In vitro propagation and secondary metabolite production in *Gentiana kurroo*

Enrollment numbers : 091551 & 091554

Name of Students : Preeti Kaura & Leena chopra

Supervisor : Dr. Hemant Sood

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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 titled "***IN VITRO* PROPAGATION AND SECONDARY METABOLITE PRODUCTION IN *GENTIANA KUROO***" submitted by "**PREETI KAURA AND LEENA CHOPRA**" in partial fulfillment for the award of degree of B.Tech of Jaypee University of Information 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 this or any other degree or diploma.

Signature of Supervisor .....

Name of Supervisor Dr. Hemant Sood

Designation Senior Lecturer

Date 23 May, 2013

ACKNOWLEDGEMENT

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We would also like to thank Mrs. Mamta Mishra, Mr. Baleshwar, Mr. Kamlesh and Mr. Ravikant for their assistance in the lab.

Preeti Kaura and Leena Chopra

SUMMARY

An efficient and rapid protocol for mass propagation of endangered medicinal herb(*Gentiana kurroo*) under in vitro conditions has been developed. The mother plants were obtained from HFRI. The motive of this study is to optimize the tissue culture conditions for its propagation and optimize the cell culture conditions for the production of medicinally important metabolites.

Among the various strengths of growth regulators tested best results were recorded when shoot apices cultured on MS basal solid media supplemented with IBA (3 mg/L) and KN (1 mg/L) for shoot proliferation. Results were less significant when different combination and concentration of auxins and cytokinins were tried along with GA3. The rooting was also optimized using auxins alone. The root induction per explants was maximum in the medium containing MS basal medium supplemented with 4 mg/L IBA. The rooted plantlets were hardened in polycups containing sterile soil, sand and vermiculite in equal ratio and were successfully acclimatized and established in soil with 75 % survival rate. Growth was best seen at 25±1°C.Continuously shaking liquid cultures with MS basal media supplemented with 1 mg/L IBA and 4 mg/L KN resulted in almost a four fold growth. Another objective was to estimate the metabolite content in this plant by using HPLC. The amount of Gentiopicroside content in the field grown mother plant(a yr old) was 3.5% whereas when *in vitro* raised plants were analysed ,the amount of accumulation of was 0.02% and Swertiamarin was found 0.11% in a month old plant. Further exploration of other cell culture conditions will help in improving the metabolite content which will improve the in vitro grown plants status for commercialization in biotech and pharmaceutical companies.

Preeti Kaura

Date: 23 May, 2013

Dr. Hemant Sood

Date: 23/5/13

Leena Chopra

Date: 23 May, 2013

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List of abbreviations:

Abbreviations	Full forms
KN	Kinetin
IBA	Indol-3- butyric acid
GA3	Gibberellic acid
MS	Murashige and Skoog
CAN	Acetonitrile
HPLC	High Pressure Liquid Chromatography

INTRODUCTION

Introduction:

India has a rich biological diversity due to its varied climatic, altitudinal variations and ecological habitats. There have been increasing rates of threats of depletion to these biological resources due to immense biotic and abiotic stresses. Indiscriminate collection of plants for their medicinal, ornamental, perfumery uses, etc. and habitat loss and degradation are potential causes of threats. Conventionally, there are two methods of conservation: *in situ* and *ex situ* conservation, both are complementary to each other. *In situ* methods allow conservation to occur with ongoing natural evolutionary processes, *ex situ* conservation via *in vitro* propagation also acts as a viable alternative for increase and conservation of populations of existing bioresources in the wild and to meet the commercial requirements.

India is one of the twelve mega diversity countries of the world with a rich diversity of biotic resources (Bapat *et al.*, 2008). Out of 34 hotspots recognised, India has two major hotspots - the Eastern Himalayas and the Western Ghats. India harbours about 47 000 species of plants of which 17 000 are angiosperms (Bapat *et al.*, 2008). A total of 560 plant species of India have been included in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened species, out of which 247 species are in the threatened category.

IUCN recognises the following categories: extinct, extinct in the wild, critically endangered, endangered, vulnerable, near threatened, least concern, data deficient and not evaluated. Critically endangered, endangered and vulnerable together form the threatened category. Each of these threatened categories can be deduced on any of the five criteria that reflect extinction risk:

1. Declining population (past or projected): this includes species with high harvests, especially in destructive fashion.
2. Narrow distribution, fragmentation and decline or fluctuation: several endemic species *prima facie* appear to be natural candidate for qualifying as threatened as per this criterion.
3. Small population size and decline: absolute population number low and rate of decline high.
4. Very small population or very restricted distribution: absolute population numbers extremely meagre.
5. Quantitative analysis of probability of extinction: simulations using deterministic

and stochastic population models.

Himalaya is known as an abode of medicinal plants which provides a variety of habitats for the luxuriant growth of potential medicinal plant species. Many of these are used by locals in remote and inaccessible areas. On account of the hilly and inaccessible terrain, the local populace has been utilizing plant resources for their sustenance since early times. Some plants are used as fuel, some prove helpful in providing raw food material, and vegetables, fruit and some are used as curatives for various ailments. Continuous overexploitation, revival of the use of herbal drugs and degradation of the habitats has brought medicinal plants on priority for conservation.
Northwest Himalaya is a distinct Himalayan region with a characteristic climate, geology and flora.

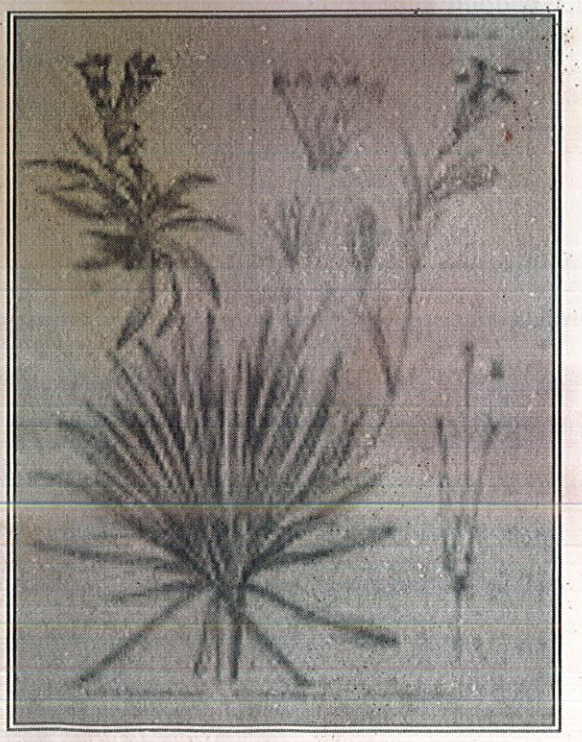
Medicinal and aromatic plants play a significant role in the life of people and are present in innumerable forms. In Indian traditions, all the plants in this earth are considered as medicinal(Satyabrata Maiti, 2001) Plants are an important source of medicines and play a key role in world health (Constabel, 1990). Almost all cultures from ancient times to today have used plants as medicine. Today medicinal plants are important to the global economy (Srivastava et al., 1995), as approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa, 1993). Generally, herbal preparations are produced from field-grown plants and are susceptible to infestation by bacteria, fungi, and insects that can alter the medicinal content of the preparations (Murch et al., 2000). It is difficult to ensure the quality control as the medicinal preparations are multi-herb preparations and it is difficult to identify and quantify the active constituents (Wen, 2000). Also, there is significant evidence to show that the supply of plants for traditional medicines is failing to satisfy the demand (Cunningham, 1993). An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of in vitro systems for the production of medicinal plants and their extracts.

