Journal of Medicinal Plants Studies

www.PlantsJournal.com

ISSN 2320-3862
JMPS 2014; 3(1): 04-09

Received: 30-11-2014
Accepted: 08-12-2014

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In vitro plant production through apical meristem culture of *Gentiana kurroo* Royle

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Abstract
Gentiana kurroo Royle is a critically endangered medicinal herb belonging to the family Gentianaceae. The roots and rhizomes of the plant have been extensively used as a bitter tonic, antiperiodic, expectorant, antibilious, anthelmintic, anticancer, immunomodulatory, antinflammatory and analgesic. A method for rapid micropropagation of *Gentiana kurroo* through apical meristem has been developed. Apical meristem was excised to a length of 0.2-2 mm from the shoot tips. Among different treatments of growth regulators either alone or in combination, the growth of meristem was best observed on Murashige and Skoog (MS) medium supplemented with 6- Benzyl amino purine (BAP) (1.0 mg/l) and Indole acetic acid (IAA) (0.5 mg/l). The maximum response for meristem proliferation was 83.3% with an average mean number of 8.1 ± 0.2 leaves /explant. Three weeks old sprouted meristems were transferred to the MS medium supplemented with 0.5 mg/l each of Kinetin (KN) and BAP for shoot elongation and proliferation resulting in 5- 6 shoots/ explant. *In vitro* regenerated shoots developed roots in six weeks when transferred to half strength MS medium supplemented with 0.5 mg/l Indole butyric acid (IBA) with a survival rate of 86%.

Keywords: *Gentiana kurroo*, apical meristem culture, MS medium, growth regulators.

1. Introduction
Gentianaceae is a family of flowering plants comprising approximately 300 species in the world [1]. In India, the family is represented by 16 genera and approximately 145 species. *Gentiana kurroo* Royle belonging to the 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. Plants of this species also occur in Pathrala thatch, Karol Tibba Solan, and Mangarh area of District Sirmour at an altitudinal range of 1700-2000 m at mean sea level [2]. In India, rhizomes and roots of this plant are used as bitter tonic, antiperiodic, expectorant, antibilious, anthelmintic, astringent, antipsychotic, anti-inflammatory, sedative, cholagogue, emmenagogue, febrifuge, refrigerant, blood purifier and carminative. The roots of this plant are a source of iridoid glycosides such as gentiopicrine, gentiamarin and the alkaloid gentianin [2]. Phytochemical screening of the plant also showed that the roots of *G. kurroo* are rich in various active ingredients like flavonoids, alkaloids and terpenoids, which are responsible for its effects as analgesic, anticancer and immunomodulatory [3, 4]. Unfortunately, the pharmaceutical industries are largely dependent on natural reserve, which lead to its extinction. Therefore, the red data book of Indian plants has listed this species as endangered and its status as critical [2]. Previously reported studies established the shoot formation using nodal segments and shoot tips [5], seedlings and leaves as explants [6], but plant regeneration through apical meristem has not been reported till date. Recently, indirect and direct organogenesis has been carried out using leaves, roots and petioles as explants, with a maximum response of 86.6% for shoot regeneration from callus cultures [7]. Meristem-tip culture is an important technique for the production of disease free plantlets and for rapid clonal multiplication. Although, many species such as *Gentiana scabra* have been found to be infected with aphid-borne viruses[8], but there is no report illustrating whether *Gentiana kurroo* is virus infected or not. In any case, meristem culture is the most effective method to raise virus free plants, not only for Gentiana species; but for all the pharmaceutical important medicinal plants. The tissue culture technique would be useful for conservation of rare and endangered plants for the production of industrially important phytochemicals. Therefore, the present study was undertaken to develop a protocol for plant regeneration through apical meristem.

2. Material and Methods

2.1 Plant material and culture conditions

Cultures of *G. kurroo* were obtained from Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan and was maintained in MS medium supplemented with 0.5 mg/l, each of KN and BAP, under controlled temperature (25 °C), humidity (70-75%) and light (10 h dark and 14 h light cycle) conditions in a growth chamber. Apical meristems were excised aseptically under the dissection microscope with the help of hypodermic needles and scalpel. The apical meristem consisting of the apical dome with one or two leaf primordia ranging from 0.1- 2 mm were gently removed from parental tissues and cultured on MS medium supplemented with different concentrations of growth regulators such as BAP (0.5-1.0 mg/l), KN (0.5-1.0 mg/l), IAA (0.5-1.0 mg/l), NAA (0.5-1.0 mg/l) and GA_3 (0.5-1.0 mg/l) as shown in the Table1. The pH of the medium was adjusted to 5.8 ± 0.1, prior to addition of 0.8 % (w/v) agar and was sterilized at 121 ºC for 15 min.

