In vitro morphogenic response of different explants of *Gentiana kurroo* Royle from Western Himalayas—an endangered medicinal plant

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Received: 6 November 2013 / Revised: 16 December 2013 / Accepted: 26 December 2013 / Published online: 12 January 2014
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Abstract Micropropagation offers a great potential to produce millions of clonal individuals through tissue culture via induction of morphogenesis. The aim of this work was to obtain an efficient protocol for callus regeneration for *Gentiana kurroo* Royle. The morphogenic response of different explants (leaves, petioles, roots) varied and responded differently for regeneration according to combinations of growth regulators. The petiole explants were best responding for callus induction and subsequently for indirect and direct regeneration. The callus induction was achieved on MS basal + 1.0 mg/l benzyladenine (BA) and 3.00 mg/l naphthalene acetic acid (NAA). MS medium supplemented with 0.10 mg/l NAA and 1.0 mg/l thidiazuron (TDZ) was recorded as the best medium for indirect regeneration. However, for direct regeneration the maximum number of shoot emergence was observed on MS basal fortified with 0.10 mg/l NAA + 0.75 mg/l TDZ. Half strength MS basal supplemented with indole-3-butyric acid (IBA) 1.00 mg/l gave best response for root induction. Subsequently, the plantlets were transferred and 100 % survival rate was recorded only on autoclaved cocopeat. No morphological variations were recorded in the callus regenerated plantlets.

Keywords Medicinal plant · *Gentiana kurroo* · Morphogenic response · In vitro rooting · Acclimatization

Introduction

Gentiana kurroo Royle (Gentianaceae) is one of the many gentian species legally protected by law in India (Raina et al.

2003). It grows on grass slopes in North Western Himalayan region at an altitude of 1,800–2,700 m. Roots and rhizomes of this species are source of secondary products: gentiopicroside, gentiarnin and gentianin (Sharma et al. 1993). It has been used as stomachic, antiperiodic, expectorant, antibilious, astringent, blood purifier and carminative (Chopra et al. 1956). Roots of this herb is especially useful in treating exhaustion from chronic diseases such as gout, dyspepsia, debility, torpid liver, spleen enlargement etc. and in all cases of weakness of the digestive system and lack of appetite (Grieve 1984; Bown 1995). The pharmaceutical industries are largely dependent on *Gentiana kurroo* for its number of medicinal properties but unfortunately, they are using natural population of this herb, as a result there is large scale depletion of the wild stands. Hence, it has been put in the negative list of exports vide Notification no. 2 (RE-98) 1997–2002 dated 13 April 1998 by Ministry of Commerce, Government of India (Raina et al. 2003). There is huge demand for this plant species which cannot be fulfilled by present practices of propagation, thus, there is need for applying biotechnological methods for enhancing its propagation and making it available on sustainable basis (Kaur et al. 2007; Tomar et al. 2011).

Plant tissue culture of this medicinal plant offers not only a means for the mass multiplication of existing stocks of germplasm but also for conservation of this endangered species. Though this plant species propagates through seeds, however, there are some associated bottlenecks. Firstly, the seeds take 6–9 months to germinate after putting in soil and germination percentage is not very encouraging. Secondly, seeds are formed after 3–4 years of pollination (Heath 1964; Abrams 1975). Thus, in vitro technique of micropropagation seems to be an apt answer for increasing the efficiency and scale up of plant production.

Regeneration of plant by organogenesis is an important method of obtaining plants by large scale vegetative propagation, induction of somatic embryogenesis and in vitro cell

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selection. There are some reports on tissue culture of *Gentiana kurroo* (Sharma et al. 1993, 1995 and Kaur et al. 2007) however our work is detailed study on morphogenesis of this plant species using different explants for direct and indirect organogenesis. Keeping in view the endangered status of *Gentiana kurroo*, the present work was undertaken with the objective to assess comparative performance of three different explants for callus formation and organogenesis.