The *in vitro*-propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Miura et al., 1987). In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang et al., 1992, 1994).

Gentianaceae is represented by more than 90 genera and 1000 species. The family is widely distributed, but more diverse in temperate and subtropical regions. The genus *Gentiana* (gentian) contains about 300 species in the world (Judd *et al.*, 1999). In India, the family is represented by 16 genera and approximately 145 species; while the genus *Gentiana* by about 62 species (Sunita & Bhattacharyya, 1982). In Kashmir Himalaya, a biotic province of the hotspot Himalaya (Mittermeier, *et al.*, 2005), the family is represented by 6 genera and 55 species. The genus *Gentiana* is believed to have high diversity in this region with 35 species. Out of these, 31 reach alpine / sub-alpine levels (Dhar & Kachroo, 1983)

From the Kashmir Himalaya, Coventry (1927) first reported *G. kurroo* from the Pahalgam area at an altitude of 1850-2000 m (a.s.l). Royle (1835) described a new plant species - *Gentiana kurroo*, on the basis of specimens collected from Kuerkoolee, Mussooree, Budraj, and Shimla areas of the northwestern Himalayas. During the last half century, despite extensive floristic exploration of the region there has been no authentic report of the collection of *G. kurroo*, even from the localities where it was previously collected.

Taxonomic description:



Gentiana kurroo Royle, *Illus. Bot. Himal.*278, 1835; Clarke in *Fl. Brit. Ind.* IV: 117. 1883; Coventry, *Wild. Fls. Kashm.* III: 81, Pl. XLI, 1927; Bla□er, *Beaut. Fls. Kashm.* II: 37, Pl. 41, 1927; Javeid, *Fl. Srinagar* I: 544, 1970; Stewart, *Ann. Cat. Vas. Pl. W. Pak. & Kashm.* 557:1972. *Pneumonthe kurroo* (Royle) G. Don, *Phil. Mag.* 75, 1836; *Gentianodes kurroo* (Royle) Omer, Ali & Qaiser, *Pak. J. Bot.* 20 : 16. 1988.

Perennial herb, with thick, stout rootstock. Flowering stems ascending to semi-erect, one to many from rootstock, simple, purplish, 30-45 cm high, 1-5 flowered. Leaves radical as well as cauline; radical ones rosulate, linear to oblong-oblanceolate, 5-10 X 0.5-1.5 cm, entire-crenate, connate at base, slightly reflexed at margins, single veined, acute; cauline leaves, opposite decussate, smaller, in pairs, linear-oblong, 2.5 X 0.3-0.6cm, entire, acute, reflexed at margins, each pair connate at base and forming a 0.4-1.0 cm long tube. Flowers 1-5 on each stem, axillary as well as terminal, on 1-3 cm long pedicels, campanulate, showy, 3-5 cm long. Calyx 2.5- 4 cm long; tube 0.8-1.2 cm long; lobes unequal, linear, acute, 1-2 cm long, sinuses between lobes obtuse. Corolla 2.5-5 cm long, bright-blue, freckled with white and yellowish inside; tube 1.5-3.5 cm long; lobes ovate, entire, acute, 0.5-1.2 x 0.4-0.9 cm. Stamens 5; filaments slender, adnate at middle of the corolla tube, slightly winged at base; anthers orsifixed, bilobed, ± sagittate, creamy-white. Ovary lanceolate, shortly stipitate, 1-2.3 x 0.3-0.7 cm; style not distinct; stigma prominent, slightly bilobed. Capsule stalked, lanceolate, 1.8-3.2 x 0.5-1 cm. Seeds numerous, oval, reticulate

Flowering: September-October

~~**Fruiting:**~~ November

Harvesting:

Ecology: A rosette-forming perennial herb, growing along sub-alpine altitudes from 1700-2300 m (a.s.l). Usually found on south-facing steeper slopes along dry and rocky sites in semi-natural grasslands and sparsely shrubby scrubs. The plant species prefers to be overshadowed by the tall grasses and shrubs. Roots are soft, creamy white, penetrate deep into the sandy-loam soil, and show a unique folding pattern with the younger ones tightly entwined all along the length of older ones (i.e. previous year). Within the rhizosphere, the roots are clumped together. The greener leaves and young flowering tops are grazed by herbivores, including insects.

Medicinal uses: The generic name of *Gentiana* has been derived from 'Gentius',

a king of Illyria (Europe), who is believed to have discovered the medicinal value of the gentian root. In fact, the specific name of *Gentiana kurroo* Royle is from the local name for the root of the plant, 'Karu' meaning bitter. The dried roots contain 20% of a yellow, transparent, and brittle resin (Coventry, 1927). The drug (rootstock) is administered in fevers and urinary complaints. It is used as a bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, antihelminthic, blood purifier and carminative (Kirtikar, & Basu, 1935). The roots are also used as ingredients in preparing a paste for flattening horses (Kaul, 1997).

Significance of the studies

There are no specific techniques available for its cultivation and propagation but having high medicinal significance led to its reckless collection, this plant is heavily extracted for root and rhizome because of their pharmaceutical importance. By far now one such report is available on in vitro multiplication of G. kurroo through shoot apex but on different media and in vitro production of metabolites of Gentiana kurroo. So the present study is carried out by having following objectives:

1) To optimize the tissue culture conditions for its propagation
2) To optimize the cell culture conditions for the production of medicinally important metabolites

Review of Literature

Chapter 2: Review of Literature

2.1 Plant

Gentianaceae is a family of flowering plants comprising of 70-80 genera and 900-1200 species. The plants of this family are annual and perennial herbs or shrubs. They are native to northern temperate areas of the world.(Daniel M and Sabnis SD,1978). Plants belonging to this genus are well known for their pharmacological properties. *Gentiana kurroo Royle* (Gentianaceae) is a critically endangered temperate bitter herb of Indian subcontinent region. It is perennial and commonly grows in Kashmir, Himachal Pradesh and adjoining hills of North-Western Himalaya between 1500–3000 m altitudes (Chaudhary and Wadhwa 1984). Commonly known karu/Indian gentian, though not mentioned by Sanskrit writers in Materia Medica (Dymock 1890), is a significant drug of ayurveda (Shahi 1993). The rhizome and roots of this herb contains some of the most known bitter compounds like gentianine (a crystalline monoterpene alkaloid), amaroswerin (Secoiridoid glycoside), gentianic acid, pectin, and uncrystallizable sugar (Anonymous 1953 and Singh 2008). Gentianine possesses anti-inflammatory, nalgesic, anticonvulsant, hypotensive, antipsychotic, sedative, diuretic, antimalarial, antiamoebic and antibacterial properties and amaroswerin gastro-protective (Singh 2008). In Indian system of medicine the root stock is valued as bitter tonic, antiperiodic, expectorant, antibilious, astringent, stomachic, anthelmintic, blood purifier, laxative, depurative, emmenagogue, galactopurifier, febrifuge and carminative (Chopra *et al.* 1956 and Anonymous 1956). It is also medicated for curing skin diseases, leucoderma, leprosy, bronchial asthma, dyppepsia, flatulence, colic, anorexia, helminthiosis, inflammations, amenorrhoea, dysmenorrhoea, strangury, haemorrhoids, constipation and urinary infections (Warrier et al. 1995). The drug is very helpful in removing

all kinds of debility and exhaustion of body from prolonged illness, improves digestive system and lack of appetite. The root is used as an ingredient in preparing feed for fattening horses (Qureshi 2007).