2.2 *In vitro* shoot multiplication, root induction and acclimatization

The proliferated meristem was multiplied and rooted on MS medium supplemented with different concentrations of KN, BAP, IAA, IBA and NAA, either alone or in combination for shoot elongation and root induction. After about 6 weeks in the rooting medium, the plantlets were transferred to the earthen pots containing a pre-autoclaved mixture of farm yard manure and clay loam (1: 1). The potted plants were covered with plastic sheet or glass beaker to maintain the humidity. Fully developed shoots with healthy roots were then acclimatized and transferred to the greenhouse for hardening. Hardening was continued for 3 weeks, or until they were successfully acclimatized. The survival rate was recorded after 45 days of plantation in the earthen pots.

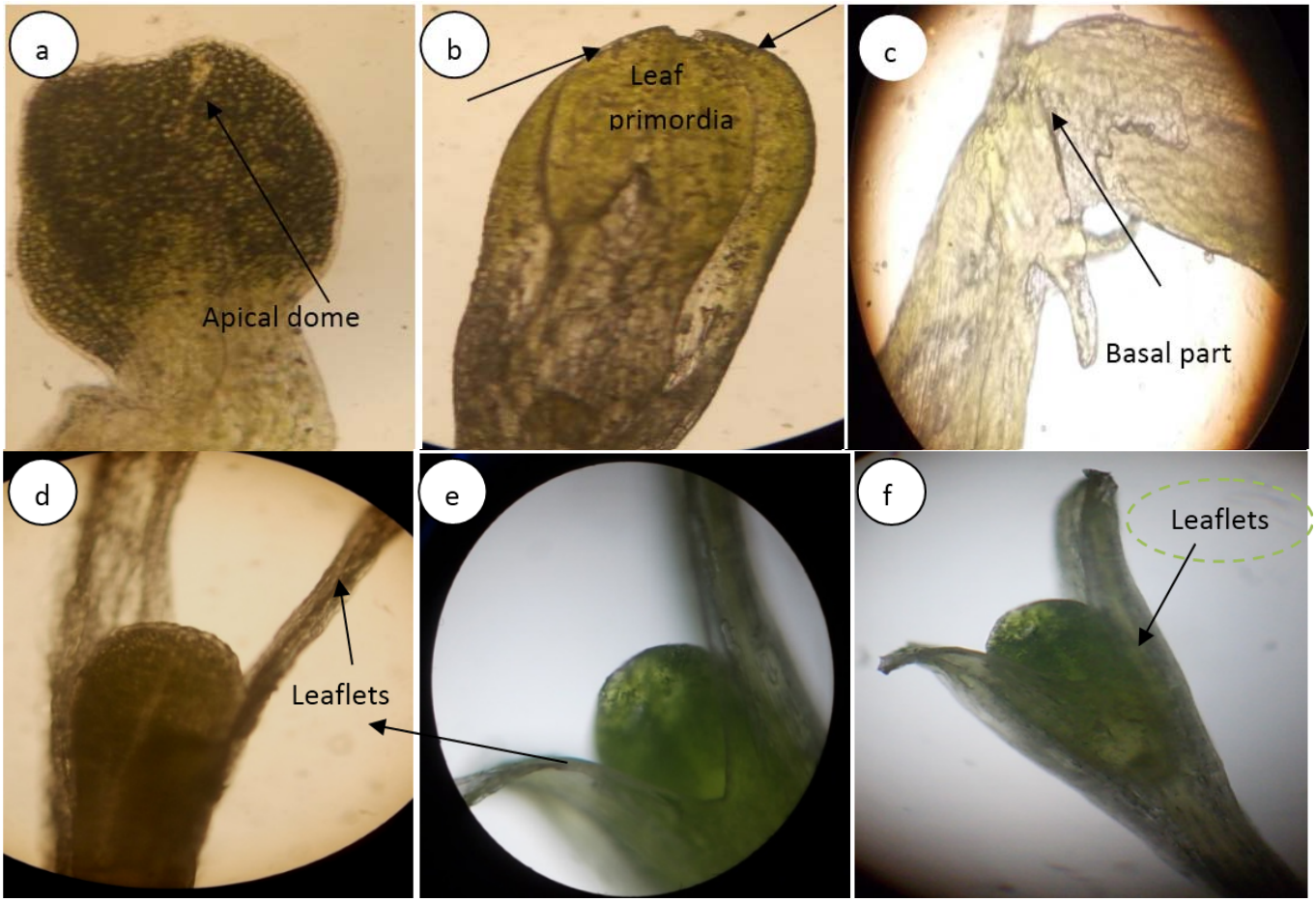
2.3 Data analysis

The data for the percentage of shoot response and average number of leaves/explant was determined after 6 weeks of subculture. Different developmental stages of excised apical meristem of *Gentiana kurroo* were observed under the light microscope. Twelve replicates were tested in each treatment and each experiment was repeated thrice. Means and standard errors were calculated for each experiment. The