Materials and methods

Source plant material

In vitro stocks being maintained for the last 15 years in the Department of Biotechnology, University of Horticulture and

Forestry Nauni, Solan were used as the source plant material. Methodology followed is given as follows:

1) INDIRECT REGENERATION

- a) Callus induction: Explants namely leaf, petiole and root from in vitro multiplying shoots were used for callus induction and for subsequent regeneration. Explants were placed horizontally on MS basal medium supplemented with various combinations of growth regulators i.e. NAA (0.75 mg/l–3.0 mg/l) and BA (0.25 mg/l–1.0 mg/l) (Tomiczak and Rybczynski 2007) and maintained at 25 ± 2 °C. Explants were incubated in light as well as in dark conditions (incubated in dark for 1 week and then shifted to 16 h light and 8 h dark photoperiod).

Fig. 1 Callus induction; **a** leaf; **b** petiole; **c** root kept for callus induction on first day of culturing; **d** callus induced on leaf explant; **e** on petiole explant; **f** on root explant on MS basal + 1.0 mg/l BA and 3.0 mg/l NAA after 4 weeks of culturing

Observations were recorded daily for growth pattern, type, colour of callus and percentage of explants forming callus and experiment was repeated thrice.

- b) Regeneration from callus: Regeneration was attempted on healthy green petiole derived calli. About 1 mm³ sized calli were cultured on MS basal medium supplemented with various combinations and concentrations of plant growth regulators such as NAA (0.10 mg/l), kinetin (0.50 mg/l), BA (0.50 mg/l–3.0 mg/l), TDZ (0.10 mg/l–1.0 mg/l). Observations were recorded for percentage regeneration of shoots and average number of shoots regenerated from each piece of callus.

2) DIRECT REGENERATION

Different explants such as leaf, petiole and root from in vitro growing cultures were tried for direct regeneration without callus formation. Explants were cultured on MS medium supplemented with various plant growth regulators like BA (0.25 mg/l–2.5 mg/l), NAA (0.10 mg/l) and TDZ (0.10 mg/l–1.0 mg/l). Observations were recorded for the average no. of explants showing regeneration and percentage of shoots regenerated directly from explants on different medium combinations.

3) MULTIPLICATION OF SHOOTS

Shoots were excised from parent culture and transferred on to multiplication medium (MS basal +

0.50 mg/l Kn and 0.50 mg/l BA) and after that shoots were transferred to MS medium fortified with three different growth regulators (0.50 mg/l NAA + 0.50 mg/l BA, 1.0 mg/l GA₃ + 0.5 mg/l TDZ and 1.5 mg/l BAP + 1.0 mg/l GA₃) for shoot elongation (Sharma et al. 1993).

4) ROOT INDUCTION FROM SHOOTS REGENERATED FROM DIRECT OR INDIRECT ORGANOGENESIS

Actively growing shoots 3–4 cm in length derived directly from petioles (direct) and multiple shoots derived from calli induced from petiole (indirect) were transferred to MS basal solid and liquid medium of different strengths supplemented with various growth regulators like NAA (0.50 mg/l), IBA (0.50 mg/l–1.0 mg/l) (Kaur et al. 2007). Observations were recorded for percentage of shoots regenerating roots, average number of roots per rootable shoot and average root length after 5 weeks of root initiation.

5) ACCLIMATIZATION OF REGENERATED PLANTLETS

Regenerated plantlets in which well developed roots were recorded, were removed from culture tubes, washed under running tap water to remove agar for 1–2 h and treated with bavistin and then they were washed with water and transferred to small plastic pots containing different autoclaved potting mixtures i.e. coco peat, sand: soil: FYM (1:1:1), soil: sand (1:1), vermiculite, coco

Table 1 Response of different explants of *Gentiana kurroo* Royle for callus induction, type, growth, colour of callus and percent of calli regenerating shoots after 4 weeks incubation on MS medium supplemented with different combinations and concentrations of plant growth regulators (PGRs)

