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STANDARDIZATION OF METHOD TO INITIATE AND ESTABLISH *IN VITRO* CULTURE OF WITHANIA SOMNIFERA

By

SUHANA 031534
GOPESH KRISHNA PANDEY 031538

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DEPARTMENT OF BIOINFORMATICS AND BIOTECHNOLOGY
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY-WAKNAGHAT
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CERTIFICATE

This is to certify that the work entitled, "Standardization of method to initiate and establish *in vitro* culture of *Withania somnifera*" submitted by SUHANA and GOPESH KRISHNA PANDEY 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

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Suhana.
Gopesh.

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LIST OF ABBREVIATIONS

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

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Fig.1 Leaf Explants of *Withania somnifera* cultured on MS media in culture tubes

Fig.2 **(A)** Root initiation from cultured leaf explants of *Withania somnifera*
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ABSTRACT

The Indian Himalayan region (IHR) is undergoing heavy extraction of wild, medicinal herbs, thus endangering many of these species. *Withania somnifera* 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 *withania somnifera* with larger objective of secondary metabolite production. Root regeneration from leaf explants, cultured on Murashige and Skoog (MS) media supplemented with IBA (1, 2 and 3 mg l^{-1}) and IAA (0.5 mg l^{-1}) was observed within a week of culturing.

CHAPTER-1

INTRODUCTION

Withania somnifera (Ashwagandha) is a small or middle sized under shrub, belongs to family solanaceae and also known as "Indian ginseng". It is distributed throughout the dry region of India, especially in wasteland ascending to an altitude of 2000 m in the Himalaya. Withania has been tapped commercially for the alkaloid withaferin which is therapeutically active, present in the roots and is of great importance in medicinal field. In animal studies, it has shown significant anticancer activity. The over exploitation of this species from natural habitats for extraction of products by destructive methods has rendered it critically endangered. Therefore this species needs to be conserved to avoid its extinction.

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 *withania somnifera* with a larger objective of developing method for producing withaniferin through *in vitro* plant tissue culture techniques.

OBJECTIVES

The objectives of this project were

1. To standardize the method for induction of root and callus culture in *Withania somnifera* 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	Tripterygium 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* 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.

There are number of reports available with respect to cell and tissue culture of *Withania somnifera*, most of which are concerned with plant regeneration and plant propagation of this species. However some workers have started exploring the possibilities of rapid multiplication of biomass of this species in large scale for the production of secondary metabolites. Wadegoankar et al 2006 reported direct rooting from leaf explants of *Withania somnifera* on half strength MS media supplemented with IAA and IBA. They multiplied the root biomass in liquid MS media and observed that the concentration of the alkaloids increased as compared to field grown roots.

Gita Rani et al 2003 obtained direct rooting from *Withania somnifera* leaf segments using IBA dip treatment and subsequent culturing on MS basal medium .The maximum rooting occurred when leaf segments were placed on half strength medium after a dip treatment with 100mg/l IBA solution for 20 minutes.

Kumar et al 2005 reported induction of transgenic hairy roots from leaf explants of *Withania somnifera* by infecting leaf explants with *Agrobacterium rhizogenes*. The induced hairy roots were grown in Murashige and Skoog (MS) solid as well as liquid media. They conducted high performance liquid chromatography analysis for withanloids which indicated the enhancement of these secondary metabolites.

CHAPTER-3

MATERIALS AND METHODS

PLANT MATERIAL

The plant material of *withania somnifera* was procured from Deptt. of forest products and utilization, Dr. Y. S. Parmar University Nauni, Solan, Himachal Pradesh. These plants were planted in medicinal plant field as well as in the pots in the lab area. The leaves from these plants were used as explants for *in vitro* culture experiments.

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 photoperiods.

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 micro 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 HgCl2 was used as a chemical sterilant at various concentrations like 0.05%, 0.1%, 0.2%, 0.5% and time period like 1,2,3 minutes. 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.

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 concentrations of plant growth regulators such as BAP, 2,4D, and Kinetin were used to initiate callus formation from the explants .In total 18 media were tried for callus induction with six replications (Table 2).

