	KN	Mean No. of auxiliary shoots formed	Avg. Shoot length (cm)	Frequency of shoot induction (%)
–	–	–	–	1.0 ± 0.00 <sup>a</sup>	2.80 ± 0.30 <sup>a</sup>	16.7
0.5	–	–	–	1.20 ± 0.2 <sup>ab</sup>	2.76 ± 0.23 <sup>a</sup>	41.7
1.0	–	–	–	1.22 ± 0.15 <sup>ab</sup>	3.77 ± 0.20 <sup>bcd</sup>	75.0
1.5	–	–	–	1.56 ± 0.24 <sup>abc</sup>	2.96 ± 0.24 <sup>ab</sup>	75.0
2.0	–	–	–	1.60 ± 0.22 <sup>abcd</sup>	3.92 ± 0.14 <sup>bc</sup>	83.3
0.5	0.2	–	–	2.00 ± 0.38 <sup>bcdef</sup>	2.70 ± 0.17 <sup>a</sup>	66.7
1.0	0.2	–	–	2.55 ± 0.31 <sup>cdefgh</sup>	3.35 ± 0.23 <sup>abc</sup>	91.7
1.5	0.2	–	–	2.20 ± 0.36 <sup>bcdefg</sup>	3.38 ± 0.22 <sup>abc</sup>	83.3
2.0	0.2	–	–	3.10 ± 0.31 <sup>ghij</sup>	4.53 ± 0.25 <sup>de</sup>	75.0
0.5	0.5	–	–	2.20 ± 0.36 <sup>bcdefg</sup>	5.24 ± 0.31 <sup>efgh</sup>	83.3
1.0	0.5	–	–	3.13 ± 0.31 <sup>ghij</sup>	5.30 ± 0.35 <sup>efgh</sup>	83.3
1.5	0.5	–	–	3.91 ± 0.24 <sup>ij</sup>	8.23 ± 0.53 <sup>m</sup>	91.7
2.0	0.5	–	–	5.58 ± 0.23 <sup>j</sup>	8.12 ± 0.33 <sup>m</sup>	100.0
0.5	1.0	–	–	1.75 ± 0.32 <sup>abcd</sup>	7.48 ± 0.51 <sup>lm</sup>	75.0
1.0	1.0	–	–	2.20 ± 0.34 <sup>bcdefg</sup>	6.99 ± 0.28 <sup>ijkl</sup>	83.3
1.5	1.0	–	–	2.50 ± 0.26 <sup>cdefgh</sup>	7.33 ± 0.59 <sup>lm</sup>	83.3
2.0	1.0	–	–	3.80 ± 0.30 <sup>hij</sup>	7.18 ± 0.26 <sup>kl</sup>	91.7
0.5	–	–	0.5	4.08 ± 0.23 <sup>ij</sup>	5.68 ± 0.16 <sup>fghi</sup>	100.0
1.0	–	–	0.5	3.45 ± 0.28 <sup>ghij</sup>	5.98 ± 0.18 <sup>ghi</sup>	91.7
1.5	–	–	0.5	3.00 ± 0.31 <sup>fghi</sup>	6.29 ± 0.25 <sup>hijk</sup>	58.3
2.0	–	–	0.5	1.56 ± 0.16 <sup>abc</sup>	6.14 ± 0.17 <sup>hij</sup>	75.0
–	0.5	–	0.5	1.60 ± 0.22 <sup>abcd</sup>	5.06 ± 0.20 <sup>efg</sup>	83.3
–	0.5	–	1.0	1.90 ± 0.26 <sup>abcde</sup>	5.57 ± 0.25 <sup>fghi</sup>	83.3
–	0.5	–	1.5	2.30 ± 0.37 <sup>cdefg</sup>	5.23 ± 0.18 <sup>efgh</sup>	83.3
–	0.5	–	2.0	2.45 ± 0.28 <sup>cdefgh</sup>	5.67 ± 0.29 <sup>fghi</sup>	91.7
0.5	–	0.5	–	2.13 ± 0.13 <sup>bcdefg</sup>	4.95 ± 0.23 <sup>ef</sup>	66.7
1.0	–	0.5	–	1.91 ± 0.25 <sup>abcd</sup>	5.30 ± 0.16 <sup>efghi</sup>	91.7
1.5	–	0.5	–	2.64 ± 0.28 <sup>defgh</sup>	5.74 ± 0.14 <sup>fghi</sup>	91.7
2.0	–	0.5	–	2.92 ± 0.39 <sup>efghi</sup>	6.15 ± 0.15 <sup>hij</sup>	100.0