Sr. no.	NAA (mg/l)	BA (mg/l)	Leaf	Root	Petiole	Type	Growth	Colour
1.	0.00	0.00	0.00 (1.00)	0.00 (0.00)	0.00 (0.00)	L R P	L R P	L R P
2.	0.75	0.25	0.00 (3.38)	0.00 (0.00)	13.33 (17.71)	– – –	– – –	– – –
3.	0.75	0.50	13.33 (4.58)	20.00 (26.57)	26.67 (30.79)	– – F	– – +	– – G
4.	0.75	0.75	20.00 (1.00)	20.00 (26.57)	40.00 (38.86)	F C F	+ + +	YG CW LG
5.	0.75	1.00	0.00 (3.38)	53.33 (47.30)	20.00 (26.57)	F C F	+ + +	YG CW LG
6.	1.50	0.25	13.33 (5.18)	26.67 (25.78)	33.33 (35.01)	– C F	– ++ +	– CW LG
7.	1.50	0.50	26.67 (3.38)	46.67 (42.70)	33.33 (35.01)	F C F	+ + +	YG CW LG
8.	1.50	0.75	13.33 (5.18)	46.67 (43.08)	60.00 (50.77)	F C F	+ + +	LG CW G
9.	1.50	1.00	26.67 (3.99)	33.33 (30.00)	60.0 (50.77)	F C F	+ + ++	YG BW G
10.	2.00	0.25	20.00 (3.99)	33.33 (35.01)	46.67 (42.70)	F C F	+ + ++	YG BW G
11.	2.00	0.50	20.00 (3.38)	53.33 (46.92)	60.00 (51.15)	F C F	+ + +	YG CW LG
12.	2.00	0.75	13.33 (2.80)	60.00 (50.77)	73.33 (59.21)	F C F	+ ++ ++	LG CW LG
13.	2.00	1.00	13.33 (2.80)	66.67 (55.37)	66.67 (60.00)	F C F	+ + +++	LG BW LG
14.	3.00	0.25	13.33 (5.18)	53.33 (47.30)	66.67 (55.37)	F C F	+ + ++	YG CW LG
15.	3.00	0.50	26.67 (5.18)	60.00 (50.77)	80.00 (63.44)	F C F	+ + ++	LG CW G
16.	3.00	0.75	26.67 (5.18)	40.00 (39.23)	73.33 (59.21)	F C F	+ + +++	LG BW LG
17.	3.00	1.00	26.67 (30.79)	73.33 (59.21)	86.67 (72.29)	F C F	+ ++ ++	G CW G
CD _{0.05}			3.14	20.0	20.5	F C F	+ ++ ++++	G BW LG

Values expressed in parentheses are arc sine transformation of percentage

C compact; F friable; G green; LG light green; CW creamish white; BW brownish white; YG yellowish green +Slow; ++Moderate; +++Vigorous; L leaf, P petiole; R root

Table 2 Indirect shoot regeneration of *Gentiana kurroo* Royle on petiole derived calli after 4 weeks of culturing of callus on MS medium supplemented with different growth regulators

Sr. no.	NAA (mg/l)	TDZ (mg/l)	Average number of shoots per callus piece	Percentage of callus regenerating shoots (%)
1	0.10	0.50	0.6	20 (21.93)
2	0.10	0.75	1.4	80 (63.44)
3	0.10	1.00	3.0	86.6 (72.29)
CD			4.08	29.0
0-				
-				
0-				
5				

Values expressed in parentheses are arc sine transformation of percentage

peat: perlite (1:1:1) and vermiculite: coco peat (1:1) and maintained under 23 °C temperature and 70 % relative humidity. To prevent evaporation and to facilitate light transportation, pots were covered by transparent polythene. After about 4 weeks, only acclimatized plantlets were transferred to glass house. Observations were recorded for percentage survival of plantlets.

Results

1) INDIRECT REGENERATION

- a) Callus induction: Leaf, root and petiole explants were cultured on MS medium Fig. 1a, b, c containing NAA, BA in different combinations. Callus initiation started after about 1 week of culturing of the explants. Leaf was the least responding explant for callus induction on all

the medium combinations followed by the response of roots Fig. 1d, f. However petiole was recorded to be the best responding explant and formed callus (86.67 %) on MS basal + 1.0 mg/l BAP and 3.0 mg/l NAA and this medium was proved as the best medium with respect to the rate of callus induction (Table 1, Fig. 1e), which was closely followed by MS basal + 0.5 mg/l BAP and 3.0 mg/l NAA on which 26.66 %, 60.00 % and 80.00 % callus induction was observed from leaf, root and petiole explants respectively. Statistically these two treatments were at par with each other.

