ESTABLISHMENT OF *IN VITRO* CULTURES OF *AZADIRACHTA INDICA* A. JUSS. AND *SPILANTHES ACMELLA* MURR. AND THEIR POTENTIAL FOR THE PRODUCTION OF SECONDARY METABOLITES

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Dedicated to my parents

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the supervision of Dr Rakhi Chaturvedi.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: June, 2011 **Mithilesh Singh**

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Establishment of *in vitro* cultures of *Azadirachta indica* A. Juss. and *Spilanthes acmella* Murr. and their potential for the production of secondary metabolites**" by Mithilesh Singh for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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ABSTRACT

Medicinal plants have always had an important place in the therapeutic arsenal of mankind. In the last few years as the demand for medicinal plants is increasing; their exploitation by the local population as well as pharmaceutical companies is also increasing continuously. It is, therefore, imperative to develop efficient methods for its large scale propagation. Besides micropropagation, *in vitro* production of useful bioactive compounds, their identification as well as quantification is also highly warranted. In this context, plant cell cultures have been proved handy for the production of high value secondary metabolites owing to the consistency in quality and quantity of the desired product. Plant cells are biosynthetically totipotent which means that each cell in culture retains the complete genetic information to produce the range of chemicals found in the parent plant.

***Azadirachta indica* A. Juss.** is a remarkable multipurpose, evergreen tree of the mahogany family, Meliaceae. The history of commercial use of neem tree is shrouded in the mystery and tradition of Vedic period of India. In the last two decades, neem has become the focus of attention due to its medicinal, agrochemical and economic uses. These properties can be attributed to several secondary metabolites present in the genus most of which, chemically, belong to the class of terpenoids. Among the entire range of diverse chemicals, one which remains the most sought after by scientists is azadirachtin, a highly oxidized tetranortriterpenoid, present in the neem seed kernels. This molecule has emerged as the safest, environment friendly and biodegradable biopesticide, unlike the chemical ones that pose problems of bioaccumulation in food chains upon long term usage. Current supply of azadirachtin from neem tree will not meet the increasing demand if the extractions from seeds remain the only source, hence, there is a need for the development of a commercially viable alternative for its enhanced and continuous production. Chemically, azadirachtin is a complex molecule and due to this complexity it is difficult to accomplish its total chemical synthesis in the laboratory. Keeping in consideration all these factors, plant cell culture can be seen as a potential alternative production system. With the cell culture methods,

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production can be more controlled and the product quality and quantity can be ensured, independent of geographical and climatic barriers. In cell culture, the culture conditions and process variable can be more easily optimized. Cell culture can also offer better selectivity and yield of the desired bioactive compound. In the present study, we made an effort towards systematic selection and screening of elite *in vitro* cell lines for constant and improved production of azadirachtin.

Spilanthes acmella **Murr.** is an indigenous herb belonging to the family Asteraceae. The genus contains several secondary metabolites which are of therapeutic value and have been widely used in traditional medicine throughout the world, since time immemorial. In spite of being a plant of potential medicinal interest there are only a few scientific reports on the properties of this species. All the biochemical studies carried out, so far, have been done using natural population. As we are aware that environmental fluxes cause alterations in type and quantity of metabolites produced, establishment of *in vitro* cultures will help to utilize the biomass and nullify the effect of seasonal variation on secondary metabolite content. The present study, examines the biotechnological worth of the plant through tissue culture. The cultures obtained have been analyzed for the production of two important compounds, spilanthol (an alkylamide) and scopoletin (a coumarin). Moreover, their practical utility has also been checked by bioassays.

The present thesis is divided into five chapters. Chapter 1, introduces and reviews all the major contributions and studies taken up till date, with regard to tissue culture, secondary metabolite production and bioassays, in both the plants. Chapter 2, groups together all the protocols and methodologies adopted for the present work. Chapter 3, presents the results obtained in the current investigation. The tables and graphs are included within the text while all the figures have been compiled at the end of the thesis, in the form of plates. Inferences drawn from the results are discussed in chapter 4, in the light of other reports available on related aspects. Chapter 5, throws light on the major highlights of the present work and its future scope. This is followed by the list of bibliography, and appendix that mentions the taxonomic classification of the plants, studied. The thesis ends with the visible research output in terms of peer-reviewed journal publications, book chapters and conference proceedings.

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LIST OF ABBREVIATIONS

ABA	☞	Abscisic acid
AO	☞	Acridine orange
ATCC	☞	American type culture collection
AUF	☞	Absorbance units at full scales
B_5	☞	Gamborg's medium
BAP	☞	N^6-benzylaminopurine
CCD	☞	Central composite design
CH	☞	Casein hydrolysate
CM	☞	Coconut milk
COX-2	☞	Cyclooxygenase-2
2,4-D	☞	2,4-dichlorophenoxyacetic acid
DCM	☞	Dichloromethane
DKW	☞	Driver's and Kuniyuki medium
DMSO	☞	Dimethyl sulphoxide
DNA	☞	Deoxyribonucleic acid
DPPH	☞	2,2-diphenyl-1-picrylhydrazyl
DW	☞	Dry weight
ESI	☞	Electrospray ionization
FDA	☞	Fluorescein diacetate
FL	☞	Fluorescence
FW	☞	Fresh weight
GA_3	☞	Gibberellic acid
GABA	☞	Gamma- aminobutyric acid
$HgCl_2$	☞	Mercuric chloride
HPLC	☞	High performance liquid chromatography
2-iP	☞	2-isopentenyl adenine
IAA	☞	Indole-3-acetic acid
IBA	☞	Indole-3-butyric acid

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IL ☞ Interleukin

iNOS ☞ Inducible NO synthase

Kinetin ☞ 6-furfurylaminopurine

LM ☞ Light microscopy

MS ☞ Murashige and Skoog’s medium

MS ☞ Mass spectroscopy

MTCC ☞ Microbial type culture collection

NAA ☞ α-naphthaleneacetic acid

NB ☞ Nitsch medium

NCTC ☞ National type culture collection

NLN ☞ Lichter’s medium

NO ☞ Nitric oxide

NSAIDs ☞ Non-steroidal anti-inflammatory drugs

NSO ☞ Neem seed oil

PB ☞ Plackett burman

PBS ☞ Phosphate buffered saline

PC-3 ☞ Prostate cancer cells

pCPA ☞ para-Chlorophenoxyacetic acid

PI ☞ Propidium iodide

PVP ☞ Polyvinylpyrrolidone

PVPP ☞ Polyvinyl-polypyrrolidone

RAPD ☞ Random amplified polymorphic DNA

RNA ☞ Ribonucleic acid

RSM ☞ Response surface methodology

Rt ☞ Retention time

SDW ☞ Sterile distilled water

SEM ☞ Scanning electron microscopy

TBA ☞ Tertiary butyl alcohol

TDZ ☞ Thidiazuron

tTCL ☞ Transverse thin cell layer

TNF-α ☞ Tumor necrosis factor- α

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UV		Ultraviolet

LIST OF UNITS

amu	☞	atomic mass unit
DF	☞	degree of freedom
µg/g	☞	microgram per gram
µl	☞	microlitre
µm	☞	micrometer
°C	☞	degree Celsius
cm	☞	centimeter
mm	☞	millimeter
g	☞	gram
h	☞	hour
l/g	☞	litre per gram
LSD	☞	least significant difference
mg/ml	☞	milligram per milliliter
min	☞	minute
mm	☞	millimeter
mM	☞	millimolar
nm	☞	nanometer
rpm	☞	revolution per minute
SD	☞	standard deviation
SE	☞	standard error
SED	☞	standard error of difference
SS	☞	sum of squares
MS	☞	mean squares
µ	☞	specific growth rate
%RS	☞	percent relative standard deviation

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Chapter 1

Introduction and Literature Review

1.1 GENERAL

***Azadirachta indica* A. Juss.** (Common names: Neem, Margosa and Indian lilac) is a remarkable multipurpose, evergreen tree of the mahogany family, Meliaceae. It is a native of Indian subcontinent (Koul *et al.* 1990) and has spread in many parts of the world. Neem is a robust, fast growing tree that can attain a height of 30 m with a trunk girth of 2.5 m and can live for over two centuries (Verkerk *et al*. 1993). It enjoys wide range of agro climatic zones and grows well in hot and dry climate where shade temperatures often reaches 50°C and annual rainfall ranges from 400 to 1200 mm (National Research Council 1992). The plant can also withstand extremes of environmental conditions like drought, infertility and poor soils but is very sensitive to cold or frost.

Natural propagation of neem occurs by seeds and the tree starts producing the yellowish ellipsoidal drupes (fruits) in about 5 years (Koul *et al*. 1990; Schmutterer 1995). The timing of flowering and fruiting varies from place to place, but in India, neem flowers in April and the fruits are ready for harvesting in July.

The history of commercial use of neem tree is shrouded in the mystery and tradition of Vedic period of India. In the last two decades, neem has become the focus of attention due to its medicinal, agrochemical and economic uses. Neem tree has been claimed to possess several biological activities, such as immune-stimulation, blood purification, anti-inflammation, anti-tumor, insect repulsion, bactericidal activity, and growth disrupting properties. These properties can be attributed to several secondary metabolites present in the genus most of which, chemically, belong to the class of terpenoids like, azadirachtin, nimbin, salannin, margosane and meliacin. Among these, azadirachtin is one of the most complex and important compound, which has been the focus of research since its isolation and characterization by Butterworth and Morgan in 1968.

With the global increase in demand for neem derived products, there has been a concomitant increase in the demand for raw material. However, the increasing

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urbanization and livestock populations have affected the status of wild plants. Further, to date, the only commercially feasible technology to produce azadirachtin is from natural seed extraction. However, this approach is very restricted because neem tree flowers once in a year, and due to operational problems and quality considerations only about 1/3 of the fruits can be collected (Jayaraj 1993; Vyas and Mistry 1996; Venkateswarlu and Mukhopadhyay 1999). Moreover, extraction from natural sources is highly variable depending on the genetic and environmental factors (Ermel *et al*. 1984, 1987; Benge 1989; Ketkar and Ketkar 1993; Ermel 1995). Collection of lower quality neem seeds has also led to inconsistent quality of products and an increase in the chances of fungal contamination during the processing of seeds. Furthermore, the chemical synthesis of azadirachtin is not an economically feasible way because of their highly complex structures and the specific stereochemical requirements. Despite advances in the science, availability of azadirachtin and other terpenoids remain a critical limitation. Hence, there is a need for the development of a commercially viable alternative for its enhanced and continuous production.

Spilanthes acmella **Murr.** (Common names: Akarkara and Toothache plant) is an indigenous species that belongs to the family, Asteraceae. There are around five species of *Spilanthes* known so far (Anonymous 1989), growing in India: *S. acmella* Murr., *S. acmella L*. var oleraceae clarke, *S. calva* L., *S. paniculata* L. and *S. mauritiana* L. Among these, *S. acmella* Murr. and *S. acmella* L. var oleraceae Clarke are rare in occurrence. It is grown as perennial plant throughout the tropics and subtropics, and can be found in damp pastures, at swamp margins, on rocks near the sea and as a weed of roadsides. *Spilanthes* is a hairy herb upto 30-60 cm tall, with numerous stem and marigold eye flower.

Flowers and leaves of the plant bear pungent taste and have been used as a spice for appetizers and as folk medicines for stammering, toothache, stomatitis and throat complains (Nakatani and Nagashima 1992; Ramsewak *et al*. 1999). This plant also possesses immune-modulatory, antioxidant and insecticidal biological properties (Ramsewak *et al*. 1999; Pandey and Agrawal 2009; Guiotto *et al*. 2008; Matthias *et al*. 2008; Prachayasittikul *et al*. 2009). Furthermore, *Spilanthes* is one of the active constituents in compositions for acute- or long-term cure of microbial infections,

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particularly, oral pathogenic microorganisms (Adler 2006). It is also effective against blood parasites at extremely low concentration and is poisonous to most invertebrates whereas harmless to the vertebrates (Watt and Brayer-Brandwijk 1962). In addition, its extract is an active constituent of beauty care cosmetics such as fast acting muscle relaxant to accelerate repair of functional wrinkles (Belfer 2007). The plant extract is also used as a nutritional supplement for taste improvement as a sweetener with high sweetness devoid of distasteful savour that does not affect the taste or odour of foods or drinks (Miyazawa *et al*. 2006). The genus contains several secondary metabolites such as alkylamides, phenolics, coumarin and triterpenoids which are therapeutic and account for its use in traditional medicines all over the world, for a long time (Prachayasittikul *et al*. 2009). In order to produce high value secondary metabolites, a constant source of planting material is required which could be utilized as a ready stock to meet the demand of the pharmaceutical industries for the production of bioactive compounds from them. Although a few reports have described chemical constituents of *Spilanthes* growing in wild, the biotechnological side is still overlooked.

Spilanthes is conventionally propagated through seeds which lose its viability within short period of time. Dependence on season and slow germination rates are some of the other major limiting factors in conventional propagation (Pati *et al*. 2006; Dobránszki and da Silva 2010). Moreover, propagation by seeds is also undesirable because of the highly heterozygous nature of the plant due to protandry, which prevents self-pollination (Reddy *et al*. 2004). Many small, bright yellow colour flowers are aggregated into capitulum (flower head) which make them attractive to insects, thus, paving the way for entomophily. The genetic variation due to insect pollination may result into high heterogeneity in quality and quantity of chemical makeup of the plant. In this context, plant cell cultures have been proved handy for the production of high value secondary metabolites owing to the consistency in quality and quantity of the desired product.

A summary of tissue culture and secondary metabolite studies, taken up till date in both the plants, is being discussed in the following sections.

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1.2. *IN VITRO* STUDIES

1.2.A. Plant Tissue Culture

1.2.A.1. *Azadirachta indica* A. Juss

In current and traditional horticulture practice, neem tree is propagated by seed, although it seldom produces a plant true to type. Moreover, the seeds of neem are recalcitrant and start losing viability after 2-3 weeks (Mohan Ram and Nair 1996). Keeping quality of the seeds is poor due to high moisture (30-35%) and oil (40-45%) content. Besides, seedlings are slow in growth and are liable to diseases and pests in the initial period. Neem seeds from different geographic regions are also known to vary considerably with respect to metabolite content and bioactivity. To maintain the genetic integrity of neem plant, vegetative propagation is very much needed. However, the vegetative methods do not ensure disease-free and healthy plants. Moreover, they typically result in low multiplication rates. In this respect, tissue culture can play an important role. Tissue culture does not only help overcome the above mentioned limitations of conventional methods, but also considerably accelerate the production of clonal material for field planting. Consequently, several investigators have attempted *in vitro* propagation of neem tree by different plant tissue culture approaches (**Table 1**). Brief overviews of two tissue culture techniques *viz*. organogenesis and somatic embryogenesis, involved in rapid multiplication of neem, are presented below:

(a) Organogenesis

Organogenesis involves adventitious and axillary shoot proliferation. It is a process by which a group of cells differentiate to form unipolar structures (either shoot or root meristems). Since recovery of plants is the usual objective, regeneration of shoots is of greater interest. Organogenesis can occur (i) directly from tissues of the excised explants without previously formed callus (direct organogenesis) or (ii) indirectly, when shoots regenerate from previously formed callus on explants or in cell cultures (indirect organogenesis). Both direct and indirect organogenesis in cell and tissue cultures require *de novo* differentiation of meristematic regions, randomly, all over the tissues other than the pre-existing meristem. Various explants like, nodal segment, shoot tip, leaf,

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cotyledon, embryo, root, anther, ovary and endosperm, have been tried with different media combinations by the scientists to obtain organogenesis in neem.

i. Meristem Culture

Meristem (apical/axillary bud or nodal segment) culture is a most popular approach for true to type clonal propagation of plants because it favours uniform plants where the bud present on the initial explant grows into a shoot. Thus, it guarantees that the characteristics of the source plant are conserved (Rao and Venkateswara 1985). Moreover, considerable gains in metabolite production can be achieved by clonal propagation of plus trees. Till date, many workers have reported clonal propagation of neem tree.

The first attempt on meristem culture was made by Drew (1993). He cultured apical and nodal segments from 6-12 months old seedlings of neem. The shoots, developed *in vitro,* were cut into one and two node segments for further multiplication. Two node cuttings gave better response than single node cuttings. On MS medium (Murashige and Skoog 1962) with N^6-benzylaminopurine (BAP; 0.1 µM), 100% cultures produced two shoots (probably one from each node) with a mean height of 10.8 mm. Recurrent multiplication of shoots was not attempted. About the same time, Joarder *et al*. (1993) cultured nodal segments from a 30-year-old tree of neem. On MS + BAP (6.6 µM), 60% of the single node cuttings exhibited bud-break, and 2 shoots developed from each node. They found that the pre-culture of nodal segments on MS basal medium for 2 weeks was essential/beneficial for bud-break to occur on MS + BAP. The rate of shoot multiplication increased in subsequent passages. For recurrent shoot multiplication, BAP was used at a lower concentration (0.44 µM). Joshi and Thengane (1996) cultured nodal segments from 2-5-year-old trees as those from 15-20-year-old trees showed negligible bud break. They found ¾ MS (major salts reduced to ¾ level) medium containing BAP (0.44 µM), 6-furfurylaminopurine (Kinetin; 0.23 µM), Calcium-pantothenate (0.05 mg/l) and biotin (0.05 mg/l) as the best medium for shoot multiplication. On this medium, the rate of shoot multiplication increased from 17-fold in the 3rd passage to 49-fold in the 5th

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passage. The shoots developed from the axillary bud as well as adventitiously from the base of the parent cutting. Gill *et al*. (1996) also reported multiple shoot formation (5-7 shoots per culture) in 80% cultures of nodal explants derived from the basal portion of the coppice shoots and cultured on MS medium supplemented with BAP (4.4 µM) and Indole-3-butyric acid (IBA; 2.46 µM). Interestingly, 39% of the cultures on MS basal medium also produced multiple shoots. Roy *et al*. (1996) found a combination of an auxin (α-naphthalene acetic acid; NAA) and a cytokinin (BAP) to be better than BAP alone for shoot proliferation in the cultures of apical and axillary buds from adult trees. However, the shoots produced on a medium containing BAP and NAA were very small and inadequate in size for rooting. Incorporation of 10% Coconut milk (CM) and 0.15% Casein hydrolysate (CH) to MS + BAP (4.4 µM) + NAA (0.5 µM) increased multiplication rate and growth of shoots (6 cm long). Islam *et al*. (1997) found synergism between BAP and Kinetin for shoot proliferation. Sarker *et al*. (1997) observed that the nodal explants responded best on MS medium supplemented with BAP (8.8 µM) and NAA (1.0 µM). Clonal multiplication of elite neem trees *via* axillary shoot proliferation has also been reported by Venkateswarlu and Mukhopadhyaya (1999). The micropropagated plants flowered after 25 months of transplantation and appeared true-to-type in terms of morphology, growth habit and azadirachtin content. Sharma *et al*. (1999) cultured nodal segments from 3-, 7- and 40-year-old trees and found the explants from younger trees to be more responsive. Shoot multiplication at a rate of five-fold in 30 days was achieved, generally, after the fifth passage on MS medium supplemented with BAP (1.1 µM) and Indole-3-acetic acid (IAA; 0.29 µM) and Adenine sulphate (81.0 µM). However, in the explants taken from 3- and 7-year-old trees, this rate of shoot multiplication was achieved even by the third subculture. Islam *et al.* (1997) recorded 4.5 shoots per explant unto fourth subculture from nodal segments of a 25-year-old tree on MS medium supplemented with BAP (4.4 µM) and Kinetin (4.7 µM). Quraishi *et al*. (2004) accomplished the micropropagation of neem by culture of nodal segments from crown branches of a mature tree, basal-sprouts of another mature tree and a single juvenile plant. A significant difference was observed in *in vitro* response of explants from these three sources. In case of crown and basal-sprouts explants, addition of

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Polyvinylpyrrolidone (PVP-40; 12.5 µM) in the establishment medium was required to control the leaching of phenols into the medium; juvenile explants did not show phenolic leaching. DKW medium (Driver and Kuniyuki 1984) with BAP (0.22 µM) was found to be significantly better than MS medium for shoot proliferation. Shoot cultures of crown branch origin did not survive and eventually died after the third subculture. In the presence of IBA (4.9 µM) in half strength DKW, 90% of the shoots from basal sprout and 100% of the shoots of juvenile origin formed roots, and plantlets survived transplantation.

