Optimization of culture conditions for production and germination of artificial seed in an important medicinal plant *Gentiana kurroo* Royle

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitlied **"**Optimization of culture conditions for production and germination of artificial seed in an important medicinal plant *Gentiana kurroo* Royle**"** pursued by **Payal Kotvi (123807)** in partial fulfillment for the award of degree in Masters of Biotechnology from Jaypee University Of Information and Technology, Waknaghat has been carried out under my supervision .This work has not been submitted partially or wholly to any other university or institute for the award of any degree or appreciation.

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Payal Kotvi

Date:

SUMMARY

Gentiana kurro Royle is a critically endangered medicinal herb. Its roots , rhizome are highly enriched with secondary metabolites (gentianin,gentiopicrin) which makes it highly important in the pharmaceutical sector. In the present study various parts of the plant i.e leaf, nodes, roots have been used as an explants, where we have optimized culture conditions for somatic embryogenesis. Different stages of Somatic embryo formation have been identified, out of which torpedo stage has been selected for the somatic seed formation. We have also used shoot apex as a raw material for the somatic embryogenesis..

Growing *Gentiana kurroo* through in vitro techniques reduces the time taken to produce seeds, reduces the number of field generations, and results in genetically stable plantlets, germplasm conservation and continuous supply of the medicinally important herb.

Plant tissue culture involving Somatic embryogenesis is the only alternative that could produce. Since it is not easy to generate plantlets through the seeds available due to seed viability and very slow germination rates, tissue culture techniques could be used to produced artificial seeds which can be directly sown in the soil and could be produced in bulk. They have similar morphological and biochemical characteristics to field produced plants. Artificial seeds also help to overcome the seasonal limitations which hinder the availability. They also enable conservation for years using cryopreservation, limiting the somaclonal variations and enabling germplasm conservation.

Payal Kotvi Dr. HemantSood

Date: Date:

LIST OF ABBREVIATIONS

Abbreviations	**Full forms**
KN	Kinetin
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
BAP	6- Benzylaminopurine
NAA	Napthalene acetic acid
2,4-D	2,4 dichlorphenoxyacetic acid
IAA	3- indoleacetic acid
MS	Murashige and skoog


Фотография Ольги Богдаревой

CHAPTER 1

INTRODUCTION

CHAPTER 1

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. 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. The roots are rich in bitter glycosides (gentiopicrin and gentianin), alkaloids (gentiomarin), which have immense medical and pharmaceutical importance. The root stock is valued as bitter tonic, antiperiodic, expectorant, blood purifier, treating urinary infections, digestive disorders and many more.

Gentiana kurroo has high medicinal potential which enables its exploitation leading to its extinction therefore it is legally protected by law and cannot be exported. It can be propagated through seeds, stem cuttings or by root divisions. Seeds requires cool, temperate climate with well-drained soil. It requires plenty of water therefore it cannot be grown in any part of India on large scale. Its stringent growth conditions makes it seed viability very less in the soil, which leads to the abortion of the seed as soon as it is sown in the soil. Hence it is necessary to find an alternative to cultivate the medicinal herb.

Morphological Characteristics

Leaves

Gentiana kurroo is a perennial herb, comprising of an aerial part which is mostly composed of radical leaves which occur in rosette. Radical leaves are long, narrow, leathery in texture and basally joined in pair to form a common sheath. They are deep green in colour and remains throughout the life cycle. The shoots are represented by flowering branches which bear cauline leaves. Cauline leave are narrow linear and in pairs united at base forming a tube around flowering shoot. They are green in colour and fade its colour with maturity.

Flowers

Flowers are large, infundibuliform, complete, hermaphrodite and deep violet blue from outside and whitish from inside. Sepals are five, gamosepalous, more or less equal in size.

Fruits

Fruit is a capsule and dehisces longitudinally.

Seeds

Seeds should be stored at low temperature (5ºC) after harvesting to avoid losing their viability. Seeds require well drained soil and low temperature for cultivation.

Propagation of *Gentiana kurroo*

Gentiana kurroo can be propagated through seeds, rhizome cuttings, microproliferation of shoot nodal segments and somatic embryogenesis.

1. **Propagation through seeds**

Ideal time for sowing seeds is June. The seeds are harvested in the first fort night of November and stored at low temperature, seeds more than one year old lose viability and do not germinate. The germination starts sixth day onwards after sowing and continues upto 28 days. Despite of numerous seed germination, seedling establishment is very low.