significance of the results was calculated using Graph pad prism 5.02 software. The overall variation in a set of data was analysed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered significant.

3. Results

3.1 Dissection and Microscopic analysis of apical meristem

Meristems were excised aseptically under the dissection microscope with the help of hypodermic needles and scalpel. The apical meristem sections without leaf primordia were excised to a range of 0.1- 2 mm in diameter and observed under a light microscope. Apical meristem appears as a shiny dome under the light microscope (Fig 1a). With the increasing number of days in the culture medium containing essential growth regulators, the excised meristem dome develops bipolar axis during reorganization (Fig b-f). Multiple leaves become evident after 10 days of culture and fully developed leaf whorl on the 14th day (Fig g- i).



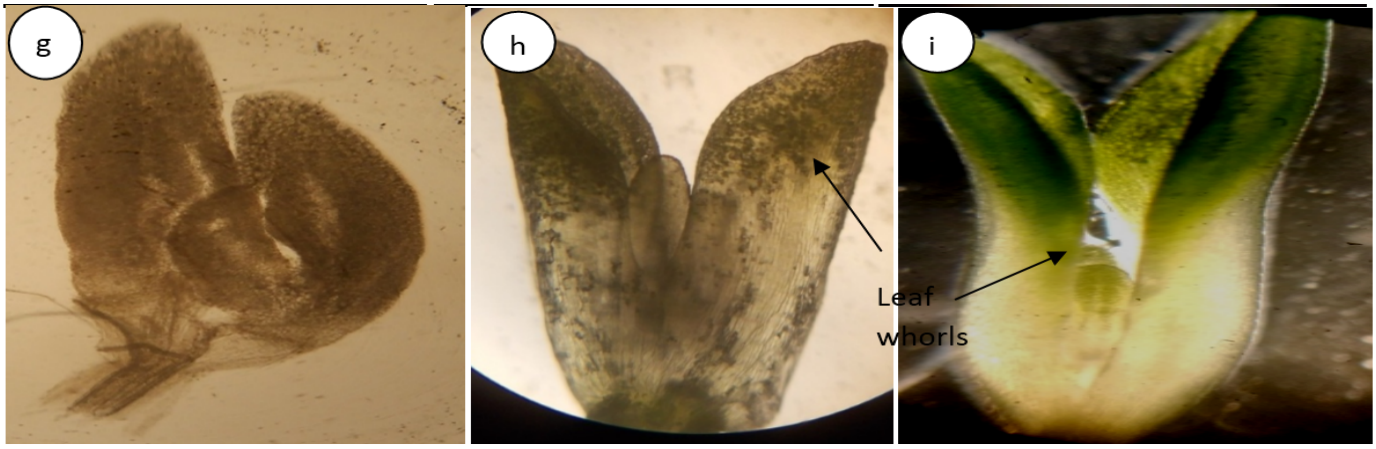


Fig 1: Developmental stages of apical meristem of *Gentiana kurroo.* (a) Light micrographs of excised meristem showing apical dome*;* (b) Leaf primordia regenerating from the apical dome; (c) Basal part of the apical meristem; (d-f) Leaflets regenerating from the apical meristem; (g-i) Formation of fully developed leaf whorls.