2.2 Morphology:

Flowers of *G. kurroo* are bracteate, pedicellate, complete, actinomorphic, hermaphrodite, hypogynous and pentamerous. Flowers are large and entomophilous. Flowering starts from the third week of August and continues till the first week of November, with the peak between 15 September

to 20 October. On average, a plant produces 20 flowers. The corolla is gamopetalous and infundibuliform, deep blue from outside up to plicae, dotted white at the throat and white at the base from inside. The flowers close during night and under low light, and reopen when the sun shines brightly. The opening and closing of the corolla continues till fertilization is complete and stigma lobes become dry . After fertilization, the corolla remains closed till it withers. The five stamens are epipetalous, with their filaments flattened at the base and fused with the corolla up to half of their length. Anthers are grouped around the stigmatic tip in bud condition, but move away as anther dehiscence starts. The gynoecium is bicarpellary and syncarpous. The ovary is unilocular and contains numerous anatropous ovules borne on parietal placentae. Initially, the two stigma lobes are adpressed and situated well below the anther level. After anther dehiscence is complete, the stigmatic lobes open out and are lifted above the level of the anthers by elongation of the pistil. The type of dichogamy noted is caused by protandry.The anthers attain maturity and dehisce to discharge pollen 1–3 days after anthesis. The anthers are placed well above stigmatic region, with the stigmatic lobes in adpressed condition at dehiscence stage. Dehiscence continues for 3–4 day. Although 94% of the pollen was viable at the beginning of dehiscence there was a gradual reduction in pollen germination as the anther dehiscence progressed. However, substantial reduction in pollen viability was observed after 72 h (73.64%) up to 108 h (3.72%) on commencement of anther dehiscence. The stigmatic lobes remain closed (in adpressed condition) till almost the completion of anther dehiscence. They start opening from the 4th to 6th day after anthesis and continue until 13th to 16th day after anthesis. This is the stage when the stigma is receptive to pollen germination. At this stage, the stigma lobes are well above the anther level . Response of *G. kurroo* to different pollination methods tested showed it to be strongly cross compatible, as was evident by significant fruit and seed set in controlled cross-pollination. Absence of fruit and seed set in bagged flowers (T2) may be due to the existence of protandry, which prevents self-pollination. A similar phenomenon has also been reported in *Gentiana newberry* L., an alpine perennial species in which no fruit and seed set was reported in caged plants4. The fact that *G. kurroo* is cross-pollinated is further strengthened by the observation that open-pollinated (T1) and open cross-pollinated (T4) flowers performed statistically at par with each other with regard to all parameters studied. The presence of anthers (in openpollinated flowers) or their absence (due to emasculation in open cross-pollinated flowers) does not make any significant difference for seed and fruit set in open and open cross-pollinated flowers, respectively. In flowers, which rely on insects for cross-pollination, the anthers and stigmas are clearly separated by at least a small gap to allow passage of insects5. In flowers of *G.kurroo*, it has been observed that anthers, which are grouped

around the stigmatic region initially when the corolla is still closed, move towards the periphery when the corolla opens, creating a small gap between the anthers and the stigma. Although no nectariferous disc is present in its flowers, insect vectors are rewarded by abundant pollen. The different insect vectors seen visiting flowers of *G. kurroo* are bumble bee (*Bombus* sp.), honeybee (*Apis mellifera*) and ladybird beetle (*Coccinella septempunctata*). Another feature that attracts the insects is the colour pattern of the corolla. The whitish interior of the corolla tube in contrast to the deep blue colour of the lobes makes the interior translucent, if held against light. This makes the interior look lighted to an insect, encouraging it to enter the flower and effect pollination. This is a common feature of many *Gentiana* species like *G. sino-ornata* and *G. acacilis*; also, the white dots at the throat region serve as guide marks for the visiting insects5.

There is generally a time lag between pollen shedding and its transfer to the stigma. During this period pollen grains are exposed to a wide range of environmental stresses, particularly of temperature and humidity, which affect their competitive ability to sire vigorous progeny6.

The factors affecting pollen viability, like the duration for which anthers continue shedding pollen and the range of environmental factors to which they are exposed, are critical for cross-pollinated species like *G. kurroo*. Availability of viable pollen over a longer period helps protandrous flowers in cross-pollination. This is more relevant in *G. kurroo*, where it has been observed (present investigation) that the stigma becomes receptive 4–6 days after anther dehiscence, and anthers continue shedding pollen for 3–4 days after initial anther dehiscence.

Whether fresh pollen grains were being shed daily or those shed at the start of anther dehiscence remain adherent to the anther surface could not be ascertained. However, there was a gradual reduction in pollen germinability from 94.0% at the beginning of anther dehiscence to 29.6% after 96 h. Even after 108 h, a small amount of pollen (3.7%) retained germinability (Table 3).