Colour and texture of callus: Quality and texture of callus was significantly affected by the presence of different growth regulators in culture medium, type of explant and light conditions (dark and light). Callus was induced in all the tried concentrations of NAA and BA. However, variability in the formation of calli was noted. High amount of callusing with green meristemoids were observed on 3.0 mg/l NAA and 1.0 mg/l BA. The same calli pieces with meristemoids were cultured on the same fresh medium and 100 % shoot regeneration was recorded. Callus induced from petiole explant was friable and green in colour, when incubated under light conditions while it was light green in colour when incubated under dark conditions during initial stages (for 1 week) which turned green again when transferred to light. It was observed that callusing was more vigorous when incubated under dark conditions for 1 week. Creamish white and brownish white coloured compact hard calli were obtained from root explants, when they were incubated under light and dark conditions respectively, whereas yellowish green and light green calli with profuse rooting

Fig. 2 Indirect and direct regeneration of *Gentiana kurroo* Royle **a** regeneration of shoots from petiole derived calli on medium (MS basal + 0.10 mg/l NAA + 1.00 mg/l TDZ) after 4 weeks of culturing; **b**

magnified view of regenerating calli, arrow showing regenerated shoot; **c** regeneration of shoots from petiole on MS basal + 0.75 mg/l TDZ + 0.10 NAA after 4 weeks of culturing

Table 3 Effect of different plant growth regulators on direct regeneration of shoots of *Gentiana kurroo* Royle after 4 weeks of culturing

Sr. no.	TDZ (mg/l)	BA (mg/l)	NAA (mg/l)	Average number of shoots per explant petioles	Percentage of shoots from petioles
1	0.00	0.25	0.00	0.06	6.66 (8.85)
2	0.00	1.00	0.10	0.00	0.00 (0.00)
3	0.00	1.50	0.10	0.46	33.3 (30.00)
4	0.00	2.00	0.10	0.8	46.6 (43.08)
5	0.00	2.50	0.10	1.0	53.3 (46.92)
6	0.10	0.00	0.10	0.6	26.6 (30.79)
7	0.25	0.00	0.10	1.0	40.0 (38.86)
8	0.50	0.00	0.10	1.2	60.0 (50.77)
9	0.75	0.00	0.10	1.6	73.3 (53.21)
10	1.00	0.00	0.10	1.30	66.6 (54.99)
CD _{0.05}				4.81	19.71

Values expressed in parentheses are arc sine transformation of percentage

was obtained from leaves when subjected to light and dark incubation respectively, during initial stages of explant callusing (Table 1).

b) Shoot regeneration from callus

Callus obtained from petiole explant was best in quality and texture and same callus was tried for indirect regeneration experiment. Callus of about 0.5–1.0 cm³ size was cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators. MS medium supplemented with 0.10 mg/l NAA and 1.0 mg/l TDZ was recorded as best medium for indirect organogenesis on which 86.6 % calli regenerated shoots which was statistically at par with MS medium supplemented with 0.10 mg/l NAA and 0.75 mg/l TDZ on which 80 % of shoots were regenerated (Table 2, Fig. 2a, b).

2) DIRECT REGENERATION

Plant regeneration via direct shoot organogenesis from petiole, leaf and root explants was screened and no shoot regeneration was recorded from leaf and root explants. MS medium with 0.10 mg/l NAA+0.75 mg/l TDZ (Table 3) was recorded to be the best medium for direct shoot induction from petiole explant on which higher number of direct organogenesis was achieved with a frequency of 73.3 % (Table 3, Fig. 2c) followed by MS

