STANDARDIZATION OF THE METHOD TO INITIATE AND ESTABLISH IN VITRO CULTURE OF GENTIANA KURROO

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MAY-2007

Submitted in partial fulfillment of the Degree of Bachelor of Technology

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MAY-2007

CERTIFICATE

This is to certify that the work entitled, "Standardization of the method to initiate and establish in vitro culture of *Gentiana kurroo*" submitted by POOJA and VAISHALLI in partial fulfillment for the award of degree of Bachelor of Technology in 2007 of Jaypee University of Information Technology 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.

Dr. Anil Kant

ACKNOWLEDGEMENT

We take this privilege to thank Dr Anil Kant for providing us an opportunity to carry out this project and for his active involvement and guidance throughout this project. We also thank to rest of the faculty members who have been instrumental in the completion of our project. This project has increased our knowledge immensely in various aspects of tissue culture.

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LIST OF ABBREVIATIONS

BA	Benzyl Adenine
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
2, 4-D	2, 4-Dichlorophenoxyacetic acid

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ABSTRACT

The Indian Himalayan region (IHR) is undergoing heavy extraction of wild, medicinal herbs, thus endangering many of these species. *Gentiana kurroo* is one of the few herbs which are used as a component of many medicines. It has been critically endangered due to it's over exploitation from natural habitats. Plant tissue culture holds a great promise for the production of secondary metabolites. It can help in conservation of endangered individual plants besides providing valuable products. Many problems of conventional cultivation can be circumvented by *in vitro* production method. In this investigation an attempt was made to initiate and establish *in vitro* culture of *Gentiana kurroo* with larger objective of secondary metabolite production. In this investigation possibility of rapid initiation and proliferation of callus and roots cultures which can be good source of biomass for secondary metabolite production was explored.

CHAPTER-I

INTRODUCTION

Gentiana kurroo is one of the most important medicinal plant of family gentianaceae. Secoiridoid glucosides are the main compounds with medicinal properties in the roots of *Gentiana kurroo.* Gentiopicroside and swertiamarin are the two important secoiridoid glucosides found in Gentiana, the former being quantitatively dominant. A new bitter acylated iridoid glucoside, 2'-(2,3-dihydroxybenzoyloxy)-7-ketologanin (1), has been isolated from the leaves of *Gentiana kurroo*. The plant has a great medicinal value as it is a bitter tonic, antiperiodic, expectorant, astringent and stomachic and is a valuable source of medicinal and many pharmaceutical products. With the increase in the demand for the crude drugs, the plant is being overexploited thereby threatening its survival.

Plant cell and tissue culture holds great promise for mass production of chemicals and secondary metabolites. This system if successful commercially can reduce the exploitation of endangered medicinal plant species from natural resources and in a way will aid in conservation of plant species. Apart from this it can also circumvent many problems and variables of conventional agriculture including environmental factors disease and pests, political and labour instabilities in producing countries, uncontrollable variations in crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. The specific cells of the plant which overproduce the product can also be selected and multiplied through in vitro culture techniques which cannot be done through conventional cultivation.

The use of plant tissue culture for production of secondary metabolites can be especially useful in case of medicinal plants. However such technologies are still being developed and despite the advantages outlined above there are variety of problems to be overcome before it can be adopted on wide scale for the production of useful plant secondary metabolites. The success of Mitsui petrochemical industry co. limited in

Japan in producing shikonin on a commercial scale from *lithospermum erythroshizone* and that of nitto denko co. ltd. Also in Japan in mass production of panax ginseng cells using 20 kl tanks have demonstrated that many problems can be overcome with perseverance. In theory it is anticipated that such large scale suspension culture will be suitable for industrial production of useful plant chemicals such as pharmaceuticals in a manner similar to that of microbial fermentation

The present work was intended to initiate and establish in vitro culture of *Gentiana kurroo* with a larger objective of developing method for producing its secondary metabolites through plant tissue culture techniques.

OBJECTIVES

The objectives of this project are

1. To standardize the method for induction of root and callus culture in *Gentiana kurroo* by using various combination of growth regulators.
2. To maintain and proliferate cultures in liquid MS media.

CHAPTER-2

REVIEW OF LITERATURE

Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, etc.), disease, political and labour instabilities in the producing countries (often Third World countries), uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Thus, the production of useful and valuable secondary metabolites in large bioreactors located in the consuming country is an attractive proposal. Additional advantages of such processes include: controlled production according to demand.