Chaturvedi *et al*. (2004a) reported a recurrent method of clonal propagation of a 50-year-old mature neem tree through axillary shoot proliferation. In this case, multiple small shoots formed on ½ MS medium (major inorganic salts reduced to half strength) supplemented with BAP (1.0 µM) and Gibberellic acid (GA_3; 0.5 µM). The number of small shoots enhanced further when cultures were transferred to ½ MS + BAP (1.0 µM) + CH (500 mg/l). Further elongation and recurrent multiplication of shoots was achieved on full MS + BAP (1.0 µM) + CH (250 mg/l) through single node segment cultures, at a rate of 7-8-fold every 5 weeks, on fresh medium of the same composition. This rate of shoot multiplication was maintained for almost 5 years. The shoots were readily rooted on ¼ MS (major inorganic salts reduced to quarter strength) medium supplemented with IBA (0.5 µM), with a frequency as high as 82% and transplantation survival of these plantlets was more than 87%. Recently, Arora *et al*. (2010) established the clonal propagation of adult neem tree through nodal segment cultures and determined the clonal fidelity of *in vitro* raised field grown plants by random amplified polymorphic DNA (RAPD). Azadirachtin content of the progeny was found similar to the mother tree, thus, confirming their chemical stability as well.

ii. Leaf Culture

Shoot regeneration from leaf explants was first reported by Narayan and Jaiswal (1985). Leaf-discs derived from an adult neem tree formed dedifferentiated callus on MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) and BAP. The calli differentiated into shoot-buds when transferred onto a medium containing BAP (0.44

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µM). On this medium, 5-8 shoots per disc appeared after 6-7 weeks. Incorporation of NAA (0.3 µM) in to the medium enhanced the frequency of shoot differentiation from 54% to 62.5%. Ramesh and Padhya (1990) cultured leaf-discs from an "elite" tree. The explants formed green nodular callus on Wood and Braun's medium (Wood and Braun 1961) supplemented with 4.0 µM each of Kinetin and BAP. After 4 weeks on the same medium, the callus differentiated 10-12 shoot-buds, adventitiously. Further, addition of Adenine sulphate (16.0 µM) to the medium increased the number of buds per culture to 18-20. Healthy shoots were obtained when the shoot-buds were transferred to GA_3 containing medium. Origin of leaf-discs affected the regeneration frequency. Discs from middle of the leaf were more regenerative than those from the basal and apical portions. Eeswara *et al*. (1998) reported direct differentiation of adventitious shoot-buds on MS + BAP (4.4 µM) + Kinetin (3.7 µM) + Adenine sulphate (32.6 µM). They have highlighted the importance of dark incubation for shoot-bud differentiation. Cultures incubated in light, right from the beginning, did not form any shoot-bud. Two weeks of dark incubation was essential for shoot-bud induction in most of the clones tested. For recurrent multiplication of shoots, it was indispensable to reduce the concentration of cytokinin ten times the original concentration. These authors also observed synergism between BAP and Kinetin for growth and multiplication of shoots. Individually, BAP or Kinetin in the medium caused increased abnormalities and vitrification of shoots. The medium recommended by the authors for recurrent shoot multiplication is MS + BAP (0.44 µM) + Kinetin (0.4 µM) + Adenine sulphate (3.3 µM). Arora *et al*. (2009) emphasized the superiority of BAP over other cytokinins for shoot organogenesis in neem. They adopted a two-step procedure whereby the leaflet segments were given a pulse treatment of high concentration of BAP (8.88 µM) along with Adenine hemisulphate (81.43 µM) followed by culture of explants in one-tenth concentration of BAP (0.88 µM) along with Adenine hemisulphate at 81.43 µM. Salvi *et al.* (2001) observed shoot regeneration from leaf explants on MS + BAP (8.9 µM) + IAA (0.6 µM).

iii. Zygotic Embryo Culture

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Rangaswamy and Promila (1972) reported indirect shoot-bud differentiation from embryo explants of neem. Embryos were grown in distilled water containing four mineral salts. They observed callus initiation at the plumular region and from which several shoot-buds differentiated. Chaturvedi *et al.* (2004b) obtained plantlets from immature zygotic embryo cultures via neomorph (abnormal structures with varied morphology) formation and adventitious shoot-bud formation. Maximum direct shoot-bud differentiation (57%) occurred from early dicotyledonous stage of embryo on MS + BAP (5.0 µM) whereas maximum neomorph formation (66%) occurred in cultures of torpedo shaped embryos on MS + 2,4-D (5.0 µM). It was possible to regenerate full plants from the neomorphs via organogenesis on MS + BAP (5.0 µM).

iv. Root Culture

Salvi *et al.* (2001) observed shoot differentiation from root explants of neem on MS medium supplemented with BAP (8.9 µM) and IAA (0.6 µM). Arora *et al.* (2011) developed a two-step procedure for shoot-bud differentiation from root segment cultures. Explants were given a pulse treatment on MS medium fortified with BAP (8.9 µM), 2-isopentenyl adenine (2-iP; 9.8 µM), IAA (5.7 µM), Adenine hemisulphate (81.4 µM) and Putrescine (2.3 µM) for two days, followed by their transfer to the same medium composition containing reduced concentrations of BAP, 2-iP and IAA.

v. Cotyledon Culture

Nirmalakumari (1993) reported both direct and indirect organogenesis from cotyledon cultures of neem. The cotyledons differentiated shoot-buds, directly, in the presence BAP alone in the medium while it was preceded by callusing when the medium contained both BAP and IAA. Abubacker and Alagumanian (1999) found cotyledons to be the best explant for regeneration. All cultures of cotyledon explants developed green, compact and nodular callus on MS + 2,4-D (6.8 µM) + IAA (2.9 µM) + BAP (6.6 µM) and on the same medium, shoots differentiated from the callus. Salvi *et al.* (2001) obtained shoot proliferation from cotyledon and cotyledonary node explants on MS medium supplemented with BAP (8.9 µM) and IAA (0.6 µM).

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vi. Anther Culture

In vitro anther culture is the most efficient, quick and dependable technique to produce haploid plants. It is highly desirable in neem trees to overcome the prevalent heterozygosity. Gautam *et al*. (1993) first cultured anthers at the uninucleate stage of microspores and observed some multicellular pollen on NB medium (Nitsch 1969) containing BAP (10.0 µM) or MS + BAP (10.0 µM) + NAA/IAA (10.0 µM). However, all the plants regenerated from anther calli were diploid. The only report on haploid production in this genus is through anther culture by Chaturvedi *et al.* (2003a). They used anthers at early-to-late uninucleate stage of microspores in the experiments and observed organogenesis through a callus phase. Callusing from anthers was induced on MS basal medium (with 9% sucrose) supplemented with 2,4-D (1.0 µM), NAA (1.0 µM) and BAP (5.0 µM) while the calli multiplied best on 2,4-D (1.0 µM) and Kinetin (10.0 µM). These calli differentiated shoots when transferred to a medium containing only BAP. For younger calli, BAP (5.0 µM) was the optimum concentration (75% cultures differentiated shoots) while older calli showed the best regeneration with BAP (7.5 µM). Histological analysis revealed that initially the anther wall cells had divided while microspore divisions occurred only after 4 weeks. In 8-week-old cultures the entire anther locules were filled with microspore calli. Of the plants that regenerated from anther callus, 60% were haploids, 20% were diploids and 20% were aneuploids.

vii. Ovary Culture

So far, there is single report on *in vitro* ovary culture of neem by Srivastava *et al.* (2009). Unfertilized ovaries, obtained from closed flower buds of an adult 54-year-old neem tree were used as explants. Ovaries were excised from flower buds of four sizes (2, 3, 4 and 5 mm) and the corresponding developmental stage of ovary was determined by paraffin sections. The MS medium with sucrose (9%), 2,4-D (1.0 µM) and BAP (5.0 µM) was the best medium for inducing calli from unfertilized ovaries. The calli were further maintained and multiplied on MS medium supplemented with 2,4-D (0.5 µM) either alone or in combination with Kinetin (4.5 µM). Concentration of BAP in the regeneration medium and stage of ovary at culture were the critical factors for shoot differentiation

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from callus. Maximum shoot regeneration (78%) was observed when calli, induced from ovaries of 4 mm size flower buds and proliferating on MS + 2,4-D (0.5 µM), were subcultured to MS medium containing BAP (5.0 µM). Histological analysis revealed that 4 mm sized flower buds correspond to a 2-nucleate stage of embryo sac. The cytological analysis by root-tip squash preparation showed that all the plantlets that regenerated from ovary callus were diploids.

viii. Endosperm Culture

In angiosperms, the endosperm is a product of double fertilisation and develops through the fusion of three haploid nuclei; therefore, it is triploid. The endosperm is a homogeneous mass of parenchymatous tissue, lacking vascular bundles, so it is suitable for morphogenic studies (Bhojwani and Razdan 1996). Plantlets that are obtained from the endosperm are usually triploids and, consequently, can be used in plant improvement programs because of their different genetic makeup (Johri and Bhojwani 1988).

There is only one report on *in vitro* production of triploid plants from endosperm tissues of neem by Chaturvedi *et al*. (2003b). As neem bears non-endospermous seeds, therefore, only immature endosperm was cultured. The seed at early-dicot stage was found convenient to excise the endosperm. In older seeds, the endocarp becomes hard rendering dissections difficult. Generally, it has been found that mature endosperm requires the initial association of embryo to form callus but immature endosperm proliferates independent of the embryo. However, Chaturvedi *et al*. (2003b) observed that the association of embryo was essential in neem to induce callusing from immature endosperm; the best explant was immature seeds. Neem endosperm showed good callus proliferation (45%) on MS + 2,4-D but best callusing (53%) occurred on MS + NAA + BAP + CH. On MS + NAA (5.0 µM) + BAP (2.0 µM) + CH (500 mg/l), the compact and nodular calli of neem endosperm differentiated into green loci. When these calli were transferred to a medium containing a cytokinin (BAP/Kinetin), shoot-buds differentiated from all over the callus. Maximum regeneration, in terms of number of cultures showing shoot-buds and number of buds per callus, occurred in the presence of BAP at 5.0 µM. A characteristic feature of the regenerants from neem endosperm callus was the

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predominance of multicellular glands on the surface of the shoots. These glands, occasionally stalked, were found in large numbers on younger leaves close to the shoot apex. Shoots were multiplied by forced axillary branching and rooted *in vitro*. Cytological analysis revealed that over 66% of the plants were triploids with 2n=3x=36 and rest were diploids with 2n=2x=24.

(b) Somatic Embryogenesis

Somatic embryogenesis is the process by which somatic cells, under inductive conditions, generate embryogenic cells, which undergo a series of morphological and bio-chemical changes resulting in the formation of somatic embryos (Komamine *et al.* 2005). Somatic embryogenesis may offer many advantages over organogenesis, such as single cell origin, possibility to automate large scale production of embryos in bioreactors and their field planting as synthetic seeds. Moreover, the bipolar nature of embryos allow their direct development into plantlets without the need of a rooting stage as required for plant regeneration via organogenesis (Bhojwani and Razdan 1996). It can also be used for the production of metabolites in species where embryos are the reservoir of important biochemical compounds. Furthermore, epidermal single cell origin of somatic embryos favours the use of this process for plant transformation. To obtain somatic embryogenesis in neem, various explants have been employed and the most common being the juvenile material, such as excised cotyledons, hypocotyls or immature embryos at various stages of development.

i. Embryogenic Cultures

The first attempt to induce somatic embryogenesis in neem was made by Muralidharan and Mascarenhas (1989) where embryo-like structures from cotyledon was obtained. On B_5 (Gamborg *et al.* 1968) medium containing BAP (4.4 µM), the explants formed nodular structures, which appeared bipolar. These structures did not show germination if left on the same medium. Upon transfer to hormone-free B_5 solid medium, the nodular structures elongated and leafy appendages developed. However, on hormone-free B_5

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liquid medium, a tap root with leaf like structures developed. No studies were conducted to confirm the embryonic nature of the bipolar structures.

Shrikhande *et al.* (1993) used immature cotyledons to test the effect of different plant growth regulators and carbohydrates on embryo induction. The best somatic embryo induction was obtained on MS medium containing 5% sucrose, BAP (4.4 µM), IAA (2.9 µM) and CH (1000 mg/l). Although these authors have furnished histological data in support of embryogenesis, the structures labelled as somatic embryos appear more like shoots, with their vascular traces running into the callus. None of the structures resembled a bipolar embryo with closed radicular and plumular poles. Moreover, on germination medium the so called "embryos" developed only a shoot, which had to be transferred to hormone-free ½ MS medium for rooting. Most surprisingly, the authors have described mature embryos with suspensor. Mature embryos generally lack a suspensor and at no stage can the suspensor be seen in macrophotographs as depicted in this paper. Islam *et al.* (1993) reported very high incidence of embryogenesis in the cultures of cotyledons on MS medium supplemented with NAA (8.0 µM). However, the germination frequency of the embryos, on MS medium containing BAP (0.9 µM) and GA_3 (0.03 µM), was very poor (maximum 11.7%) and germination was monopolar. The embryos formed only shoots. The first conclusive evidence for induction of somatic embryogenesis in neem was demonstrated by Su *et al.* (1997). They were unable to confirm the results of Shrikhande *et al.* (1993). Under the culture conditions Shrikhande *et al.* (1993) got somatic embryos, Su *et al.* (1997) observed only shoot-bud formation. The calli initiated from cotyledons or hypocotyls on MS medium supplemented with BAP (4.4 µM), NAA (2.7 µM), CH (1000 mg/l) and 5% sucrose, differentiated greenish globular detachable structures when transferred to MS + BAP + IAA + CH, in suspension cultures. In further subcultures on the same medium, the globular structures differentiated shoots and roots. However, if transferred to MS medium (5% sucrose) supplemented with only CH (1000 mg/l), the globular structures produced embryos on the spherical head. Maximum embryo differentiation (73%) occurred on MS (10% sucrose) + Zeatin (0.9 µM). In this case, germination of embryos occurred on ½ MS medium (major inorganic salts reduced to half

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strength) containing 1% sucrose. Murthy and Saxena (1998) observed somatic embryo formation in the cultures of mature seeds directly as well as via callusing. The cotyledonary callus differentiated nodular structures in suspension cultures in MS medium with or without Thidiazuron (TDZ; 1 µM). When planted on hormone-free, semi-solid medium, these structures differentiated embryos, which germinated (60-70%) on the same medium. Gairi and Rashid (2004) reported direct differentiation of somatic embryos from different regions of hypocotyl, epicotyl, cotyledonary-node, cotyledons and leaves of intact seedlings of neem on MS medium containing TDZ (1.0 µM). Individual embryos on transfer to hormone-free medium regenerated readily into plantlets.

The developmental stage of embryo at the time of culture is an important determinant of their morphogenic response (Tulecke and McGranahan 1985; Sotak *et al*. 1991; DeMarch *et al*. 1993). To identify the most responsive stage of embryos for morphogenesis, Chaturvedi *et al*. (2004b) cultured immature embryos of neem at different stages of development- globular, heart-shape, torpedo-shape and early dicotyledonous stage (early dicotyledonous embryos were 2.5 times smaller than fully developed dicotyledonous embryos) on MS medium supplemented with a range of growth regulators. The morphogenic response varied considerably with the stage of embryo at culture. Globular and heart-shape embryos generally turned brown without showing any notable change. The older embryos germinated, formed calli or differentiated into three types of organized structures: shoots, somatic embryos and neomorphs. Often the same explant differentiated more than one kind of regenerants. Maximum somatic embryogenesis and shoot-bud differentiation occurred directly from the explant on MS + BAP (5.0 µM) medium, and the most responsive embryo stage was early dicotyledonous, followed by torpedo stage. The former showed differentiation of shoots and somatic embryos at higher frequency (57%). In the combined presence of BAP (5.0 µM) and 2,4-D (1.0 µM) in MS medium, early dicotyledonous embryos showed a fairly high degree (50%) of somatic embryogenesis and neomorph formation via callusing. Since, regeneration involving a callus phase runs the risk of introducing variability due to genetic instability of the callus cells, therefore, Chaturvedi *et al*. (2004b) utilized only directly differentiated regenerants for micropropagation. Irrespective of the treatment and the stage of zygotic embryo,

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somatic embryos exhibited considerable morphological abnormalities, such as pluricotyledony, fusion of cotyledons, and absence of cotyledons. The occurrence of normal dicotyledonous embryos was extremely rare. On MS basal medium, only 3% of the embryos developed a long tap root within 4 weeks but plumular shoots were not observed. The histological examination of somatic embryos revealed a poorly differentiated radicular end, elongated hypocotyl with provascular strands and cotyledons but a distinct plumule was never seen at the junction of the hypocotyl and the cotyledon. Although somatic embryos never germinated to form complete plantlets on any of the treatments tested, secondary embryogenesis occurred on MS basal medium supplemented with BAP + IAA, or GA_3 + IAA. On MS + BAP (1.0 μM) + IAA (0.5 μM), 100% somatic embryos exhibited secondary embryogenesis with a mean number of 14 embryos per primary embryo. In this medium, secondary embryogenesis was preceded by callusing of the primary somatic embryos. However, on GA_3 (5.0 μM) + IAA (2.5 μM), secondary embryos differentiated directly from the primary somatic embryos. Secondary somatic embryos also exhibited morphological abnormalities like primary somatic embryos and failed to germinate on any of the treatments tested.

Rout (2005) also reported somatic embryogenesis from immature zygotic embryos that were cultured 40 days after anthesis. Embryogenic callus proliferated on MS medium supplemented with BAP (1.11 μM) and 2,4-D (4.52 μM). When these calli were transferred to same medium containing reduced auxin (0.45 μM 2,4-D), numerous embryos proliferated from the surface of callus. Maturation and germination of somatic embryos occurred on half strength MS salts and vitamins supplemented with Abscisic acid (ABA) and sucrose (2%); the maximum percentage (64.2%) of germination was observed with ABA (0.94 μM) within 2 weeks of culture.

ii. Axenic Cultures

Akula *et al.* (2003) cultured leaf, single nodal and root explants (1.5-2.5 mm), excised from 1-year-old *in vitro* cultures of seven clones, obtained from different countries such as China, Zambia, Indonesia, India, Kenya, Mauritius and Thailand, to study somatic embryogenesis. They reported induction of somatic embryogenesis in four out of seven

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selected clones of neem. Direct induction of somatic embryogenesis was achieved both from nodal and root segments within eight weeks of culture on MS medium without growth regulators. When these embryos were left on the induction medium, approximately 15% of the somatic embryos developed into whole plantlets after passing through a series of developmental stages. Indirect somatic embryogenesis via callusing was obtained from leaf explants grown on MS medium supplemented with a combination of TDZ (2.3-4.5 μM) and 2,4-D (0.5 μM). However, these leaf derived somatic embryos derived showed low conversion rates (< 5%).

iii. Adult Tissue Cultures

Shekhawat *et al*. (2009) utilized juvenile leaflets (3.5-7.5 cm long) from 6-year-old field grown plants to induce somatic embryogenesis in neem. Various factors, like auxins, cytokinins, sucrose, inorganic and organic salts, were examined to establish induction and maturation of embryos. Calli induced on MS medium supplemented with Kinetin (6.9 μM) and IAA (8.6 μM) were embryogenic which subsequently gave rise to somatic embryos. Maturation of embryos was accomplished on the same medium, after three subcultures. On half strength MS basal medium, 82% of the embryos germinated. More than 80% plantlets survived acclimatization.