The values are presented as mean ± SE; n = 12

Means within each column followed by the same letter are not significantly different ( $P < 0.05$ ; Duncan's multiple range test)

work is that the media volume to culture vessel volume plays a major role in shoot survival, establishment and proliferation of *G. kurroo*. A 25 mL of medium produced the lowest number of new axillary shoot formation with a 10% of survival rate, whereas any volume above 25 mL produced no shoots. The decreased number of shoots in 25 mL and loss of shoot survival above 25 mL of medium is probably due to hyperhydricity, which is caused by the complete submergence of shoots in a medium and the availability of oxygen (Sandal et al. 2001; Pati et al. 2011) Although, good axillary shoot proliferation was observed in the 10 and 15 mL media, sub culturing was required after every 20 days since the 10 mL media volume was rapidly depleted. For the 15 mL medium, it was possible

to subculture up to 40 days without hyperhydricity and without any hindrance to shoot multiplication rates. Thus, the liquid agitated culture system established in our work would be an efficient method for mass propagation of *G. kurroo* shoots.

The naturally growing shoots of *G. kurroo* produced 1.28 mg/g DW of GPD, whereas the in vitro shoots grown in liquid medium produced 9.13 mg/g DW (Fig. 5). This was 4.5-fold higher than greenhouse-grown shoots (2.03 mg/g DW) and 7.1-fold higher than the natural *G. kurroo* shoots. Similarly, *Centaurium erythraea* shoots grown under in vitro condition produced (14.5 mg/g DW) 2.9-fold higher concentration of GPD compared to the wild shoots (Piatczak et al. 2005) and the similar trend



**Fig. 2** Multiple shoot culture in liquid system: **a** shoot growing in 20 mL medium shows hyperhydricity; **b** shoots growing in 15 mL medium; and **c** single shoot showing multiple shoots

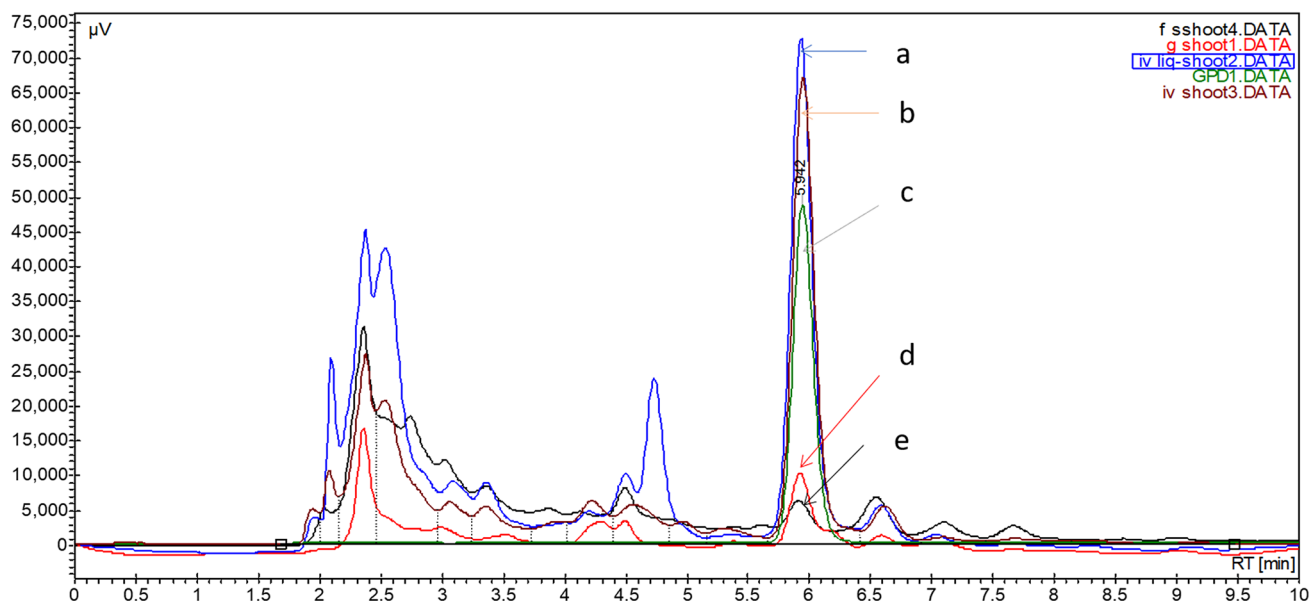
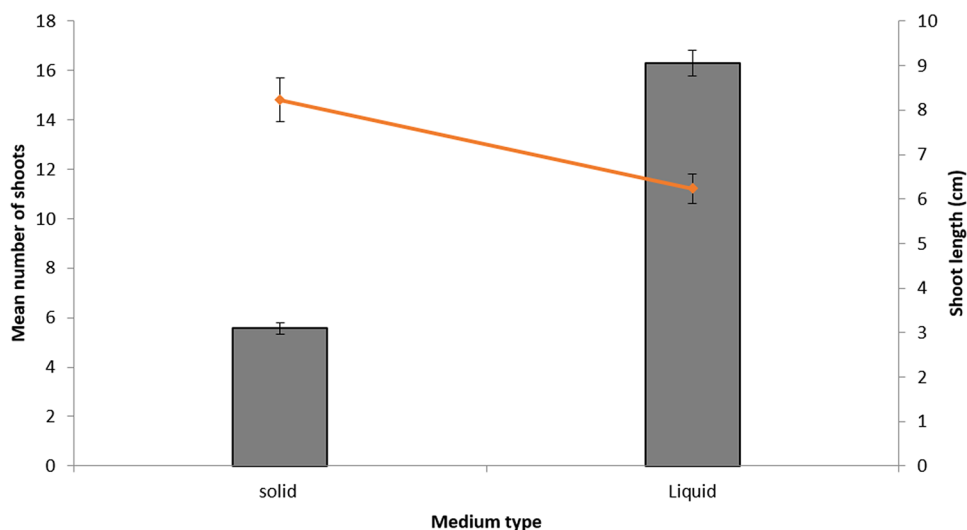
**Table 3** Influence of liquid media volume on multiple shoot formation in shake flasks