Studies on the production of plant metabolites by callus and cell suspension cultures have been carried out on an increasing scale since the end of the 1950's. The large scale cultivation of tobacco and a variety of vegetable cells was examined from the late 1950's to early 1960's by Tulecke and Nickell at Pfizer Inc., Mandels et at. at the Natick Laboratories in the U.S. Army , Street et al. at the University of Leicester and Martin et al. at the National Research Council of Canada. Their results stimulated more recent studies on the industrial application of plant cell culture in many countries. Since Japan has a highly developed fermentation technology, many industrial companies, in collaboration with some university groups have tried to apply this technology for the commercial production of useful compounds. The Japan Tobacco Inc.'s interest involved around mass-production of tobacco cells as raw materials of cigarettes; the company established 20 kl fermentors which were the largest for plant cells in 1970's. Meiji Seika in Japan also elucidated the fundamentals of production of *Panax ginseng* cells in large volumes. Researchers reported that cultured ginseng cells stimulated physiological activities in animals in a similar fashion as elicited by native ginseng roots. The work was followed by Nitto Denko Co. which has been manufacturing cell mass of ginseng commercially. The cells are used as health foods in Japan. Researchers of Kyowa Hakko

conducted extensive pharmacological screening of numerous cell cultures and found various novel products of great interest, including plasmin inhibitory proteins in *Scopolia japonica* cells and plant virus inhibitors in cultured cells of *Phytolacca americana* and other species. The virus inhibitors of *P. americana* are now being studied by many research groups in the world because of their activity against AIDS and other animal viruses.

In 1982, the 5th International Congress of Plant Tissue and Cell Cultures was held in Japan and about 70 out of 372 papers presented there related to production of secondary metabolites in cultured cells and several papers seemed to be commercially promising such as production of shikonin by Fujita et al. of Mitsui Petrochemical. International Congress on Phytotherapy held in Munich, Germany in September, 1992, many compounds were shown to be accumulated by plant cell cultures and many different strategies were presented to increase their productivity. Means for production of those compounds include not only *de novo* synthesis but also biotransformation processes. A biotransformation process to produce ß-methyl digoxin using *Digitalis lanata* cells studied by Reinhard and Alfermann in Germany was evaluated by Boehringer Mannheim Co. using 4 kl bioreactors although it has not yet been commercialized.

The recent biotechnology boom has triggered increase interest in plant cell cultures, for example, a number of firms and academic institutions in the U.S., Japan, Canada, and Europe have been investigating intensively the production of a very promising anti-tumor compound, taxol, using this technology. However, this technology is still being developed and despite the various advantages there are a variety of problems to be overcome before it can be adopted on a wide scale for the production of useful plant secondary metabolites. The success of Mitsui Petrochemical Industry Co. Ltd. in Japan in producing shikonin on a commercial scale from *Lithospermum erythrorhizon* cultivations and that of Nitto Denko Co. Ltd. also in Japan in mass production of *Panax ginseng* or ginseng cells using 20 kl tanks have demonstrated that many of the problems can be overcome with perseverance.

TABLE 1. Secondary metabolites produced in high levels by plant cell cultures

COMPOUND	PLANT SPECIES	YIELDS (% DRY WT)		CULTURE TYPE*
		CULTURE	PLANT	
Shikonin	*Lithospermum erythrorhizon*	20	1.5	s
Ginsenoside	*Panax ginseng*	27	4.5	c
Anthraquinones	*Morinda citrifolia*	18	0.3	s
Ajmalicine	*Catharanthus roseus*	1.0	0.3	s
Rosmarinic acid	*Coleus blumeii*	15	3	s
Ubiquinone-10	*Nicotiana tabacum*	0.036	0.003	s
Diosgenin	*Dioscorea deltoides*	2	2	s
Benzylisoquinoline Alkaloids	*Coptis japonica*	11	5 - 10	s
Berberine	*Thalictrum minor*	10	0.01	s
Berberine	*Coptis japonica*	10	2 - 4	s
Anthraquinones	*Galium verum*	5.4	1.2	s
Anthraquinones	*Galium aparine*	3.8	0.2	s
Nicotine	*Nicotiana tabacum*	3.4	2.0	c
Bisoclaurine	*Stephania cepharantha*	2.3	0.8	s
Tripdiolide	*Tripteryqium wilfordii*	0.05	0.001	s
* s = suspension; c = callus				

