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Research Article

Somatic embryogenesis and plant regeneration from cell suspension cultures of Gentiana kurroo Royle.

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Abstract: *Gentiana kurroo* Royle, native to North- Western Himalayas, is one of the critically endangered perennial herbs, which is being overexploited due to its multiple medicinal uses. The goal of this work was to develop a protocol for somatic embryogenesis and efficient plant regeneration through cell suspension culture. Selected ranges of plant growth regulators were experimented for somatic embryogenesis. Light micrographs of the established suspension cell cultures of somatic embryos at different developmental stages were also observed under light microscope. Somatic embryogenesis was induced from the leaf explants and the maximum induction frequency (97.2%) was obtained on Murashige and Skoog (MS) medium supplemented with 0.5 mg/l each of NAA, BAP and TDZ. Cell suspension cultures were established in MS liquid medium containing IAA (0.5 mg/l) + BAP (1.0 mg/l) with 89-93% viable cells. Transfer of cotyledonary somatic embryos to the MS agar medium containing 2, 4-D (0.4 mg/l) and KN (1.5 mg/l) resulted in somatic embryogenesis at high frequencies (80%) with an average of 28 ± 0.2 embryos/ callus piece. After transfer onto the half strength MS medium without growth regulators approximately 80-90% somatic embryos developed into complete plantlets. The protocol described here could be used for somatic hybridization, genetic transformation, isolation of protoplasts and large-scale propagation of *G. kurroo*.

Keywords: Cell suspension, *Gentiana kurroo,* Murashige and Skoog (MS), Somatic embryogenesis

Introduction

Gentiana kurroo Royle (Family: Gentianaceae) is an important native Indian species used for medicinal purposes. It is a rosette forming small perennial herb also known as Indian Gentian, Neelkanth, karu and chireta. It is mainly found in Kashmir and Himachal Pradesh with adjoining hills of North-Western Himalayas at altitudes of 1500-3400 m. In traditional and modern medicine, roots and rhizomes of this plant are valued as a bitter tonic, antiperiodic, antibilious, anthelmintic, astringent, antipsychotic, sedative, stomachic and carminative. The roots of this plant are a source of iridoid glycosides like gentiopicrine and gentiamarin and the alkaloid, gentianin (Raina *et al*., 2003). Unfortunately, the pharmaceutical industries are largely dependent on natural population of *G. kurroo* to fulfill their demands, which is depleting the wild stands of this plant. Therefore, this plant has been listed as critically endangered by the Government of India (Sharma *et al*., 1993). Many *in vitro* studies have been carried out on propagation of *G. kurroo* using shoot tips, nodal segments, seedlings, petioles, leaves and apical meristem as explants (Sharma *et al*., 1993; Fiuk *et al.,* 2003; Sharma *et al*., 2014; Kaushal *et al*., 2014). It was found that *G. kurroo* can be propagated through rhizome cuttings; shoot nodal segments, seeds and somatic embryogenesis. Suspension culture studies have been previously established from seedling explants for various Gentiana species such as *G. tibetica, G. cruciata* and *G. pannonica* (Mikuła and Rybczynski, 2001; Mikuła *et al.,* 2005). Up to the present study very few workers have established the suspension cultures with the use of embryogenic callus derived from seedling explants (Fiuk and Rybczynski, 2008 a). Hence, in the present study, we have described the light micrographs showing stages in embryo development of *G. kurroo* Royle suspensions and plant regeneration through cell suspension cultures.

Materials and Methods

Plant Material

Two-week-old authentic aseptic cultures of *Gentiana kurroo* were collected from Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan and was maintained under controlled temperature (25°C), humidity (70-75%) and light (10 h dark and 14 h light) conditions in a growth chamber.

Culture media and growth conditions

For initiating the tissue culture, leaf cuttings were used as explants for somatic embryogenesis. MS medium (Murashige and Skoog, 1962) containing 3% sucrose gelled with 0.8 % agar supplemented with varying concentrations and combinations of BAP (0.5-1.0 mg/l), TDZ (0.5-1.0 mg/l), NAA (0.5-1.0 mg/l) and 2, 4-D (0.5-2.0 mg/l) were used as shown

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in the Table 1. The pH of the medium for somatic embryogenesis and suspension culture was adjusted to 5.8 ± 0.1 and 5.3 ± 0.2 respectively and was autoclaved at 121° C and 1.05 Kg cm^{-2} for 15 min. The cultures were incubated at 16 h photoperiod provided by cool white fluorescent light (3,000 lux) at 25^{0}C in a plant tissue culture chamber.