Fig. 3 In vitro multiplication of shoots obtained by direct and indirect organogenesis; **a** subcultured shoots for multiplication on first day of culturing; **b** multiplied shoots after 1 month on medium (MS basal + 0.5 mg/l Kn + 0.5 mg/l BA); **c** in vitro rooting of elongated micro shoots on medium (MS half strength + 1.0 mg/l IBA) after 1 month of culturing; **d** Fully acclimatized plant of *Gentiana kurroo* after 1 month of hardening **e** micropropagated plant of *Gentiana kurroo* in field after 2 months of hardening

Table 4 Effect of different auxins on in vitro root induction in microshoots of *Gentiana kurroo* Royle after 4 weeks of culturing

Medium code (MS basal)	NAA mg/l	IBA mg/l	Average root length (cms)	Average number of roots per shoot	Rooting percentage
RI ₁ (full strength MS basal salts)	0.00	1.00	2.4	9.91	50.00 (45.00)
RI ₂ (half strength MS basal salts)	0.00	1.00	6.25	22.83	91.60 (82.50)
RI ₃ (half strength MS basal salts)	0.00	0.50	3.62	13.9	83.30 (75.00)
RI ₄ (half strength MS basal salts)	0.50	0.00	1.54	13.5	75.00 (67.50)
RI ₅ (half strength MS basal liquid medium)	0.00	0.50	0.5	3.6	50.00 (45.00)
RI ₆ (half strength MS basal liquid medium)	0.50	0.00	0.5	2.3	50.00 (45.00)
RI ₇ (basal medium)	0.00	0.00	1.41	7.00	58.30 (52.50)
CD _{0.05}			1.26	6.13	34.12

Values expressed in parentheses are arc sine transformation of percentage

medium with 0.10 mg/l NAA + 1.00 mg/l TDZ on which 66.6 % petioles formed shoots. In vitro regeneration using petiole explant in this herb was not attempted before in the earlier findings.

3) MULTIPLICATION OF SHOOTS

MS medium supplemented with 0.50 mg/l BA and 0.50 mg/l Kn was observed to be the best medium for shoot multiplication Fig. 3a, b. The emergence of multiple shoots occurred in 20–25 days. After multiplication, shoots were transferred to MS medium supplemented with 0.50 mg/l NAA and 0.50 mg/l BA on which maximum increase in length of shoots was observed as compared to other growth regulators like (1.0 mg/l GA₃ + 0.5 mg/l TDZ) and (1.5 mg/l BAP + 1.0 mg/l GA₃).

4) ROOT INDUCTION FROM THE SHOOTS

In vitro shoots from both callus and petiole were excised from their origin and transferred to MS solid and liquid medium of different strengths supplemented with various concentrations of auxins such as IBA and NAA. Root initiation was started about 15 days of culturing of shoots and it was observed that rooting response varied in terms of percentage roots per shoot, length and number of roots. The best medium for rooting was MS half strength containing 1.0 mg/l IBA on which 91.6 % of shoots developed roots with an average of 22.83 roots per shoot with 6.25 average root length followed by MS half strength containing 0.50 mg/l IBA on which 83.30 % of shoots developed roots with an average of 13.9 roots per shoot with 3.62 average root length (Table 4, Fig. 3c).

5) ACCLIMATIZATION OF REGENERATED PLANTLETS

The rooted plantlets were carefully taken out without injuring the roots, thoroughly washed with running tap water to remove the adhered agar by gentle shaking, and dipped in an antifungal solution (bavistin 0.1 %) for 5 min. Then, they were washed and planted in different potting mixtures. After 6 weeks of hardening healthy

plantlets were shifted to glasshouse. No morphological variations were recorded in callus regenerated plantlets and survival rate of hardened plants observed to be 100 % on cocopeat (Table 5; Fig. 3d, e) as compared to other potting mixtures on which 66.6 %, 60 %, 50 %, 41.6 and 40 % survival of plants was recorded on vermiculite, sand: soil: FYM, vermiculite: cocopeat: perlite, cocopeat: perlite and soil: FYM respectively. The plants were successfully transferred to field conditions and 80 % survival was recorded in field after 7 to 9 weeks. Thereafter the plants are luxuriantly growing in the field.

