INNOVARE JOURNAL OF AGRICULTURAL SCIENCE

Vol 4, Issue 4, 2016

ISSN - 2321-6832

Research Article

OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION AND GERMINATION OF ARTIFICIAL SEED IN AN IMPORTANT MEDICINAL PLANT, *GENTIANA KURROO*

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Received: 09 August 2016, Revised and Accepted: 12 August 2016

ABSTRACT

Background: *Gentiana kurroo* is a critically endangered herb which is known as a bitter drug in Indian medicine. The present study was conducted to optimize the production and germination of artificial seeds in *G. kurroo* for its mass propagation.

Methods: This study utilized somatic embryogenesis as a source for the artificial seed production of *G. kurroo* royle. Morphogenetic capabilities of leaf explants have been optimized on Murashige and Skoog (MS) media containing different concentrations and combination of kinetin (KN)+2,4-dichlorophenoxyacetic acid (2,4-D). Upon callus induction, torpedo shaped somatic embryos were selected for artificial seed production. The artificial seed were produced using the different concentrations of sodium alginate and calcium chloride solution followed by germination on MS media supplemented with indole-3-butyric acid (IBA)+KN+gibberrelic acid (GA3).

Results: The study revealed that KN (1 mg\L) and 2,4-D (0.5 mg\L) are most appropriate for callus initiation and its proliferation. The artificial seeds were successfully produced with 3% sodium alginate and 100 mM calcium chloride solution. The 90-95% germination of artificial was achieved in 7-10 days on MS media supplemented with IBA (1 mg/L)+KN (3 mg/L)+GA_3 (2 mg/L).

Conclusions: Artificial seed production has led to increase in the viability rates and germination potential of *G. kurroo* royle seeds in soil, thereby providing efficient means of germplasm conservation, easy transportation, and large scale propagation of endangered species.

Keywords: Synthetic seeds, Somatic embryogenesis, *In vitro* encapsulation, *Gentiana kurroo*, Calcium alginate beads, Artificial seed germination, Callus induction, Mass propagation.

INTRODUCTION

Gentiana kurroo Royle, commonly known as 'Karu' or 'Kutki' belongs to the family Gentianaceae, is a critically endangered perennial herb which occurs in the Himalayan region of India at latitude of 1500-3300 [1]. This herb is used in Ayurveda and sometimes substitute by the endangered medicinal herb, *Picrorhiza kurroa* which is also used for the treatment of liver disorders [2-5]. The genus Gentiana contains about 360 species out of which 62 species are observed in India. The dried roots and rhizomes are important in the pharmaceutical codex [6]. The roots are rich in bitter glycosides (gentiopicrin and gentianin), alkaloids (gentiomarin), which have immense medical and pharmaceutical importance [1,6]. The root stock is valued as bitter tonic, antiperiodic, expectorant, blood purifier, treating urinary infections, digestive disorders, and many more [1,7,8].

Due to overexploitation and lack of organized cultivation, the wild populations have declined fast. In addition, there are number of constraints mentioned for the propagation and conservation through seed propagation. The major ones are variations in climatic factors, low percentage seed germination, and seasonal dormancy [1,9]. The abovementioned causes prompted to find an alternate method of rapid micropropagation of this species. Hence, synthetic seed technology provides large-scale production of viable materials that are later converted into plants [8]. Because the naked micropropagules are sensitive to desiccation and/or pathogens when exposed to natural environment, it is envisaged that for large scale mechanical planting and to improve the success of plant (*in vitro* derived) delivery to the field or greenhouse, the somatic embryos useful in synthetic seed production would necessarily require some protective coatings [1,10,11]. Encapsulation is expected to be the best method to provide protection and to convert the *in vitro* derived propagules into synthetic seeds [12]. In this study, leaf explants were used to carry out studies on exploring morphogenic capabilities of the medicinal herb. In the present study, callus induction and regeneration into shoots was optimized under culture conditions. Somatic embryogenesis was induced to produce artificial seeds which were reported for the first time and tested for germination. This developed technology could be utilized for large scale production of rich planting material high in quality and for the production of phytochemicals.

METHODS

Plant material and culture establishment

The *G. kurroo* plantlets were procured from the Himalayan Forest Research Institute Shimla, Himachal Pradesh, India, and planted in pots in a polyhouse at the experimental area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat, India.