Several products were found to be accumulated in cultured cells at a higher level than those in native plants through optimization of cultural conditions (Table 2). For example, ginsenosides by *Panax ginseng*, rosmarinic acid by *Colleus blumei*, shikonin by *Lithospermum erythrorhizon*, diosgenin by *Dioscorea*, ubiquinone-10 by *Nicotiana tabacum* were accumulated in much higher levels in cultured cells than in the intact plants. However, many reports have described that yields of desired products were very low or sometimes not detectable in dedifferentiated cells such as callus tissues or suspension cultured cells. In order to obtain products in concentrations high enough for commercial manufacturing, therefore, many efforts have been made to stimulate or restore biosynthetic activities of cultured cells using various methods. The following are typical approaches that may increase productivity of cultured plant cells. These factors include media components, phytohormones (growth regulators), pH, temperature, aeration, agitation, light, etc. Sucrose and glucose are the preferred carbon source for plant tissue cultures. The concentration of the carbon source affects cell growth and yield of secondary metabolites in many cases. The maximum yield of rosmarinic acid produced by cell suspension cultures of *Salvia officinalis* was 3.5 g/L when 5% of sucrose was used but it was 0.7 g/L in the medium containing 3% sucrose (Whitaker et al. 1984).

Phytohormones such as auxins and kinetins have shown the most remarkable effects on growth and productivity of plant metabolites. In general, an increase of auxin levels, such as 2, 4-D, in the medium stimulates dedifferentiation of the cells and consequently diminishes the level of secondary metabolites. This is why auxins are commonly added to the medium for callus induction, but they are added at a low concentration or omitted for production of metabolites. Decendit reported that cytokinins stimulated alkaloid synthesis which was induced by removing auxin from the medium of a cell line of *C. roseus*. However, productions of L-DOPA by *Mucuna pruriens* (Brain, 1976), ubiquinone-10 by *N. tabacum* (Ikeda, 1976) and diosgenin by *Diocorea deltoidea* (47) were stimulated by high levels of 2,4-D. Gibberellic acid is also effective on plant cell cultures. DiCosmo et al. reported that the growth of callus of a taxol-producing

plant, *Taxus cuspidata*, was significantly promoted by addition of gibberellic acid into the solid medium.

Most products are generally accumulated intra-cellularly by cultured plant cells, but some compounds were reported to be secreted into the media. *Chinchona ledgerina* cells excrete anthraquinones in the liquid medium. Robins et al. reported that addition of a resin, XAD-7, into its suspension culture stimulated the production of anthraquinones up to 539 mg/L which was approximately a 15 times increase compared to the medium without resin. In 1976, Zenk and his colleagues in Germany obtained cell lines of *Catharanthus roseus* which accumulated higher levels of ajmalicine and serpentine. Yamada et al. reported cell cloning using cell aggregates of *Coptis japonica*, and obtained a strain which grew faster and produced a higher amount of berberine and cultivated the strain in a 14 L bioreactor. The selected cell line increased growth about 6-fold in 3 weeks and the highest amount of the alkaloid produced was 1.2 g/L of the medium. The strain was very stable, producing a high level of berberine even after 27 generations.

Addition of appropriate precursors or related compounds to the culture media sometimes stimulates secondary metabolite production. This approach is advantageous if the precursors are inexpensive. Since Chan and Staba initially examined the production of alkaloids with this approach in the 1960's, many similar experiments have been carried out. For example, amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids, and ephedorin and some stimulative effects have been observed. It is true that some amino acids are precursors of various alkaloids, but generally the biosynthetic steps from amino acids to alkaloids are so complicated that the author doubts whether amino acids added were incorporated into the alkaloids directly in cell culture. Perhaps, they affected not only alkaloid biosynthesis directly as precursors, but also indirectly through other metabolic pathways in the cells.

CHAPTER-3

MATERIALS AND METHODS

PLANT MATERIAL

Seeds and seedlings of *G. kurroo* were collected from Department of Forest Products and Utilization of Dr.Y.S Parmar University Of Horticulture And Forestry, Nauni, Solan H.P. The seedlings were transferred to pots. As the plant grows at high altitudes and needs moderate amounts of sunlight, it was periodically exposed to sun and at other times kept in shade. The leaves from these plants were used as explants for in vitro culture initiation.

MEDIA AND CULTURE CONDITIONS

The basal MS media (Murashige and skoog 1962) with 3% sucrose solidified with 0.8% agar-agar was used in all the experiments. The pH of media was adjusted to 5.8 before autoclaving. The media was supplemented with various growth regulators depending upon the experiment. All the cultures were incubated at 25 ± 2°C in culture room with 16:8 light and dark photoperiod.