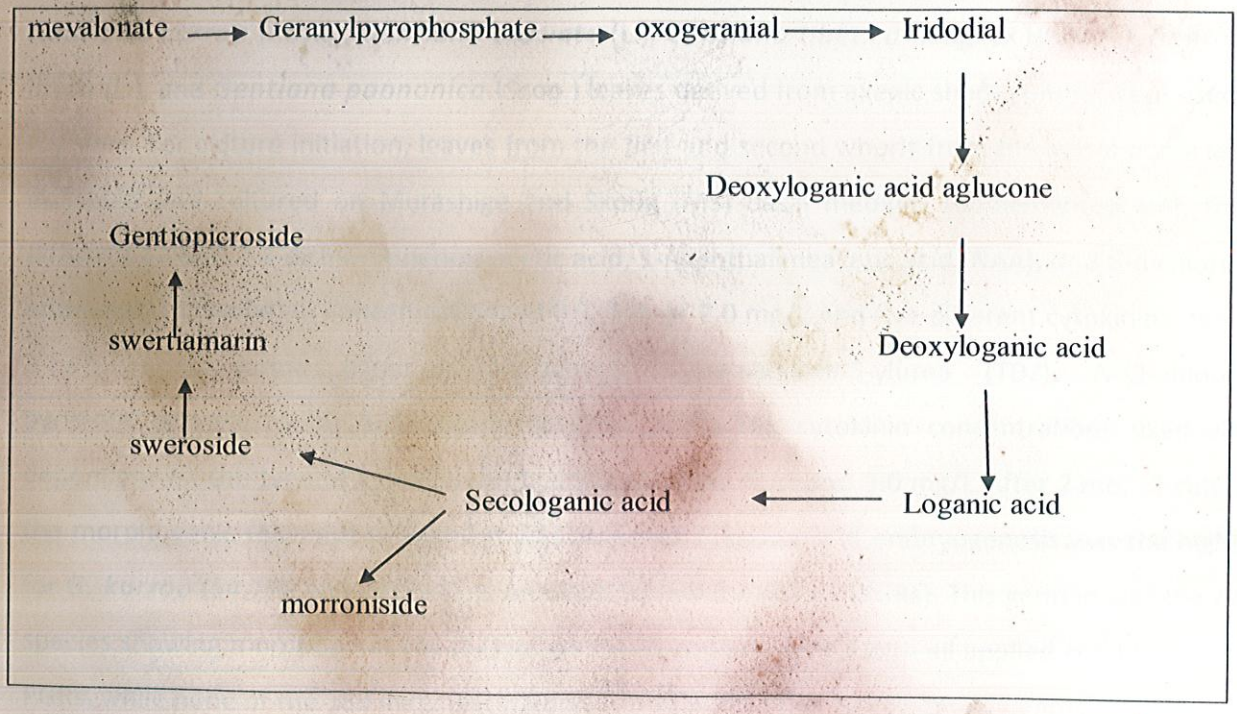
Although *G. kurroo* seems to be chiefly crosspollinated, as is indicated by no fruit and seed set in bagged flowers and statistically similar results obtained in open and open cross-pollinated flowers, 7.24% fruit set in controlled selfing (selfing effected manually at stigma receptive stage) indicates that some selfpollination is still possible. A small amount of pollen remaining viable even after 108 h may be responsible for fruit set. The first fortnight of November is the ideal time for seed harvest after which the capsules open up, scattering the seed. 70–75% of seeds germinate and June is the ideal month for seed sowing. The seed has to be stored at low temperature (below 5°C) after harvesting. Otherwise, there is considerable reduction in germination percentage. Seeds more than one-year-old lose viability and do not germinate.

2.3 Phenology:

Shoot of this peremial herb is represented by flowering branches only. Flowering occurs during September-october with 1-9 inflorescences per plant. Flowers are cross pollinated. Different insect vectors seen visiting flowers of gentiana kurroo are bumble bee, honey bee, lady bird beetle. Capsule takes 18-20 days to mature after fertilization. The first fort night of November is the ideal time for seed harvest. Seeds are light in weight and 100 seeds weighs 0.1275gm.

2.4 Biosynthetic pathway of gentiopicroside:

The biosynthetic pathway for gentiopicroside is available [Jensen et al., 200 The biosynthetic pathway leading to the iridoid glucosides commonly found in Gentianaceae.



2.5 Drugs obtained so far:

1.Quingann capsules from Sun pharmaceuticals which is used in treatment of liver fibrosis contains 98% of Gentiopicroside in the capsule.

2. D worm from Rajasthan aushadhi is helpful in intestinal worms as well as bacteria responsible for blood and skin disorders, cough, anemia, urticaria & abdominal pain.

2.6 *In vitro* propagation of the plant

In vitro propagation of *Gentiana kurroo* was studied by Neelam Sharma et.al (1993) where shoot multiplication of ***Gentiana kurroo*** Royle, a threatened medicinal plant species, was achieved ***in vitro*** using shoot tips and nodal segments as explants. Fifteen-fold shoot multiplication occurred every 6 weeks on Murashige and Skoog's medium (MS) containing 8.9 µM benzyladenine and 1.1 µM 1-naphthaleneacetic acid. Rooting was accomplished successfully in excised shoots grown on MS basal medium containing 6% sucrose.

Agnieszka Fiuk, Jan J. Rybczyński (2008) studied different genotype and plant growth regulator-dependent response of somatic embryogenesis from G*entiana* spp. By using leaf explants where *Gentiana kurroo* (Royle), *Gentiana cruciata* (L.), *Gentiana tibetica* (King. ex Hook. f.), *Gentiana lutea* (L.), and *Gentiana pannonica* (Scop.) leaves derived from axenic shoot culture were used as explants. For culture initiation, leaves from the first and second whorls from the apical dome were dissected and cultured on Murashige and Skoog (MS) basal medium supplemented with three different auxins: 2,4-dichlorophenoxyacetic acid, 1-naphthaleneacetic acid (NAA), or 3,6-dichloro-*o*-anisic acid (dicamba) in concentrations of 0.5, 1.0, or 2.0 mg/l; and five different cytokinins: zeatin, 6-furfurylamonopurine (kinetin), *N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea (TDZ), *N*-(2-chloro-4-pyridyl)*N'*-phenylurea, or 6-benzylaminopurine (BAP). The cytokinin concentrations used were dependent on the type of cytokinin and varied between 0.25 and 3.0 mg/l. After 2 mo. of culture, the morphogenic response of explants was assessed. Frequency of embryogenesis was the highest for *G. kurroo* (54.7%) and dependent on plant growth hormones (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 *G. lutea* explants. *G. tibetica* and *G. cruciata* both produced an average of 6.6 somatic embryos per explant, while *G. pannonica* and *G. kurroo* regenerated at 15.7 and 14.2 somatic embryos per explant, respectively. Optimum regeneration was achieved in the presence of NAA combined with BAP or TDZ. This auxin also stimulated abundant rhizogenesis. Somatic embryos were also regenerated from adventitious roots of *G. kurroo*, *G. cruciata*, and *G. pannonica*. Somatic embryos converted into plantlets on half strength MS medium. Effect of sucrose concentration on

photosynthetic activity of in vitro cultures *Gentiana kurroo* (Royle) germlings reported by Jan J. Rybczyński et al (2007)where the effect of sucrose on photosynthetic activity during in vitro culture was studied. Experiments were carried out using uniform somatic embryo-derived germlings of *Gentiana kurroo*(Royle) confirmed by chromosome counting and flow cytometry technique. Photosynthetic activity was measured by chlorophyll ***a*** fluorescence and gas exchange method. The efficiency of photosynthetic apparatus as measured by the ratio F_v/F_m, Yield and qP (light phase of photosynthesis) was the highest when the medium was supplemented with 0.3% sucrose which well corresponded with plant gas exchange. Taking all data into consideration for the best development of photosynthetic apparatus and the most efficient of net photosynthesis of studied germlings would be medium supplemented with 0.2–0.4% of sucrose.