Initiation and establishment of suspension cultures

Cell suspensions were initiated by inoculating the friable callus into liquid medium. Individual cells or cell aggregates were maintained in suspension by agitation or aeration (Five replicates). Pieces of friable callus of approximately two-gram weight were removed from the petridishes and transferred into 250 ml conical flasks containing 50 ml of culture medium. The culture flasks were sealed with two layers of aluminium foil and paraflim. Cultures initiated from friable callus and embryogenic callus were incubated in the dark and 16 h photoperiod respectively on a rotary shaker at 110 r.p.m. at 25 ± 1°C.

Subculture and maintenance of cell suspensions

The first subculture was performed 10 days after initiation of the culture. The suspended cells were filtered through a double layer of 250 µm nylon meshe into a 100 ml measuring cylinder and allowed to settle down for 5-10 minutes. The supernatant was poured off and the cells were sub-cultured into a 250 ml conical flask containing 50 ml of fresh medium. After incubation for 10 days on a rotary shaker at 110 r.p.m. at 25 ± 1°C, the cell suspension was again filtered through a double layer of nylon mesh. At this time, only 15 ml of filtrate was used as inoculum for every 50 ml of fresh medium. Subsequent subcultures were made at 7-day intervals and were incubated in the same way. Light micrographs showing stages in embryo development of *G. kurroo* Royle suspensions were developed. For plant regeneration, large embryogenic masses were eliminated from cell suspension and were transferred to the MS medium supplemented with different concentrations of BAP (1.0-3.0 mg/l), KN (1.0-3.0 mg/l), NAA (0.5-2.0 mg/l), 2, 4-D (1.0-2.0 mg/l) and IAA (0.5-2.0 mg/l).

Statistical analysis

The data for the percentage of somatic embryogenesis, initiation time, and number of embryos per explants, regeneration frequency of suspension culture and the average number of shoots/explants were determined after 6 weeks of subculture. Twelve replicates were tested in each treatment and each experiment was repeated thrice. Means and standard errors were calculated for each experiment. The overall variation in a set of data was analyzed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

Results and Discussion

Somatic embryogenesis

Many factors including choice of explants and suitable combination of growth regulators are responsible for successful somatic embryogenesis. Induction of somatic embryogenesis directly from leaf without undergoing callus induction was tried on MS medium with various concentrations of 2, 4- D (0.5- 1.5 mg/l), BAP (0.25- 0.8 mg/l), NAA (0.5- 1.0 mg/l) and TDZ (0.25- 0.8 mg/l) (Table 1). When the leaves were cultured on MS medium supplemented with various concentrations of TDZ, NAA and BAP (0.2-1.0 mg/l), somatic embryogenesis was initiated in about 8-12 days followed by rapid growth. Of the various concentrations of TDZ tested, the maximum response was observed on MS medium supplemented with 0.5 mg/l NAA, BAP and TDZ where maximum percentage of embryogenesis was recorded as 97.2 % (Table 1) and (Fig.1a-f). It has been reported that TDZ has a cytokinin and auxin like activity for promoting cell division, differentiation and somatic embryogenesis (Aboshama, 2011). Greenish globular embryos were produced from the explants after 6 weeks of incubation in continuous light. The globular embryos started further development in subsequent days. The embryos were soft, friable and organogenic in all concentrations of TDZ which further developed into heart and torpedo shaped after 3 weeks of subculture (Fig. 1 g, h). A similar study was reported in *G. kurroo*, where the best morphological quality of embryos was observed in the presence of 2.0 mg/l NAA with 2.0 mg/l each of TDZ or BAP in which highest frequency of embryogenesis was 54.7% which has been increased in our present study ((Fiuk and Rybczynski, 2008 b). Also, the concentration used in their study, was very high as compared to the concentration used in our study. This variation in *G. kurroo* response might be due to the difference in the genotypes and difference in the physiological state of the explants used in the two studies. 2, 4-D (0.8 mg/l) in combination with 0.8 mg/l each of BAP and TDZ was also effective in inducing somatic embryogenesis up to 83.3% (Table. 1). 2, 4- D in combination with cytokinins for inducing somatic embryogenesis have been reported in many plant species such as *Bacopa monnieri* and *Curculigo orchioides* (Tiwari *et al.,* 1998; Nagesh *et al.*, 2010).