Media preparation and culture conditions

MS medium supplemented with different concentrations and combinations of growth hormones such as 2,4-D (0-2) mg/L, naphthalene acetic acid (NAA) (0-3) mg/L, and IBA (0-1) mg/L with sucrose 3% (w/v). The pH of the media was adjusted to 5.7 using 0.1 N hydrochloric acid (HCl) and 0.1 N sodium hydroxide (NaOH) and finally agar-agar 0.8% (w/v) was added as a gelling agent before autoclaving at 121°C. After surface sterilization of leaf explants of *G. kurroo* by using 0.5% bavistin and 0.1% mercuric chloride followed by 4-5 washings with sterile water, the axenic cultures were incubated at 16 hrs light/8 hrs dark cycle at 25±2°C in plant tissue culture chamber of the department.

Callus induction and regeneration

The leaves explants were given small incisions are given using an autoclaved blade were cultured on MS medium supplemented with

Table 1: MS media supplemented with different concentrations of growth hormones for callus induction in *G. kurroo*

MS+Growth hormones						Parameters for callus induction	
IBA (mg/l)	KN (mg/l)	BA (mg/l)	NAA (mg/l)	TDZ (mg/l)	2,4D (mg/l)	Days to initiation of callus formation	Percent explants forming calli
3	1	-	-	-	-	30-40	20
-	-	3	2	-	-	15-17	40
-	-	-	-	2	1	14-16	60
-	-	-	-	3	2	16-20	55
-	1	-	-	-	0.5	7-10	85

different concentrations and combinations of growth hormones such as 2,4-D=0.5 mg/L, NAA=2 mg/L, and IBA=1 mg/L with sucrose 3% (w/v). The cultures were kept under observation for 30 days. The cultures were sub-cultured after every 15-20 days on callus induction media for 2 months so as to obtain good growth. After the formation of calli, the sub-culturing was carried out on the best medium to good mass. Then, the calli were cultured on regeneration medium having MS supplemented with IBA (0-1) mg/L, KN (0-3) mg/L, and GA_3 (0-2 mg/L) to generate shoot biomass. Data were recorded on days to initiation of callus formation, per cent explants forming calli days to regeneration of shoots and number of shoots.

Encapsulation of mature embryos

The sub-cultured callus is observed under high definition microscope for the selection of torpedo shaped somatic embryos. These embryos have been encapsulated under *in vitro* conditions using solution of sodium alginate 3% dissolved in 100 ml of Luke warm water and calcium chloride 100 mM dissolved in 100 ml of water. Both of these solutions are sterilized by autoclaving at 121°C. Developed somatic embryo from calli were selected and added to the sodium alginate solution under sterile condition. The sodium alginate solution containing the callus was added drop wise to the chilled calcium chloride solution. The beads encapsulating the callus were kept in the calcium chloride solution for 30-45 minutes. Obtained seeds were cultured on the MS media supplemented with growth hormones and tested for germination. Seeds were also stored at 4°C.

Somatic seed germination

Encapsulated somatic seeds were cultured on the regeneration media containing MS media supplemented with growth hormones such as IBA=1mg/L, KN=3mg/L, GA_3=2 mg\L, and 0.5% activated charcoal. The cultures were kept at 25°C±1°C temperature, 16 hrs light, 8 hrs dark photoperiod to initiate the germination from the somatic seeds. Data recorded for day to germination and number of shoots formed.

Statistical analysis

The results were obtained by calculating the mean ± SD from data in triplicate.

RESULTS

Callus induction

The best callusing (85%) was obtained on MS media supplemented with 2, 4-D (0.5 mg/L) and KN (0.1 mg/L) in 10-15 days of culturing on the respective medium. In contrast, shoot regeneration was observed on 30-35 days with 2-3 leaf primodia on MS medium containing IBA=1 mg/L, KN (3 mg/L), and GA_3 (2 mg/L). The shoots were sub-cultured for increase their shoot mass. Calli were sub-cultured on the best medium to obtain somatic embryos. According to the experiments conducted in the present study callusing and regeneration is reported best in MS supplemented with MS media containing 2,4-D=0.5 mg/L and KN=1 mg/L. There were two types of somatic embryos obtained. One was the green embryogenic callus and the other was the non-green embryogenic callus. Callusing was reported within 10-15 days from leaf explants and regeneration of the shoots started 40-50 days from callus induction (Tables 1 and 2; Fig. 1).