2.7 Secondary Metabolites

The dried roots and rhizomes that are official in Indian pharmaceuticals. It contains many classes of secondary metabolites.They are intensely bitter and is valuable remedy for digestive system ailments. The medicinal value is due to presence of bitter glycosides (Hostettmann-Kaldas M, et al., 1981).the interest among potential medicinal plants of gentianaceae has been revived and significant phytochemicals are being studied for drug development (Brahamchari G, et al., 2004). Potential phytochemicals of gentianaceae are amarogentin, amaroswerin, gentianine, gentianadine, gentiopicrin, sweroside, swertiamarin.In vitro production of these secondary metabolites has been studied by Vinterhalter Branka et al where they carried out study of Quantitative determination of secoiridoid and xanthone glycosides of *Gentiana dinarica* Beck cultured in vitro and found that *Gentiana dinarica* Beck, which is native to the Balkan Dinaric Mountains, was established in vitro from axillary shoot buds. It was maintained in the form of shoot cultures on MS medium supplemented with 1.0 mg/L 6-benzyladenine (BA) and 0.1 mg/L α-naphthaleneacetic acid and excised root cultures were maintained on ½ MS medium with 0.5 mg/L indole-3-butyric acid (IBA). Shoot cultures, adventitious roots and excised root cultures were analysed by HPLC techniques for the presence of secoiridoids and xanthones. Gentiopicrin and swertiamarin, the dominant components of shoot cultures, could not be detected in root cultures. Xanthones were present in both shoot and root cultures with norswertianin-1-***O***-primeveroside as the dominant metabolite. The secoiridoid and xanthone content, although characteristic for certain plant organs, was dependent on the concentration of plant growth regulators (BA and IBA) added to the medium. BA in the shoot multiplication stage strongly increased the secondary metabolite (SEM) content of shoot cultures. IBA had little effect on SEM accumulation in shoots during rooting, while it moderately stimulated SEM accumulation in excised root cultures.

2.2.1.Amarogentin:

It is secoiridoid glycoside, and is the most bitter substance known. It tastes even bitter at a dilution of 1:58,000,000. It can obtained from Gentiana lutea L(Van Haelen ,1983). Swertia chirayita(Roxb ex. Flem), swertia japonica(Roem. & Schult). It possesses topoisomerase inhibition(Ray et al.,1996), chemo-preventive(Saha P and Dass, 2005) and antileishmanial properties(Ray S et al.,1996)

2.2.2 Amaroswerin:

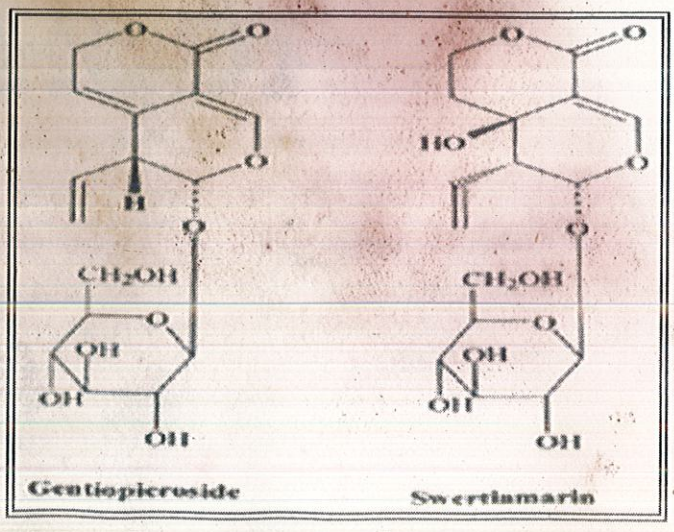
It is a secoiridoid glycoside obtained from Gentiana kurroo Royle(Niiho Y et al., 2005), swertia chirayita(Roxb ex. Flem). It has been found to be gastro-protective (Niiho Y et al., 2005)

2.2.3.Gentianine:

Gentianine is widely distributed in plants belonging to the family Gentianaceae. It is a bitter, crystalline monoterpene alkaloid. The biological sources include Gentiana kurroo Royle, Gentiana lutea L., Gentiana macrophylla(Zhong JF and Jin h,1988). It possesses anti-inflammatory, antihistaminic, antipsychotic and antibacterial properties.

2.2.4.Gentiopicrin:

It is a crystalline monoterpene gentiopicroside. It is a bitter glycoside widely distributed in plants of Gentianaceae.



MATERIALS AND METHODS

CHAPTER 3

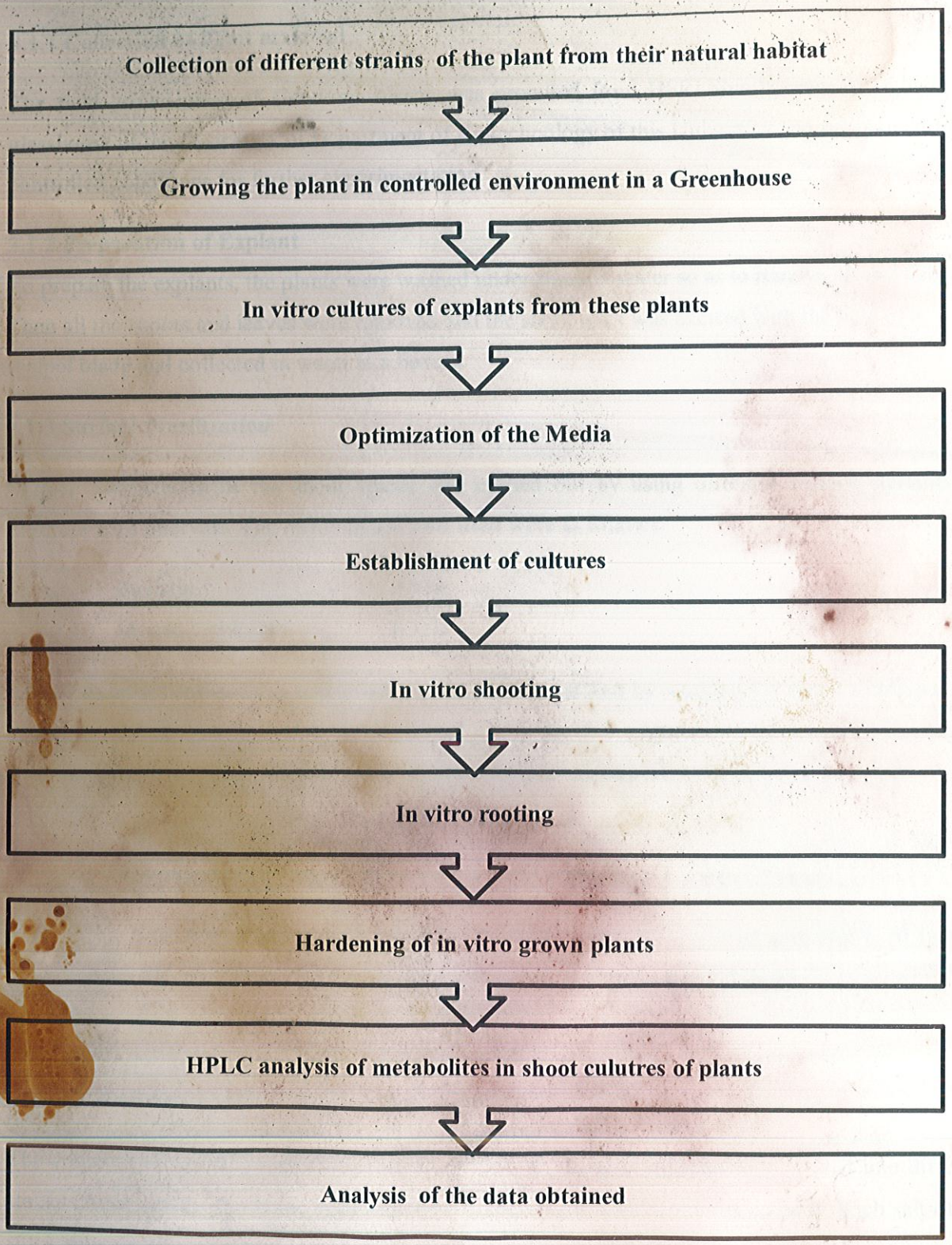
MATERIALS AND METHODS

The present experiments of establishment of *in vitro* shoot cultures of *Gentiana kurroo*, regeneration of plantlets & hardening of *in vitro* grown plantlets were carried out in the Department of Biotechnology, Jaypee University of Information Technology, Waknaghat, during 2012-2013..