2. **Propagation through rhizome cutting**

Rhizomes of 5-8 cm diameter are longitudinally split into two parts and the cutting are treated with the root hormones (IAA or IBA) and planted in raised bed. IBA is more preferred and give better results.

3. **Micropropagation**

- **Clonal propagation through shoot proliferation**

Auxiliary branching is a simple procedure for propagation of *Gentiana kurroo.* Shoot tips and nodal stems of mature plants are taken and treated with detergent followed by distilled water, which are thereafter treated with surface disinfectant (0.1% Hgcl2) for 10 min and rinsed with sterile distilled water. The shoot and nodal explants are then supplemented with the MS media with 6% sucrose (pH should be 5.8). Clonal plantlets thus produced are genetically stable and invitro propagation can be done on commercial basis.

- **Somatic embryogenesis**

Seedlings, callus tissue cell suspension or solid media and isolated protoplast serve for high potential of somatic embryo formation and shoot regeneration.

Hence due to the low potency of seeds in the soil and its stringent need of environment for the cultivation, enhancement in the methods for its cultivation is required to meet the medicinal requirements with the increasing demands.

Pharmacological importance

Root extracts of G. kurroo have been used by diverse cultures since ancient times as a bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, anti-inflammatory, antibacterial, anthelmintic, blood purifier and carminative . It is also reported to be used for curing skin diseases (leucoderma), bronchial asthma, urinary infections, inflammations, leprosy, helminthiosis, dyspepsia, exhaustion from chronic diseases and in all cases of weakness of digestive system and loss of appetite. The anti-inflammatory properties of G. kurroo flower tops extract. Air-dried flower tops of G. kurroo have been tested for the anti-inflammatory activity. The result of the studies have indicated that the flower tops of G. kurroo possess a maximum anti-inflammatory effect of 68.58% against the inflammation induced by carrageenan in acute phase which significantly inhibited paw edema. The two bitter compounds i.e. Gentianin and Amaroswerin from the root extracts of G. kurroo. He found that Gentianin possessed anti-inflammatory, analgesic, anticonvulsant, hypotensive, antipsychotic, sedative, diuretic, anti-malarial and anti-amoebic properties, whereas, Amaroswerin possessed gastro-protective properties. The phytochemical analysis have revealed the presence of tannins, alkaloids, saponins, cardiac glycosides, terpenes, flavonoids, phenolics, and carbohydrates. The antioxidant activity was evaluated by DPPH, hydroxyl radical, lipid peroxidation and protective oxidative DNA damage assays. The anti-proliferative activity has been determined by the sulphorhodamine B (SRB) assay against lung carcinoma cell line . The phytochemicals identified by liquid chromatography–tandem mass spectrometry were found to be iridoid glucosides (iridoids and secoiridoids), xanthones and flavonoids. Loganic acid, Sweroside, Swertiamarin, Gentiopicroside, Gentisin .From these studies it has been concluded that this plant can be used as an alternative in the treatment of autoimmune diseases like arthritis. The antioxidant and antibacterial activity of methanolic extract of leaves and roots of G. kurroo have been revealed. The extracts of G. kurroo roots and leaves possessed antibacterial activity against both Gram positive and Gram negative bacteria. The results of their study indicated that the antioxidant and antibacterial activity of the root extract was found to be comparatively higher than that of the leaf extract which is in accordance with the total phenolic and flavonoid content of the two extracts.

IMPORTANCE OF THE PROJECT

Gentiana kurroo Royle, a critically endangered bitter herb holds high medicinal potential due to which it is highly exploited. Hence there is an immense need to plant the medicinal herb. The low potency of the seeds in the soil, enables low availability of the herb, hence alternative method to cultivate the herb is needed. Production of artificial seeds of *Gentiana kurroo* through somatic embryogenesis helps in regeneration of the plantlet through calcium alginate beads in the invitro conditions.

These artificial seeds will enable in increasing the germination potency of the medicinal herb through somatic embryogenesis and yield an entire plant. Artificial seed production will enable the storage of seeds for a longer time, reduce variability and germplasm conservation. This will lead to the availability of the herb throughout the year overcoming its seasonal limitations.

OBJECTIVES OF THE STUDY

The objectives of the present study are as following :

- Optimization of culture conditions for inducing indirect somatic embryogenesis for *Gentiana kurroo.*

- Optimization of culture conditions for production of artificial seeds and germination.