Medium volume in 250 mL flask (mL)	Initial days taken for shoot formation	No of Auxiliary shoot formed	Frequency of shoot formation (%)	Avg. shoot length (cm)	Observation
10	7.10 ± 0.23 <sup>a</sup>	6.50 ± 0.58 <sup>a</sup>	100%	5.26 ± 0.33 <sup>c</sup>	Good multiplication, but media depleted within 20 days
15	7.90 ± 0.26 <sup>a</sup>	16.30 ± 0.49 <sup>b</sup>	100%	6.35 ± 0.34 <sup>c</sup>	Good multiplication
20	10.66 ± 0.42 <sup>b</sup>	15.66 ± 0.61 <sup>b</sup>	60%	3.61 ± 0.25 <sup>b</sup>	Shoots are pale green in color and small
25	12.33 ± 1.2 <sup>c</sup>	5.33 ± 0.67 <sup>a</sup>	30%	2.50 ± 0.50 <sup>a</sup>	Poor shoot formation and are pale green in color
30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	No shoot formation, fully died
35	0 ± 0	0 ± 0	0 ± 0	0 ± 0	No shoot formation, fully died
40	0 ± 0	0 ± 0	0 ± 0	0 ± 0	No shoot formation, fully died

The values are presented as mean ± SE;  $n = 12$

Means within each column followed by the same letter are not significantly different ( $P < 0.05$ ; Duncan's multiple range test)