Characterization and optimization of suspension culture medium

Optimum subculture regimes, culture conditions and concentrations of the different media components were evaluated in pre-experiments. A total of ten combinations of the suspension induction medium components listed in Table 2 were assessed in combination with the MS-macro and micronutrients. Five flasks with liquid medium were placed on shaker for cell suspension studies at 110 rpm (25 ± 2° C). For suspension culture, somatic embryos induced on 0.5 mg/l each of NAA, BAP and TDZ were

transferred to the MS liquid medium containing IAA (0.5 mg/l) + BAP (1.0 mg/l) for agitation on a rotary shaker at 100 rpm (Fig. 2 a-c). Previously reported studies have established auxin-cytokinin combination for somatic embryo formation in Gentiana species (Mikuła *et al.*, 1996; Bach and Pawłowska, 2003). Embryogenic cell clumps were filtered through stainless sieves for uniform size. Suspension cultures were subcultured in the same medium after 7 days. The cell suspension was found to consist of small, round isolated cells with dense cytoplasm, an apparent nucleus, and rich in plastids with starch (Fig. 2 d, e). A few days after culture, 89-93% of the cells released were viable as assessed by cell counting method using erythrosine B stain. Average numbers of viable cells per square of haemocytometer were 10-11 and concentration of viable cell/ ml was 2.20×10^5 cells/ml which is an optimum concentration to grow single cells. Previous suspension studies with *G. kurroo* were performed with the use of embryogenic callus derived from seedling explants (Fiuk and Rybczynski, 2007).

Fig. 1. Direct somatic embryogenesis of *G. kurroo*: (a) & (b) Establishment of somatic embryogenesis from the leaf explant on MS medium supplemented with 0.5 mg/l each of BAP, NAA and TDZ after 8th and 20th day of inoculation; (c) & (d) somatic embryo proliferation after 35th and 45th day; (e) & (f) fully differentiated somatic embryos after 50th and 60th day of inoculation; stages of somatic embryo: (g) early globular, cotyledonary, heart and torpedo stages of somatic embryo; (h) somatic embryo.

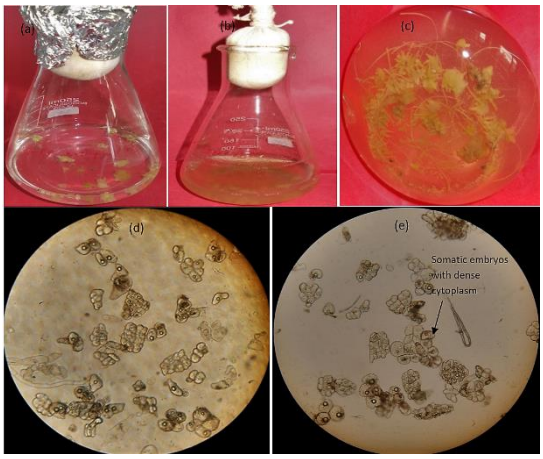

Fig. 2. Suspension cultures of *G. kurroo* : (a) Somatic embryos transferred to MS liquid medium containing IAA (0.5mg/l) and BAP (1.0 mg/l); (b) & (c) somatic embryo suspension cultures after 10 and 20 days respectively; (d) & (e) single suspended round isolated cells of somatic embryos with dense cytoplasm.