ROOT INDUCTION EXPERIMENT

This experiment was done to standardize the media and conditions for rapid regeneration of root from the cultured explants. In this case the full strength and half strength MS media supplemented with various combinations of growth regulators were used. This experiment was conducted by using 36 media and each treatment was replicated six times (Table 3A & 3B).

Table 2. MS media and growth regulators used for callus induction

S. No.	Media Code	BAP(mg/l)	2,4D(mg/l)	Kn (mg/l)
1	C1	1.00	0.10	0.00
2	C2	2.0	0.10	0.00
3	C3	3.0	0.10	0.00
4	C4	1.0	0.25	0.00
5	C5	2.0	0.25	0.00
6	C6	3.0	0.25	0.00
7	C7	1.0	0.50	0.00
8	C8	2.0	0.50	0.00
9	C9	3.0	0.50	0.00
10	C10	1.0	1.00	0.10
11	C11	2.0	1.00	0.10
12	C12	3.0	0.00	0.10
13	C13	1.0	0.00	0.25
14	C14	2.0	0.00	0.25
15	C15	3.0	0.00	0.25
16	C16	1.0	0.00	0.50
17	C17	2.0	0.00	0.50
18	C18	3.0	0.00	0.50

Table 3 (A) MS half strength media and growth regulators used for root induction

S. No.	Media Code	IBA (mg/l)	IAA (mg/l)	BA (mg/l)
1	R1	1.00	0.10	0.00
2	R2	2.00	0.10	0.00
3	R3	3.00	0.10	0.00
4	R4	1.00	0.25	0.00
5	R5	2.00	0.25	0.00
6	R6	3.00	0.25	0.00
7	R7	1.00	0.50	0.00
8	R8	2.00	0.50	0.00
9	R9	3.00	0.50	0.00
10	R10	1.00	0.10	0.10
11	R11	2.00	0.10	0.10
12	R12	3.00	0.10	0.10
13	R13	1.00	0.25	0.10
14	R14	2.00	0.25	0.10
15	R15	3.00	0.25	0.10
16	R16	1.00	0.50	0.10
17	R17	2.00	0.50	0.10
18	R18	3.00	0.50	0.10

Table 3 (B) MS Full strength media and growth regulators used for root induction

S. No.	Media Code	IBA (mg/l)	IAA (mg/l)	BA (mg/l)
1	R19	1.00	0.10	0.00
2	R20	2.00	0.10	0.00
3	R21	3.00	0.10	0.00
4	R22	1.00	0.25	0.00
5	R23	2.00	0.25	0.00
6	R24	3.00	0.25	0.00
7	R25	1.00	0.50	0.00
8	R26	2.00	0.50	0.00
9	R27	3.00	0.50	0.00
10	R28	1.00	0.10	0.10
11	R29	2.00	0.10	0.10
12	R30	3.00	0.10	0.10
13	R31	1.00	0.25	0.10
14	R32	2.00	0.25	0.10
15	R33	3.00	0.25	0.10
16	R34	1.00	0.50	0.10
17	R35	2.00	0.50	0.10
18	R36	3.00	0.50	0.10

CHAPTER-4

RESULTS AND DISCUSSIONS

In present investigation an attempt was made to standardize methods and protocol to initiate and maintain the *in vitro* cultures of Withania somnifera with a larger objective of investigating the *in vitro* production of secondary metabolites under cultural conditions. The leaf explants of *Withania somnifera* 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. The surface sterilization with 0.1% mercuric chloride for one minute gave adequate culture establishment without contraindications where as higher concentrations of $HgCl_2$ and exposure time proved injurious to explants too. However late onset of the infection in all the cultures were observed after culture establishment after 15-20 days of culturing. It was observed that this infection does not arise from the explants in most of the cases. The culture vessel used in this experiment was plastic magenta boxes. The lids of these boxes used to get loose after their transfer to lower temperature in culture room. This might be the reason for this recurrent contamination after culture establishment.

Callus induction from leaf experiment was attempted to by culturing the leaf explants on full strength MS media supplemented with combination and concentrations of BAP, 2, 4-D and kinetin (Table 2). In total 18 different media with varying growth regulator composition were tried for callus induction. The observations were recorded after 10-15 days. It was observed that none of the media tried induced callus formation from cultured explants during this time period. Due to the late onset of infection in the cultures as well as scarcity of time these culture could not be observed for longer duration for any kind of response.