3.2 Meristem establishment and shoot multiplication

The apical meristem was cultured on MS medium supplemented with different concentrations and combinations of growth regulators. No morphogenic response was observed when nodal explants were cultured on MS medium devoid of plant growth regulators. About 83.3% of the apical meristem sprouted within 10-12 days of culture in MS medium supplemented with 0.5 mg/l IAA and 0.8 mg/l BAP (Table 1; Fig 2b &c). Also, IAA (0.5 mg/l) in combination with KN (0.8 mg/l) showed excellent growth with a success rate of 66.6% with an average number of 5.6±0.2 leaves/ explant after 20 days of inoculation (Table 1). After three weeks, the established primary meristem culture was aseptically subcultured on semisolid MS medium supplemented with different concentrations of KN and BAP. BAP and KN (0.25, 0.5, 0.75 and 1.0 mg/l) were used at equimolar concentrations and tested either alone or in combination for shoot elongation and multiplication (Fig. 3). Among different combinations, the MS media with 0.5 mg/l each of BAP and KN was the best combination for shoot elongation and proliferation with an average of 5.0± 0.2 shoots/ explants (Fig. 2d-f & Fig. 3). BAP alone was significantly more efficient (4-5 shoots/explant) than KN alone (1-2 shoots/ explant) for promoting shoot multiplication, which resulted in two fold increase in the number of shoots/ explant (Fig. 3). All other concentrations, except 0.5 mg/l each of KN and BAP showed a marginal increase in the number of shoots/ explant (5.0± 0.2) as compared to higher and lower concentrations. When KN and BAP were used in combination at higher concentration (1.0 mg/l), the shoot number and shoot length was reduced (3.9 ± 0.2 shoots/explant) with callusing at the basal ends (Fig. 3).

Table 1: Effect of growth regulators on development stages of apical meristem of *G. kurroo*.

	Growth regulators (mg/l)	***Average mean number of leaves/explant**	**Percentage (%)of shoot response**
Control (basal)		0.0 ± 0.0	0.0
	0.25 + 0.25	0.0 ± 0.0	0.0
NAA + KN	0.25 + 0.75	1.08 ± 0.2	8.3
	0.30+ 1.0	4.1 ± 0.2	41.6
	0.25 + 0.25	0.0 ± 0.0	0.0
NAA + BAP	0.25 + 0.75	3.9 ± 0.18	50.0
	0.3+ 0.8	5.10 ± 0.12	58.3
	0.25 + 0.25	0.0 ± 0.0	0.0
BAP + KN	0.25 + 0.50	3.7 ± 0.18	8.3
	0.5+ 0.5	5.9 ± 0.2	50.0
	0.8 + 0.8	6.2 ± 0.21	58.3
	0.25+ 0.5	0.0 ± 0.0	0.0
IAA + BAP	0.25+ 0.75	4.12 ± 0.17	50.0
	0.5 + 0.8	**8.16 ± 0.2**	**83.3**
	0.25 + 0.5	2.3 ± 0.2	8.3
IAA + KN	0.25 + 0.75	3.7 ± 0.23	25
	0.5+ 0.8	5.16 ± 0.2	66.6
	0.1 + 0.25	0.0 ± 0.0	0.0
GA_3 + BAP	0.25 + 0.25	1.08 ± 0.2	16.6
	0.25+ 0.5	3.67 ±0.14	41.6
	0.1 + 0.25	0.0 ± 0.0	0.0
GA_3 + NAA	0.1 + 0.3	1.08 ± 0.2	16.6
	0.25 + 0.5	3.3 ± 0.13	33.3

* Each value represents mean ± SE of 12 replicates per treatment.