The Experiments and methodology followed to carry out the above-mentioned experiments have been described under the following headlines.

3.1 Micropropagation of *Gentiana kurroo*

3.1.1 collection of Plant material
3.1.2 Preparation of explants
3.1.3 Surface Sterilization
3.1.4 Media Preparation
3.1.5 Cleaning of glassware
3.1.6 Culture conditions
3.1.7 Inoculation of prepared explant
3.1.8 *In vitro* shoot multiplication on prepared MS media
3.1.9 Induction of *in vitro* rooting on prepared MS media
3.2 Estimation of gentiopicroside and Swertimarin using HPLC
3.3 Hardening and acclimatization of *in vitro* rooted plantlets in glass house


Collection of different strains of the plant from their natural habitat
Growing the plant in controlled environment in a Greenhouse
In vitro cultures of explants from these plants
Optimization of the Media
Establishment of cultures
In vitro shooting
In vitro rooting
Hardening of in vitro grown plants
HPLC analysis of metabolites in shoot culutres of plants
Analysis of the data obtained

3.1.1 Collection of Plant material

The field grown plant of *Gentiana kurroo* was procured from HFRI,Shimla were brought to the glasshouse of the laboratory of Department of Biotechnology of this University and maintained under controlled conditions for further experimentation.

3.1.2 Preparation of Explant

To prepare the explants, the plants were washed under running water so as to remove all soil debris. Then all the shoots and leaves were removed and the shoot apex was excised with the help of a scalpel blade and collected in water in a beaker.

3.1.3 Surface Sterilization

Surface Sterilization of the shoot apices was carried out by using different surface sterlants for different time intervals. The different sterlants used were as follows

i) Bavistin - 0.5 %

ii) Mercuric chloride - 0.1 %

The shoot apices collected in beaker were surface sterilized first by washing 3-5 times with tap water, later treated with Labolene (2-3 drops/100 ml water) for 2-3 minutes and then washed thoroughly with distilled water. Henceforth, the surface sterilization was carried out in Laminar air hood.

i) The washed shoot apices were then treated with Bavistin (0.5 %) for 2-3 minutes. Subsequently, the shoot apices were washed with distilled water 1-2 times.

ii) After being treated with Bavistin, the shoot apices were treated with $HgCl_2$ (0.1%) for 0.5-1 minute. Finally, the shoot apices were washed with distilled water 3-4 times.

3.1.4 Media Preparation

The composition of Media was based on Murashige & Skoog (1962) medium divided into different stocks (Annexure I).The Stock Solutions were prepared and kept in the refrigerator. Each salt of the stock solution was dissolved separately in distilled water so as to avoid precipitation. The Chemicals used were of reliable grade and were obtained from reliable firms like Sigma Aldrich. Each Stock solution of MS Media was added one by one to prepare the desired medium. After addition of sucrose

(30g/l), growth hormones, desired volume was made with distilled water and the pH of the medium was adjusted to 5.6-5.7 by using pH meter. Then, agar-agar (9g/l) was added and dissolved by boiling the medium till it became homogenous. The medium was finally dispensed in 250 ml culture jars for culturing. These were closed with the jar caps and autoclaved at a pressure of 15 psi, 121 °C for 20 minutes for proper sterilization of culture medium. This medium was allowed to solidify and was used after 2 days of preparation. In all 10 different combinations of media was prepared using different concentrations of appropriate growth hormones like KN, BA, IBA, GA3 for each of the experimental setups.

3.1.6 Cleaning of Glassware

All the glassware which were not used before were cleaned in a dilute solution of Labolene with test tube brush and rinsed thoroughly in tap water. The Glassware was then kept for drying in hot dry air oven at 80-100 °C for 2-3 hours.

The used glassware with contaminated cultures were first autoclaved and molten media was disposed off and the culture jars were cleaned.

3.1.7 Culture Conditions

All the operations were carried out aseptically in a Laminar Air Hood filled with UV Light. The Laminar Flow Chamber was thoroughly wiped with rectified spirit prior to use and the UV Light was kept on for 15-20 minutes in Laminar Flow Chamber. Thereafter, the airflow was allowed to run and the UV Light was switched off. Before starting with aseptic manipulations, hands and arms were washed with soap and water. Hands were frequently wiped during manipulations with rectified spirit which was allowed to evaporate. All the metallic tools like scalpel blade, forceps and needles were first autoclaved and wiped with rectified spirit and flame sterilized at the time of use. The rims of tubes and jars were quickly flame sterilized before and after inoculations. Subsequently, the jars were kept in trolleys in culture room at 25 ± 2 °C under 16 hours photoperiod and 3000 flux light intensity.

3.1.8 Inoculation of prepared ex plant

For the establishment of the cultures, the surface sterilized explants were inoculated onto the MS Medium supplemented with various combinations of growth hormones. The cultures were then incubated for the proliferation of shoot apex. The proliferation of shoot apex was indicated by the emergence of micro shoots and leaflets.

Observations

Average number of micro shoots proliferated per shoot apex on prepared MS media was recorded.

3.1.9 *In vitro* shoot multiplication on prepared MS media

The shoots obtained from the shoot apex were subcultured on prepared MS media containing various combinations of growth hormones including GA3, IBA & KN. Subculturing for multiplication was carried out at 3 weeks interval up to 4-5 subcultures.

Observations

Average number of shoots, shoot length after 3 -4weeks of sub culturing was recorded.

3.1.10 *In vitro* rooting on prepared MS media

Rootable shoots were excised from lavishly multiplying shoot cultures having at least 2 cm length and were transferred singly to separate culture jars containing MS Media enriched with rooting growth hormones KN and IBA of different concentrations.

Observations

Average number of roots and root length after 3-4 weeks was recorded.

3.2 Estimation of gentiopicroside and Swertimarin using HPLC

The *in vitro* biosynthsis and accumulation status of gentiopicroside and Swertimarin was determined in shoot and root cultures of *G. kurroo* by subjecting fresh samples from different experiments to chemical analysis. The shoots including leaf material and roots were harvested separately while the leafy material was green, actively-growing, and non-senescent stage. The quantification of

gentiopicroside and Swertimarin was carried by reverse phase High Performance Liquid Chromatography (HPLC Waters 515) through C18 (5µ) 4.6 x 250mm Waters Symmetry Column using PDA detector (Waters 2996). Fresh shoots and roots were ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was sonicated at 25°c for 15mins and was filtered through 0.22µ filter. The filtrate was diluted 10x and 20x and injected into above mentioned column. Two solvent systems were used for running the test samples i.e. Solvent **A** (0.05% trifluoroacetic acid) and Solvent **B** (70% acetonitrile in 30% water). Solvent **A** and **B** were used in the ratio of 70:30 (v/v). The column was eluted in the gradient mode with a flow rate of 1.0 ml/min. Gentiopicroside and Swertimarin were detected at absorbance of 270 nm wavelength in a cycle time of 30 min at 30^0C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex Inc. The quantification was done and the data were subjected to statistical analysis.