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Seed germination studies in Gentiana kurroo Royle-An endangered medicinal herb

Gentiana kurroo Royle is a critically endangered medicinal plant of north-western Himalayas. The roots and rhizome of this plant are valued for their bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, anthelmintic, blood purifying and carminative properties. Mature capsules of G. kurroo fruits were collected and observations on morphological parameters were recorded. Seed germination under laboratory conditions commenced from sixth day after sowing and continued up to 28 days. The total germination recorded was 68.0 % of which the maximum germination (25.0 %) was observed in the second week and the minimum germination of 8.0 % occurred in the first week of sowing under laboratory conditions. Seeds sown achieved a peak value of 2.62, mean daily germination of 2.43 %, and germination value of 6.36. Seeds of G. kurroo are very minute and hence it is quite difficult to collect seeds in quantities sufficient for planting. Moreover though the plants set numerous seeds, the seedling establishment is also very poor. Premature anther development is the reason behind this .Seeds should be stored at low temperature (below 5º C) after harvesting otherwise there is considerable reduction in germination percentage. Seeds more than one year old lose viability and do not germinate .Seed germination studies in Gentiana kurroo Royle. June is the ideal time for seed sowing. Furthermore, all the seeds may not reach the level of maturity at the same time causing difficulty in getting seedling stocks for transplanting. Despite a valuable and threatened medicinal plant, G. kurroo is not cultivated on a large scale in its native habitat.

Macroproliferation of *Gentiana kurroo* Royle

Gentiana kurroo is a small perennial herb, with a stout rhizome bearing decumbent flowering stems, commonly found in North–Western Himalayas, at altitudes of 5,000−11,000 ft. The dried rhizome and roots of *G. kurroo*, described under the name Indian Gentian are exported from the hills. The plant does not seem to have been cultivated on a large scale anywhere in India. The flowers are hermaphrodite and are pollinated by bumblebees and butterflies. It takes some years to produce flowers and considerable time elapses before the roots reach marketable size. Flowering starts from the third week of August and continues till the first week of November, with the peak between 15 September and 20 October and on an average, a plant produces 20 flowers. The flowers close during night and under low light, and re-open when the sun shines brightly. The first fortnight of November is the ideal time for seed harvest after which the capsules open up, scattering the seeds. About 70−75 % of seeds germinate and June is the ideal month.

Gentiana kurroo Royle – A critically endangered bitter herb

Gentiana kurroo Royle a member of this family is a critically endangered medicinal herb of western and northwestern Himalayan biomes. The bitter glycosides (gentiopicrin & gentianin), alkaloids (gentiomarin) etc. The root stock is valued as bitter tonic. It is also used for curing skin diseases, urinary infection.

Genotype and plant growth regulators –dependent response of somatic embryogenesis from *Gentiana* leaf plants.

Gentiana kurroo Royle *Gentiana crutiata* (L), *Gentiana tibetica, Gentiana lutia* and *Gentiana pannonica* leaves derived from axenic shoot culture were used as explants. For culture initiation, leaves from the first and the seconds whorls from the apical dome wre dissected and cultured on MS media. Basal medium supplemented with three different auxins ; 2,4 –D, NAA, dicamba in concentration of 0.5,1,or 2 mg\l. Five different cytokinins Zeatin , Kinetin, TDZ ,BAP were used. The cytokinin concentrations used were dependent on the type of cytokinin and varied between 0.25 & 3 mg\l. Frequency of Embryogenesis was highest for Gentiana kurroo (54.7%) and dependent on PGRs. This gentian was the only species showing morphogenic capabilities on media supplemented with all applied combinations of PGRs while none of the 189 induction media permutations stimulated somatic embryogenesis from *Gentiana lutia explants. Gentiana crutiata* (L), *Gentiana tibetica* both produced 6.6 somatic embryos per explants .*While Gentiana pannonica & gentiana kurroo* regenerated at 15.7and 14.2 somatic embryos per explant respectively. Optimum regeneration was achieved in the presence of NAA combined with BAP or TDZ.

Morphogenic capabilities of *Gentiana kurroo (*Royle *)* seedling and leaf explant.

Experiments have been carried out on seedling and primary leaf explants of Gentiana kurroo Royle. Morphogenic capacities of cotyledons ,hypocotyles, and roots were investigated using MS medium supplemented with 4.64 uM kinetin & 2.26,4.52 or 9.04 uM 2,4-D.Percentage of callusing explants for each combination was inversely proportional to numbers of obtained embryos. Cotyledons show the highest number of morphogenic capabilities. The presence of NAA with BAP and dicamba with Zeatin produced greatest number of differentiated somatic embryos. The best embryo conversion into germlines was obtained on MS media containing 4.64 um kinetin ,1.44 um GA3 & 2,68 um NAA or half MS.