Fig 2: Propagation of *Gentiana kurroo* through apical meristem. (a) Asceptically excised apical meristem (~ 2 mm) as an explant inoculated on MS medium supplemented with 0.5 mg/l IAA and 0.8 mg/l BAP; (b) & (c) Differentiated meristem with leaf primordia after 10 and 20 days of culture respectively; (d) & (e) Shoot induction from *in vitro* established meristem on MS semisolid medium with 0.5 mg/l KN and 0.5 mg/l BAP after 30 and 40 days respectively; (f) Shoot proliferation on MS medium supplemented with 0.5 mg/l KN and 0.5 mg/l BAP after 60 days of inoculation (g) Root induction from *in vitro* established meristem derived shoot on half strength MS semisolid medium with 0.5 mg/l IBA after 45days of culture in the rooting medium; (h) Plantlet with healthy roots kept in a mixture of sterilized FYM and Clay loam (1:1) under glass beaker to maintain the humidity; (i) Meristem derived hardened plant of *Gentiana kurroo* in pot after 60 days of hardening.

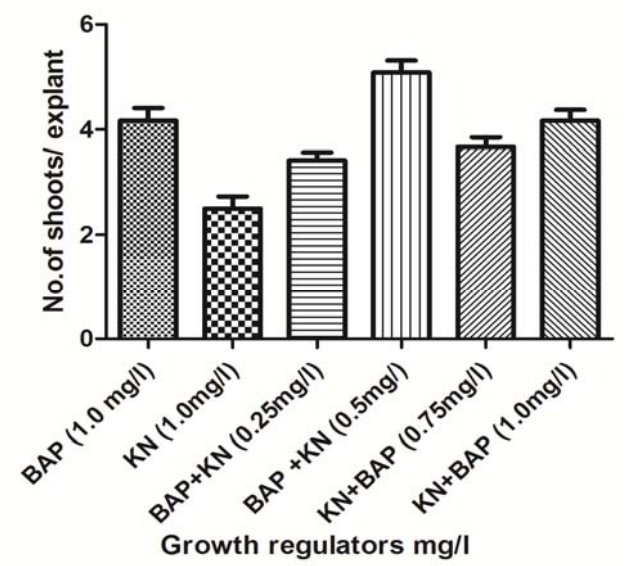


Fig 3: Effect of different concentrations of growth regulators on number of shoots/ explant of *G. kurroo*. Number of shoots/ explant was determined based on the data as shown in Fig 2f. Data represents an average and standard error of experiments performed in triplicate; $^{***}p < 0.05$.

3.3 *In vitro* rooting and Hardening

Elongated shoots were excised individually and rooted in the half strength MS medium supplemented with different types of auxins (IAA, IBA and NAA). IBA was clearly the best of three auxins tested, and each plantlet developed 24-25 roots/explant with 88.8% root response followed by IAA (69.4%), NAA (60.8%) and 30% on MS half basal salts (Fig. 4a). Well developed rooted plantlets were hardened in a mixtures containing sand and soil (1:1); soil and farm yard manure (FYM) (1:1); sand, soil and farm yard manure (1:1:1); clay loam and FYM (1:1) and coco peat. The survival rate after six weeks of acclimatization was highest in clay loam and FYM (86.0 %), followed by coco peat (80.5%), sand, soil and FYM (44.4 %), soil and FYM (38.8 %) (Fig. 4b). No morphological aberrations were observed after 2 months of hardening.