3.3 Hardening and acclimatization of *in vitro* rooted plantlets in glass house

Transplantation and hardening of *in vitro* regenerated plants was carried out in plastic pots. After growth of 5 weeks on the rooting medium, roots were well developed and the plantlets from all rooting media were taken out of culture jars. Precaution was taken to avoid any possible damage to delicate root system. The agar medium from roots was washed gently under running tap water. After removal of the adhering medium, the plantlets were kept in Bavistin solution (0.5%) for 15-20 minutes.

Plantlets were transferred to plastic pots containing pre-autoclaved mixture of sand, soil and perlite, vermiculite and coco-peat in combinations. The root portion of plantlets was then gently placed in the pots containing the various rooting mixtures and sand at the bottom. The plantlets were watered and covered with glass jars to maintain high humidity.

After a week, when the plantlets showed initial signs of establishment in pots, with the appearance of new leaves, the pots were initially uncovered for overnight. Then after 2-3 weeks of transfer to pots, the plantlets were totally uncovered.

Observations

Percentage of survived plants in different potting mixtures was recorded.

RESULTS AND DISCUSSIONS

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1. Induction of multiple shoots in shoot apices

The surface sterilized axillary shoot tips were cultured on MS media containing IBA and KN in different combinations. Out of 10 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (1mg/l) + IBA (3mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) was found to be the best with 60 % of shoot apices proliferating into multiple shoots within 5-6 days of inoculation (Table 4.1).

The same medium was found suitable for obtaining maximum shoots (22.4) per explant. However different concentrations of IBA and KN resulted in increased shoot length and less frequency of shoots. With the addition and increase in concentration of GA3 in the medium, there was a profused growth of the shoots with no increase in the shoot length but small leaves were observed when cultures were incubated at 25±1^{0}C (Fig. 4.1). The modifications in MS media by changing the concentrations and combinations of auxins and cytokinins did not help in obtaining shoots with good growth and well developed leaves.

4.2 Proliferation of shoot apex on prepared MS media

After 3 weeks of inoculation of shoot apex, we observed 3 to 8 micro shoots emerging from each inoculated explants.

4.3 *In vitro* shoot multiplication on prepared MS media.

4.3.1 Solid Media

After 3-4 weeks of growth, the shoots thus proliferating from the shoot apex were excised under aseptic conditions and subcultured on the prepared MS medium enriched with different concentrations of GA3, KN & IBA growth hormones (Table 1) The maximum shoot multiplication was seen in MS Media containing (MS + KN (1mg/l) + IBA (3mg/l) +Sucrose 30g/l + agar-agar 9g/l),but the shoot length did not increase.

The maximum shoot length was seen in MS media containing (MS + KN (3mg/l) + IBA (1mg/l) +Sucrose 30g/l + agar-agar 9g/l) whereas minimum growth was observed in MS media containing (MS+ Sucrose 30g/l + agar-agar 9g/l)

TABLE 1: *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins at 25°C

IBA	GA3	KN	No. of days	Average leaf size(cm^2)	Avg No. of Shoots per explant	Avg shoot length per explant (cm)
0	0	0	15-20	2.6 *0.5	3.1	1.3
0	1	0	9-10	2.2 *0.4	5.2	1.5
1	1	0	9-10	2.3*0.4	5.2	1.5
1	0	3	8-9	3.2 *0.6	6.0	3.2
1	2	0	8-9	2.3*0.4	6.0	1.7
0	2	1	6-8	2.4*0.5	6.2	1.6
1	0	2	6-8	3.0 *0.8	6.7	1.9
2	0	1	5-6	3.3*0.7	7.0	1.8
2	0	2	5-6	3.3*0.9	7.8	1.7
3	**0**	**1**	**6-7**	**4.1* 1.0**	**9.5**	**2.1**

4.3.2 Liquid Media

After 3-4 weeks of growth, the shoots thus proliferating from the shoot apex were excised under aseptic conditions and subcultured on the prepared MS medium enriched with different concentrations of GA3, KN & IBA growth hormones (Table 2) The maximum shoot multiplication was seen in MS Media containing (MS + KN (4mg/l) + IBA (1mg/l) +Sucrose 30g/l),and the shoot length also increased as compared to solid media.

TABLE 2: *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins in liquid media at 25°C and 120rpm

IBA	KN	No. of Days	Average leaf size(cm^2)	Avg No. of Shoots per explant	Avg shoot length per explant (cm)
0	0	15-20	2.6 *0.5	3.1	1.3
0	0	9-10	2.2 *0.4	5.2	1.5
1	0	9-10	2.3*0.4	5.2	1.5
1	**4**	**8-9**	**3.2 *0.6**	**11.5**	**4.5**
1	0	8-9	2.3*0.4	6.0	1.7
0	1	6-8	2.4*0.5	6.2	1.6
1	2	6-8	3.0 *0.8	6.7	1.9
2	1	5-6	3.3*0.7	7.0	1.8
2	2	5-6	3.3*0.9	7.8	1.7
3	1	6-7	4.1* 1.0	9.5	3.2

4.4 *In vitro* rooting on prepared MS media

4.4.1 Solid Media

The Rootable shoots which were excised and put in separate culture jars containing MS Media enriched with rooting growth hormones IBA of different concentrations (Table 2) were observed after 1-2 week(s) and the best results for number of roots and root length was seen in media containing (MS + IBA 4mg/l + Sucrose 30 g/l + agar-agar 9 g/l).

TABLE 3: *In vitro* root induction on different combinations and concentrations of auxins

MS + Growth Hormones (mg/l)		Days to root formation	Avg No. of Roots per explant	Avg root length per explant (cm)
KN	IBA			
0	0	18-20	2.1	0.3
0	1	14-16	2.7	0.45
0	3	12-14	3.8	0.45
1	3	12-14	4.3	1.5
0	**4**	**8-10**	**5.5**	**2.1**

4.4.2 Liquid Media

The rootable shoots which were excised and put in separate culture jars containing MS Media enriched with rooting growth hormones IBA of different concentrations (Table 2) were observed after 1-2 week(s) and the best results for number of roots and root length was seen in media containing (MS + IBA (3mg/l) + KN (1mg/l)+ Sucrose 30 g/l).

TABLE 4: *In vitro* root induction on different combinations and concentrations of auxins on liquid media at 25°c and 120rpm

MS + Growth Hormones (mg/l)		Days to root formation	Avg No. of Roots per explant	Avg root length per explant (cm)
KN	IBA			
0	0	18-20	2.1	0.3
0	1	14-16	2.7	0.45
0	3	12-14	3.8	0.45

1	3	12-14	5.5	2.1
0	4	8-10	4.3	1.5

4.4 Estimation of gentiopicroside and Swertimarin using HPLC

The *in vitro* growing cultures were analysed for gentiopicroside and swertiamarin content using HPLC. The content of gentiopicroside in a month old plant shoots cultured on MS+IBA (3mg/L)+ KN(1mg/L) was found to be 0.02% whereas the content of swertiamarin was 0.11%. No significant content was found in roots of the cultures grown on same media composition and in shoot cultures growing on MS supplemented with KN(3mg/L) +IBA(1mg/L).