SURFACE STERLIZATION OF EXPLANTS

The explants used for the initiation of in vitro culture need to be surface sterilized because these contain inoculums of various organisms. Overzealous treatment with chemical sterilants removes the organisms but is also lethal to plant tissue. So it is important to determine the optimal conditions for each plant, species and tissue used. In this investigation various surface sterilants were tested at different concentrations and time period for their effectiveness as surface sterilants. The explants were rinsed thoroughly under running tap water and then washed in a mild detergent before treatment with chemical sterilants. The explants were then rinsed with autoclaved distilled water to remove the traces of chemicals.

IN VITRO SEED GERMINATION

The seeds of *G. kurroo* were surface sterilized with varying concentrations of mercuric chloride and then cultured on basal MS media. The cultured seedlings were then incubated in the culture room at 16 hour photoperiod.

CALLUS INDUCTION EXPERIMENT

The callus induction experiment was conducted to standardize the media for the initiation and proliferation of callus from cultured explants. MS media supplemented with various combinations and concentration of the growth regulators (Table1) were investigated for the initiation of callus. In total 11 media were tried for callus induction. Each treatment was replicated six times.

ROOT INDUCTION EXPERIMENT

This experiment was designed to standardize the media for rapid regeneration of roots from cultured explants. In this case various combination and concentration of plant growth regulators in MS media were investigated for the initiation of roots from explants. The various types of media tried for root induction have been given in Table 2.

Table 1. MS media used for Callus Induction

S. No.	Media Code	BAP(mg/l)	NAA(mg/l)	IBA(mg/l)
1	C1	0.5	0.10	0.10
2	C2	1.0	0.50	0.50
3	C3	2.0	1.00	1.00
4	C4	2.0	0.50	0.50
5	C5	3.0	1.00	1.00
6	C6	0.5	0.10	0.00
7	C7	1.0	0.50	0.00
8	C8	2.0	1.00	0.00
9	C9	0.0	0.50	0.50
10	C10	0.0	1.00	1.00
11	C11	0.0	1.00	0.00

Table 2 MS media used for Root Induction

S. No.	Media Code	NAA (mg/l)	IBA(mg/l)
1	R1	0.10	0.00
2	R2	0.25	0.00
3	R3	0.50	0.00
4	R4	0.00	0.10
5	R5	0.00	0.25
6	R6	0.00	0.50
7	R7	0.10	0.10
8	R8	0.25	0.25
9	R9	0.10	0.25

RESULTS AND DISCUSSION

In present investigation an attempt was made to standardize methods and protocol to initiate and maintain the in vitro cultures of *Gentiana kurroo* with a larger objective of investigating the in vitro production of secondary metabolites under cultural conditions.

In vitro seed germination experiment was conducted to have axenic seedlings under in vitro conditions which could be used for rapid establishment of the in vitro cultures. The explants taken from such plants do not require the surface sterilization hence result in rapid establishment of *in vitro* cultures because the surface sterilization treatments are injurious to plant tissues and cells and these take some time to recover from the shock. However the seed germination was very poor. There can be many reasons for this poor seed germination. First of all the seeds of this species were very small rather seems to be the powder. So the surface sterilization treatments tries may have proved detrimental. The other reason could be the chilling requirement. *G. kurroo* is a native of New Zealand and it has been reported that it germinates in response to chilling temperatures (Simpson & Webb 1980). Being a plant of temperate region it has a low temperature requirement for seed germination which may be related to the extent of winter cold. It has been reported that seeds of this species require dark for the proper germination. Therefore seed germination did not take place under illuminated conditions with a 16 h photoperiod. Seeds germinate best if given a period of cold stratification and quickly lose viability when stored, with older seed germinating slowly and erratically. Thus to enhance the seed germination under in vitro conditions needs further investigation. It may be advantageous to keep the seed at about 10°c or below for a few. GA3 has also been shown to substitute for the cold temperature requirements of many dormant seeds. For G .*corymbifera* 100 mg/liter GA3 gave maximum seed germination in contrast to the 300 mg/liter GA3 reported by Bicknell (1984) for *G. Scabra.* In both sets of experiments higher GA3 concentrations were tested for their effects on dormancy breaking and were found to be less effective so it is concluded that excessive GA3 concentrations can suppress seed germination in *Gentiana.* Germination was always

significantly lower in the absence of GA3 compared to when GA3 was present. Thus GA3 can be tried at low concentration in media to improve the seed germination.

The leaf explants of G. kurroo were surface sterilized with varying concentrations of mercuric chloride. Exposure time of plant tissue to mercuric chloride was also varied between 1 to 4 minutes. But all the concentrations and exposure time of mercuric chloride was found detrimental for the plant tissue as it turned brown and eventually died within 15-20 days. This forced us to try other surface sterilants. Sodium hypochlorite at 1% concentration was tried at varying exposure times. Although it appear to be better surface sterilant to this sensitive plant but the time of treatment need to be standardized.

