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A novel cost-effective medium for *in vitro* cultivation of *Gentiana kurroo* Royle using sugarcane bagasse

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ABSTRACT

Objective: To develop a simple, cost-effective, and efficient medium by using sugarcane bagasse (SB) as a base material to replace the conventional Murashige and Skoog (MS) medium.
Materials and Methods: Water extracts of SB along with some macronutrients and plant growth regulators were gelled with 0.7% agar-agar powder. Nodal segments of *Gentiana kurroo* were used as explants and inoculated in the medium and placed in a growth chamber under standard conditions of light and temperature. Out of the tested combinations of plant growth regulators, 0.5 mg/l each of kinetin (KN) and 6-Benzylaminopurine (BAP) showed the excellent shoot multiplication and proliferation rate on the bagasse medium with the same potential as on the MS medium with an average of 5–6 shoots/explant. *In vitro* rooting was obtained on half strength MS medium supplemented with IBA (0.5 mg/l) with an average length of 7–8 cm and 20–25 roots/explant. The plants were hardened in a mixture of clay loam and farmyard manure in 1:1(w/w) with 70%–80% survival rate without any phenotypic aberrations.
Conclusion: The results from the present investigation indicate that SB can be used as a cost-effective substitute of MS medium for *in vitro* propagation of *G. kurroo*.

1. INTRODUCTION

Gentiana kurroo Royle is a medicinal plant belonging to the family Gentianaceae. It is mainly found in the northwestern Himalayas and in some pockets of Sirmaur district in the state of Himachal Pradesh, India at the altitudes of 1,500–3,400 m [1]. In India, rhizomes and roots of this plant are valued as a bitter tonic, antiperiodic, expectorant, antibilious, anthelmintic, astringent, antipsychotic, anti-inflammatory, sedative, antibacterial, stomachic, and cholagogue [2]. The roots of this plant are a good source of gentiopicrine, gentiamarin, and the alkaloid, gentianin [3]. Unfortunately, the pharmaceutical industries are largely dependent on the natural population of *G. kurroo* to fulfill their demands, thus, depleting the wild stands of this plant. Because of its restricted distribution and widespread extraction from its natural habitats coupled with nil cultivation, this plant has been listed as critically endangered by the Government of India [1].

Research in plant tissue culture has played an important role in the production and conservation of many medicinal and ornamental plants. Many *in vitro* studies have also been carried out on the propagation of *G. kurroo* using seedlings, petioles, leaves, apical meristem, and somatic embryo as explants [4]–[7]. Generally, Murashige and Skoog (MS) medium [8] is used for the cultivation of *Gentiana kurroo*, which is costly, therefore, some new simple and cost-effective alternative of MS medium should be developed for the successful *in vitro* cultivation of *G. kurroo*.

Agricultural wastes which contain large amounts of residual nutrients and are available in large quantities may be explored for the *in vitro* cultivation of medicinal or ornamental plants. Sugar industries produce tons of sugarcane bagasse (SB) every year which is rich in cellulose, hemicellulose, and lignin [9], [10]. Despite its potential to be used for the development of some value-added products, it is burned in sugar mills and alcohol distilleries for energy generation or is used as animal feed [11], [12]. SB has been used as a substitute of agar for the *in vitro* root induction in apple and strawberry [13], [14]. It has also been used for the successful cultivation of some plant and human pathogenic fungal

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Table 1: Composition and cost analysis of MS and SB media.

Ingredients	Strength in MS basal medium (mg/l)	Cost/liter (INR) of MS medium	Strength in SB medium (mg/l)	Cost/liter (INR) of SB medium	Modified MS medium (Himedia)
I. STOCK-A					
1. KNO_3	1,900	0.99	500	0.26	
2. $MgSO_4.7H_2O$	370	0.17	250	0.11	
3. KH_2PO_4	170	0.21	100	0.12	
II. STOCK-B					
1. NH_4NO_3	1,650	0.61	-	-	
III. STOCK-C					
$CaCl_2.2H_2O$	440	0.71	-	-	
$CaNO_3$	-	-	200	0.10	
IV. STOCK-D					
1. Na_2EDTA	37.3	0.08	25.0	0.05	
2. $FeSO_4.7H_2O$	27.5	0.01	25.0	0.01	
V. STOCK-E					
1. KI	0.83	0.57	-	-	
VI. STOCK-F					MS salts, sucrose, vitamins and Agar
1. H_3BO_3	6.2	0.00	-	-	
2. $COCl_2.6H_2O$	0.25	0.04	-	-	
3. $ZnSO_4.7H_2O$	8.6	0.00	-	-	
4. $CuSO_4.5H_2O$	0.25	0.00	-	-	
5. $MnSO_4.4H_2O$	22.3	0.03	-	-	
$MnSO_4$(anhydrous)	(1.69)			-	
6. $Na_2MoO_4.2H_2O$	0.25	0.03	-	-	
VII. STOCK-G					
1. m-inositol	100	0.24	-	-	
2. Glycine	2	0.00	-	-	
VIII. STOCK-H					
1. Pyridoxin-HCL	0.50	0.02	-		
2. Nicotinic Acid	0.50	0.00	-		
3. Thiamine HCL	0.10	0.00	-		
Sucrose	30 g/l	24.69	10 g/l	8.23	
Agar	8 g/l	58.67	6 g/l	44.00	
Whatman filter No. 1				6.00	
Total cost		87.03		58.8	97