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Table 1: Summary of tissue culture work in *Azadirachta indica*

S.No.	Mode of Propagation	Explant used	Adult / Juvenile	Observations	References
1.	**Organogenesis**	Nodal segments	Juvenile	Axillary shoots→ Plantlets	Drew 1993; Quraishi *et al*. 2004
			Adult	Axillary shoots→ Plantlets	Joarder *et al*. 1993; Gill *et al*. 1996; Joshi and Thengane 1996; Islam *et al*. 1997; Sarker *et al*. 1997; Venkateswarlu and Mukhopadhyaya 1999; Sharma *et al*. 1999; Chaturvedi *et al*. 2004a; Arora *et al*. 2010
		Apical and axillary buds	Adult	Axillary shoots→ Plantlets	Roy *et al*. 1996
		Nodal segments from crown branches	Adult	Axillary shoots→ did not survive	Quraishi *et al*. 2004
		Nodal segments from basal sprouts branches	Adult	Axillary shoots→ Plantlets	
		Leaves	Juvenile	Callus→ Shoots→ Plantlets	Sarker *et al*. 1997
				Adventitious shoots→ Plantlets	Eeswara *et al*. 1998
				Adventitious shoots→ Plantlets	Salvi *et al*. 2001
		Leaf-discs	Adult	Callus→ Shoots→ Plantlets	Narayan and Jaiswal 1985; Ramesh and Padhya 1990
		Leaf tips	Not Mentioned	Callus→ Shoots	Abubacker and Alagumanian 1999
		Leaflets	Adult	Adventitious shoots→ Plantlets	Arora *et al*. 2009
		Zygotic embryos	Juvenile	Callus→ Shoots	Rangaswami and Promila 1972
				Shoot-buds→ Plantlets	Chaturvedi *et al.* 2004b
				Callus→ Shoot-buds→ Plantlets	
		Cotyledonary nodes	Juvenile	Adventitious shoots→ Plantlets	Salvi *et al*. 2001
		Cotyledons	Juvenile	Shoots→ Plantlets	Nirmalakumari *et al.* 1993
				Callus→ Shoots→ Plantlets	Nirmalakumari *et al.* 1993; Abubacker and Alagumanian 1999
				Adventitious shoots→ Plantlets	Salvi *et al*. 2001
		Roots	Juvenile	Adventitious shoots→ Plantlets	Salvi *et al*. 2001; Arora *et al.* 2011

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		Anthers at uninucleate stage microspores	Juvenile	Callus→ Plantlets (diploid)	Gautam *et al*. 1993
		Anthers at early-late-uninucleate stage microspores	Juvenile	Callus→ Shoots→ Plantlets (haploid)	Chaturvedi *et al*. 2003a
		Ovaries	Juvenile	Callus→ Shoots→ Plantlets (diploid)	Srivastava *et al*. 2009
		Endosperms	Juvenile	Callus→ Shoots→ Plantlets	Chaturvedi *et al*. 2003b
2.	**Somatic Embryogenesis**	Cotyledons	Juvenile	Embryo like nodular structures→ Plantlets	Muralidharan and Mascarenhas 1989
				Callus→ Embryos→ Plantlets	Shrikhande *et al*. 1993
				Callus→ Embryos→ Shoots	Islam *et al*. 1993; Su *et al*. 1997
				Callus→ Embryos→ Plantlets	Murthy and Saxena 1998
				Embryos→ Plantlets	Murthy and Saxena 1998; Gairi and Rashid 2004
		Hypocotyls	Juvenile	Callus→ Embryos→ Shoots	Su *et al*. 1997
				Embryos→ Plantlets	Gairi and Rashid 2004
		Epicotyls	Juvenile	Embryos→ Plantlets	Gairi and Rashid 2004
		Cotyledonary nodes	Juvenile	Embryos→ Plantlets	Gairi and Rashid 2004
		Leaves	Juvenile	Callus→ Embryos→ Plantlets	Akula *et al*. 2003
				Embryos→ Plantlets	Gairi and Rashid 2004
		Leaflets	Juvenile	Callus→ Embryos→ Plantlets	Shekhawat *et al*. 2009
		Nodes	Juvenile	Callus→ Embryos→ Plantlets	Drew 1993; Akula *et al*. 2003
				Embryos→ Plantlets	Akula *et al*. 2003
		Roots	Juvenile	Callus→ Embryos→ Plantlets	Akula *et al*. 2003
				Embryos→ Plantlets	
		Immature zygotic embryos (early dicot stage most responsive)	Juvenile	Callus→ Embryos	Chaturvedi *et al*. 2004b
				Callus→ Neomorphs→ Shoot-buds→ Plantlets	
				Callus→ Embryos→ Plantlets	Rout 2005

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1.2.A.2. *Spilanthes acmella* Murr.

Despite the profound economic value of *Spilanthes*, very little scientific work is done in this species. Tissue culture studies on *S. acmella* are in its infancy and so far only seven reports are available on micropropagation of this species (**Table 2**). A majority of these reports (Saritha *et al*. 2002; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh *et al*. 2009b) deal with *in vitro* plant regeneration by adventitious shoot proliferation from seedling explants, leaf or hypocotyls or thin cell layer sections of nodal segment, which may runs the risk of change in ploidy due to *de novo* differentiation of shoot-buds resulting into chimera formation. Furthermore, conditions for micropropagation were not optimized in either of these studies. Saritha *et al*. (2002) were first to report the successful tissue culture of *Spilanthes.* They reported multiple shoot proliferation (10 ± 0.6) from hypocotyl explants of 1-week-old seedlings on MS medium supplemented with BAP (2.2 µM) and NAA (0.54 µM). About 95% of the *in vitro* developed shoots rooted on half strength (½) MS medium containing IBA (4.9 µM). After 6 years, Saritha and Naidu (2008) reported shoot regeneration from leaf explants obtained from the above mentioned multiple shoots. Maximum number of shoots per explants (20 ± 0.47) was recorded on MS medium containing BAP (13.2 µM) and IAA (5.7 µM). An anatomical study confirmed that shoot regeneration was via direct organogenesis. Micropropagation of *Spilanthes* by leaf-disc culture was also reported by Pandey and Agrawal (2009). They obtained green and compact callus on MS medium supplemented with BAP (10.0 µM) and NAA (1.0 µM), in 15 days. The fresh subculture of callus on the same medium resulted into the differentiation of an average of 12.9 shoot-buds, in 50% cultures after every 30 days. Elongation of shoot-buds occurred only if they were transferred to MS + BAP medium devoid of NAA. On MS + BAP (10.0 µM), an average of 4.22 shoots and 15 shoot-buds per explants were obtained in 70.3% culture in 30 days. Shoots were rooted on ½ MS + IBA (0.1 µM) within 2 weeks. The plantlets were successfully hardened and established in soil where they flowered and set viable seeds. Later, Singh *et al.* (2009b) established *in vitro* propagation system of *Spilanthes* using nodal segment transverse thin cell layer (tTCL) culture system. MS medium fortified with BAP (5.0 µM) was optimal for shoot regeneration from tTCL. On this medium, the explant inoculated in

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the upright orientation exhibited a high frequency (97%) of shoot regeneration from the edge of the explants, and the highest number of shoots (an average of 31.5) per explant. In contrast to this, intact node (1.0-1.5 cm) cultured on MS + BAP (5.0 µM) had significantly lower shoot multiplication ability with only 4.5 shoots per responsive explants. Incorporation of Kinetin or NAA in BAP containing medium did not increase shoot multiplication from tTCL nodal segments. Rooting of shoots was achieved on growth regulator free full-strength MS medium. Singh *et al.* (2009a) also carried out plant regeneration from alginate encapsulated shoots tips of *Spilanthes*. They accomplished the best gel complexation for encapsulation of shoot tips using sodium alginate and calcium chloride. From encapsulated shoot tips both shoots and roots emerged simultaneously on growth regulator-free full-strength MS liquid medium.

Haw and Keng (2003) attempted *in vitro* clonal propagation of *Spilanthes* by axillary shoot proliferation. The aseptic axillary buds formed multiple shoots within five weeks when cultured on MS medium supplemented with BAP (8.8 µM) and NAA (0.54 µM). The addition of IBA had no significant effect on the multiple shoot formation. However, the study lack crucial information on percent culture response, the rate of proliferation in recurrent cycles of shoot multiplication, frequency of rooting, etc., and transplantation was not attempted. A recent report (Singh and Chatuvedi 2010) on systematic clonal propagation by nodal segment culture is published from the present thesis work whereby, detailed description on *in vitro* shoot multiplication, rooting and hardening are described. Additionally, the study revealed the accumulation of scopoletin, a phytoalexin, in *in vitro* obtained leaves, the amount of which was comparable to those of field-grown mother plants. Thus, signified the effectiveness of *in vitro* methodology for true-to-type plant regeneration of *Spilanthes* and their later utility for biosynthesis and constant production of scopoletin throughout the year.

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Table 2: Summary of tissue culture work in *Spilanthes acmella*

S. No.	Explant	Direct/Indirect Organogenesis	Medium + PGR		References
			Shoot Multiplication	Rooting	
1.	Hypocotyls	?	MS + BAP (2.2 µM) + NAA (0.54 µM)	½ MS + IBA (4.9 µM)	Saritha *et al*. 2002
2.	Axillary buds	Direct	MS + BAP (8.8 µM) + NAA (0.54 µM)	Not Mentioned	Haw and Keng 2003
3.	*In vitro* leaves	Direct	MS + BAP (13.2 µM) + IAA (5.7 µM)	½ MS + IBA (4.9 µM)	Saritha and Naidu 2008
4.	*In vitro* leaves	Indirect	MS + BAP (10.0 µM) + NAA (1.0 µM)	½ MS + IBA (0.1 µM)	Pandey and Agrawal 2009
5.	Shoot tips	Direct	Growth regulator-free full-strength MS liquid medium		Singh *et al.* 2009a
6.	Nodal segment transverse thin cell layer sections	Direct	MS + BAP (10.0 µM)	MS basal medium	Singh *et al*. 2009b
7.	Nodal segments	Direct	MS + BAP (5.0 µM)	½ MS + Sucrose (5%)	Singh and Chaturvedi 2010

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1.2.B. Secondary Metabolite Production

In spite of advances in synthetic organic chemistry, plant still contributes significantly to the bulk of the market products, such as secondary metabolites. Use of wildly growing plant for commercial purpose has certain limitations like, plants need a seasonal period of growth before harvesting is possible. Other concerns include short growing season, disease and insect predation. On the other hand, chemical synthesis of several compounds is not feasible. Thus, the major limitation to the commercial use of potential metabolites is their very scarce supply from the field grown plant. Therefore, an alternative system for desired secondary metabolites is of great interest. In this respect, plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites. Plant cells are biosynthetically totipotent which means that each cell in culture retains the complete genetic information to produce the range of chemicals found in the parent plant. Plant cell cultures possess a number of advantages over conventional methods, for example, it is not subjected to the limitation of soil, water, season and environment conditions, and the cells can grow at a relatively fast rate. Moreover, production can be more reliable, simpler and more predictable. In addition to this, interfering compounds that occur in field-grown plant can be avoided in cell cultures. Using this system, novel compounds can also be produced that are not normally found in parent plant. To date, cell cultures have been established from many plants, but often do not produce sufficient amounts of the required secondary metabolites and only a few plant metabolites have been produced at the industrial level. The reason is not fully understood, but it may be due to slow growth rates of plant cells and low yields of secondary metabolites (Kolewe *et al*. 2008). In such cases, strategies to improve the production of secondary metabolites must be considered. In this regard, selection of high-producing cell lines and medium optimization strategies can lead to an enhancement in secondary metabolite production.

1.2.B.1. *Azadirachta indica* A. Juss

In neem, so far, callus and cell suspension cultures containing azadirachtin have been established from various explants **(Scheme 1)**. The first reports of azadirachtin

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production are of Van der Esch *et al*. (1993). They established the cell suspension cultures from hypocotyl derived callus. The authors observed that cell suspension cultures raised in light, produced higher amount of azadirachtin (0.66 µg/g DW) than those grown in dark condition (0.19 µg/g DW). After that, Kearney *et al*. (1994) studied the effect of permeabilizing agent on azadirachtin secretion in suspension cultures. In this study, callus, cell suspension and micropropagated shoots of neem were found to be highly antifeedant to the desert locust, *Schistocera gregaria.* However, when Allan *et al*. (1994) analyzed the callus it showed extremely low azadirachtin content (0.0007% of the DW of callus as compared to 0.6-0.8% in the seeds). Similarly, Zypman *et al*. (2001) demonstrated the anti-feedant effects of extracts from whole plants as well as from callus cultures but the compounds responsible for this effect was not identified by them. Wewetzer (1998) reported azadirachtin production from callus cultures of leaf and bark, established from trees of different origin. Analysis of callus cultures showed that the production of azadirachtin A was dependent on the source of cell line (plant origin/explant type), the nutrient medium and sucrose concentration. He observed higher azadirachtin production in White's medium as compared to MS medium from the same cell lines. Nicaraguan cell line (from leaves) shows the highest azadirachtin content (<0.5-64 µg/g DW) than those of tongo cell line (<0.5-11 µg/g DW) on White's medium while the azadirachtin content was found to be three times higher on medium supplemented with 15 g/l sucrose (<0.5-64 µg/g DW) as compared to those having 30 g/l sucrose (<0.5-22 µg/g DW), suggesting the effect of genetical and culture condition on azadirachtin production.Veersham *et al.* (1998) reported surprisingly high production of azadirachtin in callus cultures of leaves (26800 µg/g DW) and flowers (24600 µg/g DW). Such unexpected values have never been stated in any available reports on azadirachtin production. The presence of azadirachtin in hairy root cultures of leaves (3.6 µg/g DW) and stem (2.7 µg/g DW), bark derived callus (1200 µg/g DW), *in vitro* roots and shoots from embryo (4-8 µg/g DW), callus and suspension culture from leaves (5.36 mg/l) and callus from shoot-tips (0.5 µg/g DW) have also been reported (Sundaram *et al*. 1996; Bajagopal and Ramaswamy 1996; Srividya *et al*. 1998; Kuruvilla *et al*. 1999; Schaaf *et al*. 2000). Besides azadirachtin, Bajagopal and Ramaswamy (1996) detected nimbin

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(2100 µg/g DW) and gedunin (1300 µg/g DW) in callus cultures of bark.

Allan *et al*. (2002) established hairy root cultures from stem and leaf explants of neem and reported 0.007% azadirachtin, 0.012% 3-acetyl-1-tigloyl-azadirachtin and 0.002% 3-tigloyl-azadirachtol in the cultures. Raval *et al*. (2003) studied the effect of major nutrient on growth and production of azadirachtin-related limonoids in shoot cultures of neem with an aim to enhance their yield. They reported 250 µg/g DW of azadirachtin production in MS medium containing modified concentrations of nitrate, phosphate and sucrose. Prakash *et al*. (2005) observed a large variation in azadirachtin content in seeds (collected from different parts of India) and their respective callus cultures. They selected the highest azadirachtin (1890 µg/g DW) producing cell line developed from the seed kernels of a tree growing in Trivandrum, southern part of India and established shake flask suspension cultures. Afterwards, they came out with statistically optimized media for cell growth and azadirachtin production in suspension cultures. By statistical optimization, azadirachtin production was increased from 1890 µg/g DW to 2980 µg/g DW (Prakash and Srivastava 2005). The studies were extended further to establish the kinetics of cell growth/azadirachtin production and substrate consumption of neem suspension cultures in low shear steric impeller bioreactor with statistically optimized conditions (Prakash and Srivastava 2006). Further, Prakash and Srivastava (2007) compared azadirachtin production in stirred tank bioreactor with different types of impellers. Prakash and Srivastava (2008) also studied the role of elicitors like salicylic acid, chitosan, jasmonic acid, methyl jasmonate and yeast extract at different concentrations in shake flask suspension cultures of *A. indica*. Satdive *et al*. (2007) investigated the effect of different culture media and elicitor on growth and production of azadirachtin by hairy root cultures of neem. Hairy root cultures that were established from 3-week-old germinated seedling produced 0.14% DW of azadirachtin after elicitation. Rafiq and Dahot (2010) studied the effect of explant source, carbohydrates and major nutrients on production of azadirachtin related limonoids in callus and cell suspension cultures of neem. The variability in azadirachtin content was found in cell lines raised from different explants. The highest azadirachtin content (254.2 µg/g DW) was produced from immature flower cultures while the lowest azadirachtin content (16.1 µg/g DW) was

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observed in cultures raised from leaves. Azadirachtin content was significantly increased (373.1 µg/50 ml) in suspension cultures by optimization of carbohydrate and nitrogen source concentrations. Recently, Srivastava and Chaturvedi (2011), for the first time, reported azadirachtin accumulation in microspore derived cell lines. They have observed the beneficial effect of organogenesis on azadirachtin production. In their study, maximum 728.42 µg/g DW of azadirachtin was accumulated in a redifferentiated haploid cell line.

1.2.B.2. *Spilanthes acmella* Murr.

In *Spilanthes*, so far, there is no report on *in vitro* metabolite production. Although few reports have described chemical constituents of *Spilanthes* plants, growing in the wild, the field grown plants are not desirable for constant metabolite production at the commercial level because of environmental fluctuations, climatic and geographical variations, disease and pathogen attack. In contrast to this, plant cell culture technology shows promise for the large-scale production of high-value secondary metabolites. This technique offers uniform secondary product synthesis by eliminating effect of unforeseen climatic conditions and diseases as observed in field grown plants. In addition, plant cell can perform stereo- and regiospecific biotransformation for the production of novel compounds from low cost precursors. All these factors make plant cell cultures an attractive option for producing important and novel metabolites all the year round. Till date, the utilization of *Spilanthes* was not explored by biotechnological means. Present study, therefore, is an attempt to realize the potential of *Spilanthes* using tissue culture techniques.

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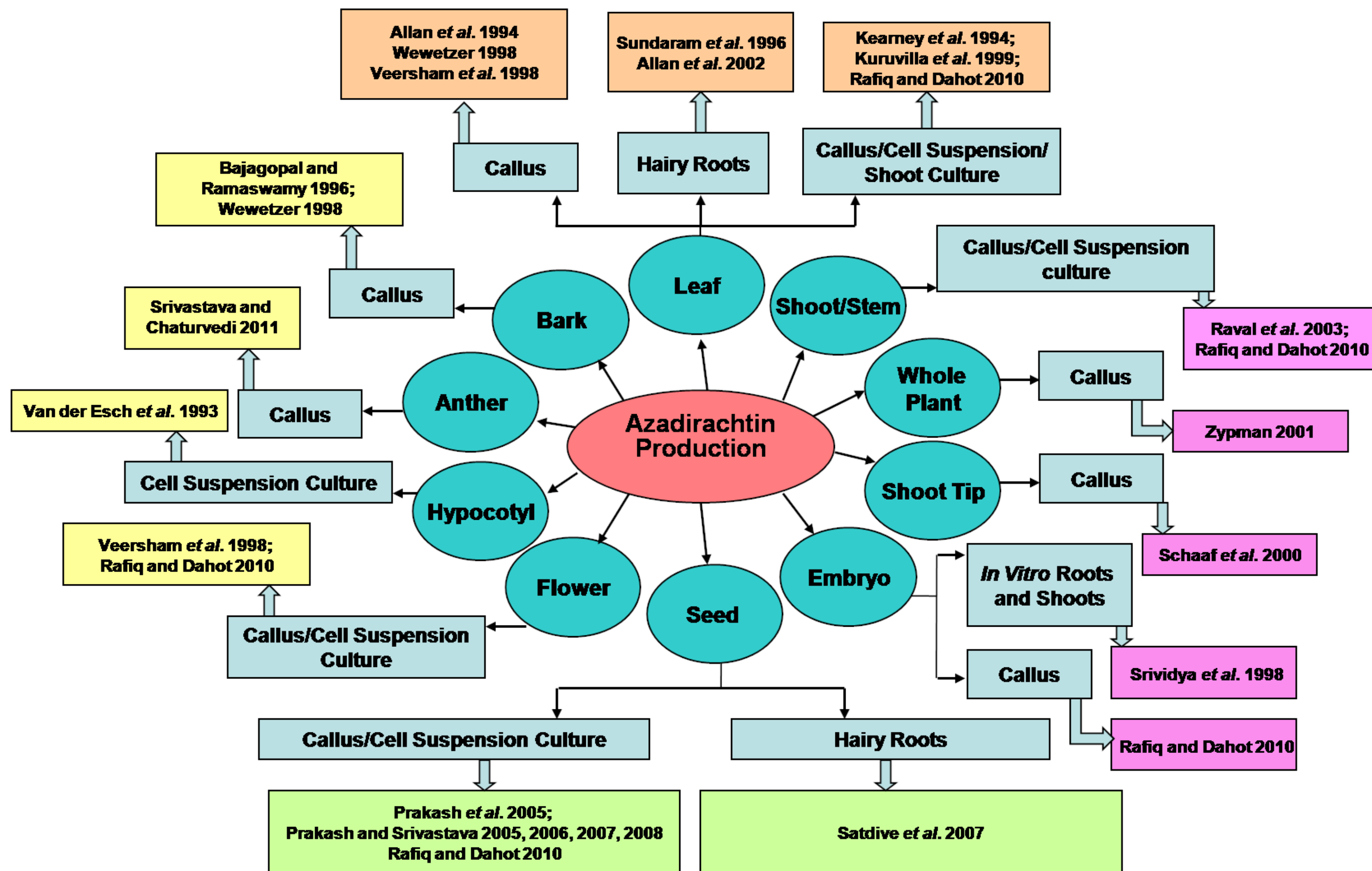

Scheme 1: Reports on azadirachtin production in tissue cultures of *Azadirachta indica*

1.3. PHARMACOLOGICAL APPLICATIONS OF SECONDARY METABOLITES

Plants are able to synthesize an array of secondary metabolites, which are used as pharmaceuticals, biopesticide, flavours, fragrance, colours and food additives. The compounds do not participate in the vital metabolic processes of the living cells, but are involved in the interaction of the producing plant with its environment. Each plant species has its own specific set of secondary metabolites. The spectrum of chemical structures synthesized by the plant kingdom is broader than that of perhaps any other group of organisms (Rao and Ravishankar 2002). Secondary metabolites possess various bioactivities and contribute essentially to the persistence of plants, being involved in processes such as defence against phytopathogens (bacteria, fungus and virus), response to abiotic stresses, regulation of oxidative stress, and probably hormonal regulation (Bourgaud *et al*. 2001). In the present scenario, a large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. In addition, in the form of natural products or as functional foods, medicinal plants and their extracts offer an alternative to specifically targeted drugs in the treatment and prevention of many diseases.