In *vitro* propagation of Gentiana kurroo – an indigenous threatened plant of medicinal importance

Shoot multiplication of *Gentiana kurroo* Royle , a threatened medicinal plant species, was achieved in vitro using shoot tips and nodal segments as explants. Shoot multiplication was achieved on MS containing 8.9Um benzyladenine and1.1 Um NAA. Rooting was accomplished successfully in excised shoots grown on MS basal medium containing 6% sucrose**.**

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 SELECTION OF PLANT MATERIAL AND ESTABLISHMENT OF AXENIC CULTURE-

The *Gentiana kurroo* plant cuttings 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.

3.2 SURFACESTERILIZATION OF EXPLANTS-

Different explants such as leaves, roots, nodes etc were cut into small segments using autoclaved blades and were washed with sterile water to remove dirt and debris and were surface sterilized in 0.5% Bavistin and 0.1 % Mercuric Chloride followed by 4-5 washings with sterile water. Both bavistin and mercuric chloride are known to be antibacterial and antifungal.

3.3 SELECTION AND CULTURE OF EXPLANTS-

Young leaves were taken from *in vitro* grown plantlets of *Gentiana kurroo* and cultured on MS medium supplemented with different concentrations and combinations of growth hormones such as 2,4-D, IAA, NAA and IBA with sucrose 3% (w/v). The pH of the media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The cultures were incubated at 16 h light /8 h dark cycle at 25 ± 2°C in plant tissue culture chamber. Data were recorded on days to initiation of callus formation, per cent explants forming calli, days to compete callus formation. The data were statistically analyzed for test of significance. The cultures were sub-cultured after every 15 – 20 days on callus induction media for 2 months so as to obtain good growth.

3.4 CALLUS INDUCTION

Leaf explants given were were cultured on MS media supplemented with different concentration of auxin and cytokinin such as 2,4-D, KN with 3% of sucrose. The pH of the media was adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and finally agar-agar 0.8% (w/v) was added as a gelling agent. The cultures were incubated at 16 h light /8 h dark cycle at 25 ± 2°C in plant tissue culture chamber. Leaf explants were given incisions and cultured on the MS media supplemented with growth hormones to induce callus formation (Table 3.4). The cultures were established at 25°C ± 1°C under dark conditions. The data was recorded for percentage of callus formation and days to callusing. (Table 4.1)

Table 3.4. **MS media supplemented with different concentrations of growth hormones for callus induction**

MS MEDIA	IBA(mg/l)	KN(mg/l)	BA(mg/l)	NAA(mg/l)	TDZ(mg/l)	2,4D(mg/l)
MS1	3	1	-	-	-	-
MS2	-	-	3	2	-	-
MS3	-	-	-	-	2	1
MS4	-	-	2	1	3	2
MS5	-	1	-	-	-	0.5

3.5 SOMATIC SEED FORMATION

Embryogenic green callus containing the pre-embryonic determined cells(PEDC) were visualized under high definition microscope, where different stages of somatic embryos were identified. Torpedo shaped somatic embryos were selected for the encapsulation in the sodium alginate and calcium chloride solution. Synthetic seeds were successfully produced by having 3% Sodium alginate by using 100mM solution of chilled calcium chloride solution .fragments of callus and shoot tips were encapsulated using Calcium alginate beads. Culture conditions for artificial seed production was successfully optimized(temperature 25ºC ± 1°C and 16 hrs light ,8hrs dark photoperiod) on the MS media.The seeds were thereafter stored at 4ºC.

Table 3.5. MS media supplemented with different concentrations of growth hormones for somatic seed formation.

Components	Required amount	Final volume
Sodium alginate	1% , 2%, 3%	100ml/L
Calcium chloride	100mM	100ml/L

3.6 ARTIFICAIL SEED PRODUCTION

Different stages of somatic embryos have been identified under high definition microscope out of which torpedo shaped embryos have been selected for the somatic seed seed formation. The somatic embryos have been encapsulated by using calcium alginate beads and cultured on MS media. The produced artificial seeds were cultured in different media combinations supplemented with activated charcoal (0.5%).the cultures were incubated under the same culture conditions as mentioned above. The data was recorded days to germination and percentage of germination.(Table

The Artificial seed formation is requires the following components:

Physical requirements :

- Sterile beakers
- Droper
- Measuring cylinder
- Glass rod

Chemical requirements:

- Sodium alginate
- Chilled Calcium chloride
- Autoclaved water

Steps involved in the artificial seed formation are as follows:

1. 3 grams of sodium alginate was dissolved in 100ml of luke warm water.
2. 100 mM of Calcium chloride was dissolved in the 100 ml of autoclaved water and kept at -180 to become chilled.
3. Green callus and shoot apex were taken as raw material and was added to the sodium alginate solution.
4. The sodium alginate solution containing the callus and shoot apex was added drop wise to the chilled calcium chloride solution.
5. The beads encapsulating the callus and shoot apex were taken out from the calcium chloride solution and kept on the MS media supplemented with different hormones.
6. The cultured were incubated at temperature 37ºC and 16 hrs light ,8hrs dark photoperiod.
7. The seeds were stored at 4°C.

3.7 MEDIA FOR CALLUS REGENERATION INTO SHOOTS

Embryogenic green callus of *Gentiana kurroo* was taken and cultured on the regeneration media containing different concentration of auxin and cytokinin and observed for the shoot regeneration under the optimized conditions (25° C± 1 ° C temperature, 16 hours light, 8 hours dark photoperiod).

Table 3.7. MS media supplemented with different concentrations of growth hormones for callus regeneration into shoots.

MS MEDIA	IBA(mg/l)	KN(mg/l)
MS 1	2	3
MS 2	1	3

3.8 MEDIA FOR DIRECT REGENERATION (DIRECT SHOOTING)

The shoot apex of *Gentiana kurroo* were used as an explants for direct shoot formation and their further multiplication is carried out in the below mentioned media acquired from the callus regeneration into shoots were incised and cultured *in vitro* on the regeneration media containing MS media supplemented with different growth hormones. The cultures were expected to show increase in shoot length when kept under optimized conditions (25° C ± 1 ° C temperature, 16 hours light, 8 hours dark photoperiod).(Table 3.8)

Table 3.8.MS media supplemented with different concentrations of growth hormones for Direct shoot regeneration of *Gentiana kurroo.*

MS MEDIA	BA(mg/l)	KN(mg/l)	GA3(mg/l)
MS 1	-	3	2
MS 2	0.5	3	1
MS 3	1	3	1
MS 4	1	3	2

3.9 SOMATIC SEED GERMINATION

Encapsulated somatic seeds were cultured on the regeneration media containing MS media supplemented with growth hormones such as IBA(1), KN(3),GA3(2) mg\l and 0.5% activated charcoal. The cultures were kept at 25° C ± 1 ° C temperature, 16 hours light, 8 hours dark photoperiod to initiate germination from the somatic seeds. (Table 3.8) Researchers have reported successful somatic seed germination (Kumar vijay Chandra) in various other plant species where they have utilized the potential of MS media supplemented with growth hormones such as IBA alone and in combination with KN and NAA.(Table 3.9)

Table 3.9.MS media supplemented with different concentrations of growth hormones for somatic seed germination of *Gentiana kurroo.*

MS MEDIA	BA(mg/l)	KN(mg/l)	GA3(mg/l)	% OF ACTIVATED CHARCOAL
MS 1	-	3	2	0.5
MS 2	0.5	3	1	0.5
MS 3	1	3	1	0.5
MS 4	1	3	2	0.5

RESULT & DISCUSSION

CHAPTER 4

CHAPTER 4

RESULTS

4.1 ESTABLISHMENT OF CALLUS CULTURES-

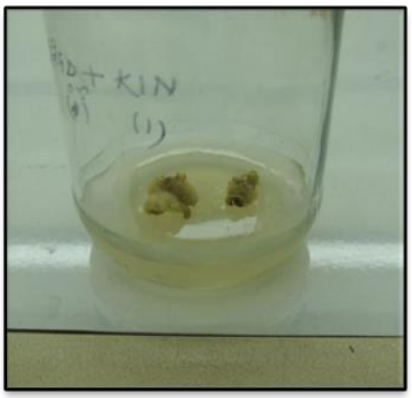
Callus cultures were initiated from different explants such as leaf discs, nodal and root segments of *Gentiana kurroo* on MS salts supplemented with different concentrations of growth hormones as shown in Table 3.4. Callus formation initiated in all explants within 10 days at the cut surfaces in all test media combinations (Figure 1). The explants were transformed into complete callus mass within 10-12 weeks of culture. Researchers (Agnieszka & Rybczyn ski) have also reported the callus induction on MS media supplemented with growth hormones (NAA, BAP, KN, Zeatin). Overall, in this study MS medium supplemented with 2,4-D(0.5 mg/l) + KN (1mg/l) was found to be the best for callus induction with frequencies of 98% (Fig 4.1). Callus cultures derived from leaf explant were sub-cultured for 3-4 weeks so as to proliferate the callion suitable callus induction media. The data was recorded days to callusing and percentage of callusing (Figure4.1)