Artificial seed formation and germination

The mature somatic embryos from the callus were seen under high definition microscope and the Torpedo shaped mature somatic

Table 2: MS media supplemented with different concentrations of growth hormones for shoot regeneration from calli in *G. kurroo*

MS+growth hormones		Parameters for shoot formation	
KN (mg/l)	IBA (mg/l)	Days to generation of shoots	Number of shoots (mean±SE)
1	0.5	30-35	1±0.8165
1	1	29-30	1.75±0.9574
2	1	20-25	2.75±0.9574
3	1	10-12	4.75±1.708

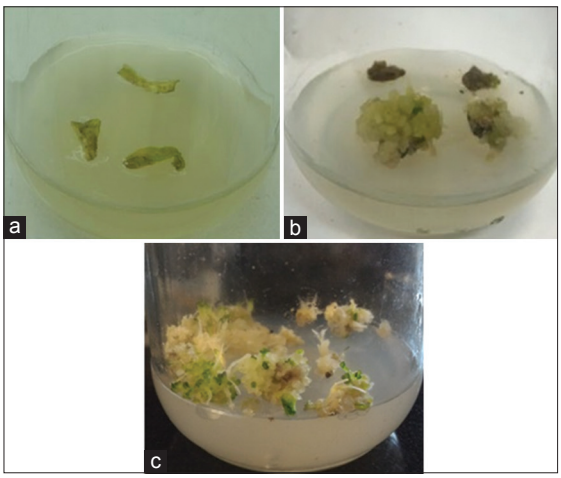

Fig. 1: Callusing and regeneration of *Gentiana kurroo*, (a) leaf explants on callusing medium, (b) callus induction within 15-20 days of explants, (c) shoots regeneration from 30-40 days of callus induction

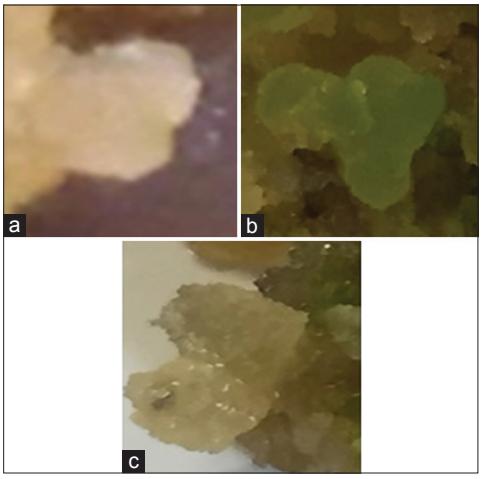

Fig. 2: (a-c) Somatic embryos under high definition microscope

Table 3: MS media supplemented with different concentration of growth hormones for germination of artificial seeds

MS+growth hormones	Parameters for seed germination			
	Days to germination	% of activated charcoal	% of germination	No of shoots (Mean±SE)
MS+BAP+GA_3 (2:1)	15-25	0.5	40-50	3±1.000
MS+BAP+IAA (1:1)	30-32	0.5	50-55	4±1.000
MS+IBA+KN+GA_3 (1:3:2)	7-10	0.5	90-95	5.66±1.528

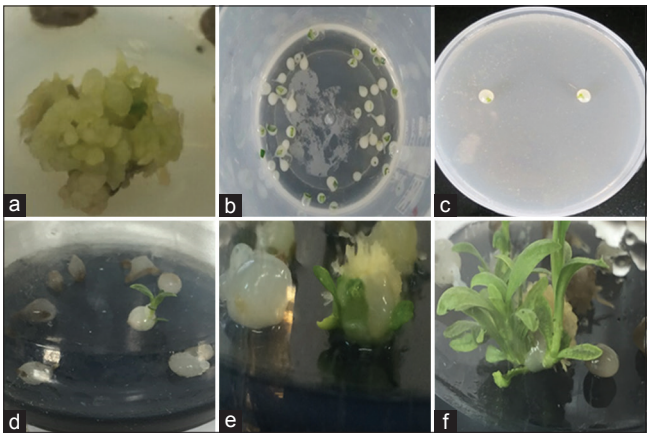

Fig. 3: Artificial seed production and germination of *G. kurroo*, (a) somatic embryo, (b) synthetic seed encapsulated with sodium alginate and complexed with calcium chloride, (c) Somatic embryo cultured on MS media for germination, (d) emergence of shoot from a germinating synthetic seeds from 10 days of culture establishment, (e) artificial seed after 1 month, (f) shoot formation after 45 days of germination