***Azadirachta indica* A. Juss.** synthesizes a lot of medicinally important secondary metabolites with a diverse range of bioactivity. Bioactive principles isolated from different parts of the plant include: azadirachtin, nimbin, nimolinone, dihydronimbin, salannin, meliantriol and many other derivatives of these principles **(Scheme 2)**. Among all, azadirachtin has received the most attention because it is relatively abundant in neem kernels and the main component responsible for both antifeedant and toxic effects in insects. Azadirachtin, a complex tetranortriterpenoid, is in fact a combination of seven isomeric compounds azadirachtin-A to azadirachtin-G with azadirachtin-A being present in the highest quantity and azadirachtin-E considered as the most effectual insect growth regulator (Verkerk *et al*. 1993). The biosynthesis of azadirachtin starts with a steroid precursor (e.g. tirucallol, azadirone, azadiradione) and C-ring opening (eg. nimbin, salannin), after which it proceeds by main level of structural complexity i.e. furan ring formation (e.g. modification yield azadirachtin) (Ley *et al*. 1993).

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Neem insecticides, which are extracts of neem seeds, contain many related triterpenoids, but, their efficacy is directly related to azadirachtin content. The natural mixtures of azadirachtin in neem insecticides may usefully mitigate against the development of resistance compared to azadirachtin alone (Feng and Isman 1995). Apart from its action against insects, bacteria, fungi, viruses and protozoa that account the major enemy of crops are also reported to be sensitive to azadirachtin (Mordue *et al*. 1993). As antibiotics, neem seed oil showed bactericidal activity against pathogenic bacteria like *Staphylococcus aureus* and *Salmonella typhi*. As fungicides, over 14 common fungi species are sensitive to neem preparations (Khan and Wassilew 1987). They include the genera *Trichophyton* (athlete's foot), *Epidermophyton* (ringworm of skin and nails), *Microsporum* (ringworm of skin and hair) and *Candida* (thrush). Apart from this, antiviral activity of aqueous neem leaf extracts against vaccinia, chikungunya, group-B Coxsackie and measles virus has also been reported (Rao *et al*. 1969; Gogati *et al.* 1989; Badam *et al*. 1999).

Besides, its properties as biopesticides, the new potential of azadirachtin are being explored including *in vitro* inhibition of sexual development of malarial parasites (Jones *et al*. 1994) and inhibitory potential on dengue virus type-2 replication (Parida *et al*. 2002). Some investigators also studied the efficacy of extracts of neem against Trypanosomes. Yanes *et al.* (2004) observed that fractions of chloroformic extract of neem leave inhibit growth of *Trypanosoma cruzi* epimastogotes with ultrastructural changes such as vacuolization, organelle degeneration and cell division disruptions. Similarly, De Azambuja and Garcia (1992) reported that azadirachtin has potential to block the development of *T. cruzi*.

Currently, studies on effect of neem solution on cancer, diabetes, heart disease and AIDS have been carried out. Anticarcinogenic activity of neem leaf extracts was observed in murine system. Induction of apoptosis in rat oocytes was seen when treated with neem leaf extracts (Chaube *et al*. 2006). The ethanolic leaf extract of neem also caused cell death of prostate cancer cells (PC-3) by inducing apoptosis (Kumar *et al*. 2006). Furthermore, acetone-water neem leaf extract showed antiretroviral activity through inhibition of cytoadhesion. The extract increased haemoglobin concentration, mean CD^{+},

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cell count and erythrocyte sedimentation rate in HIV/AIDS patients (Udeinya *et al*. 2004).

Azadirachtin is present in all parts of the tree but its highest concentration is present in mature seeds. All commercial formulations and other products based on azadirachtin, contain azadirachtin which is extracted from the seeds of naturally grown plants, this approach has the disadvantages of heterogeneity in azadirachtin content depending upon the plant genotype and environment. Seasonal variation on azadirachtin content in seeds of neem has also been reported (Sidhu and Behl 1996). Moreover, the geographical distribution of neem tree is limited, also the seeds do not have longer shelf life and azadirachtin percentage dropped upto 32% within four months of storage (Yakkundi *et al*. 1995). Besides, azadirachtin is a complex molecule and this complexity precludes a synthetic production system. Keeping in consideration of all these factors, researchers all over the world are focusing on tissue culture and scale up studies to harvest this important compound from *in vitro* raised cultures. With the cell culture method, production can be more controllably ensured in terms of the product, its quality and quantity, independent of geographical and climatic barriers. Although the lower yield of azadirachtin in cell cultures has restricted the commercial utilization of this approach, several strategies have been proposed to enhance product yield and productivity in the cell culture system.

As far as ***Spilanthes*** is concerned, it contains a wide array of compounds with a diverse range of bioactivity such as alkylamides (spilanthol), phenolics (ferulic acid and vanillic acid), coumarin (scopoletin) and triterpenoids like β-sitostenone and stigmasterol (**Scheme 2**) (Prachayasittikul *et al*. 2009). The plant has been used as folk medicine since ancient times to cure severe toothache, infections of throat and gums, stomatitis, paralysis of tongue, and psoriasis (Anonymous 1989). In Amazon, it has been used for the treatment of tuberculosis by laymen (Storey and Salem 1997). This plant is well known for antiseptic, analgesic, and diuretic properties (Jondiko 1986; Ramsewak *et al*. 1999). Ratnasooriya *et al.* (2004) investigated the diuretic potential of *Spilanthes* using rats. *Spilanthes* extract is also effective against malarial parasites, specifically malarial spirochetes, either as a prophylactic or as a treatment for malarial paroxysms (Richard 1996). Recently, Prachayasittikul *et al*. (2009) evaluated antimicrobial, antioxidant and

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cytotoxic activities of *Spilanthes* extracts. They showed that fractions from the chloroform and methanol extracts can inhibit the growth of many microorganisms e.g. *Corynebacterium diphtheriae* (NCTC 10356) and *Bacillus subtilis* (ATCC 6633). In addition, studies on *Spilanthes* immune stimulating properties are available, which find applications in local buccal mucosa preparations indicated for painful mouth tissues and minor mouth ulcers. Besides, *Spilanthes* is well known for larvicidal, and insecticidal properties (Ramsewak *et al*. 1999; Saraf and Dixit 2002; Pandey *et al*. 2007).

In *Spilanthes*, these properties are mainly due to the most abundant principle antiseptic alkylamide, (2E, 6Z, 8E)-deca-2,6,8-trienoic acid N-isobutyl amide, commonly known as spilanthol (Khadir *et al.* 1989). Spilanthol has been found harmless to majority of vertebrates and lethal to invertebrates (Watt and Brayer- Brandwijk 1962). The analgesic activity of spilanthol has been attributed to an increased gamma-aminobutyric acid (GABA) release in the temporal cerebral cortex (Rios *et al*. 2007).

Interestingly, spilanthol has been demonstrated to inhibit nitric oxide (NO) production in a murine macrophage cell line, to efficiently down-regulate the production of inflammatory mediators interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF-α), and to attenuate the expression of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) (Wu *et al*. 2008). Other investigations have also confirmed the down regulation of some pro-inflammatory cytokines by bioactive alkylamides under various experimental conditions (Cech *et al*. 2006; Guiotto *et al*. 2008; Wang *et al*. 2008; Woelkart and Bauer 2007). These findings suggest that spilanthol can be a useful inhibitor of inflammatory mediators and is a potential new lead compound for COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs). Spilanthol clinical trial has also been done for anti-aging property. It stimulates, reorganize and strengthen the collagen network, notably those of the face, and can be used as an anti-wrinkle product.

Apart from the spilanthol, other isobutylamides have also been reported which includes 2E-N-(2-methylbutyl)-2-undecene-8,10-diynamide, 2E,7Z-N-isobutyl-2,7-tri-decadiene-10,12-diynamide, 7Z-N-isobutyl-7-tridecene-10,12-diynamide (Nakatani and Nagashima 1992), undeca-2E,7Z,9E-trienoic acid isobutylamide and undeca-2E-en-8,10-diynoic acid

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isobutylamide (Ramsewak *et al.*1999). These alkylamides also contribute to the *spilanthes* properties.

Among others, the scopoletin (coumarin) and ferulic acid (phenolics) found in this plant are reported to be of immense pharmacological interests (Prachayasittikul *et al*. 2009). Scopoletin is a phytoalexin, its production is mainly seen upon pathogenic infection and is considered as an important defense mechanism against bacteria and fungi (Smith 1996). It has attracted the most attention because of its use in cardiovascular disease, and antitumor and antithyroid treatment. In addition to this, scopoletin also possesses antioxidant, antimicrobial, anti-inflammatory, antipyretic and hepatoprotective properties. Ferulic acid is most highly regarded for its antioxidant property. Additionally, it exhibited a wide range of therapeutic effects against cancer, diabetes, cardiovascular and neurodegenerative diseases.

In spite of multifaceted uses of *Spilanthes*, only a few reports have described the chemical constituents from its various parts. The plant is being overexploited by the local population as well as pharmaceutical companies. It is, therefore, imperative to develop more efficient methods for its large scale propagation. Besides micropropagation, *in vitro* production of bioactive compound, their identification as well as quantification is also highly warranted. The already attempted and future possible biotechnological interventions in *Spilanthes* are presented in **Scheme 3.**

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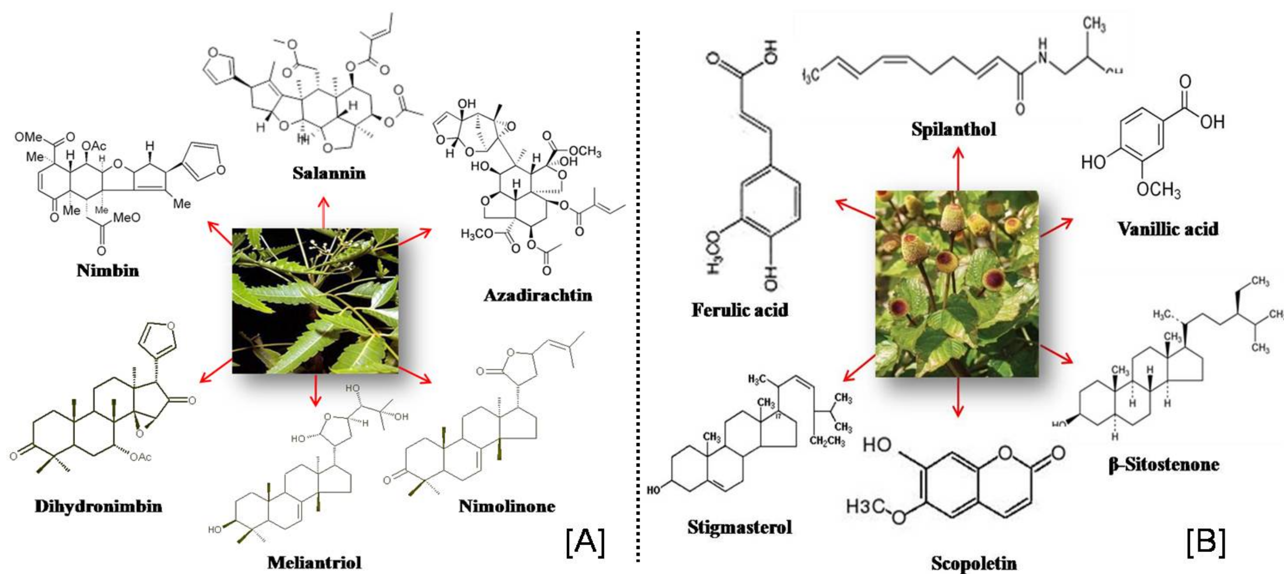

Scheme 2: Chemical structures of medicinal compounds present: [A] *Azadirachta indica* [B] *Spilanthes acmella*

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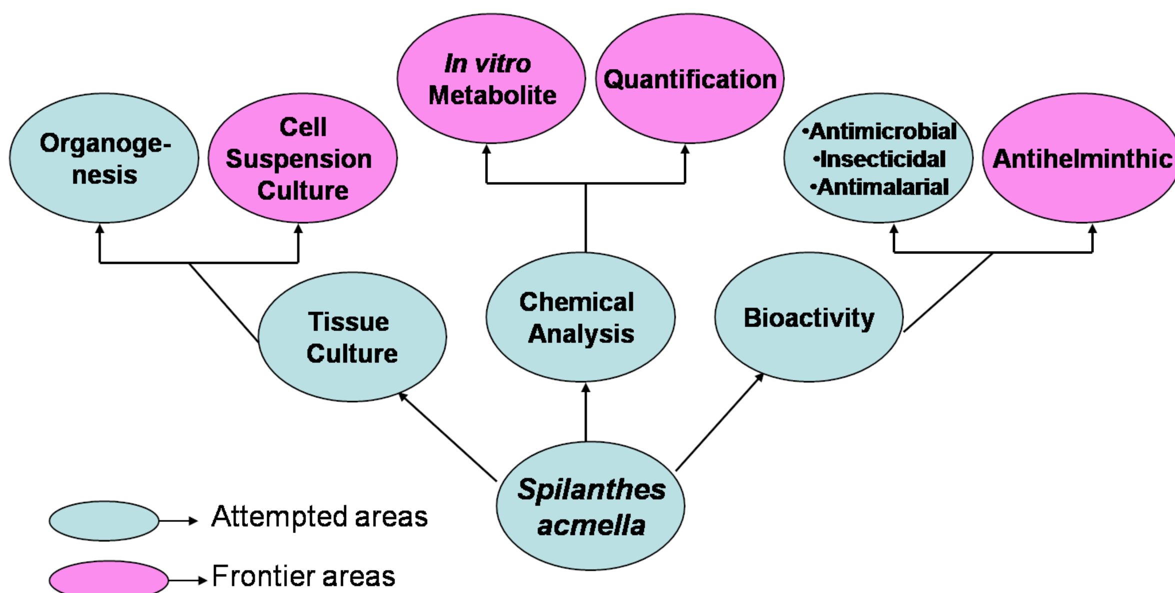

Scheme 3: Biotechnological interventions in *Spilanthes acmella*

1.4. OBJECTIVES OF THE PRESENT STUDY

The discussions in the above sections have sufficiently described that *Azadirachta indica* A. Juss. and *Spilanthes acmella* Murr., two medicinal plants selected for the present study, hold important places in Indian and global scenario due to their economic and medicinal properties. From the last few years as the demand for bioactive compounds has been increasing constantly, exploitation of these plants has also increased. Hence, there is an urgent need to develop an alternative method for the large scale production of metabolites and quality plants. In this respect, plant cell and organ cultures offer an attractive alternative to whole plant extraction for homogeneous, controlled production, especially, when we take the commercial demand into picture. It also results in more consistent yield and quality of the products, irrespective of the seasons and the regions.

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The major impediments in neem, with regard to availability of metabolites like, azadirachtin, from tissue culture lie in its variable and low productivity. In the present study, we made an effort towards systematic selection and screening of elite *in vitro* cell lines for constant and improved production of azadirachtin. Experiments related to neem bioactivity were not performed because the plant is well known for its biological properties and enough reports are available on this aspect. As for *Spilanthes,* little scientific work is done on this genus. Inspite of being a plant of potential medicinal interest there are a very few reports that document the properties of this plant scientifically. All the biochemical studies carried out, so far, have been done on plants growing in wild. As we are aware that environmental fluxes cause alterations in type and quantity of metabolites produced, establishment of *in vitro* cultures will help to utilize the biomass and nullify the effect of seasonal variation on secondary metabolite content. With this background, the present study was undertaken with the following specific objectives.

Specific Objectives

- Establishment of *in vitro* cultures of *Azadirachta indica* A. Juss. and *Spilanthes acmella* Murr.
- Bioassay based selection of *in vitro* cultures.
- Identification and quantification of bioactive compounds in *in vitro* cell lines.
- Optimization of media constituents for improved production of metabolites.

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Chapter 2

Materials and Methods

Materials used in the present study as well as the methodologies followed have been grouped into two broad sections. **Section 2A** is devoted to *Azadirachta indica* A. Juss. while **section 2B** is dedicated to *Spilanthes acmella* Murr. Further, **section 2A** is divided into two subsections, first subsection contains tissue culture studies and the second subsection groups together all the methodologies related to biochemical analysis. Similarly, **section 2B** is divided into three subsections. First one deals with tissue culture studies while second and third subsections report methodologies of chemical analysis and bioassay experiments, respectively.

2A *AZADIRACHTA INDICA*

2A.1. Materials

Immature fruits, leaves and flower-buds were collected from a mature 35-year-old neem tree, growing near the campus of Indian Institute of Technology Guwahati, during the months of April-June. Early-late dicotyledonary stages of embryos were used for zygotic embryo culture establishment. To raise ovary cultures, closed flower-buds of 4 mm size were selected and for establishment of leaf cultures, newly developed leaves were used. Various callus lines, obtained from these cultures, were utilized for azadirachtin content analysis.

The plant growth regulators, used in the present study, were procured from Sigma, USA. MS (Murashige and Skoog 1962) basal medium constituents were purchased from Merck, India. Glassware and plasticware were obtained from Borosil, India and Tarsons, India, respectively. For ploidy analysis, propidium iodide and RNAase were obtained from Sigma, USA.

For chemical analysis, HPLC grade methanol, analytical grade dichloromethane and methanol were purchased from Merck, India. Water used for HPLC analysis was purified by Milli-Q system. Standard compound of azadirachtin was purchased from sigma, USA.