species [15]. Till date, there is no report of *in vitro* cultivation of *G. kurroo* using SB as a growth medium. The present study was conducted with the aim to develop a simple, cost-effective, and efficient medium by using SB as a base material to replace the conventional MS medium.

2. MATERIALS AND METHODS

2.1. Plant Material and Culture Conditions

Two-week-old aseptic cultures of *G. kurroo* were obtained from Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan and maintained under controlled temperature (25°C), humidity (70%–75%), and light (10 hours dark and 14 hours light) conditions in a growth chamber. Nodal segments were used as explants for plantlet production.

2.2. Optimization of Medium

SB was procured from local sugarcane juice vendor free of cost and was shade dried under a muslin cloth to protect from house flies. After drying, the internal part (medulla parenchyma) was extracted manually. The internal part was ground to fine powder in a steel kitchen grinder. The refined or crushed powder thus obtained was strained twice through a muslin cloth. Twenty-five grams of this powder was added to 400 ml distilled water and was placed on a shaker at 180 rpm for 1 day. After 1 day, the contents of the flask were filtered first through a muslin cloth and then through Whatman filter paper number 1. The filtrate (200 ml) was taken

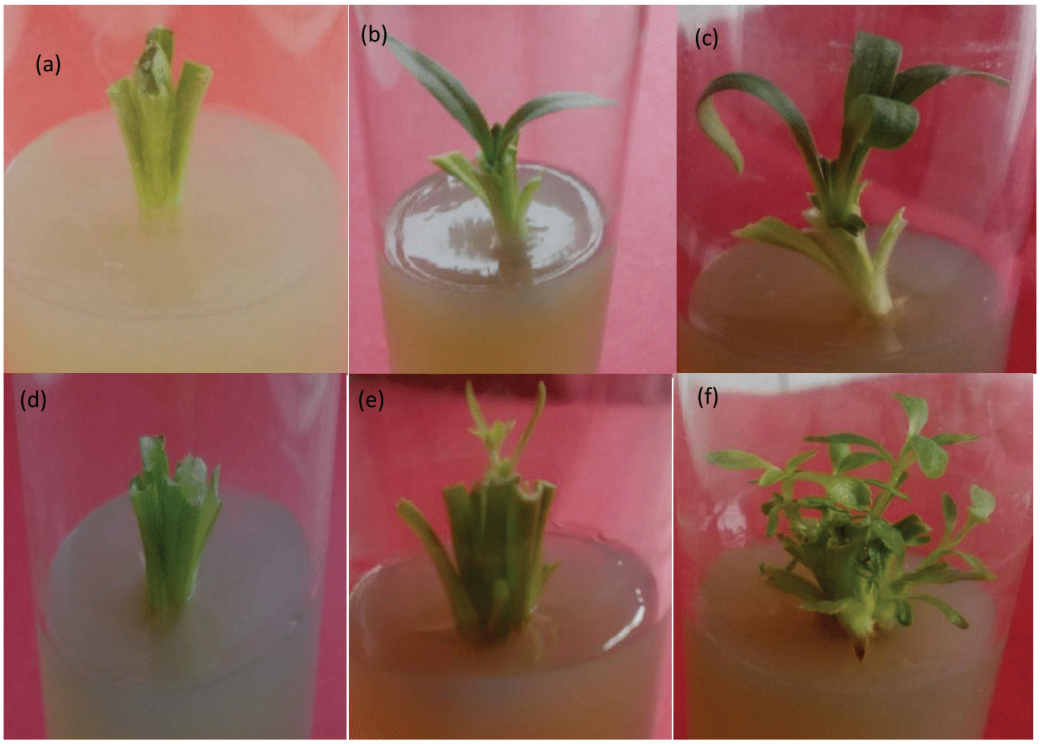

Figure 1: *In vitro* cultivation of *G. kurroo* on varying concentrations of sucrose. (a) Establishment of nodal explant on SB medium supplemented with 3% sucrose and growth regulators; (b) & (c) plantlet regeneration after 3 and 6 weeks with stunted growth; (d) establishment of nodal explant on SB medium supplemented with 2% sucrose and growth regulators; (e) & (f) shoot regeneration after 3 and 6 weeks with hyperhydration.