**Fig. 3** Effect of solid media and liquid media for multiple shoot induction

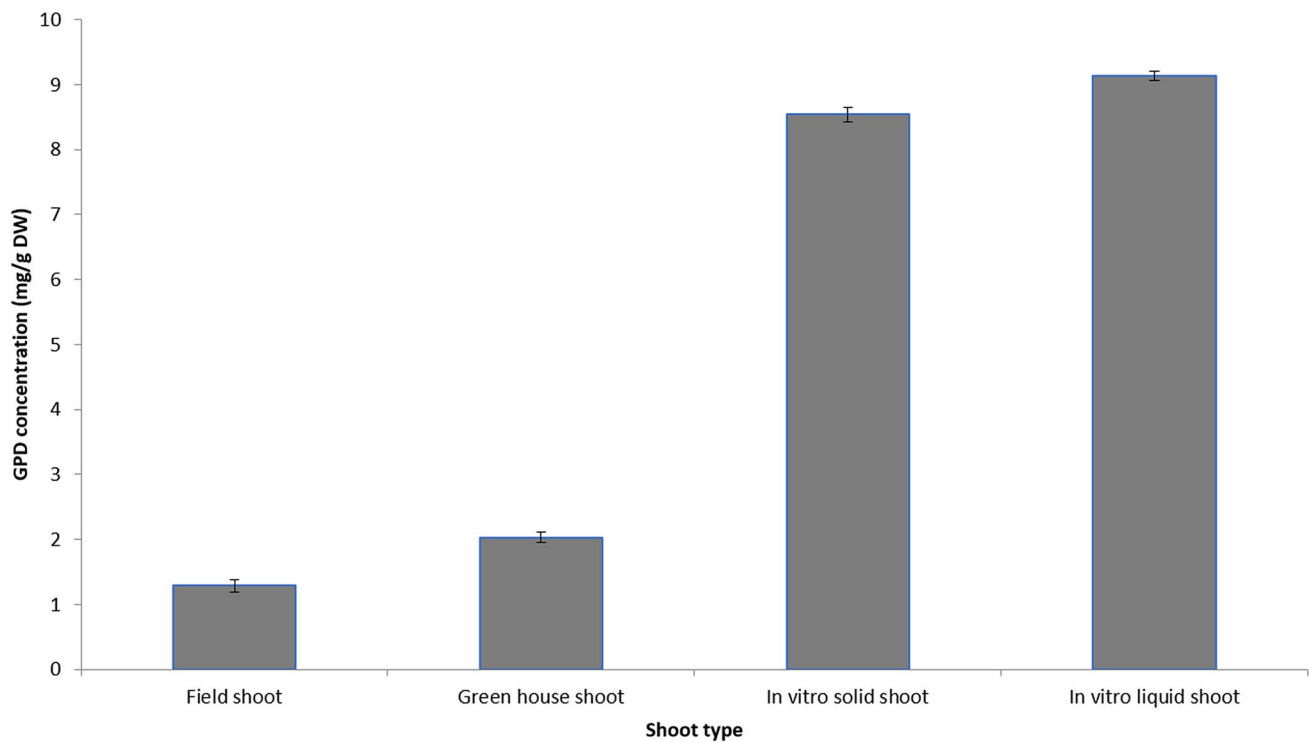


**Fig. 4** HPLC chromatogram of different shoot extracts of *G. kurroo*: **a** in vitro liquid-medium-grown shoots; **b** in vitro solid-medium-grown shoots; **c** standard GPD; **d** greenhouse-grown shoot; and **e** field shoot

was also observed in *Gentiana lutea* where in vitro shoot contained (11.46%) highest GPD (Cvetković et al. 2020). The increased production of GPD under in vitro conditions may be due to the effect of added BAP to the culture medium (Cai et al. 2009). There was no great difference in GPD content whether the shoots were cultured in a liquid or solid medium (Fig. 5). A similar effect was observed in *C. erythraea* where both solid (14.50 mg/g DW) and liquid shoots (14.48 mg/g DW) produced a similar amount of GPD (Piatczak et al. 2005).

This study showed that the 15 mL liquid culture volume improved the shoot multiplication rate and increased the concentration of secoiridoid glucoside GPD and the in vitro shoots contained higher amount of GPD than naturally growing plants or plants grown under greenhouse conditions. Thus, *G. kurroo* shoots can be propagated on a large-scale using a liquid agitated culture system to harvest the economically important GPD for the pharmaceutical industry.





**Fig. 5** GPD concentration in *G. kurroo* shoots grown in different conditions: field shoot—collected at flowering stage; greenhouse shoot—30 days old; in vitro solid shoot—shoot grown in solid media

harvested after 40 days; in vitro liquid shoot—shoot grown in liquid media harvested after 40 days

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**Author Contributions** MA executed the work and prepared the manuscript; RC and KT designed the research; SR and DPF provided technical support and revised the manuscript; RC, KT, and RR collected the plant and established the culture.

#### Declaration

**Conflict of interest** All authors declare that they have no conflict of interest.

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