Microscopic analysis

Established cell suspensions of somatic embryos at different developmental stages were observed under light microscope. The embryos developed from single cells, which by unequal divisions formed two, four and eight celled stage (Fig. 3 a-c). After that, it formed a globular structure also known as a young embryo (Fig. 3 d). A young embryo by unequal divisions gave rise to a large mass i.e. proembryogenic mass or late proembryo stage (Fig 3. e, f). Further differentiation of proembryogenic mass led to the formation of early and late heart shaped embryos and finally to the cotyledonary stage with prominent shoot apices (Fig. 3 g-l). In the earlier report of cell suspension culture of *G. kurroo*, authors observed the ultrastructure of *G. kurroo,* indicating the presence of amyloplasts and starch grains (Fiuk and Rybczynski, 2008 a). In their study, the best qualities of embryos were observed in the presence of 0.5–1.0 mg/l KN, 0.5 mg/l GA_3 and 80.0 mg/l AS. Flow cytometry analysis revealed 100% uniformity for cotyledon suspensions with increased amounts of DNA in about one third of the regenerants. Serial stages in embryo formation by an isolated single cell from tissue culture of *G. kurroo* were also observed. Cell first divided by an unequal division forming a large vacuolated cell and small richly cytoplasmic cell, which, by a series of divisions gave rise to a tissue mass from which embryos were, differentiated (Fig. 4 a-e).

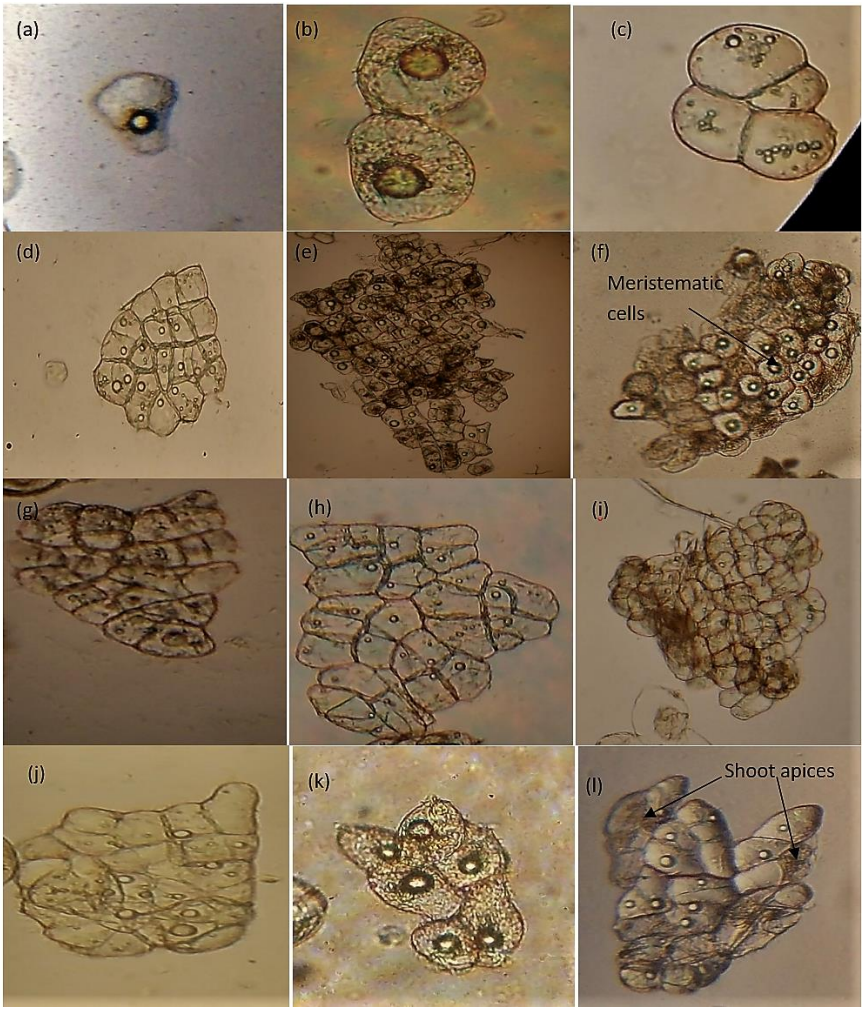

Fig. 3. Light micrographs showing stages in embryo development of *G. kurroo* suspensions: (a) and (b) Showing a single and pair of cytoplasm-rich somatic cells; (c) 4- celled cytoplasm rich proembryo; (d) a young embryo; (e) and (f) late globular proembryo showing central core of meristematic cells; (g) early heart shaped proembryo; (h) and (i) late heart shaped embryo; (j) and (k) early cotyledonous embryo; (l) late cotyledonous embryo with shoot apices.