and macronutrients K, Mg, Fe, P, and N were added (Table 1). Different concentrations of sucrose (1%, 2%, and 3%) gelled with 0.7% agar-agar supplemented with varying concentrations of growth regulators were tested. The pH of the medium was adjusted to 5.7 ± 0.1 and was autoclaved at 121°C and 1.05 kg cm^{-2} for 15 minutes. The cultures were incubated at 16 hours photoperiod provided by cool white fluorescent light (3,000 lux) at 25°C in a plant tissue culture chamber.

2.3. *In Vitro* Shoot Multiplication, Root Induction, and Acclimatization

Nodal segments were cultured on SB medium supplemented with different concentrations of BAP (0.5–1.0 mg/l) and KN (0.5–1.0 mg/l) for shoot elongation and multiplication. Well-developed shoots were excised and transferred to the half strength MS medium supplemented with various concentrations of root initiating growth regulators viz., NAA (0.1–0.5 mg/l), IBA (0.1–0.5 mg/l), and IAA (0.1–0.5 mg/l). *In vitro* grown rooted shoots were removed from the culture tubes after 7–8 weeks and then transferred to the earthen or plastic pots containing an autoclaved mixture of clay loam and farmyard manure (FYM) (1:1 w/w). The potted plants were kept under polythene or glass beaker to maintain the humidity for 2 weeks. Hardening was continued for 3 weeks with irrigation at 2–4 days interval supplied with ¼ strength MS major salts until these were successfully acclimatized. The survival rate (%) was recorded after 45 days of the transfer. Observations on percentage survival and number of shoots developed on the SB and MS media were recorded after 6 weeks of subculturing (subculturing done at 3-week intervals). A minimum of seven cultures was raised in each set, and each experiment was repeated thrice.

2.4. Data Analysis

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. A value of $p < 0.05$ was considered significant. Calculations were done using the software GraphPad Prism 5.02.

3. RESULTS AND DISCUSSION

3.1. Standardization of SB Medium

Dried SB powder (25 g) in 400 ml distilled water after continuous shaking on a rotary shaker for 1 day produced 200 ml of opaque filtrate**.** Controls consisted of culture jars containing MS medium supplemented with 0.5 mg/l each of KN and BAP and 3% sucrose and 0.8% agar-agar. Three concentrations for sucrose (3%, 2%, and 1%) were compared and growth regulators were added to each concentration. Out of these, the optimal concentration of sucrose for shoot multiplication was 1%, whereas, in 2% and 3% sucrose, the shoot length was stunted with a little hyperhydration (Fig. 1a–f).

3.2. *In Vitro* Propagation of *Gentiana kurroo* on SB Medium

Plant tissue culture provides a platform for the mass propagation of plants which helps in making easy availability and conservation of rare, threatened, and endangered species. However, this is

Table 2: Effects of different concentrations and combination of growth regulators on average number of shooting (shoots/explant) of *G. kurroo* on SB and MS media.

Treatment: plant growth regulators (mg/l)		SB medium	MS medium
		*Average no of shoots/explant	*Average no. of shoots/explant
1.	Control (Basal medium)	0	0
2.	BAP (1.0)	3.9 ± 0.1	4.2 ± 0.2
3.	KN (1.0)	2.0 ± 0.2	2.1 ± 0.2
4.	KN (0.5) + BAP (0.5)	5.76 ± 0.1	5.8 ± 0.1
5.	NAA (0.25) + BAP (0.5)	3.6 ± 0.4	3.7 ± 0.2
6.	BAP (0.5) + IAA (0.25)	3.7 ± 0.2	3.9 ± 0.2
7.	KN (0.5) + IAA (0.25)	2.4 ± 0.1	2.5 ± 0.1

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

Figure 2: *In vitro* propagation of *G. kurroo* on SB and MS medium. (a) Nodal explant establishment on SB medium supplemented with KN and BAP (0.5 mg/l); (b) shoot regeneration after 20 days of culture (c) plantlet formation with 4- 5 shoots on SB medium after 6 weeks; (d) nodal explant establishment on MS medium supplemented with KN and BAP (0.5 mg/l); (e) shoot regeneration after 20 days of culture (f) plantlet formation with 4- 5 shoots on MS medium after 6 weeks; (g) & (h) acclimatization of in vitro grown plantlets after six weeks in clay loam and farmyard manure (1:1) after 6 weeks and 2 months respectively.