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2A.2. Methods

2A.2.1. Establishment of In Vitro Culture of A. indica

i. Zygotic Embryo Culture

To establish cultures from zygotic embryo explants, immature fruits were thoroughly washed with 1% (v/v) savlon (Johnson-Johnson, India) solution for 10 min, followed by rinsing with sterile distilled water (SDW). Fruits were then rinsed by 90% ethanol for 30 seconds before surface sterilizing, using 0.1% (m/v) mercuric chloride ($HgCl_2$) for 10 min in the laminar-air-flow cabinet (Saveer Biotech, India). After washing with SDW three times, the fruits were dissected with the aid of a stereo-microscope (NIKON, SMZ-645, Japan). Embryos at early-late dicotyledonary stages were cultured on MS medium supplemented with various concentrations of 2,4-D, BAP and TDZ either alone or in combinations with NAA, IAA, Kinetin, ABA, GA_3 and CH, for callus induction and morphogenesis. Four zygotic embryos were placed in one Petridish with 16 dishes for each treatment. Data were collected as number of responding zygotic embryos relative to total number of zygotic embryos cultured. After 5 weeks of culture, the calli were transferred to fresh medium of parental composition for further multiplication.

ii. Leaf-Disc Culture

Leaves were washed with a mixture of 1% savlon solution and 3 drops of tween-20 (Merck, India) for 20 min, followed by rinsing with SDW thrice. Thereafter, the remaining steps were carried out inside the laminar-air-flow cabinet. Leaves were surface sterilized with 0.1% (w/v) $HgCl_2$ for 8 min, followed by three rinses with the SDW. Leaf-disc explants were prepared by punching the sterilized leaves with 5 mm sized cork borer and cultured with the abaxial side in contact with the medium. MS basal medium supplemented with different combinations and concentrations of auxins and cytokinins, including BAP, Kinetin, Zeatin, TDZ, NAA, IAA, IBA and CH was used to induce callusing from explants. Each experiment contained at least twenty four replicates. Data were collected as number of responding explants relative to total

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number of leaf-discs cultured. After 5 weeks, leaf-discs calli were transferred to the fresh medium of same composition for further multiplication and shoot regeneration.

iii. Ovary Culture

For establishing ovary cultures, the flower buds of 4 mm size were sterilized with 0.1% (m/v) $HgCl_2$ for 7 min, followed by three washings in SDW inside the laminar-air-flow cabinet. After dissecting the flowers, ovaries were gently taken out and cut transversely into two halves of 0.4-0.5 mm size and implanted with the basal cut end touching the medium. Four ovary-slices from two ovaries were placed on each Petridish with 16 dishes for each treatment. For callus induction, MS medium was tested with 2,4-D or BAP either alone or in combination with Kinetin, Glutamine and Serine. A temperature pre-treatment of 4^oC or 33^oC for 15 days was given to the cultures before transferred to 25^oC, to study their callusing response. Some cultures were maintained continuously at 25^oC as control. Data were collected as number of responding explants relative to total number of sliced ovaries cultured. After 4 weeks of culture, ovary calli were transferred to different sets of media combinations for further multiplication and regeneration of shoots from them.

iv. Shoot Elongation, Multiplication, and Rooting

For elongation, individual small shoots (*ca.* 1.5 cm long) were excised and transferred to lower concentration of BAP at 0.5 µM. For further multiplication, the shoots, which had grown fairly long were cut into single node segments and cultured on MS medium containing BAP (1.0 µM) and CH (250 mg/l). The number of propagules obtained at the end of a multiplication cycle was regarded as the rate of shoot multiplication.

For rooting, terminal 3 cm long shoots were transferred to ¼ MS medium (major inorganic salts reduced to quarter strength) supplemented with low concentration of IBA (0.5 µM). Remaining portion of the shoot was again cut into single nodes and utilized for multiplication.

v. Culture Media

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All the cultures were raised on MS basal medium (***see* Table 3**). The medium was variously supplemented with growth regulators and other adjuvants (**Table 4**). Unless mentioned otherwise, all the media contained 3% sucrose and were gelled with 0.8% agar (HiMedia, India). Analytical grade chemicals and Milli-Q water were used to prepare stock solutions and culture media. Stock solutions of macronutrients (× 20), micronutrients (× 200), iron (× 200) and vitamins (× 200) were made separately and stored at 4°C till further use. The stock solutions (1×10^{-3} M) of growth regulators were prepared and stored in refrigerator. Myoinositol and sugar were weighed and directly added to the culture medium at the time of media preparation. Required quantities of various stock solutions, growth adjuvants and sugar were added to molten agar and final volume made up with Elix water (Millipore, USA). After adjusting the pH to 5.8, by using 0.1N HCl or 0.1N NaOH, the medium was dispensed into 150 x 25 mm Borosil rimless glass tubes with 20 ml medium per tube. The culture tubes were plugged with nonabsorbent cotton wrapped in cheesecloth and autoclaved at 121°C at 15 psi for 15 min. For initiation of zygotic embryo and ovary cultures, the media were autoclaved in Erlenmeyer flasks and allowed to cool down to *ca.* 50°C, before dispensing into 60 mm pre-sterilized Petriplates under aseptic conditions with 10 ml per Petriplate. The thermolabile substances such as IAA, ABA, GA_3 and TDZ were filter sterilized using Whatman cellulose nitrate filters (0.45 µm pore size) and added to the autoclaved medium cooled to 50°C. The medium was then dispensed into glass tubes inside a laminar-air-flow cabinet.

vi. Inoculation

All aseptic manipulations of the plant material, including inoculations, were made inside a laminar-air-flow cabinet. Before starting the work, the platform and the inner sides of the cabinet were cleaned with rectified spirit and exposed to UV light for 15 min. The instruments used for inoculation (forceps, scalpels and needles etc.) and Petriplates used for dissection and chopping the material at subculture were wrapped in aluminum foil and autoclaved. During inoculation, at regular intervals, the instruments were dipped in 90% ethanol, flamed and cooled before use. At the time of raising fresh cultures or subcultures, the rim of the glass tube was flamed, the plug

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was removed and after planting the material on the medium the plug was replaced in quick succession.

vii. Culture Conditions

The cultures were generally incubated at 25 ± 2°C and 50-60% relative humidity. Depending on the experiment, the cultures were maintained in diffuse light (1000-2000 lux) with 16/18 h (light/dark) photoperiod, provided by cool day light fluorescent tubes (Philips TL 40W) or in continuous darkness. To study the effect of temperature on callusing the ovary cultures were kept at 4°C and 33°C for 15 days before being transferred to 25°C temperature. Unless mentioned otherwise, at least twenty four cultures were raised for each treatment and each experiment was repeated at least three times.

Table 3: Constituents of MS (Murashige and Skoog 1962) basal medium.

Medium Components	Concentrations (mg/l)
Major elements	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor elements	
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25

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$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic constituents	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine.HCl	0.5
Thiamine.HCl	0.1
Glycine	2
Iron Constituents	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Sucrose	30,000
Agar	8,000

Table 4: Growth regulators, amino acids and other compounds used as additives to MS basal medium.

Additives	Manufacturer
Auxins	
2,4-D	Sigma, USA
pCPA	Sigma, USA
NAA	Sigma, USA
IAA	Sigma, USA
IBA	Sigma, USA
Cytokinins	
BAP	Sigma, USA
Kinetin	Sigma, USA
Zeatin	Sigma, USA

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TDZ	Sigma, USA
2-iP	Sigma, USA
Aminoacids	
L-Glutamine	Sigma, USA
L-Serine	SRL, India
Other compounds	
GA_3	Sigma, USA
ABA	Sigma, USA
Casein hydrolysate	HiMedia, India
Maltose	Merck, India
Fructose	Merck, India
Glucose	Merck, India

viii. Observation of Cultures

The cultures were observed periodically, and the morphological changes were recorded at weekly intervals or whenever necessary. Final observations were taken after 5 weeks. In primary cultures of zygotic embryo, leaf and ovary the number of explants showing callus formation was recorded and percent callusing response was calculated. Regeneration in the subcultures of callus is expressed as percent response.

ix. Histological Studies

For histological analysis, regenerating calli were sampled and wax sections were cut to trace the developing vascular strands in the tissue. Material was fixed in FAA (5:5:90 v/v/v formalin: acetic acid: 70% ethanol) for 48 hours and stored in 70% alcohol. The material was passed through tertiary-butyl alcohol (TBA) series for dehydration, infiltrated with paraffin wax (melting point 60°C, E. Merck, Germany) and, finally, embedded in pure paraffin wax. Paraffin blocks were mounted on wooden stubs and 8-10 µm thick sections were cut using Rotary Microtome (Leica, Germany), attached with a steel knife. The sections were mounted on microslides, dewaxed and double stained with safranin (1%) and astra-blue (1%).

x. Scanning Electron Microscopy

Somatic embryos obtained from zygotic embryo cultures were fixed in 2.5% glutaraldehyde and dehydrated through a graded alcohol series. After drying, the

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samples were sputter-coated with gold and observed using a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

xi. Ploidy Analysis

The ploidy level of the regenerants, derived from leaf, ovary and zygotic embryo cultures, was determined by flow cytometry. The nuclear suspension from fresh plant material was prepared by chopping the tissues with scalpel in cold woody plant buffer (**Table 5**; Loureiro *et al*. 2007). The suspension was then filtered through a 30 µm nylon filter membrane (Millipore, USA) to remove fragments and large tissue debris. For staining nuclei, 50 µg/ml propidium iodide (PI; Sigma) was added to the filtered suspension. To prevent staining of double stranded RNA, 50 µg/ml of RNAase (Fluka) was also added.

Table 5: Constituents of woody plant buffer (Loureiro *et al*. 2007).

Buffer Components	Concentrations
Tris-HCL	0.2 M
$MgCl_2.6H_2O$	4 mM
EDTA $Na_2.2H_2O$	2 mM
NaCl	86 mM
Sodium metabisulfite	10 mM
Triton X-100	1% (v/v)
pH	7.5

2A.2.2. Analysis of In Vitro Cell Lines for Azadirachtin Production

i. Selection of Cell Lines

The calli, induced from the three explants, *viz*. zygotic embryos, leaf-discs and ovary slices, were broadly categorized into dedifferentiated (non-regenerative) and

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redifferentiated (regenerative) lines. Five-week-old, six best cell lines, three dedifferentiated and three redifferentiated, grown on various media combinations were analyzed for azadirachtin production profile. Besides, seeds, leaves and ovaries from adult parent plant were also utilized for azadirachtin analysis and served as control I, control II and control III, respectively.

ii. Preparation of Azadirachtin Standard

The stock solution (1000 µg/ml) of azadirachtin (Sigma, USA) was prepared by dissolving 0.5 mg of the standard compound in 0.5 ml of HPLC grade methanol (Merck, India). The solution was then stored at -20°C. The stock solution was serially diluted with HPLC grade methanol to make samples with concentrations of 250, 125, 62.5, 31.3, 15.6, 7.8 µg/ml. Each concentration of standard was filtered through a 0.22 µm nylon membrane filters (Millipore, USA) before HPLC analysis.

iii. Preparation of Sample Solution

To prepare samples, cultured plant cells were harvested from various media, washed with distilled water and filtered under vacuum. Thereafter, washed cell lines, seed, leaf and ovary samples were dried separately, in an oven at 30 ± 2°C until a constant weight was achieved. The drying temperature was kept low to prevent thermal decomposition of metabolites. The dried samples were dipped in methanol (analytical grade, Merck, India) overnight and, thereafter, sonicated for 45 min at 35% amplitude with 5 seconds pulse on and off. Samples were centrifuged in a high speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5000 rpm for 10 min. The supernatant was pooled and water was added in the ratio of 40:60 (40% water and 60% methanol). After addition of water, the solution was partitioned against 100 ml dichloromethane (DCM) in separating funnels. Solution was mixed thoroughly and separating funnels were kept aside for 10 min to separate two immiscible solvents (methanol + water and DCM). Later, upper water-methanol layer was discarded and DCM layer was collected and then evaporated to dryness at 40°C temperature in a rotary vacuum evaporator (Buchi Rotavapor R-200, Japan). The DCM fraction residue, thus, obtained was redissolved in HPLC grade methanol, filtered through a 0.22 µm nylon membrane filter (Millipore, USA) prior to analysis and aliquots of 20

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µl of clean solution were injected into HPLC system. Extraction from seeds consisted of an additional step of defattening with hexane prior to extraction with methanol.

iv. High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) was conducted for quantitative estimation of azadirachtin using Varian Prostar HPLC system (Varian, USA) equipped with UV visible spectrophotometer detector, a prostar binary pump, a 20 µl injection loop, and Hypersil BDS RP-C18 column (Thermo, USA) of dimensions 250 × 4.6 mm. The mobile phase used were 90% of methanol and 10% of water at a flow rate of 0.5 ml/min. UV detection was carried out at 210 nm with attenuation of 0.1 absorbance units at full scales (AUF). The chromatographic peaks of the analytes were confirmed by comparing their retention time with those of the azadirachtin standards (≈ 95 %, Sigma, USA).

v. Linearity and Precision

Linearity of developed method was checked by running the standard compound at different concentrations. Calibration curve was generated by plotting the peak area (y) against concentration in µg/ml of standard solutions (x) on Microsoft Office (Excel) Professional Edition 2003. The standard equation obtained from curve, was used for quantification of the compound in unknown samples. The correlation coefficients (R^2) were also generated in Excel by fitting the linear trendlines to the standard curve obtained. Precision of developed assay was evaluated by running same concentration of standard compounds at least three times on the same day (intraday) and twice at one day interval (interday). The values were calculated in terms of percent relative standard deviation (% RSD) which is calculated as: (Standard Deviation/ Mean) x 100.

vi. Mass Spectroscopy

MS detection was carried out on Waters quadrapole-Tof premier mass spectrometer with micro channel plate detector (Waters, USA). Samples were analyzed in positive mode with a probe temperature of 400˚C and a source block temperature of 150˚C. The source was operated with a corona pin voltage of 3.50 kV, and a cone voltage of 25 V. The MS data were obtained in full scan mode (mass range 100–1000 amu). A

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comparison of mass spectra of the standard compound obtained from Sigma, with that of sample isolated from HPLC, confirmed the presence of azadirachtin.

vii. Plackett-Burman Design (PBD)

The Plackett–Burman design was used to evaluate the relative importance of various nutrients on azadirachtin production. Plackett–Burman experimental design is based on the first order polynomial model:

$$Y = \beta_0 + \Sigma \beta_i x_i$$

where Y is the response, β_0 is the model intercept and β_i is the linear coefficient, and x_i is the level of independent variable. This model does not describe interaction among factors and it is used to screen and evaluate the important factors that influence the response. In the present work, five assigned variables were screened with twelve treatment combinations. Each independent variable was tested at two levels, a high (+1) level and low (-1) level. The high level of each variable was set far enough from the low level, to identify, which ingredient of the medium has significant influence on the azadirachtin production. From the regression analysis, the variables, which were significant at 95% level ($p < 0.05$), were considered to have greater impact on azadirachtin production. These variables were further optimized by a central composite design. **Table 6** shows the factors, their levels used and other details of the experimental design. The experimental design and statistical analysis of the data were developed by using Minitab 15.5 statistical software package.

Table 6. The two levels of medium components used in plackett-burman design.

Component	Symbol	Lower (-1) level	Higher (+1) level
Sucrose	X_1	1.0 %	5.0 %
MS Major Salts	X_2	0.5 (half)	2.0 (double)
BAP	X_3	5.0 µM	13.0 µM
IAA	X_4	2.5 µM	7.5 µM
CH	X_5	250 mg/l	1000 mg/l

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viii. Response Surface Methodology (RSM)

Central composite design (CCD) was used for determining the optimum concentration level of three significant factors screened in Plackett–Burman design. The experimental data to correlate the relationship between the response value (azadirachtin content) and the variables were explained by the following second-order polynomial equation:

$$Y = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ii} X_i^2 + \Sigma \Sigma \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient and X_i and X_j are the coded independent variables or factors.

The experimental design protocol for RSM was developed using Minitab 15.5 statistical software package. The analysis of variance (ANOVA) tables were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significance of all the terms in the polynomial was judged statistically by computing the F-value at a probability (p) of 0.05. The regression coefficients were used to make statistical calculations to generate response surface curves from the regression models.

To test the model accuracy, R^2, adjusted-R^2 (R^2_{adj}) and predicted-R^2 (R^2_{pred}) were estimated. For normality assumption, Kolmogorov–Smirnov normality test was performed and outliers were checked by studentized residual values. The second order polynomial equation was maximized by using the Minitab response optimizer under global solution of desirability equal to one to obtain the optimal levels of the independent variables and the predicted maximum azadirachtin production. The accuracy of the values was verified by comparing the predicted values obtained with the mathematical model, and the measured values obtained after the experiments under the same conditions.

2A.2.3. Statistical Analysis

Unless mentioned otherwise, twenty four cultures were raised for each treatment and all the experiments were repeated at least three times. Data were subjected to ANOVA

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and means were compared using Duncan's multiple range test by SPSS 16.0 software. P-values less than 0.05 were considered statistically significant. Arcsine transformation was done for the percent values before proceeding for ANOVA. For azadirachtin estimation, all results are an average of three separate analyses. Results are represented as mean ± standard deviation.

2B *SPILANTHES ACMELLA*

2B.1. Materials

Nodal segments and leaves were collected from plants, growing at Indian Institute of Technology Guwahati campus, at monthly intervals to establish *in vitro* cultures. Constituents of MS medium were purchased from Merck, India. The plant growth regulators, propidium iodide, RNAase, acridine orange and fluorescein diacetate (FDA), used in this study, were purchased from Sigma, USA. Glassware and plasticware were procured from Borosil, India and Tarsons, India, respectively.

For chemical analysis and bioassays, HPLC grade acetonitrile and methanol, analytical grade methanol, ethyl acetate and hexane were purchased from Merck, India. Water used for HPLC analysis was purified by Milli-Q system. Dodeca-2(E), 4(E)-dienoic acid isobutylamide standard, used for spilanthol quantification, was obtained from chromadex and scopoletin was purchased from Sigma, USA. For antioxidant assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma, USA. Folin–Ciocalteu reagent and gallic acid standard, used for phenolic estimation, were procured from Merck, India.

The reference strains of *Staphylococcus aureus* (MTCC 7443), *Pseudomonas aeruginosa* (MTCC 741), *Klebsiella pneumonia* (MTCC 109), *Escherichia coli* (MTCC 443) and *Streptococcus mutans* (MTCC 890) were purchased from IMTECH, Chandigarh. Live parasites (amphistomes) were obtained from freshly slaughtered host (pig).

2B.2. Methods

2B.2.1. Establishment of In Vitro Cultures of S. acmella

i. Nodal Segment Culture

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After removing the leaves, nodal segments, bearing two opposite axillary buds, were thoroughly washed in 1% (v/v) solution of savlon (Johnson-Johnson, India) with two drops of tween-20 (Merck, India) for 20 min, followed by rinsing with sterile distilled water (SDW). All subsequent operations were carried out inside a laminar-air-flow cabinet. The cuttings were given a quick rinse (30 seconds) in 90% ethanol before surface sterilizing with 0.1% (w/v) $HgCl_2$ solution for 6 min. Each treatment was followed by repeated washing (three washings) in SDW. The cuttings were slightly trimmed at both ends to expose the fresh tissue, leaving behind 1.5 to 2.0 cm of internodal portions on either side of the node, and planted on different nutrient media.

Initially, basal medium was examined with three carbon sources *viz*. sucrose, glucose or maltose. Subsequently, modified MS media with major inorganic salts either reduced to half strength (½ MS) or increased to double strength (2 MS) were also tested. The medium was variously supplemented with cytokinins, BAP, Kinetin or 2-iP, individually, or BAP in combination with an auxin or GA_3 to evaluate their effect on *in vitro* axillary shoot proliferation.

Observations were taken at weekly intervals. Final observation on the number of shoots per axillary bud (on nodal explants), number of nodes per shoot, shoot length, percent axillary-bud proliferation were recorded at week five from total aseptic nodal segment cultures. The rate of shoot multiplication was determined as the average number of shoots per axillary buds (on nodal explants) × the average number of axillary buds per *in vitro* shoots, at the end of a multiplication cycle (growth period).

ii. Leaf-Disc Culture

Leaves were washed with 1% (v/v) savlon for 20 min, followed by three rinses in SDW. Thereafter, the remaining steps were carried out inside the laminar-air-flow cabinet. Leaves were surface sterilized with 0.1% $HgCl_2$ for 10 min and rinsed thrice with SDW. Leaf-disc explants were prepared by punching the sterilized leaves with 5 mm sized cork-borer before being cultured with the abaxial side in contact with the media. MS basal medium supplemented with varying combinations and concentrations of auxins and cytokinins, including NAA, IAA, pCPA, 2,4-D, BAP, TDZ and Kinetin was used to induce callusing or regeneration of shoots directly from the explants. Observations were taken at weekly intervals. Final observation on the

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percent explants showing callus induction or direct shoot proliferation, number of shoots per explant and shoot length were recorded at week five from total aseptic leaf-disc cultures.

After callus induction, the cell biomass was multiplied constantly, by inoculating 0.1g of calli onto fresh medium of parental composition, at every 5-week intervals. For multiplication of shoots from various experiments, two approaches were followed. The shoots, which were small (*ca.* 1.5 cm long), transferred in a bunch of 4-5 shoots after 5 weeks to fresh medium of parental composition for sufficient elongation. However, the shoots, which had grown fairly long, were either cut into single node segments and transferred to shoot multiplication medium or cultured in rooting medium to obtain complete plantlets.

iii. Establishment of Suspension Culture

After 4-5 passages of subcultures, healthy, friable and soft calli, maintained on responding semi-solid media, were utilized to establish suspension cultures. Cultures were initiated in Erlenmeyer flasks (250 ml), containing 50 ml of liquid medium and inoculated with approximately 0.1 g of fresh calli. The cultures were incubated in an orbital shaker under shaking conditions at 120 rpm at 25 ± 2°C and maintained in diffused light (1000-2000 lux) with 16 h photoperiod or in continuous darkness. The cell biomass was subcultured regularly into fresh medium at every 18th day.

iv. Rooting and Transplantation

For *in vitro* rooting, 5-week-old *in vitro* developed shoots were excised, given an oblique cut at the base to increase the surface area of absorption for the nutrients from the medium and cultured on MS and ½ MS (major salts reduced to half strength) basal medium containing 10, 30 or 50 g/l sucrose. Excised shoots were cultured in 25×150 mm culture tube (Borosil, India) containing 20 ml of medium.