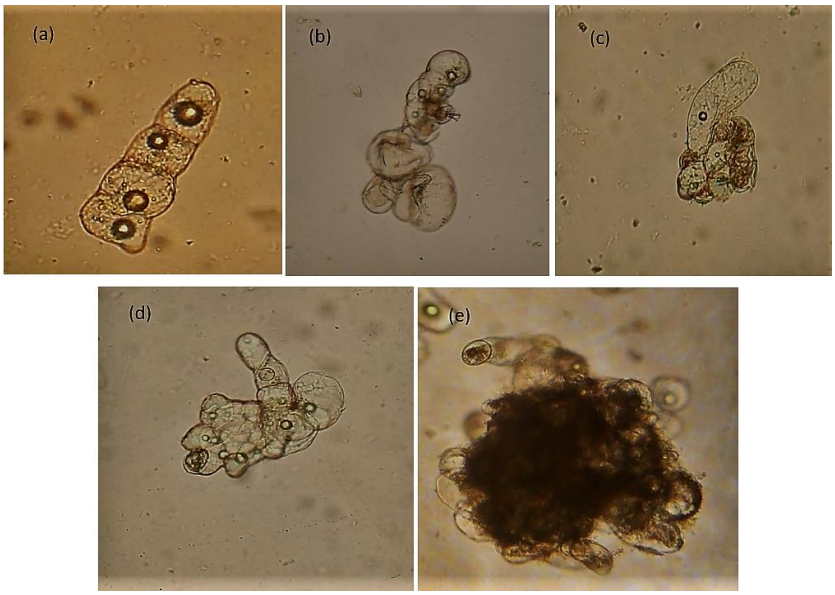

Fig. 4. Different stages of development of embryo from single cell of *G. kurroo:* (a) Single cell with large vacuolated cells and small richly cytoplasm; (b)-(e) the latter, by a series of divisions, gives rise to a tissue mass from which embryos were differentiated.

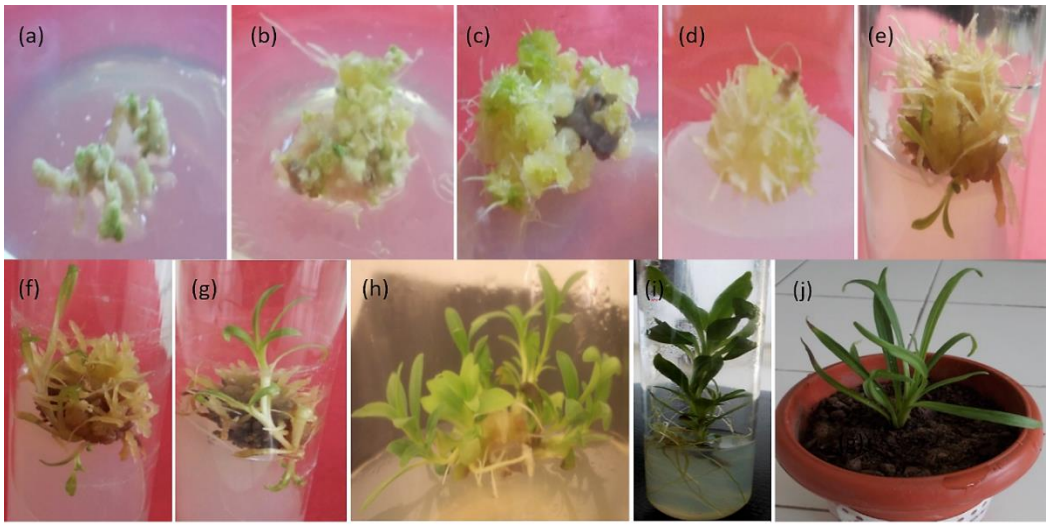

Fig. 5. Plant regeneration through suspension cultures of somatic embryos of *G. kurroo*:
(a) Large mass of somatic embryo eliminated through suspension culture; (b) sub cultured on 2, 4-D (0.4 mg/l) and KN (1.5 mg/l) after 30 days; (c) fully differentiated somatic embryo after 60 days; (d) somatic embryo subcultured on MS ½ strength basal medium after 60 days; (e) and (f) small plantlets generating from somatic embryos after 25 and 35 days respectively; (g) 2- 3 shoots developed after 45 days of culture on MS ½ strength basal salts; (h) multiplication of shoots on MS medium supplemented with 0.5mg/l each of KN and BAP after 60 days; (i) root induction on MS ½ IBA (0.5mg/l) after 4 weeks; (j) 3 months old acclimatized plants grown in greenhouse.