Table 4.1 MS media for callus induction in Gentiana kurroo

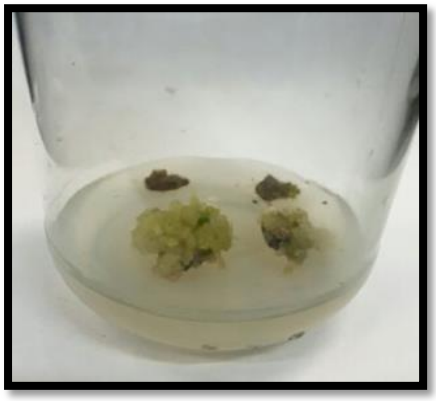
MEDIA COMPOSITION	DAYS OF CALLUSING	% OF CALLUSING
MS +TDZ+2,4 D(2:1)	1 WEEK- 2 WEEKS	NOT RESPONDING
2,4 D+ BAA+ NAA(2:1:3)	1 WEEK- 2 WEEKS	50- 70%
BA + NAA (3:2)	1 WEEK-2 WEEKS	50-80%
2,4-D+ KN(0.5:1)	10 DAYS-2 WEEKS	95%

CALLUS INDUCTION

(a)



(b)



(c)

(d)

Figure4.1(a)Incised leaf explants cultured on the MS media containing growth hormones. (b) Callus induction seen within 15-20 days of culture establishment. (c) Increase in callus size within 30-35 day of culture establishment. (d) Green embryogenic callus formation within 40-50 days.

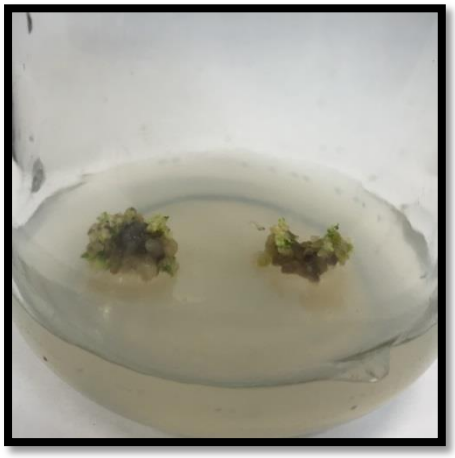
4.2 REGENERATION OF CALLUS INTO SHOOTS-

Proliferating callus cultures or parts thereof were subcultured onto regeneration media containing MS salts supplemented with different concentrations and combinations of BA, GA_3, BAP, KN and IAA Media containing BA and KN induced shoot primordia formation in the form of green nodular structures. Various report where researchers(Behera & Raina) have also shown growth hormones such as NAA, BAP, KN in different concentrations showing response in shoot regeneration from callus on other species. The MS supplemented with BA (1 mg/l) + KN (3 mg/l) + GA_3 (2 mg/l)was found to be the best for regeneration (Fig 4.2).The shoots regenerated from callus cultures were allowed to grow on regeneration medium for better growth. The data was recorded days to regeneration and percentage of regeneration.(Figure4.2)

Table 4.2 Media for callus regeneration into shoot in Gentiana kurroo

MEDIA COMPOSITION	DAYS TO REGENERATION	% OF REGENERATION
MS+BAP+GA_3(2:1)	30 – 32 days	50-60%
MS+BAP+IAA(1:1)	30-32 days	50-70%
MS+BA+KN+ GA_3 (1:3:2)	28- 32 days	100%

CALLUS REGENERATION INTO SHOOTS



(a)

(b)