Four weeks old rooted shoots were washed to remove the agar, transferred to the plastic pots (5 cm) containing a mixture of autoclaved vermiculite, perlite and garden soil (4:1:4) and maintained initially at standard culture room conditions. The plants were acclimatized by covering the pots with polythene bags to maintain high humidity for 6-7 days and irrigated with major salt solution of MS medium, every alternate day. After 7 days, 3-4 small holes were made in the bag and plantlets were irrigated as

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before but at frequent intervals. After 25 days, polythene bags were removed and the acclimatized plants were transferred to a shaded area under natural conditions.

v. Culture Media

Sterilized nodal and leaf explants were cultured on MS basal medium consisting of macro and microsalts, vitamins, iron and 100 mg/l myoinositol. The basal medium was variously supplemented with plant growth regulators and other adjuvents. Unless mentioned otherwise, all media contained 30 g/l sucrose and were solidified with 0.8% agar (Hi Media Laboratories, India). After adjusting the pH to 5.8 with 0.1 N NaOH or 0.1 N HCL, 20 ml medium was dispensed into each 150×25 mm Borosil rimless glass tubes. The subculture experiments of leaf-discs were raised in 250 ml Erlenmeyer flasks where each flask contained 50 ml of medium. The culture tubes and flasks were plugged with nonabsorbent cotton wrapped in cheesecloth before autoclaving at 15 psi and 121 ^{O}C for 15 min. The thermolabile substances such as IAA and TDZ were filter sterilized using Millipore filters (0.45 µm pore size) and added to autoclaved medium cooled to 50ºC. The medium was then dispensed into each culture vessel inside the laminar-air-flow cabinet. The cultures were maintained at 25±2^{O}C temperature with 50-60% relative humidity and a 16/8 h (light/dark) photoperiod provided with diffuse light (1000-2000 lux). At least twenty four cultures were raised for each treatment and each experiment was repeated at least three times. Observations were recorded at weekly intervals.

vi. Anatomical Studies

Fresh specimens of stem segments were taken from *in vitro* grown shoots. The sections, passing through the nodes, were cut manually with the help of Gillette razor blades. After staining with 0.1% acridine orange for 5 min, the sections were observed under Nikon Fluorescence Microscope.

vii. Scanning Electron Microscopy

Leaf-discs of *Spilanthes* showing shoot-bud differentiation were fixed in 2.5% glutaraldehyde and dehydrated through a graded alcohol series. After drying, the samples were sputter-coated with gold and observed under a scanning electron microscope (Leo 1430vp, Carl Zeiss, Germany).

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viii. Ploidy Analysis

The ploidy stability of the regenerants, derived from nodal segments and leaf cultures, was determined by flow cytometry and cytological analysis. For flow cytometry, the nuclear suspension from fresh plant material was prepared by chopping the tissues with scalpel in cold woody plant buffer. The suspension was then filtered through a 30 µm nylon filter membrane (Millipore, USA) to remove fragments and large tissue debris. For staining nuclei, 50 µg/ml propidium iodide was added to the filtered suspension. To prevent staining of double stranded RNA, 50 µg/ml of RNAase was also added.

For cytological analysis, root tips (*ca.* 1 cm) from *in vitro* raised plantlets and field grown plants were excised at around 10.00 AM. After washing the samples with distilled water, the material were pretreated with 0.02% 8-hydroxyquinoline (Merck, India) at 4°C for 4 h. This was followed by fixation in Carnoy's fluid containing absolute alcohol, chloroform and glacial acetic acid (7:4:1) for 48 h. The fixed material was placed in a mixture of nine drops of 1% aceto-orcein and one drop of 1 N HCl in a watch glass and heated gently. After cooling, the material was placed in a drop of fresh stain on a glass slide, and a cover slip was placed over it. The slide was warmed gently and the material squashed. The slides were observed using a light microscope (80i, Nikon, Japan), and the cells showing a good separation of chromosomes were photographed.

2B.2.2. Analysis of Metabolites in Cultures of S. acmella

i. Determination of Dry Cell Weight

The calli maintained on responding semi-solid medium were harvested at the end of the growth period of 5 weeks, washed with distilled water and filtered under vacuum. Subsequently, its fresh weight was taken on high precision analytical balance (Sartorius, India). Thereafter, the cells were dried in oven at 30 ± 2°C until a constant weight was achieved. The drying temperature was kept low to prevent decomposition of thermolabile compounds. The dried cell mass was utilized for biochemical studies. For batch kinetic studies, cells were harvested at a regular interval of three days from liquid medium and dried in a similar manner as described above.

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ii. Preparation of Standard Solution

Stock solutions (1000 µg/ml) of scopoletin and dodeca-2(E), 4(E)-dienoic acid isobutylamide (a reference standard for spilanthol) were prepared by dissolving 5 mg of the compound in 5 ml of HPLC grade methanol. The solution was then stored at -20 °C. Quantification was carried out using 5 levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 0.110 µg/ml to 0.006 µg/ml for scopoletin, and from 250 µg/ml to 15 µg/ml for dodeca-2(E), 4(E)-dienoic acid isobutylamide. Each concentration of standard was filtered through a 0.22 µm nylon membrane filter (Millipore, USA) before HPLC analysis and run at least thrice to check the repeatability and precision of results.

iii. Preparation of Sample Solution

To prepare samples, dried powdered cell mass and leaf samples from *in vivo* and *in vitro* grown plants were separately soaked in methanol (analytical grade, Merck, India) for 12 h. The methanolic samples were then centrifuged in a high speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 5000 rpm for 10 min. The supernatant was transferred into new tube and the residue was re-extracted thrice with 10 ml methanol. Thereafter, the residue was discarded and the supernatant was pooled, filtered and evaporated to dryness in a rotatory evaporator (Buchi Rotavapor R-200, Japan) at 40°C. The methanolic fraction, thus, obtained was redissolved in HPLC grade methanol, filtered through a 0.22 µm nylon membrane filter prior to analysis and aliquots of 20 µl of clean solution were injected into HPLC system.

iv. High Performance Liquid Chromatography

Detection and quantification of scopoletin and spilanthol were carried out using Varian Prostar HPLC system (Varian, USA) equipped with Ultraviolet (UV) and Fluorescence (FL) detector, a prostar binary pump, a 20 µl injection loop, and Hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6 × 250 mm.

The mobile phase used for scopoletin was 50% A (50% methanol + 50% acetonitrile + 0.1% phosphoric acid) and 50% B (Milli Q water + 0.1% phosphoric acid) with a flow rate of 0.8 ml/min. The eluted samples were detected by UV detector at 346 nm.

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FL detector was used with an excitation wavelength at 385 nm and an emission wavelength at 445 nm. The chromatographic peaks of the scopoletin were confirmed by comparing their retention time with those of the scopoletin standard and by spiking samples with standard scopoletin.

For spilanthol analysis, mobile phase used was 93% A (acetonitrile) and 7% B (Milli Q water) with a flow rate of 0.5 ml/min. The eluted samples were detected by UV detector at 237 nm. Spilanthol peaks obtained in HPLC were identified by electrospray ionization mass spectra and by comparing with published data. As there was no commercially available standard for spilanthol, it was tentatively quantified on the basis of another compound; dodeca-2(E), 4(E)-dienoic acid isobutylamide that is structurally similar to spilanthol. Both the compounds have isobutylamide group and long carbon chain.

v. Linearity and Precision

Linearity of developed method was checked by running the standard compound at five different concentrations. Calibration curve was constructed by plotting the peak area (y) against concentration in µg/ml of standard solutions (x). The standard equation obtained from the curve was used for quantification of the scopoletin and spilanthol in the unknown samples. Scopoletin and spilanthol contents were reported as µg/g DW of sample. The correlation coefficients (R^2) were also generated in Microsoft Ofiice (Excel) Professional Edition 2003 by fitting the linear trendlines to the standard curves obtained for each of the two compounds.

Precision of developed assay was evaluated by running same concentration of standard compounds at least three times on the same day (intraday) and twice at one day intervals (interday). The values were calculated in terms of relative standard deviation (RSD).

% RSD = (Standard Deviation/ Mean) x 100

vi. Mass Spectroscopy (MS)

MS detection of spilanthol and scopoletin was carried out on Water quadrapole Tof Premier mass spectrometry with micro channel plate detector (Waters, USA). The analysis was done with an ESI probe source in positive mode and with collision

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energy of 3V. The cell entrance and exit voltage was set at 2V and -10V, respectively. For spilanthol identification, all peaks that appeared in HPLC were collected, concentrated and than redissolved in methanol prior to analysis. Confirmation of the spilanthol peak was done by comparing the mass spectra of samples and that reported in published literature. A comparison of mass spectra of scopoletin standard compound, obtained from Sigma, with those of the individual samples isolated from HPLC, confirmed the presence of scopoletin.

vii. Batch Kinetics of Cell Suspension Cultures

a. Batch Kinetics Studies

To establish the kinetics of cell growth and nutrient uptake, cells were harvested from liquid medium at an interval of three days, washed and dried with same procedure as mentioned in section 2B.2.2.i. The pH and residual nutrients (phosphate, nitrate and sugar) of the suspension cultures were monitored at every third days. Phosphate was estimated by the standard calibration curve made from dihydrogen sodium phosphate (NaH_2PO_4); to 0.5 ml of standard or sample solution, 4 ml of reagent [acetone (CH_3COCH_3), sulphuric acid (H_2SO_4) 2.5 M and ammonium molybdate tetrahydrate ($(NH_4)_2MoO_4.4H_2O$) 10 mM, mixed in the ratio of 2:1:1] was added. After thoroughly mixing the solutions, 0.4 ml of 1M citric acid was added and absorbance was taken at 355 nm. Similarly, for nitrate estimation, standard curve was made from 0.01N stock solution of potassium nitrate (KNO_3), preserved in chloroform. After acidification of samples with hydrochloric acid, absorbance was recorded at 275 nm in a UV visible spectrophotometer (Cary 100, Netherlands). For sucrose estimation, anthrone reagent was prepared as described by Trevelyan and Harrison (1952) by dissolving 0.2 g of anthrone in 100 ml of H_2SO_4. The reagent was allowed to stand for 30-40 min, and then to 1 ml of standard or sample solution, 5 ml of anthrone reagent was added. After incubating the reaction mixture in boiling water bath for 5 min, absorbance was taken at 620 nm. Standard curve was made by glucose. The specific growth rate (μ) was calculated by:

$$\mu = \ln (MT_2 - MT_1) / T_2 - T_1; T_2 > T_1$$

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where, MT_2 and MT_1 are biomasses at the different time points (T_1 and T_2), respectively.

b. Carbon Source

Three carbon sources, glucose, fructose and sucrose were tested at 3% concentration to understand the growth and production profile of cells in suspension cultures. The *Spilanthes* cells were inoculated such that each 250 ml Erlenmeyer flasks containing 50 ml of medium had 0.1 g of the cells. The cell biomass was harvested at the end of the growing phase of 15 days, to analyse dry cell weight and metabolite content.

c. Agitation Speed

Effect of agitation speeds was evaluated on fresh and dry weight of cells and their viability, at the end of each passage. Callus cells weighing approximately 0.1 g were harvested at the end of growth period and re-inoculated in 50 ml of fresh medium of the same composition. The cultures were incubated in shaking conditions at 60, 90, 120, 150 and 180 rpm, under darkness, for a period of 18 days and their fresh and dry weights were recorded. The viability of cells under each condition was checked with 1% FDA solution.

2B.2.3. Bioassays

For bioassays, 50 g dried, finely ground, cell mass and *in vivo* leaf samples were soaked in different solvents (hexane, ethyl acetate, methanol and water) and extracted by following the protocol described in 2B.2.2.iii. The percentage yield of extracts was calculated relative to weight of dried cells.

i. Antioxidant Assay

a. DPPH radical scavenging activity

Free radical-scavenging activity of the plant extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. Stock solutions (10 mg/ml each) of the extract were prepared in methanol. About 0.5 mg/ml solution of DPPH in methanol was prepared and 500 µl of this solution was added to 2.0 ml of extract solution. Thirty minutes later, the absorbance of these solutions were recorded on an ultraviolet and visible (UV-Vis) spectrophotometer (Cary 100, Netherlands) at 517

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nm using a blank containing the same concentration of DPPH radicals. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage (I%) of free radical by the sample and was calculated using the following formula:

$$\mathbf{I\% = [(A\ blank - A\ sample)/A\ blank] \times 100}$$

Where A blank is the absorbance value of the control reaction (containing all reagents except the extract) and A sample is the absorbance values of the extract. Ascorbic and vanillic acid were used as the reference compound.

b. Assay for total phenolics

Total phenolic constituents of extracts were determined by literature methods involving Folin–Ciocalteu reagent and gallic acid standard (Djeridane *et al*. 2006). The solutions of each extract (200 µl; 5 mg/ml) were taken individually in test tubes. To this solution, 500 µl of distilled water and 500 µl of Folin–Ciocalteu reagent were added, and the flasks were thoroughly shaken. After 1 min, 800 µl of 7.5 % Na_2CO_3 solution were added and the mixtures were allowed to stand for 30 min with intermittent shaking. Absorbencies were taken at 760 nm. The same procedure was repeated for all the standard gallic acid solutions, and a standard curve obtained. Total phenols of the extract, as gallic acid equivalents, were determined by using the absorbance of the extract measured at 760 nm as input to the standard equation. All tests were carried out in triplicate, and phenolic contents as gallic acid equivalents were reported.

ii. Antimicrobial Assay

a. Culture Media

All the reference strains were grown in nutrient agar (agar-agar 20 g) or nutrient broth (without agar) media (Titan Biotech, India). Both the media consisted of peptone- 5 g, beef extract- 3 g, NaCl- 5 g, and distilled water-1000 ml at pH-7.0. The bacterial strains were incubated for 24 h at 37°C. Thereafter, the cultures were passed into peptone broth and incubated for 4-5 h at same temperature. After adjusting the concentration with McFarland no. standard 0.5, bacterial colonies were swabbed on Muller-Hinton agar plates (Rankem, India; acid hydrolysate of casein 17.5 g, beef

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infusion 2.0 g, starch 1.5 g bacteriological agar 17 g, distilled water-1000 ml at pH 7.3) in three directions according to CLSI (Clinical and Laboratory Standards Institute).

b. Disc Diffusion Assay

Antibacterial susceptibility test was done by Bauer-Kirby disc diffusion method (Bauer *et al*. 1966). Filter paper discs of 5 mm diameter were made from Whatman filter paper number 3 and sterilized. Each disc was about 4.7-4.8 mg in weight. Plant extracts were dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration of 10 mg/ml. The samples were filtered by 0.22 µm nylon filter membrane (Millipore, USA) prior to use. Each pre-sterilized disc was loaded with 20 µl of the extract, allowed to dry and placed on swabbed agar plates with the help of pre-sterilized forceps.

iii. Anthelmintic Assay

Live parasites (amphistomes) were collected in 0.9% phosphate-buffered saline (PBS; 8 g NaCl, 0.34 g KH_2PO_4, and 1.21 g K_2HPO_4 in 1 l of distilled water, pH 7 ± 0.3) from freshly slaughtered hosts at local abattoirs.

The adult amphistome parasites were incubated at 37 ± 1°C in PBS supplemented with 1% DMSO buffer containing no extract (control) or crude extract at 10, 20 and 30 mg/ml. Three replicates were used for each concentration. The time required for complete inactiveness or paralysis and death of the parasite was recorded.

2B.2.4. Statistical Analysis

For tissue culture studies, twenty four replicates were raised and each experiment was repeated thrice. Standard deviation of the mean was calculated and is represented as bars in the graph. All the investigated parameters were analyzed using analysis of variance (ANOVA) and significance was determined at $p < 0.05$. The data was analyzed statistically using SPSS (version 16) software and significant differences among the mean values were assessed on the basis of the least significant difference (LSD) and Duncan's multiple range tests. Percentages have been transformed using arcsine transformation before statistical analysis.

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For secondary metabolite estimation from callus cultures, observations are an average of three separate analyses. For batch kinetics studies, all experiments were done in triplicate to check the reproducibility of the results. Results are represented as mean ± standard deviation. For bioassays, all experiments were done in triplicates, along with positive and negative controls. Results are expressed as mean ± standard deviation.

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Chapter 3

Results

In the present chapter, two medicinal plants were investigated for tissue culture studies and important metabolite production. The outcomes of the experiments have been divided into two broad sections; **section 3A** presents the results on *Azadirachta indica* A. Juss. and **section 3B** deals with *Spilanthes acmella* Murr. Each section is further subdivided into: subsection 1 which presents results related to tissue culture studies, subsection 2 which groups together the biochemical analysis work. An additional subsection 3 is added to section 3B which reports results on bioactivity of *S. acmella*. The experiments on bioactivity of *A. indica* are not presented in this thesis since the plant is very well known for its biological properties and numerous reports are available supporting such properties.

3A. *AZADIRACHTA INDICA*

3A.1. Establishment of *In Vitro* Cultures

In vitro cultures of neem (*A. indica*) were established from three explants e.g. zygotic embryo, leaf and ovary. The results of these experiments are depicted below:

3A.1.1. Zygotic Embryo Culture

To establish cultures, zygotic embryos at early-late dicotyledonary stages **(Fig. 1A)** were cultured on MS basal medium variously supplemented with growth regulators. The explants showed different types of morphogenic responses. They either callused or differentiated into somatic embryos directly from the explants. MS basal medium alone did not show any response. Out of the various treatments tested, dedifferentiated (non-regenerative) cultures were obtained from late dicotyledonary stage of embryos on MS + BAP (5.0 µM) + 2,4-D (1.0 µM) + NAA (1.0 µM). On this medium, profusely growing light green, fresh and friable callus proliferated from explants in 100% cultures, within four weeks (**Fig. 1B**). Callus proliferation rate was increased in subsequent subcultures on the same medium. However, the calli remained in dedifferentiated state. For redifferentiated cultures, MS + BAP (9.0 µM) + IAA (5.0

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μM) + CH (500 mg/l) medium was found to be the best. On this medium, calli proliferated within one week in 78% cultures, which after 5 weeks turned hard and nodulated. Histological analysis of nodulated callus cultures showed the development of vascular strands within the calli (**Fig. 1C**). On subsequent subcultures of callus on the same medium, shoot proliferation started from these calli, within three weeks (**Fig. 1D)**. Both the cell lines, dedifferentiated and redifferentiated, were maintained for more than 3 years.

Besides above, zygotic embryo cultures also showed somatic embryogenesis directly from explants on MS medium supplemented with TDZ alone or in combination with GA_3 or ABA (**Table 3A.1**). Maximum somatic embryogenesis occurred on MS medium supplemented with TDZ (1.0 μM) and GA_3 (1.0 μM), where 100% cultures responded and somatic embryos were differentiated directly from hypocotyl and plumular regions of the zygotic embryos **(Fig. 2A).** In some of the cultures, on this medium, shoots were also proliferated along with somatic embryos at plumular regions (**Fig. 2B**). On another medium, MS + TDZ (1.0 μM) + ABA (1.0 μM), 37% cultures showed embryogenesis with 32.3 ± 0.4 somatic embryos per culture. However, on this medium, most of the somatic embryos proliferated from cotyledons of germinated zygotic embryos (**Fig. 2C,D**). Overview of various developmental stages of embryos had been recorded by scanning electron microscope (SEM); somatic embryos at globular, early heart and cotyledonary stages were visible during this study (**Fig. 3A-D).** Somatic embryos never germinated to form the complete plantlets.

3A.1.2. Leaf-Disc Culture

In the present study, leaf-disc explants of 5 mm size were cultured on a range of media. The treatments involved MS basal medium and varying concentrations and combinations of cytokinins and auxins like BAP, TDZ, Kinetin, Zeatin, 2,4-D, NAA, IAA, IBA and additives like CH **(Table 3A.2).** Leaf-disc cultures showed no response in the absence of growth regulators. Presence of at least one cytokinin or one auxin was found obligatory for caulogenic induction.