Therefore it requires further investigation for the induction of the callus with different composition of growth regulators as well as maintaining the explants on media for longer duration. The withanloids generally accumulates in the roots of this plant under natural condition so much of the efforts are concentrated towards the induction and culture of root biomass of this species under in *vitro* conditions also. Most of the *in vitro* plant secondary metabolite production methods involve the suspension culture of plant cells which are generally derived from the callus cultures induced and maintained on the solid media. The cell suspension cultures are preferred because these can be maintained and handled like microorganisms in the bioreactors. So it will be worth while to induce the callus culture of *Withania somnifera* and check whether withanloids accumulate in callus culture. Otherwise also the efforts can be made to elicit their accumulation by modifying the culture conditions, modifying media and use of the elicitators owing to the advantages of suspension culture for secondary metabolite production.

The root induction experiment was conducted to standardize the composition of growth regulators in the MS media. In this experiment half strength MS media as well as full strength MS media supplemented with combinations of IBA, IAA and BAP were used. The leaf explants were cultured on 36 media treatments each of which was replicated for the six times. The observations were recorded on daily basis. The number days taken to root initiation and percentage of explants rooted were recorded at the end of the experiment. The root initiation occurred in the explants cultured on the MS half strength media R7, R8 and R9 (Table 4). All these media contains 0.5mg/l IAA and 1, 2, 3 mg/l IBA respectively. Wadegoankar et al 2006 have also reported the induction of root initiation from leaf explants using IBA and IAA in the half strength MS media whereas Rani et al 2004 induced rooting from leaf explants by giving a dip in IBA solution and there subsequent culture on half strength MS

media. So, half strength media with concentration of IBA 1mg/l and IAA 0.5mg/l is best suited for the *in vitro* root initiation of Ashwagandha since percentage of explants responded was highest in minimum number of days with this media composition.

Table-4 Media treatments and there response toward initiation of roots from cultured leaf explants

S.No.	Media	No. of days taken for root initiation	Percentage of explants rooted
1	**R7** (MS Half strength +1.0mg/l IBA and 0.5mg IAA)	15 days	25%
2	**R8** (MS Half strength +2.0mg/l IBA and 0.5mg IAA)	15 days	15%
3	**R9** (MS Half strength +3.0mg/l IBA and 0.5mg IAA)	19 days	10%

Fig.1 Leaf Explants of *Withania somnifera* cultured on MS media in culture tubes

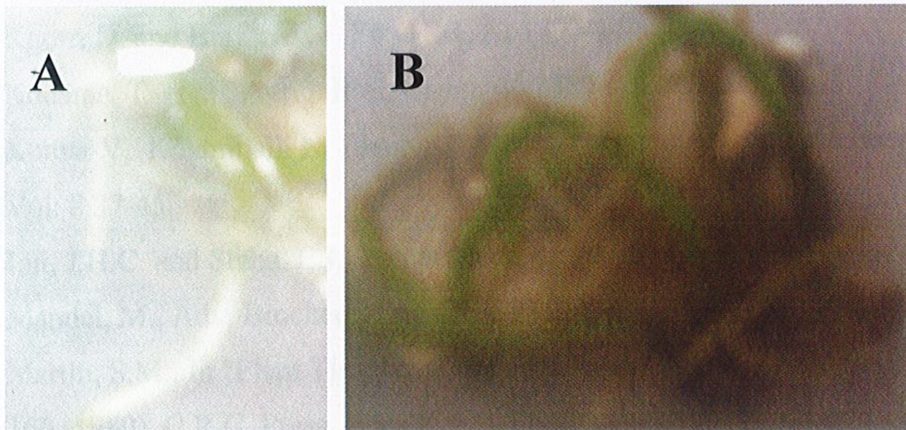

Fig.2 (A) Root initiation from cultured leaf explants of *Withania somnifera*
(B) Proliferation of regenerated roots from leaf explants of *Withania somnifera*

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