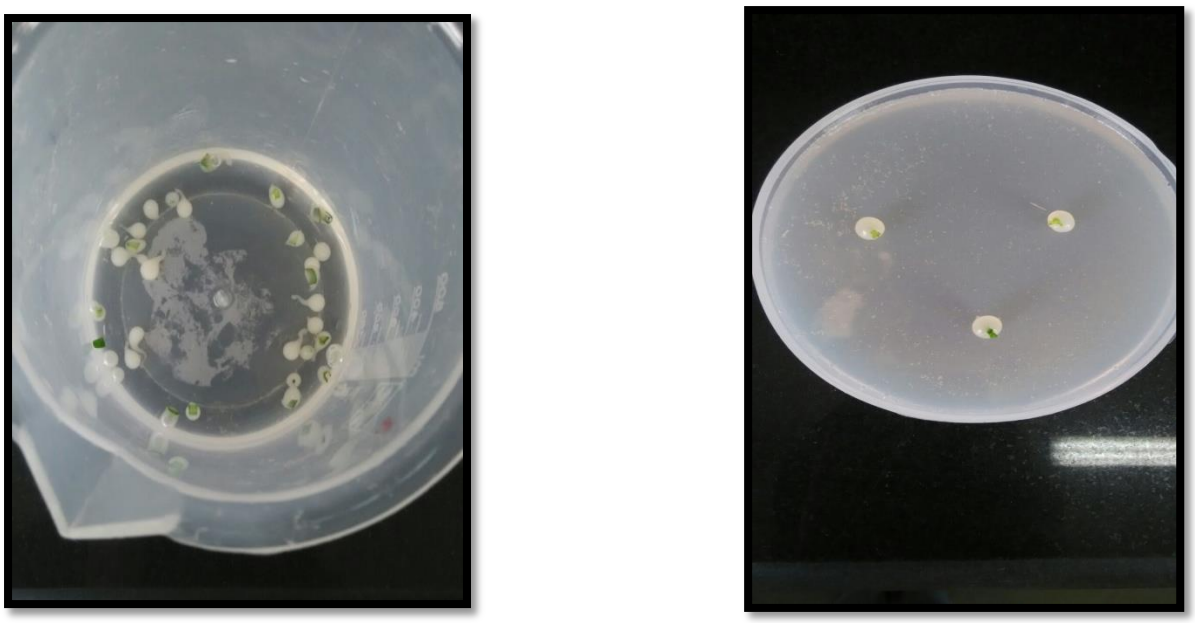
(c)

(d)

Figure 4.2 (a) Leaf explant inducing callus formation when cultured on MS media supplemented with growth hormones. Callus induction was seen within 15-20 days of culturing. (b) Shoot regeneration from callus (30-35 days). (c) Increase in shoot formation was seen within 40-50 days of culture establishment. (d) Shoot grown into full length after 1 month of shoot formation.

4.3 SOMATIC SEED FORMATION

The selected somatic embryos were encapsulated with sodium alginate (2%, 3%,4%) and calcium chloride (80mM, 100mM) in various concentrations. Various experiments have been conducted on many species where the researchers (V. Kumar and S. Chandra) have generated data on the successful production of artificial seeds on MS media with different concentrations of sodium alginate (2%, 3%, 4%) and calcium chloride. In the present study the best results were observed with 3% sodium alginate and 100 Mm calcium chloride. The somatic seeds were kept on MS media with different concentrations of growth hormones as shown in table 3.5 .The cultures were kept at 25° C ± 1 ° C temperature, 16 hours light, 8 hours dark for optimizing the culture conditions.



(a) **(b)**

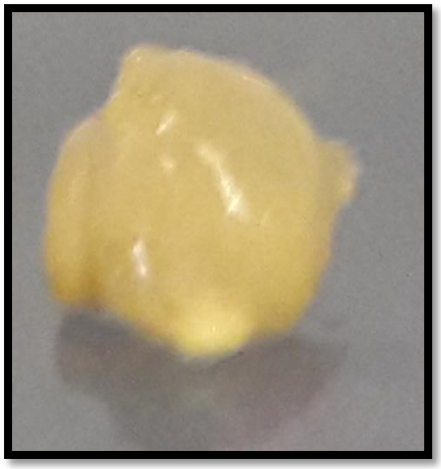
Figure 4.3 (a) Calcium alginate beads encapsulating somatic embryo of *Gentiana kurroo.* (b) Artificial seeds showing germination when cultures on the MS media supplemented with growth hormones.