Table 1. Effect of plant growth regulators on initiation of somatic embryogenesis of *G. kurroo* after 60 days.

S. No	Growth regulators (mg/l)		Initiation time (d)	% Response
1.		(0.25) + (0.25)	-	-
2.		(0.5) + (0.5)	18	58.3
3.	**NAA + TDZ**	(0.75) + (0.75)	15	66.6
4.		**(1.0) + (1.0)**	**11**	**83.3**
5.	**NAA + BAP + TDZ**	(0.25) +(0.25) +(0.25)	21	75
6.		**(0.5) + (0.5) + (0.5)**	**8**	**97.2**
7.		(0.5) + (0.25)	-	-
8.	**2,4-D + BAP**	(0.75) + (0.5)	-	-
9.		(1.0) + (0.5)	25	33.3
10.		(1.5) + (0.5)	11	58.3
11.		(0.25) + (0.25)	-	-
12.	**BAP + TDZ**	(0.5) + (0.5)	21	44.4
13.		(0.75) + (0.75)	17	69.4
14.		**(1.0) + (1.0)**	**14**	**80.5**
15.		(0.25) + (0.25) + (0.25)	-	-
16.	**2,4-D + BAP +TDZ**	(0.5) + (0.5) + (0.5)	16	75
17.		**(0.8) + (0.8) + (0.8)**	**10**	**83.3**

Table 2. Effect of plant growth regulators on the survival percentage of somatic embryogenesis and number of embryos /explants of *G. kurroo* after 8 weeks.

S.No.	Growth regulators (mg/l)		Initiation time (days)	*Mean No. of embryos/ callus piece	Survival Percentage of S E (%)
1.		0.25 + 0.25	0.0	0.0	0.00
2.	NAA + KN	0.25 + 0.75	0.0	3.4 ± 0.09	0.0
3.		0.5 + 1.0	55	6.5 ± 0.1	5.0
4.		0.25 + 0.25	0.0	0.0	0.00
5.	NAA + BAP	0.25 + 0.75	0.0	0.0	0.0
6.		0.5 + 1.0	45	13.5 ± 0.2	15.0
7.		1.0 + 1.0	50	1.9 ± 0.2	10.0
8.	BAP + KN	1.5 + 1.0	49	7.5 ± 0.1	25.0
9.		2.0 + 1.0	33	8.9 ± 0.1	50.0
10.		1.0 + 0.25	0.0	0.0	0.00
11.	IAA + KN	1.0 + 0.5	55	7.9 ± 0.2	5.0
12.		2.0 + 1.0	44	11.6 ± 0.2	50.0
13.		4.0 + 2.5	38	17.4 ± 0.1	60.0
14.	2,4-D + BAP	0.25 + 1.0	53	7.2 ± 0.2	20
15.		0.4 + 1.5	35	12.8 ± 0.2	45
16.	2,4-D + KN	0.25 + 1.0	50	12.3 ± 0.1	55
17.		0.4 + 1.5	39	28.6 ± 0.2	80

*Each value represents mean ± SE of 12 replicates per treatment. Results represents the average and standard error of experiments performed in triplicate; ***$p < 0.05$.

Table 3. Regeneration frequency of suspension culture for plantlet regeneration after 24 days of culture on a rotary shaker at 110 rpm in dark at 25˚C ± 2˚C

S.No.	Growth regulators (mg/l)		Regeneration frequency of suspension culture on regeneration medium (%)
1.	**2, 4-D + BAP**	(0.25) + (0.5)	46.6
2.		(0.5) + (1.0)	60.0
3.	**IAA + BAP**	(0.25) + (0.5)	40.0
4.		**(0.5) + (1.0)**	**66.6**
5.	**2,4- D + KN**	(0.25) + (0.5)	26.6
6.		(0.5) + (1.0)	46.6
7.	**IAA + KN**	(0.25) + (0.5)	26.6
8.		(0.5) + (1.0)	40.0
9.	**NAA + BAP**	(0.25) + (0.5)	20.0
10.		(0.5) + (1.0)	33.3

Plant regeneration through suspension cultures of somatic embryos.