Table 5: HPLC results showing the data analysed for Gentiopicroside and Swertiamarine content

Sample	Plant part analysed	Gentiopicroside % of fresh shoot weight	Swertiamarine % of fresh root weight
1.	*in vitro* shoot, 25°C (IBA:KN in 3:1 ratio)	0.02	0.11
2	*in vitro* shoot,25°C (KN:IBA in ratio 3:1)	-	-
3.	*in vitro* root, 25°C (KN:IBA in 3:1)	-	-

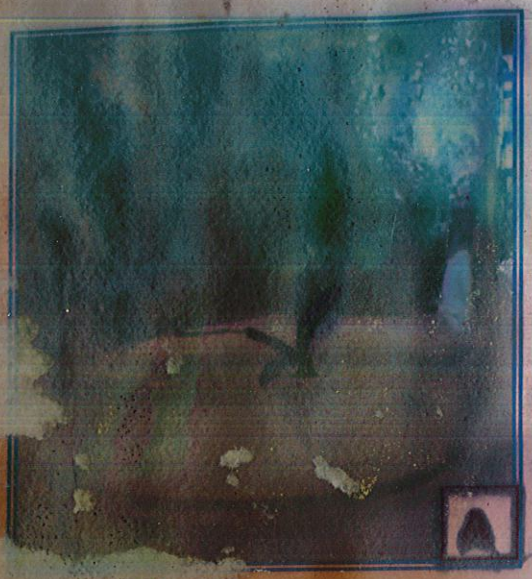
4.5 Hardening of *in vitro* rooted plantlets in glass house

After 5 weeks of growth on rooting medium, roots were well developed and the plantlets from all rooting media was taken out from all culture jars and after the treatment already mentioned, were potted in potting plastic pots containing different potting mixtures (Table 3). After a week, when the plants showed initial signs of establishment in pots, with the appearance of new leaves, the pots were

initially uncovered for overnight. After 2 weeks of transfer to pots, and when the plantlets were totally uncovered, we observed that the potting mixture of sand, soil with perlite, vermicultite, coco peat in ratio 1:1:1 gave us the best results in terms of plant survival and growth.

Table 6: Growth and Survival of hardened plants in different potting mixtures under glass house conditions.

Potting Mixture (Sand+Soil)	Ratio	No. of Leaves	Percentage Survival of plants(%)
Vermiculite	--	17	25
Perlite	--	18	25
Coco-peat	--	15	50
Perlite+Vermicultie	1:1	18	50
Perlite+coco-peat	1:1	21	75
Vermiculite+coco-peat	1:1	23	50
Perlite+Vermiculite +coco-peat	**1:1:1**	**25**	**75**



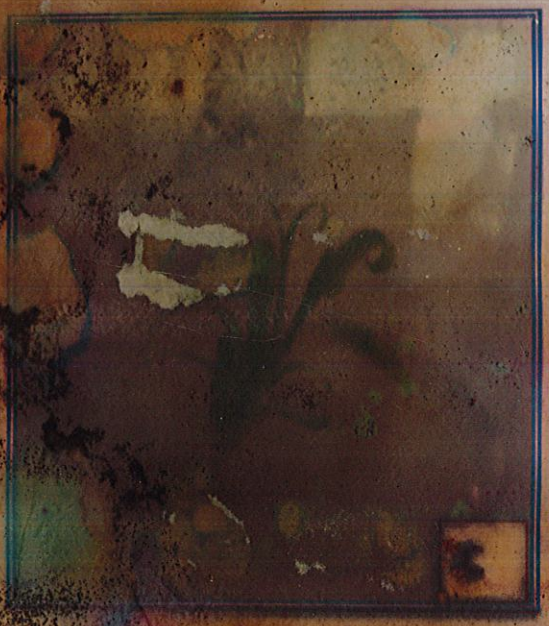


FIGURE 1: *In vitro* shoot multiplication using shoot apex as starting culture.

a) shoot apex cultured in the medium

b) shoot proliferation after 2 weeks

c) shoot proliferation after 3-4 weeks

d) shoot proliferation after 5-6 weeks

(B)

FIGURE 2: Subculturing done on different growth hormone combinations of MS media.

(A)Multiple shoots on IBA:KN (3:1) at 25ºC

(B) shoot elongation on KN:IBA (3:1) at 25ºC.

Conclusion

Conclusion:

As of now there is no such work done on the similar lines which have been attempted by us where we have developed *in vitro* grown shoots from shoot apex with 8-9 shoots growing on MS media containing IBA(3mg/L) +KN(1mg/L) + sucrose (30 g/L) + agar-agar (9g/L) out of ten different concentrations of growth hormones tried.

We have also tried other types of media where comparison for shoot growth have been studied by comparing these parameters of solid and liquid media. It was found that the growth of the shoots was much better and fast in liquid media as compared to the solid media.

As it is reported for the first time where these medicinally important metabolites have been produced through plant tissue culture where the content of secondary metabolite accumulated and biosynthesised in one and a half month old *in vitro* grown shoot of *Gentiana kurroo* cultured on IBA(3mg/L) +KN(1 mg/L) at 25°c.

In vitro developed plantelets were successfully hardened in the glasshouse in potting mixture containing cocopeat:vermiculite:perlite:sand:soil in the ratio 1:1:1:1. Plants are now ready to be transferred to the field.

So we would like to conclude that we have developed successful protocol for *in vitro* propagation of *Gentiana kurroo* and produce importanat secondary metabolites through shoot cultures. This study holds potential in pharmaceutical and biotech industry.

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ANNEXURE

ANNEXURE

Composition of used Murashige and Skoog (1962) basal medium (MS Medium)

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME
A - 10X	KNO_3	1900	19	}
	$MgSO_4.7H_2O$	370	3.7	} 100ml/l
	KH_2PO_4	170	1.70	
B - 20X	NH_4NO_3	1650	33	50ml/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	}
	$CuSO_4.5H_2O$	0.025	0.0025	10 ml/l

	$MnSO_4 .4H_2O$	22.3	2.23	
	$Na_2MbO_4 .2H_2O$	0.25	0.025	
G - 100X	m-INOSITOL	100	10	} 10ml/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	

BIO-DATA:

Name: Preeti Kaura

CGPA: 7.5

Stream: B.Tech Biotechnology

Industrial training: Biotech study and research centre, Chandigarh

Achievements: Presented a poster in "National symposium on plant tissue culture and biotechnology for food and nutritional security" at CFTRI, Mysore (11-13 march, 2013)

Email Id: kaurapreeti@yahoo.in

Name: Leena Chopra

CGPA: 6.9

Stream: B.Tech Biotechnology

Industrial training: Ranbaxy Laboratories pvt.ltd, Gurgaon

Achievements: Presented a poster in "National symposium on plant tissue culture and biotechnology for food and nutritional security" at CFTRI, Mysore (11-13 march, 2013)

Email Id: leena_1992chopra@yahoo.co.in

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