Discussion

The main aim of the present study was to focus on high frequency plant regeneration by direct and indirect organogenesis. There are few reports on tissue culture of *Gentiana kurroo* Royle (Sharma et al. 1993; Sharma 2001; Fiuk and Rybczynski 2008). However, there is no report on multiplication of plant by direct and indirect organogenesis. Therefore,

Table 5 Hardening percentage of regenerated plants of *Gentiana kurroo* Royle

Sr. no.	Different potting mixtures	Ratio of different compounds of potting mixtures	Survival percentage percentage of hardened plants
1.	Sand: soil: FYM	1:1:1	60.00
2.	Soil: FYM	1:1	40.00
3.	Coco peat	Full strength	100.00
4.	Vermiculite	Full strength	66.66
5.	Coco peat: perlite	1:1	41.66
6.	Vermiculite: cocopeat: perlite	1:1:1	50.00

propagation technique by direct and indirect organogenesis is particularly needed in *Gentiana kurroo*. The protocol for direct and indirect regeneration presented in the current study could be useful for large scale multiplication of this medicinally important endangered plant.

The present investigation was carried out to explore morphogenic potential of different explants (leaf, petiole and root) of *Gentiana kurroo* and it was recorded that all the explants responded significantly different from each other on similar combinations of growth regulators (Hosokawa et al. 1996 and Mikula and Rybczynski 2001). Petiole explants performed best of all for callus initiation and subsequently the petiole derived callus also proved best for indirect regeneration of shoots.

The callus induction was quickly and effectively achieved from petiole explant on MS medium supplemented with 1.00 mg/l BAP and 3.00 mg/l NAA combination. The present finding supports the earlier finding reported by (Kaur et al. 2009). The petiole explant showed good response in direct organogenesis in MS medium fortified with 0.10 mg/l NAA + 0.75 mg/l TDZ. Similar results were obtained in *Artemisia vulgaris* (Sujatha and Kumari 2007). In the present study; petiole derived calli showed maximum shoot regeneration on MS medium supplemented with 0.10 mg/l NAA and 1.00 mg/l TDZ. Several studies reported that improved regeneration obtained when TDZ was used for regeneration of plant species (Lane et al. 1998; Leblay et al. 1991). TDZ is a potent growth regulator in inducing direct and indirect organogenesis which was demonstrated by previous studies (Kadota and Numi 2003; Mundhara and Rashid 2006). It has been reported that BA being considered as one of the most useful cytokinin for multiplication and used for propagation of different plants like *Dregea volubilis* (Yogananth et al. 2011), *Gentiana dinarica* (Vinterhalter et al. 2012), *Centella asiatica* (Thangapandian et al. 2012), *Mentha piperita* (Mehta et al. 2012), *Ajuga bracteosa* (Kaul et al. 2013) and *Stevia rebaudiana* (Lata et al. 2013). For shoot elongation BA and NAA was more suitable than BA alone. Lui and Li (2001) noted that low BA concentration appeared to be key growth regulator for successful shoot elongation. For the induction of strong and stout root systems the elongated shoots were individually cultured on half strength MS medium with 1.0 mg/l IBA which gave the best response with 91.60 % of roots produced and on an average of 22.83 roots per shoot were recorded within 4 weeks. The effectiveness of IBA in rooting has been reported for many medicinal plants, viz; *Aegle marmelos* (Puhan and Rath 2012), *Alpinia zerumbet* (Rakkimuthu et al. 2011), *Clitoria ternatea* L. (Barik et al. 2007), *Murraya koenigii* (L.) Spreng (Bhuyan et al. 1997) and *Ocimum basilicum* L. (Sahoo et al. 1997). This is due to an imbalance between endogenous auxin and exogenously supplied auxin IBA (Manickam et al. 2000). However, a similar conclusion was also seen in the work of Usha and Swami (1998) and Anand et al. (1997). In vitro derived plantlets with

well-developed shoots and roots were successfully transferred to pots containing autoclaved potting mixtures and regenerated plantlets showed a survival percentage of 100 % in the cocopeat and 80 % survival in field without any abnormal morphological changes.

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