Among individual cytokinin/auxin treatments, MS + Zeatin (5.0 μM) and MS + IBA (5.0 μM) evoked maximum callus induction (100%) and produced profuse

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brownish-green callus from all over the surface of the explants within 5 weeks. Individually,

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Table 3A.1. Effect of TDZ alone or in combination with ABA and GA_3 on somatic embryogenesis from zygotic embryo cultures of neem.
Growth period: 4 weeks. Control: MS basal medium.

S. No.	Media	Percent somatic embryogenesis	Number of somatic embryos per culture
1.	MS Basal Medium	0.0h	0.0i
2.	TDZ (0.5 µM)	15.0f	6.1 ± 0.6g
3.	TDZ (1.0 µM)	36.0d	26.4 ± 0.6b
4.	TDZ (2.0 µM)	30.0e	23.6 ± 1.0c
5.	TDZ (0.5 µM) + ABA (1.0 µM)	0.0h	0.0 ± 0.0i
6.	TDZ (1.0 µM) + ABA (1.0 µM)	37.0d	32.3 ± 0.4a
7.	TDZ (2.0 µM) + ABA (1.0 µM)	0.0h	0.0 ± 0.0i
8.	TDZ (0.5 µM) + ABA (4.0 µM)	10.0g	18.0 ± 0.7d
9.	TDZ (1.0 µM) + ABA (4.0 µM)	64.0b	12.0 ± 0.7e
10.	TDZ (2.0 µM) + ABA (4.0 µM)	14.0fg	10.0 ± 0.4e
11.	TDZ (0.5 µM) + GA_3 (1.0 µM)	68.0b	6.3 ± 0.4g
12.	TDZ (1.0 µM) + GA_3 (1.0 µM)	100a	9.4 ± 0.3ef
13.	TDZ (2.0 µM) + GA_3 (1.0 µM)	38.0d	3.1 ± 0.6h
14.	TDZ (0.5 µM) + GA_3 (4.0 µM)	30.0e	5.3 ± 0.5gh
15.	TDZ (1.0 µM) + GA_3 (4.0 µM)	45.0c	7.2 ± 0.4fg

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16.	TDZ (2.0 µM) + GA_3 (4.0 µM)	18.0f	5.5 ± 0.3gh

Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p<0.05$) according to Duncan's multiple range test.

Table 3A.2. Effect of various growth regulators alone or in combinations, on callusing response from leaf-disc explants of neem.
Growth period: 5 weeks. Control: MS basal medium.

S. No.	Media	Percent callusing response
1.	MS Basal Medium	0.0i
2.	**MS + BAP (5.0 µM)**	**76.0c**
3.	MS + TDZ (5.0 µM)	40.0e
4.	MS + Kinetin (5.0 µM)	0.0i
5.	MS + Zeatin (5.0 µM)	100a
6.	MS + 2, 4-D (5.0 µM)	19.7g
7.	MS + NAA (5.0 µM)	84.7b
8.	MS + IAA (5.0 µM)	0.0i
9.	MS + IBA (5.0 µM)	100a
10.	**BAP (2.2 µM) + NAA (21.5 µM) + CH (500 mg/l)**	**100a**
11.	MS + BAP (5.0 µM) + 2,4-D (1.0 µM) + NAA (1.0 µM)	11.3h

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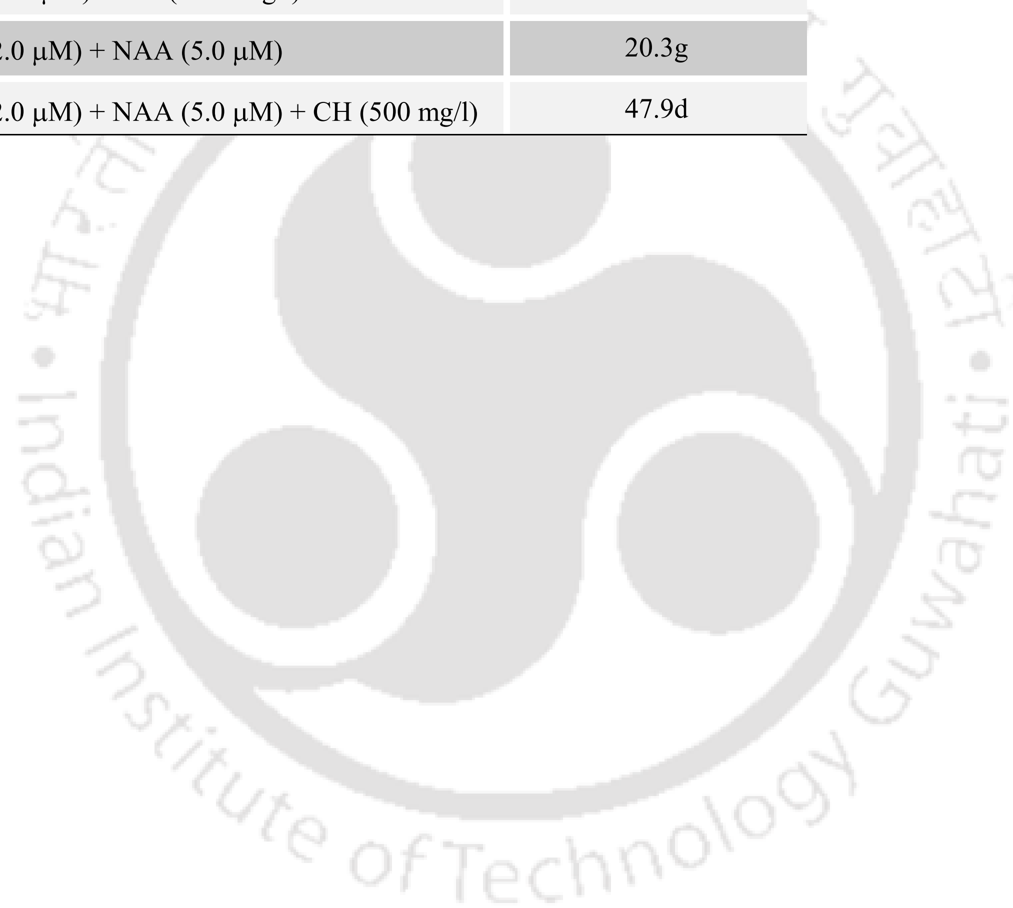
12.	MS + BAP (2.0 µM) + 2,4-D (5.0 µM)	39.7e
13.	MS + 2,4-D (5.0 µM) + CH (1000 mg/l)	29.7f
14.	MS + BAP (2.0 µM) + NAA (5.0 µM)	20.3g
15.	MS + BAP (2.0 µM) + NAA (5.0 µM) + CH (500 mg/l)	47.9d



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Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p<0.05$) according to Duncan's multiple range test.

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TDZ, NAA and IBA at 5.0 μM concentration induced callus and produced slow growing yellowish-brown calli. However, none of these treatments exhibited any kind of organogenesis. Moreover, in subcultures on the original media, the calli did not show sustained growth and the rate of callus proliferation declined after 3 subcultures, each of 5 week duration. Leaf-disc explants showed no response on kinetin or IAA supplemented medium.

The interactive effect of auxin and cytokinin has also been evaluated. The combined presence of one auxin and one cytokinin supported sustained callus growth. The best treatment for callusing, in terms of the number of explants showing callusing (100%) and the degree of callusing, was MS + BAP (2.2 μM) + NAA (21.5 μM) + CH (500 mg/l). On this medium, callus growth was significant ($p<0.05$) and fast growing, fresh and friable callus proliferated in 5 weeks which remained dedifferentiated even after 17 subcultures, each of 5 weeks duration (**Fig. 4A,B**).

Organogenesis from leaf-disc explants was achieved on MS medium supplemented with BAP at 5.0 μM. On this medium, brownish-green and compact calli were developed at the cut end of the explant, within 3 weeks, which later turned into brown and dark-green, nodulated callus after 5 weeks of culture initiation, in 76% of the cultures. On subculture to the original medium, the nodular structures organized into well developed shoots within 3 weeks (**Fig. 4C);** an average of 7 green shoots developed per explant. Histological sections of the regenerating calli, passing through the nodular regions, showed the presence of developing vascular strands within the tissue, indicating the points of origin of shoot-buds. A nest of vascular bundles can be seen in the section **(Fig. 4D)**.

3A.1.3. Ovary Culture

As an alternative approach for high frequency regeneration and secondary metabolite production, *in vitro* ovary culture method was followed. For initiation of ovary cultures, flower buds of 4 mm size (**Fig. 5A)** were selected, ovaries were excised (**Fig. 5B)**, and either 0.4-0.5 mm thick transverse slices of ovaries or entire ovaries were cultured on MS basal medium or basal medium supplemented with a range of growth regulators. To induce callusing from explants, cultures were exposed to low (4°C) and high (33°C) temperature treatments for 0 or 15 days prior to their transfer to standard

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culture room conditions. Exposure of slice sections at 33°C was found to be very effective for caulogenic induction (**Table 3A.3**). While, slice sections exposed at 4°C did not show any response. In control (25°C), initially explants showed slight callusing but after first subculture calli turned brown without showing any further proliferation. In all cases, the response of ovary slice cultures was shown to be better than that of entire ovary cultures, in terms of time taken for callus initiation and degree of callusing. In slice cultures, callusing started after 1 week and explants were completely covered with the proliferating callus by the end of 4th week (**Fig. 5C,D)** while for entire ovary culture, it took 4 weeks to start callus initiation.

Of various growth regulator combinations tested, MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM) + Glutamine (800 mg/l) + Serine (200 mg/l) though induced callusing in maximum (92.3%) number of cultures during the initial period of 4 weeks but, it did not promote sustained proliferation of callus in successive subcultures. In terms of biomass and sustained rate of callus proliferation, best response was observed on MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM) followed by MS + 2,4-D (0.5 µM) and MS + 2,4-D (1.0 µM) + Kinetin (10.0 µM), in a single growth cycle of 4 weeks. Moderate to fast growing, cream, soft and friable calli obtained on these three combinations, were multiplied and maintained for more than 1 year.

Since calli on above three multiplication media remained undifferentiated, to achieve regeneration, ovary calli, from the three media were transferred to MS medium comprising of different combinations and concentrations of BAP, IAA and CH (**Table 3A.4**). Out of the three multiplication media, the calli from MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM) only showed regeneration in regeneration media and shoot regeneration was achieved on only two combinations: MS + BAP + IAA or MS + BAP + IAA + CH. The calli in the regeneration media initially turned bright green in colour and later on within 4 weeks compact nodular structures developed which after subculture to the same medium differentiated into shoot-buds in the next 4 weeks. On the first combination, MS + BAP (5.0 µM) + IAA (0.5 µM), on an average 25.5% cultures formed 2.5 shoot-buds per explants in 4 weeks (**Fig. 5E)**. In comparison to this, the second medium, MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l) was found to be the best for shoot regeneration where 50% of the callus cultures developed

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on an average 5.5 shoot-buds per explant in 4 weeks **(Fig. 5F,G)**. Histological analysis of regenerating calli showed the presence of well formed tracheary elements **(Fig. 5H)**.

Table 3A.3. Effect of temperature pre-treatments and different growth regulator combinations on callus induction from ovary slice cultures. Growth period: 4 weeks. Control: MS basal medium.

Media	Temperature pre-treatments		
	25 °C (control)	4 °C	33 °C
MS basal medium	0.0d	0.0	0.0e
MS + 2,4-D (0.5 µM)	36.7a	0.0	86.5b
MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM)	38.2a	0.0	89.0ab
MS +2,4-D (1.0 µM)+ Kinetin (10.0 µM)	30.3b	0.0	50.3d
MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM) + Glutamine (800 mg/l) + Serine (200 mg/l)	38.3a	0.0	92.3a
MS + BAP (5.0 µM) + Glutamine (800 mg/l) + Serine (200 mg/l)	21.4c	0.0	77.3c

Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p<0.05$) according to Duncan's multiple range test.

Table 3A.4. Effect of three induction media on shoot-bud differentiation from ovary calli in the regeneration medium. Growth period: 4 weeks. Control: MS basal medium.

Regeneration treatments (µM)	Induction media and their effect on nature and growth of calli			Percentage of cultures showing shoot regeneration
	MS + 2,4-D (0.5µM)	MS + 2,4-D (0.5µM) + Kinetin (4.5µM)	MS + 2,4-D (1.0µM) + Kinetin (10.0µM)	
MS basal medium	Nil			Nil
MS + CH (500 mg/l)	Nil			Nil
MS + BAP (5.0 µM)	bright green, friable callus			Nil
MS + BAP (9.0 µM)	bright green, friable callus			Nil
MS + BAP (9.0 µM) + IAA (5.0 µM)	bright green, friable callus			Nil
MS + BAP (5.0 µM)	dark green, friable callus	**bright green, moderately hard; fresh nodulated**	dark green, friable callus	**25.5 (2.5)***

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+ IAA (0.5 µM)		**callus with shoot-buds**		
MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l)	bright green, moderately hard callus	**bright green, with massive growth, moderately hard; fresh nodulated callus with shoot-buds**	bright green, moderately hard callus	**50 (5.5)***

* Values in parentheses represent average number of shoot buds formed per explants.

3A.1.4. Elongation, Multiplication and Rooting of Shoots

Irrespective of the explants used, the shoots regenerated from various calli did not grow beyond 1 cm while attached to the parent tissues. Therefore, individual shoots were excised and transferred to MS basal medium supplemented with a lower concentration of BAP for elongation. On MS + BAP (0.5 µM), shoots attained a height of 4-6 cm with each shoot having an average of 4-5 nodes, after 5 weeks **(Fig. 6A, Table 3A.5)**. Once the shoots acquired sufficient length, further multiplication was achieved on MS medium supplemented with BAP and CH. At the end of the passage, each long shoot from elongation medium was cut into single nodal segments and planted on MS + BAP (1.0 µM) + CH (250 mg/l). Each node again produced a single, 6-7 cm long, multinodal shoot, which provided 6-7 cuttings after 6 weeks **(Fig. 6B)**. Thus, 7-fold shoot multiplication could be achieved in every 6 weeks on MS + BAP + CH by cutting the solitary shoot into single node segments and culturing them on fresh medium.

For rooting, 3 cm long terminal portions of 6-week-old elongated shoots were cut and transferred to ¼ MS medium (major salts reduced to quarter strength) supplemented with IBA (0.5 µM). Remaining part of the shoots were further utilized for multiplication by nodal segment culture. MS basal medium did not support rooting. Direct rooting was attained in cultures, from the basal cut end of shoots on ¼ MS + IBA (0.5 µM). On this medium, root initiation occurred within 3 weeks, and after 5 weeks 100% shoots had formed 4-7 roots. Thus, routinely, the shoots were rooted on ¼ MS + IBA (0.5 µM) (**Fig. 6C, Table 3A.6**).

3A.1.5. Ploidy Analysis

The ploidy stability of *in vitro* regenerants, derived from leaf, ovary and zygotic embryo cultures, was determined by flow cytometry. Leaves from field grown parent

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plant and *in vitro* plants were utilized for ploidy analysis. **Figure 7A-D** shows representative histograms of parent plant (control) and *in vitro* shoots from zygotic embryo, leaf and ovary cultures. Similar channel position (around 200) and the presence of single peak in *in vitro* plants corresponded to the peak obtained from parent plant suggested that the *in vitro* plants maintained their genetic stability and no changes in ploidy levels occurred during regeneration process.

Table 3A.5. Elongation of shoots obtained from various explants on MS medium supplemented with BAP at 0.5 µM. Growth Period: 5 weeks.

***In vitro* shoots**	**Percent explants showing shoot elongation**	**Shoot length (cm)**	**Number of nodes per shoot**
In vitro shoots from leaf-disc culture	100	4.5 ± 0.2	4.1 ± 0.2
In vitro shoots from zygotic embryo culture	100	5.5 ± 0.3	4.3 ± 0.2
In vitro shoots from ovary culture	100	5.0 ± 0.3	4.5 ± 0.5

Values are represented as Mean ± SE

Table 3A.6. Rooting response of *in vitro* shoots on ¼ MS medium supplemented with IBA at 0.5 µM. Growth Period: 5 weeks.

Parameters	***In vitro* shoots from leaf-disc culture**	***In vitro* shoots from zygotic embryo culture**	***In vitro* shoots from ovary culture**
Percentage of rooted shoots	100 ± 0.0	100 ± 0.0	100 ± 0.0
Number of roots per shoot	5.0 ± 1.0	6.0 ± 1.0	6.6 ± 0.4
Number of laterals	8 ± 1.4	10 ± 0.9	12 ± 0.4
Length of longest and smallest root (cm)	4.0 and 1.5	6.0 and 2.8	5.0 and 1.2

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Length of laterals (cm)	0.8 ± 0.1	0.7 ± 0.2	0.8 ± 0.3

Values are represented as Mean ± SE

3A.2. Analysis of *in Vitro* Cell Lines for Azadirachtin Production

In order to select an elite *in vitro* cell line for azaditrachtin production, redifferentiated and dedifferentiated calli obtained from various explants of neem, *viz.* zygotic embryo, leaf and ovary, were analyzed by HPLC.

3A.2.1. Detection and Estimation of Azadirachtin

i. Calibration Curve Analysis

With the protocol adopted, azadirachtin peak was obtained at retention time of 6.0 ± 0.39 min (**Table 3A.7, Fig. 8A**). Calibration curve for azadirachtin standard showed good linearity with high reproducibility and accuracy at all tested concentrations (7.8 µg/ml to 250 µg/ml). Regression analysis of the calibration curve data points showed excellent correlation coefficient (R^2) of 0.9638. The linear regression equation for standard azadirachtin was: $y = 1.0194\ x + 54.22$, where x is the concentration of standard and y is the total peak area. The equation, thus, generated from the curve by external standard method was used to calculate the amount of compound present in crude samples.

The precision of the developed method, as mentioned in materials and methods, was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard samples, at same concentration, were analyzed at least three times within the same day and the RSD value obtained was 2.48 %. Similarly, for inter-day variability, same concentration of the standard was run at least twice at one day interval and the values for the same figured out to be 3.14 % (**Table 3A.7)**.

Table 3A.7. Standard curve analysis for azadirachtin.

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Compound	Retention time (min) ± SD	Standard equation	R^2	% RSD	
				Interday	Intraday
Azadirachtin	6.0 ± 0.39	y = 1.0194x + 54.22	0.9638	3.14 %	2.48%

ii. Identification and Quantification of Azadirachtin by HPLC

By following the protocol as described in materials and methods, three controls, *viz.* seeds (control I), leaves (control II) and ovaries (control III), and three dedifferentiated and three redifferentiated cell lines, obtained from explants, were analyzed for azadirachtin accumulation. Presence of azadirachtin was confirmed in all the control samples (**Fig. 8B-D)** and cell lines (**Fig. 9A-F)** by HPLC peak obtained at 6.0 ± 0.39 min retention time.

From the standard equation obtained, the amount of azadirachtin in different callus lines was calculated and listed in **Table 3A.8**. The estimation of azadirachtin in different control samples revealed that the seed extract contained azadirachtin (7.41 ± 0.07 mg/g DW) in amount that were orders of magnitude higher than control leaf (5.49 ± 0.08 mg/g DW) and ovary (1.38 ± 0.02 mg/g DW) extracts. However, the highest azadirachtin production (2.33 ± 0.03 mg/g DW) occurred in redifferentiated zygotic embryo callus, while the least (0.52 ± 0.01 µg/g DW) was obtained from dedifferentiated leaf callus (**Table 3A.8**).

iii. Analysis of Azadirachtin by Mass Spectroscopy

The fraction of crude extracts eluted from HPLC at a retention time of 6.0 ± 0.39 minutes was collected, analyzed by mass spectrometry and the fragment characteristics were compared with that of standard azadirachtin procured from Sigma, Aldrich. Spectra were obtained in full scan mode. The positive ESI mass spectrum has a base peak at m/z 743 corresponding to the $[M+Na]^+$ ion. The ion at m/z 759 were formed due to potassium ion adduct formation $[M+K]^+$. Characteristic fragments seen at the m/z 685 and m/z 703 may be due to elimination of water, $[MH-2H_2O]^+$ and $[MH-H_2O]^+$, respectively (**Fig. 10A,B**). Similar m/z fragments in standard compound and HPLC fraction confirmed the presence of azadirachtin in *in vitro* cell lines.