usually constrained by the high cost of nutrient media. The present study was undertaken to develop a low-cost medium for *in vitro* propagation of *G. kurroo.*

SB medium regenerated plantlets with the same potential as MS medium. The nodal segments were taken as explants and were propagated on MS and SB medium supplemented with different concentrations and combinations of BAP (0.5–1.0 mg/l), KN (0.5–1.0 mg/l), NAA (0.1–0.5 mg/l), and IAA (0.2–0.5 mg/l) (Table 2). The *in vitro* propagation of shoots was strongly influenced by the types of macronutrients and cytokinins employed. BAP in combination with KN (0.5 mg/l each) showed excellent shoot multiplication and proliferation with the same potential as on the MS medium with an average of 5–6 shoots per explant (Fig. 2a–f). Shoot multiplication was better on media with BAP + IAA than with KN + IAA. Shoot multiplication was better on the medium when IAA (0.25 mg/l) was used in combination with BAP (0.5 mg/l) with 4–5 shoots/explant as compared with the combination of IAA (0.25 mg/l) + KN (0.5 mg/l). An increased shoot length with decreased proliferation rate was observed with an average of 2 or 3 shoots after 6 weeks of culture (Table 2).

Strategies to reduce the cost of tissue culture media have been reported by many researchers on other plants. Fernando and Subasinghe [16] used Albert's solution, which is a water-soluble fertilizer containing most of the macro and micronutrients medium as a cost-effective alternative of MS medium, whereas Raghu *et al*. [17] experimented with household sugar and tap water as a substitute for laboratory sugar and double distilled water. Locally available low-cost fertilizers have also been used as a substitute for conventional MS salts in sweet potato seedlings [18]. Despite these achievements in cost reduction in tissue culture media for other plants, to our best knowledge, no work has been reported so far in regard to *G. kurroo*, and hence, this study was carried out to check whether the powdered SB along with additional nutrients and growth regulators is able to support the plant growth.

A good root system is of great significance for the acclimatization of the plantlets [19], [20] since roots and rhizomes facilitate the absorption of nutrients from the soil. Well-developed plants were excised and transferred to the half strength MS and SB 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) to test rooting.

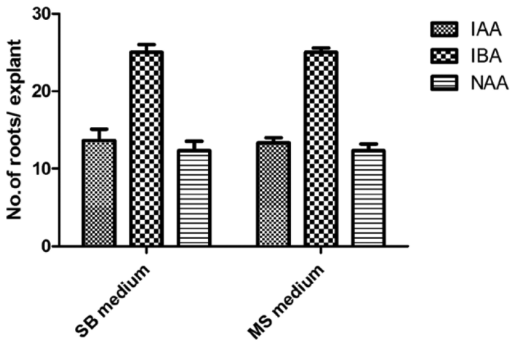

Figure 3: Effect of auxins on SB and MS medium during *in vitro* rooting of *G. kurroo* after 6 weeks. Results represents the average and standard error of experiments performed in triplicate; ***p < 0.05.

Out of these three auxin levels 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 7–8 cm which was same as that of MS medium (Fig. 3). Shoots with well-developed roots were transferred to earthen pots containing a mixture of clay loam and FYM (1:1 w/w) (Fig. 2g). The rooted plants were hardened and after 6 weeks of hardening, the plants were transferred to the greenhouse where 70%–80% of plantlets are surviving without any phenotypic aberrations (Fig. 2h). Findings from this study have shown that it is possible to reduce the cost of *in vitro* plantlet production through tissue culture by using agroindustrial residues as an alternative support system. The total cost for 1 l MS medium and conventional modified MS medium was about INR 87 and 97 and the total cost of 1 l SB medium was INR 58 only (Table 1).

4. CONCLUSION

Conventional media used for the *in vitro* cultivation of plants are expensive. It becomes a difficult task to cultivate and repeatedly subculture large number of plants. Utilization of agricultural wastes as a base material for the *in vitro* cultivation and conservation of important plants is a step forward toward the solution of this problem. In the present investigation, the potential of SB as a substitute of MS medium for the cultivation of *G. kurroo* Royle was evaluated. It was observed that SB medium regenerated plantlets with the same potential as MS medium without any phenotypic aberrations.

5. CONFLICT OF INTERESTS

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

6. ACKNOWLEDGMENTS

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