4.4 SOMATIC SEED GERMINATION

Somatic embryos when encapsulated with different concentrations of sodium alginate and calcium chloride ,forming the calcium alginate beads were cultured on MS media containing various concentrations of growth hormones. Out of the the different concentrations germination was effectively seen on the MS media containing IBA(1), KN(3), GA3(2) mg\l and 0.5 % activated charcoal as mentioned in . Sodium alginate (3%) gave the best results when in reaction with 100Mm calcium chloride as mentioned in figure 4.3. The somatic seeds were kept under temperature 25 ± 1°C,16 hrs light, 8 hrs dark photoperiod for optimum germination. The data has been recorded as days to germination and were 10-15 days. (Table 4.4)

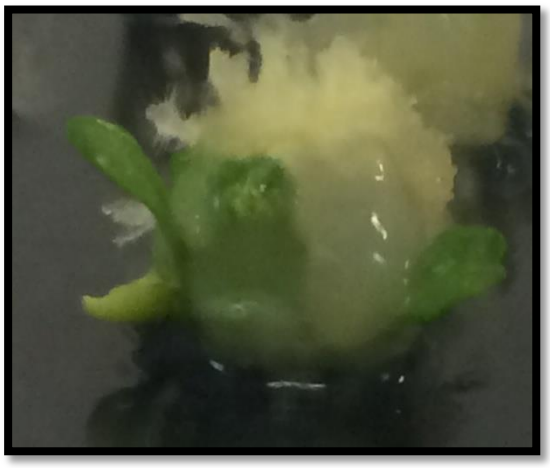
Table 4.4 Media for callus regeneration into shoot in Gentiana kurroo

MEDIA COMPOSITION	DAYS TO GERMINATION	% OF ACTIVATED CHARCOAL	% OF GERMIRATION
MS+BAP+GA_3(2:1)	15-25 days	0.5	40-50
MS+BAP+IAA(1:1)	30-32 days	0.5	50-55
MS+BA+KN+ GA_3 (1:3:2)	7-10 days	0.5	90-95



(a)

(b)



(c)

Figure 4.4 (a) Somatic seeds kept for germination. (b) Somatic seed showing germination on the media containing activated charcoal. Days to germination 10-15 days. (c) Somatic seeds days to germination 20-25 days.

CHAPTER 5

CONCLUSION

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 .Due to roadblocks in production of *G. kurroo* due to the low seed potency in the soil, emphasis on finding an alternative to cultivate the medicinal herb has to be targeted. 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, globular. For culture initiation Basal medium supplemented with three different auxins 2,4 –D, KN in concentration of 0.5,1mg\l. Different concentrations of cytokinins and auxin such as 2,4-D, Kinetin, TDZ ,BAP were used to initiate callus induction. Optimum regeneration achieved in the presence of 2,4-D combined with KN and TDZ has also been reported to initiate callus induction. Interesting fact is that callus induction was seen on MS medium with 2,4D (0.5mg/l) and KN(1mg/l) .

Other interesting fact is that while growing callus on 2,4-D + KN media darkening of callus occurred within 30-35 days. The callus was cultured on the MS media containing IBA and KN. Interesting fact is that dark brown callus were changed to green. Shooting was induced after 30-40 days of callus sub cultured on regeneration media with 80% frequency, which is quiet rapid . Induced shoots length was reached upto after 20 days and shoots multiplied up.

MS medium supplemented with different growth regulators such as TDZ+ BAP+ IBA both singly and in combination, has been reported. Induced mass of adventitious shoot buds with callus was removed gently and the mass of shoot buds was cut, which were subsequently cultured on multiplication medium containing MS medium with IBA + KN and hence shoot regeneration has been reported with MS containing IBA+KN+GA3.The use of explants as leaf discs cultured in basal MS medium failed to give any response and turned yellow subsequently whereas our results were best using leaf discs as explants.

Encapsulation of callus and shoots tips of *G.kurroo* in calcium alginate beads has been reported by Islam & Bari ,2012 .In the current experiment easy to handle, uniform beads were formed with Sodium alginate (3%)and chilled Calcium chloride(100 Mm).

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, limiting variability and also providing stratergy for multiplying plantlets due to low seed viability.

FUTURE PROSPECTS

1. Storage of artificial seeds and plant recovery.

2. Studying the molecular aspects for secondary metabolite production in *Gentiana kurroo*.

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ANNEXURE

ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH(mg/l)	STOCK(g/l)	FINAL VOLUME
A-10X	KNO_3	1900	19	
	$MgSO_4.7H_20$	370	3.7	100 ml/l
	KH_2PO_4	170	1.70	
B-20X	NH_4NO_3	1650	33	150 ml/l
C-100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D-100X	Na_2EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	27.85	2.28	
E-100X	KI	0.83	0.083	100 ml/l
F-100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.3	2.23	
	$Na_2MbO_4.2H_2O$	0.25	0.025	
G-100X	m-INOSITOL	100	10	10 ml/l
	GLYCINE	2	0.2	
H-100X	PYRIDOXINE-HCL	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	