embryos were selected for encapsulation (Fig. 2). Out of the various concentrations of sodium alginate and calcium chloride solution used for encapsulation, the 3% sodium alginate, and 100 mM calcium chloride solution was obtained as optimum concentration for artificial seed production in *G. kurroo*. The germination of the above artificial seeds were obtained on IBA (1 mg/L), KN (3 mg/L), and GA_3 (2 mg/L) supplemented with 0.5% activated charcoal (Fig. 3). Interestingly, 90-95% germination was achieved in 7-10 days, which could enhance the mass propagation of *G. kurroo* in future (Table 3).

DISCUSSION

In various medicinal plants, poor seed germination has contributed toward reduced cultivation of endangered species. This might be due to various factors *viz*. unfavorable climatic conditions, low percentage seed set, seasonal dormancy, heterozygosity of seed, minute seed size, and presence of reduced endosperm [6,13]. Embryogenesis is a critical stage in the life of plants. A prevalence of studies reported the highest somatic embryogenesis on MS medium supplemented with 2,4-D in combination with 0.75 mg/L NAA (β-naphthoxyacetic acid) and 1 µM TDZ (thidiazuron) [14,15]. Further, optimum somatic embryogenesis was also observed on MS medium supplemented with 2,4-D (0.5 mg/L) and IBA (0.5 mg/L) [16-17]. Moreover, Chervathur *et al*. [18] showed the optimum somatic embryo formation on MS medium supplemented with 4 mg/L 2,4-D and 0.5 mg/L IBA. However, in the present study, decreased concentration of 2,4-D (0.5 mg/L) with KN (1 mg/L) showed enhanced somatic embryo induction and maturation. Similarly, Kumar and Chandra [19] reported highest embryogenic response on MS medium supplemented with 2,4-D (0.5 mg/L) and KN (0.5 mg/L). Vinterhalter *et al*. also reported that 2,4-D contributing toward low somatic embryo formation [20].

Despite of the enormous potential of synthetic seeds, they have still not been reported in *G. kurroo*. Generally, synthetic seeds have been prepared in sodium alginate (0-5%) and calcium chloride (75-100 mM) solution. Kumar and Chandra [19] reported somatic embryos encapsulated in 4% sodium alginate and 100 mM calcium chloride solution. Contrary, in the present study, 3% sodium alginate and 100 mM calcium chloride solution have been found to be best suited for uniform bead formation. Similar results were obtained in the studies conducted by Cheruvathur *et al*. [18] and Ghanbarali *et al*. [21]. However, Saha *et al*. [22] reported optimum gel encapsulation of shoot tips using 3% sodium alginate and 75 mM calcium chloride solution.

Further, the artificial seeds produced in this study were germinated on MS media supplemented with IBA (1mg/L), KN (3 mg/L), and GA_3 (2 mg/L). In contrast to this, sodium alginate encapsulated beads cultured on MS media supplemented with IBA (1 mg/L) and NAA (0.5 mg/L) were found to be most suitable [19]. Moreover, encapsulated somatic embryos on MS media supplemented with GA_3 have been shown to exhibit single root formation [17]. Conversely, divergent results have been observed and recorded in the present study where MS media supplemented with GA_3 has shown to exhibit multiple shoot emergence.

CONCLUSION

The overall objective of the current study was to develop an *in vitro* system for optimizing culture conditions for artificial seed formation in *G. kurroo* through somatic embryogenesis and regeneration of the entire new plant through calcium alginate beads. To the best of our knowledge, this is the first report of artificial seed production and germination of *G. kurroo*. Artificial seeds have been reported by many authors. Due to roadblocks in production of *G. kurroo* and the low seed potency in soil, emphasis on finding an alternative to cultivate the medicinal herb has to be embattled. Therefore, rapid callusing regeneration and seed viability is necessary which has been a major problem in *G. kurroo*. Callus produced was soft, very friable, compact, and globular. The developed protocol can be utilized for large scale production of seeds of related species which can be further cryopreserved. It ensures developing raw material for the germplasm conservation, developing new plant lines, limiting variability, and also providing strategy for multiplying plantlets due to low seed variability.

ACKNOWLEDGMENT

This work was supported by the Department of Biotechnology and Bio-informatics, Jaypee University of Information Technology for providing essential facilities and environment for research.

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