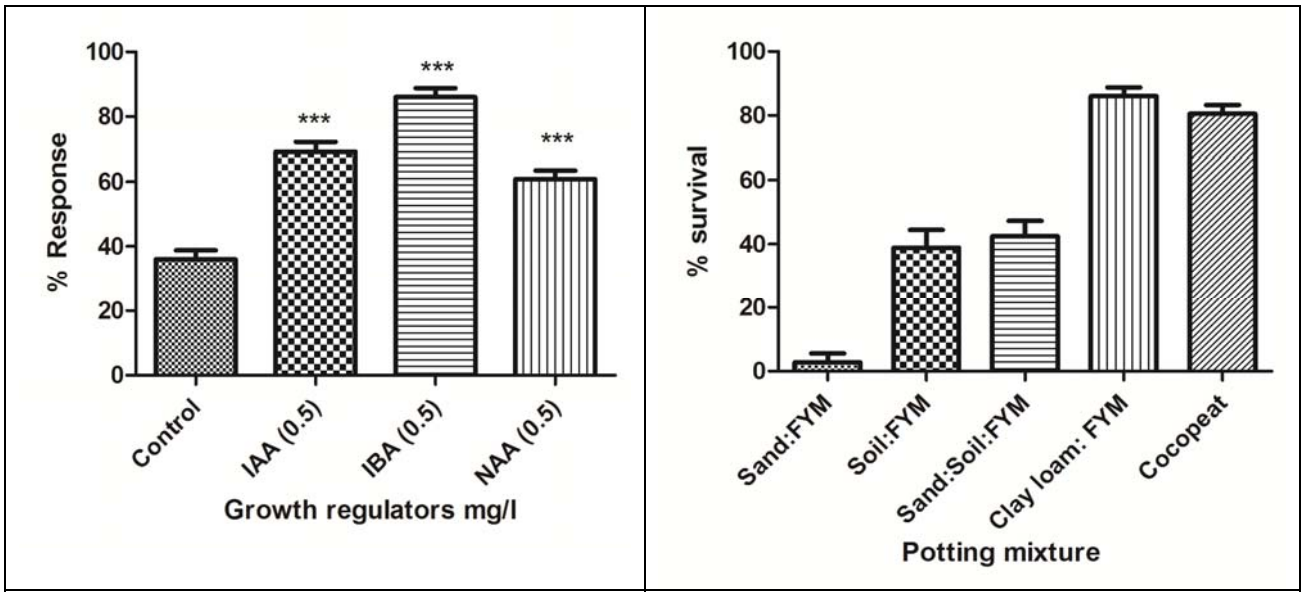


Fig 4: Effect of auxins on rooting of *Gentiana kurroo*.(a) In half strength MS medium after six weeks; (b) Percentage survival (%) of *in vitro* plantlets in different potting mixture after 2 months. Results represents the average and standard error of experiments performed in triplicate; $^{***}p < 0.05$.

4. Discussions

Apical meristem culture has recently become an important technique for virus elimination in plant tissue culture technology. Shoot development directly from the apical meristem avoids callus formation, ensuring that genetic instability and somaclonal variation are minimized [9, 10]. In the present study, apical meristem was excised and cultured on MS medium, which successfully developed into a new plantlet. Previously reported studies have explained the use of shoot tips and nodal segments as explants for *in vitro* propagation of *Gentiana kurroo* [5]. Recently, indirect and direct organogenesis has been carried out using leaves, roots and petioles as explants with maximum response (86.6%) of shoot regeneration from callus cultures [7]. Despite these studies for *in vitro* propagation of *Gentiana kurroo*, nothing has been reported dealing with plant regeneration through apical meristem. Hence, this study was carried out to describe the morphogenetic events through microscope during the development of plantlet through meristem culture. Shoot elongation and subsequent proliferation was effectively achieved on MS medium supplemented with a combination of KN and BAP. Similar observations have been reported in *Dendrocalamus strictus* and *Arundinaria callosa* [11, 12]. Shoot proliferation was declined when the KN and BAP concentration was increased beyond 1.0 mg/l in the medium. Similar results were also reported in *Stevia rebaudiana* [13]. The lower percentage of average number of leaves/ explant was observed on medium containing BAP and NAA due to the profuse callusing at the basal part of differentiated shoot buds. Similar observations were also reported in *Jatropha curcas* [14].

For the root induction, elongated shoots were individually excised and cultured on half strength MS basal salts supplemented with 0.5 mg/l IBA resulting in 91.6% root response with an average of 24.5±0.2 roots/ explant. IBA has successfully been used in root induction for other species like *Arbutus unedo*, *Hydrastis canadensis* and *Aegle marmelos* [15, 16, 17]. *In vitro* rooted shoots were successfully transferred to the plastic pots containing sterilized potting mixture of clay loam and farmyard manure (1:1), with a survival percentage of 86% after two months of acclimatization without any somaclonal variations. Similar report on acclimatization of *G. kurroo* has been achieved on coco peat with 80% survival rate [7].

5. Conclusion

The present study established the technique of meristem culture which may be exploited in situations where the donor plant is infected with viral, bacterial or fungal pathogens. The protocol described here could be used for large scale propagation of *G. kurroo* through apical meristem, thereby, ruling out the dependence on natural stands to fulfill the growing demands for this species.

6. Acknowledgment

The authors are grateful to Prof. D. R. Sharma for the scientific guidance and Shoolini University of Biotechnology and Management Sciences, Solan for providing the infrastructure and facilities to conduct this research.

7. Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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