Individual cotyledonary somatic embryos obtained from cell suspension were cultured on MS medium supplemented with 2, 4-D in combination with BAP and KN. The percentage of somatic embryo survival was 80% with 28 ± 0.2 embryos/ callus piece of 100 µm (Table 3). In 2, 4-D (0.4 mg/l) and KN (1.5 mg/l) supplemented medium, the totipotent calli regenerated into plantlets within 39 days of culture; while 2, 4-D (0.4 mg/l) and BAP (1.5 mg/l) supplemented medium took 35 days for regeneration (Fig. 5a, b). The calli became embryogenic only in the presence of KN. Many reports indicated that *in vitro* organogenesis required a high cytokinin/ auxin ratio in several plant systems like *Eucalyptus grandis*, *Coffea arabica* and *Eleusine indica* (Luis et al., 1999; Zoriniants *et al.,* 2003; Yemets *et al.,* 2003). In the present study, NAA (0.25- 0.5 mg/l) in combination with BAP and KN (0.25- 1.0 mg/l) did not find a significant response in survival percentage of somatic embryos (5 % and 10 %). KN (0.25- 2.5 mg/l) in combination with IAA (1.0- 4.0 mg/l) and BAP (1.0- 2.0 mg/l) also elicited a good response with a survival percentage of 50% and 60% respectively (Table. 3). After 60 days of culture in the 2, 4- D and KN supplemented MS medium, fully differentiated somatic embryos were formed and were sub cultured on MS ½ strength basal medium (Fig. 5 c). It was observed that complete removal of 2, 4-D from the medium favored plant regeneration. Small plantlets were obtained from somatic embryos after 25th day and 2- 3 shoots developed after 45 days of culture (Fig. 5 d-g). 80-90% somatic embryos developed into complete plants after 6 weeks of culture. Similar study was reported in *G. kurroo* using seedlings and leaf explants, where, 56-71 % of somatic embryos developed into germlings and plants, when sub-cultured in half strength MS medium supplemented without plant growth regulators ((Fiuk and Rybczynski, 2008 c). Many reports indicate that plant development from somatic embryos is usually associated with the elimination of auxin from the medium. Similar studies have been reported by many authors in *Astragalus adsurgens, Coffea canephora and Eleusina caracana* (Hatanaka *et al.,* 1991; Eapen and George, 1989; Luo and Jia, 1998). Small plantlets were excised from the somatic embryos and inoculated in the MS medium supplemented with 0.5 mg/l each of KN and BAP for the shoot proliferation (Fig.5h). Many reports, on propagation of plant species such as *Dendrocalamus strictus and Arundinaria callosa* indicated that, KN in combination with BAP was the best plant growth regulator for the shoot multiplication and proliferation (Chowdhury *et al.*, 2004; Devi and Sharma, 2009). For *in vitro* root induction, well-developed plants were excised and transferred to the half strength MS medium supplemented with various concentrations of NAA (0.1 - 0.5 mg/l), IBA (0.1 - 0.5 mg/l) and IAA (0.1-0.5 mg/l). Out of these three auxins tried, half strength MS + IBA (0.5 mg/l) showed the best response with an average of 20-25 roots per plant with a length of 7 to 8 cm (Fig. 5 i). Shoots with well-developed roots were transferred to earthen pots containing a mixture of clay loam and farmyard manure (1:1 w/w). The rooted plants were hardened and after six weeks of hardening, the plants were transferred to the greenhouse where 70 to 80 % of plantlets are surviving without any somaclonal variations (Fig. 5 j). Findings of this study have shown a high frequency of somatic embryogenesis in *G. kurroo* and plant regeneration through suspension cultures which opens up vistas for large scale micropropagation of this valuable medicinal plant, vis-à-vis *in situ* conservation.

Conclusion

The present study established somatic embryogenesis and plant regeneration from cell suspension cultures of *Gentiana kurroo*. The protocol described here could be used for somatic hybridization, genetic transformation, isolation of protoplasts and large-scale propagation of *G. kurroo* which is highly valuable, critically endangered medicinal herb thereby ruling out the dependence on natural stands to fulfill the growing demands for this species. The present investigation opens up vistas for

large scale micropropagation of this valuable medicinal plant, vis-à-vis in situ conservation.

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