Callus induction from leaf experiment was attempted to by culturing the leaf explants on various combination and concentrations of growth regulators (Table 2). This experiment was conducted in parallel with standardization of surface sterilization treatments. All the surface sterilization treatments given proved detrimental to the leaf explants which died within 10-15 days after inoculation. Thus response of various media tried for callus induction from leaf explants could not be recorded. Similarly the response of different root induction media tried was not available due to the same reason. In the literature various authors such as Fiuk and Rybczynski, Tomiczak et al who have worked on various aspects of plant tissue culture e.g. protoplast isolation and culture and genetic transformation of different species of *Gentiana* have used the established culture derived from in vitro grown seedlings. Thus to initiate and maintain the in vitro culture from field grown plant as well as from seed culture of *Gentiana kurroo* need further investigation for longer duration so that a reproducible method of callus and root proliferation can be documented. This would be step towards the exploring the possibility of *in vitro* production of secondary metabolites from this species.

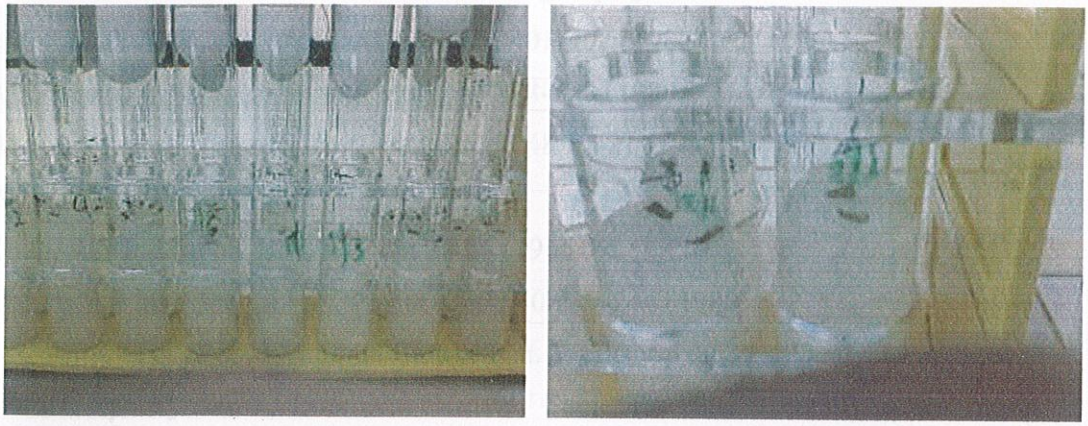

Fig.1 Leaf Explants of *Gentiana kurro* cultured on MS media in culture tubes

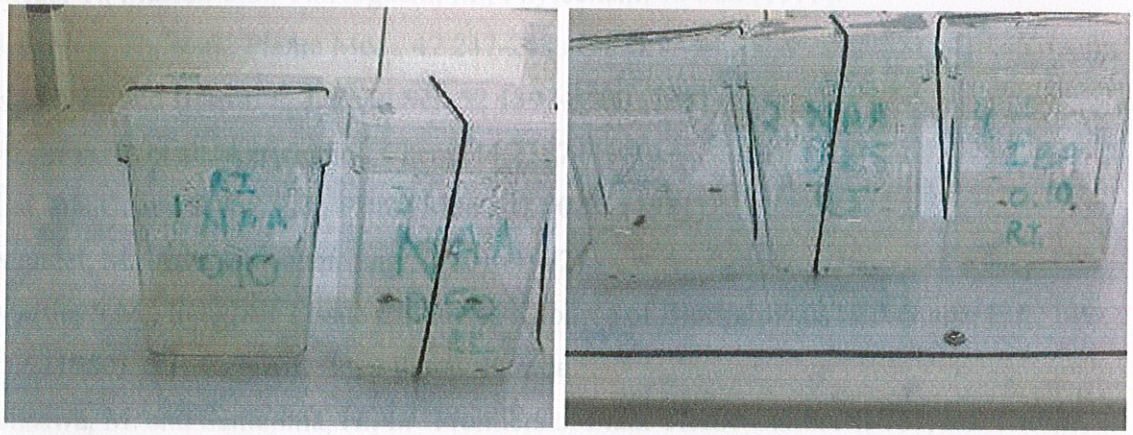

Fig.2 Leaf Explants of *Gentiana kurro* cultured on MS media in Magenta boxes

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