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3A.2.2. Statistical Optimization

The highest azadirachtin (2.33 mg/g DW) producing redifferentiated cell line, obtained from zygotic embryos on MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l), was utilized for statistical optimization study. Five major media components, BAP, IAA, CH, MS major salts and sucrose, were chosen for the study.

i. Evaluation of Factors Affecting Azadirachtin Production

The first optimization step was a twelve run Plackett–Burman design to identify the significant factors affecting azadirachtin production in redifferentiated zygotic embryo callus. The data in **Table 3A.9** indicated that there was a wide variation in azadirachtin content from 0.03 mg/g DW to 4.55 mg/g DW in the twelve trials. This variation reflected the significance of factors. The analysis of regression coefficients

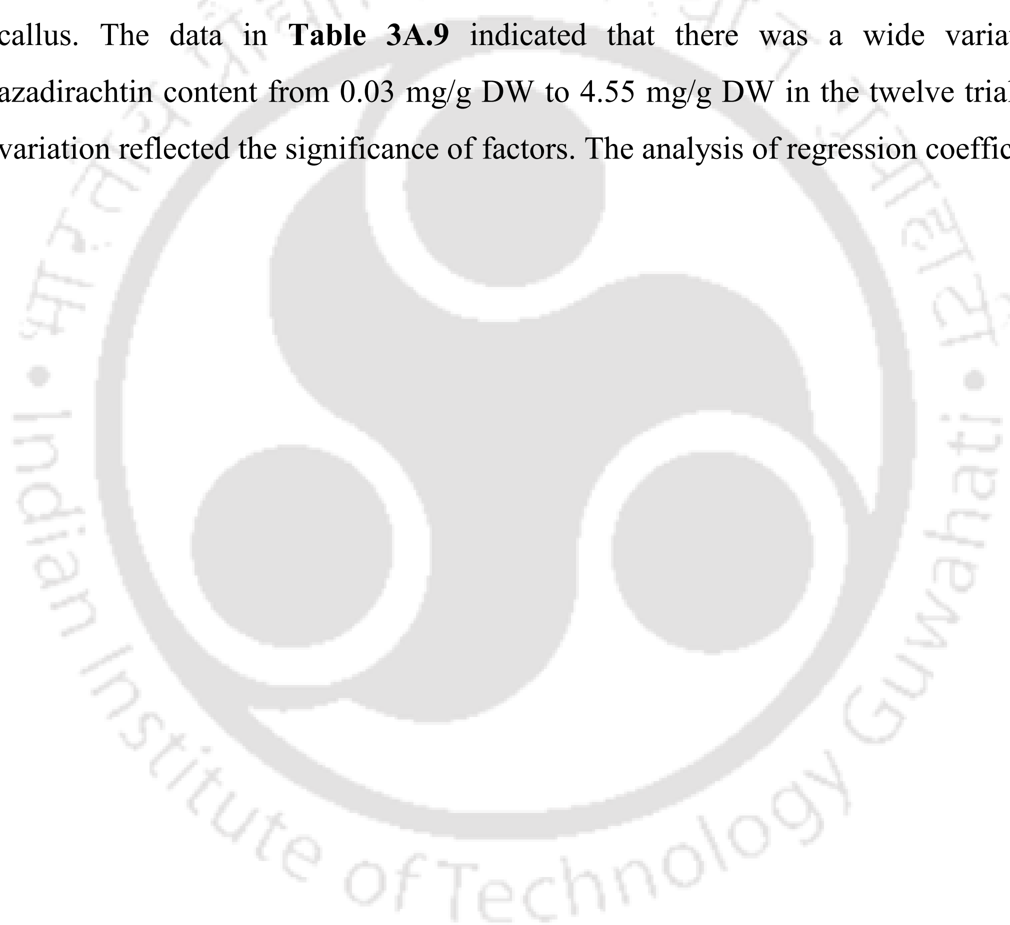

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Table 3A.8. Effect of explant source and organogenesis on azadirachtin content.

Source	Medium	Culture	Amount of azadirachtin (mg/g DW) ± SD
Zygotic embryo	Seed (Control I)		7.41 ± 0.07
	MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l)	Redifferentiated	2.33 ± 0.03
	MS + BAP (5.0 µM) + 2,4-D (1.0 µM + NAA (1.0 µM)	Dedifferentiated	1.15 ± 0.01
Leaf	Leaf (Control II)		5.49 ± 0.08
	MS + BAP (5.0 µM)	Redifferentiated	1.6 ± 0.01
	MS + BAP (2.2 µM) + NAA (21.5 µM) + CH (500 mg/l)	Dedifferentiated	0.52 ± 0.01
Ovary	Ovary (Control III)		1.38 ± 0.02
	MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l)	Redifferentiated	1.28 ± 0.02
	MS + 2,4-D (0.5 µM) + Kinetin (4.5µM)	Dedifferentiated	1.03 ± 0.01

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The values are mean ± SD

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and t-value of five medium components are shown in **Table 3A.10.** It can be seen that sucrose (X_1), MS major salts (X_2) and BAP (X_3) have significant effect on azadirachtin production. CH (X_4) and IAA (X_5) were found insignificant with positive coefficients. Neglecting the variables which were insignificant, the first order model equation for azadirachtin production can be written as:

$$Y = 3.20 + 1.24 X_1 - 0.73 X_2 + 0.85 X_3$$

With the help of relative ranking, MS major salts, sucrose and BAP were selected for further optimization, which had the most significant effects on azadirachtin production.

ii. Optimization of Culture Conditions by RSM

The three above-mentioned important components (MS major salts, sucrose and BAP) were optimized using response surface methodology. **Table 3A.11** shows various combinations of three screened factors and corresponding measured and predicted azadirachtin content. The amounts of other variables were same as those in the basal media.

Data were analyzed by using Minitab 15.5 statistical software package and mathematical expression of relationship to the azadirachtin production with variables is shown below:

$$Y = 4.85 + 0.69 X_1 + 0.30 X_2 + 0.37 X_3 - 0.06 X_1 X_2 + 0.00 X_1 X_3 + 0.14 X_2 X_3 - 0.65 X_1^2 - 0.75 X_2^2 - 0.56 X_3^2$$

To test the fit of the model equation, the regression based determination R^2 coefficient was evaluated. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The model presented a high determination coefficient (R^2 = 0.9582) explaining 95.82% of the variability in the azadirachtin production. The adjusted determination coefficient (R^2_{adj}) and predicted determination coefficient (R^2_{pred}) were 0.9207 and 0.6776, respectively. The adjusted R^2_{adj} corrects the R^2 value for

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the sample size and for the number of terms in the model. The normality test was also carried out for judging the model adequacy which showed the p value > 0.15, thus, confirming the normality assumption. To check the outliers, the studentized residual values were calculated. All the values lie within the range of −2 and +2, thereby validating the model (*see* **Table 3A.11**). Studentized residual values outside of −3.5 and +3.5 are considered as an outlier.

The correlation plot between measured values of azadirachtin content and the predicted (modeled) values determined by the model is presented in **Graph 3A.1**. Model coefficients estimated by regression analysis for each variable is shown in **Table 3A.12**. The significance of each coefficient was determined by t-values and P-values. The larger t-value and smaller p-value indicate the high significance of the corresponding coefficient. Value of p less than 0.05 implies the model is significant. The results revealed that sucrose concentration, MS major salts and BAP had a significant effect on azadirachtin production. Positive coefficients of sucrose, MS major salts and BAP variables indicated a linear effect for the increase in azadirachtin production. Among the interactions, sucrose × BAP ($p<1.00$) and major salts × BAP ($p<0.271$) had positive coefficients while sucrose × major salts ($p<0.622$) had negative coefficients. To validate the regression coefficient, analysis of variance for azadirachtin production was performed (**Table 3A.13).**

The graphical depiction provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables in order to deduce the optimum conditions. In this design the response surface curves for the three variables are shown in **Fig. 11A-C**. The response surface representing azadirachtin production was a function of concentrations of the two medium components with third nutrient being at an optimum level. A steep slope or curvature shows that the azadirachtin production is sensitive to that factor.

The model predicted a maximum azadirachtin content of 5.13 mg/g DW by solving the regression equation and also by analyzing the response surface plot by Minitab software. The optimum levels of the significant variables were: sucrose – 5.68%, BAP – 10.42 µM and full MS major salts. To validate the predicted model three experiments were conducted using this optimum medium composition. Azadirachtin content of 4.97 mg/g DW was observed at this medium composition, which agreed

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well with the predicted value (5.13 mg/g DW). As a result, the developed model was considered to be accurate and reliable for predicting the production of azadirachtin from *in vitro* cell lines of neem.

Graph 3A.1. Linear correlation plot between measured vs predicted azadirachtin content (mg/g DW)

Table 3A.9. Plackett-Burman design showing five variables with real values along with the observed results of azadirachtin content.

Run order	Sucrose (%)	MS major salts	BAP (µM)	IAA (µM)	CH (mg/l)	Azadirachtin amount (mg/g DW)
	Low 1 High 5	Low 0.5 High 2	Low 5 High 13	Low 2.5 High 7.5	Low 250 High 1000	
1	5.00	2.00	5.00	2.50	250.00	0.25
2	5.00	2.00	13.00	7.50	250.00	3.02
3	1.00	0.50	5.00	2.50	250.00	0.13
4	1.00	2.00	5.00	7.50	1000.00	1.53
5	1.00	2.00	13.00	2.50	1000.00	0.03

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6	1.00	2.00	13.00	2.50	250.00	0.04
7	5.00	0.50	13.00	7.50	250.00	3.56
8	5.00	0.50	5.00	2.50	1000.00	2.16
9	1.00	0.50	13.00	7.50	1000.00	2.08
10	5.00	2.00	5.00	7.50	1000.00	4.55
11	5.00	0.50	13.00	2.50	1000.00	3.38
12	1.00	0.50	5.00	7.50	250.00	0.65

Table 3A.10. Statistical analysis of Plackett–Burman design showing coefficient values, t- and p-value for each variable for azadirachtin production.

Term	Coefficient	t	p
Constant	3.20	15.97	0.000
Sucrose (X_1)	1.24	6.19	0.001
MS Major Salts (X_2)	-0.73	-3.65	0.011
BAP (X_3)	0.85	4.25	0.005
IAA (X_4)	0.09	0.43	0.680
CH (X_5)	0.14	0.70	0.510

Table 3A.11. CCD experimental design matrix of three variables in real units and amount of azadirachtin production.

Run order	Sucrose	MS major salts	BAP	Azadirachtin amount (mg/g DW)		Studentized residuals
				Measured	Predicted	
1	2.25	1.50	5.00	1.89	2.05	-1.00
2	4.50	1.00	9.00	4.86	4.86	1.23
3	4.50	1.00	9.00	4.90	4.86	1.28
4	4.50	1.00	9.00	4.89	4.86	1.27
5	6.75	0.50	13.00	3.86	3.57	-0.66
6	2.25	0.50	5.00	1.98	1.6	-0.70
7	4.50	1.00	9.00	4.88	4.86	1.26
8	6.75	0.50	5.00	3.43	3.12	-0.44
9	4.50	1.00	15.72	4.10	3.88	-0.23
10	4.50	1.84	9.00	3.48	3.23	-0.67
11	2.25	0.50	13.00	1.93	2.07	-1.18
12	4.50	1.00	9.00	4.82	4.86	1.18

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13	4.50	1.00	9.00	4.82	4.86	1.18
14	8.28	1.00	9.00	3.86	4.17	-0.88
15	6.75	1.50	13.00	4.08	4.32	-0.82
16	2.25	1.50	13.00	2.87	3.06	-0.90
17	0.72	1.00	9.00	1.98	1.85	-0.80
18	4.50	1.00	2.27	2.24	2.65	-0.90
19	4.50	0.16	9.00	1.80	2.23	-1.28
20	6.75	1.50	5.00	3.58	3.31	-0.66

Table 3A.12. Results of regression analysis of CCD for azadirachtin production.

Term	Coefficient	t	p
Constant	4.85	35.69	0.000
X_1	0.69	7.66	0.000
X_2	0.30	3.28	0.008
X_3	0.37	4.05	0.002
X_1^2	-0.65	-7.43	0.000
X_2^2	-0.75	-8.55	0.000
X_3^2	-0.56	-6.42	0.000
$X_1 X_2$	-0.06	-0.51	0.622
$X_1 X_3$	0.00	-0.00	1.000
$X_2 X_3$	0.14	1.17	0.271

Table 3A.13. Analysis of variance of CCD for optimization of azadirachtin production.

Source	DF	SS	MS	F	p
Regression	9	25.53	2.84	25.50	0.000
Linear	3	9.54	3.18	28.61	0.000
Square	3	15.80	5.27	47.36	0.000

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Interaction	3	0.18	0.06	0.54	0.666
Residual Error	10	1.11	0.11		
Pure Error	5	0.01	0.00		
Total	19	26.65			

R^2 = 95.82%; R^2_{adj} = 92.07%; R^2_{pred} = 67.76%; SS, sum of squares; DF, degrees of freedom; MS, mean square.

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3B. SPILANTHES ACMELLA MURR.

3B.1. Establishment of *In Vitro* Cultures

3B.1.1. Nodal Segment Culture

i. Effects of Season on Culture Establishment

The extent of contamination as well as bud-break was highly dependent on the season. The cultures initiated during spring season (January to April) showed best response not only in terms of the frequency of bud-break but also in the vigor of the shoots; significantly ($p<0.05$) higher aseptic culture establishment (87.4%) and bud-break (64%) was observed during this season with least contamination rate (12.5%) (**Graph 3B.1**). Around 23% of the aseptic cultures did not respond and turned brown after 2 weeks of inoculation in the medium. Since, summer (May to August) is the period that concurs with rainy season in the north-east India, 79% cultures raised in these months were prone to infection. By winter (September to December) the shoots become old and it was difficult to break the dormant state of the buds. Therefore, routinely, the cultures were raised in January-April because of the higher number of aseptic culture establishment and best shoot growth recorded in the season.

ii. Effect of Carbon Source on Shoot Proliferation

For shoot induction from axillary buds, three carbon sources, sucrose, glucose and maltose were evaluated at a fixed concentration of 30 g/l. The incorporation of maltose or glucose to the MS medium was not helpful. The growth of shoot, number of nodes per shoot and the rate of shoot multiplication were significantly higher ($p<0.05$) on sucrose than on the other two carbon sources (**Table 3B.1**). Consequently, in all subsequent experiments only sucrose was used as carbon source. On sucrose containing medium 64% cultures showed bud-break with 6.4-fold multiplication, every 5 weeks.

iii. Effect of Major Inorganic Salts on Shoot Proliferation

Shoot proliferation from nodal segments of *S. acmella* varied considerably at different major inorganic salt concentrations. Double strength of major salts (2MS) inhibited shoot induction completely (**Table 3B.2**); only rooting was noticed at the base of the explant. Results on shoot induction in half (½MS) and full strength of major salts (MS) were significantly ($p<0.05$) different where 60% and 64% cultures, respectively,

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showed bud-break. MS basal medium also supported multiple shoot proliferation

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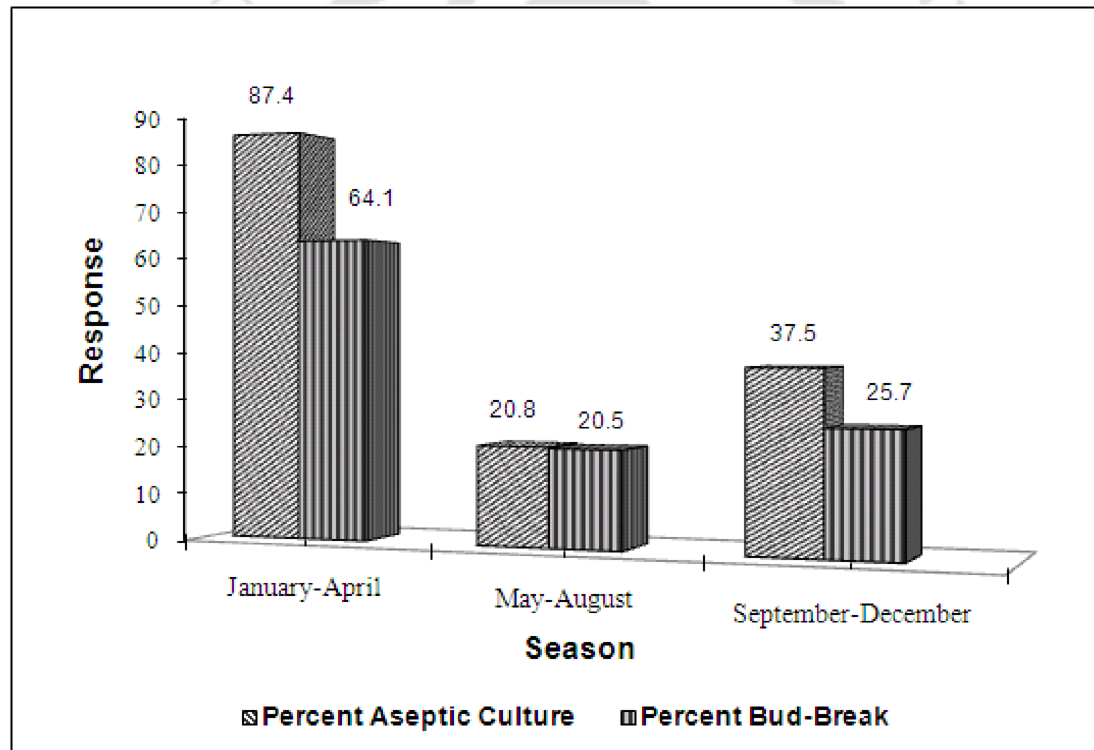
Graph 3B.1. Effect of season on percent aseptic culture establishment and bud break response from nodal explants cultured on MS basal medium. Values are mean of three independent experiments. Data was recorded after 5 weeks of culture.



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Least significant difference (LSD) at 5%, for percent aseptic culture = 5.90, and for percent bud-break = 4.67; Degree of freedom (DF) = 6

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Table 3B.1. Effect of carbon source on shoot proliferation from nodal segments of *Spilanthes acmella.* Growth period: 5 weeks; Control: MS medium with 3% sucrose.

Carbon source (3%)	Percent bud-break	Shoot length (cm)	Number of shoots per explant	Number of nodes* per shoot	Rate of shoot multiplication**
Glucose	41.6	1.1	1.2	1.0	2.4
Maltose	59.3	1.5	1.3	1.0	2.6
Sucrose	64.1	3.5	1.6	2.0	6.4
5% LSD 6 DF	0.84	0.18	0.18	0.30	0.40

*Each node bears 2 opposite axillary buds; **Unit for rate of shoot multiplication is number of axillary buds (on nodes) per explant per multiplication cycle; Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means. DF, Degree of freedom

Table 3B.2. Effect of major inorganic salt concentration on shoot proliferation from nodal segments of *Spilanthes acmella*. Growth period: 5 weeks; Control: MS medium with full strength of inorganic salts.

Strength of medium	Percent bud-break	Shoot length (cm)	Number of shoots per explant	Number of nodes* per shoot	Rate of shoot multiplication**
Half	60.0	3.5	1.0	1.5	3.0
Full	64.1	3.5	1.6	2.0	6.4
Double	0.0	0.0	0.0	0.0	0.0
5% LSD 6 DF	1.58	0.26	0.11	0.17	0.64

*Each node bears 2 opposite axillary buds; **Unit for rate of shoot multiplication is number of axillary buds (on nodes) per explant per multiplication cycle; Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means. DF, Degree of freedom

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(1.6 shoots/axillary bud) with highest rate of shoot multiplication as 6.4 axillary buds/explant, every 5 weeks (**Table 3B.2**). Therefore, for all subsequent experiments MS basal medium was selected with full strength of major inorganic salts.

iv. Effect of Growth Regulators on Shoot Proliferation

Nodal explants of *S. acmella* bearing two opposite axillary buds were cultured on MS basal medium or basal medium supplemented with BAP, Kinetin or 2-iP at 3.0 µM concentration. Although on basal medium, the frequency of bud-break was appreciable (64%), and incorporation of BAP to the basal medium had further improved the incidence of bud-break and promoted multiple shoot formation on the nodes (2 shoots/axillary buds). While BAP favoured 100% shoot-bud induction from axillary buds, only 20% response was observed on Kinetin supplemented medium, and 2-iP was noted as being inhibitory for axillary bud proliferation. The addition of a low concentration of GA_3 (1.0 µM) to the BAP supplemented medium promoted multiple shoot formation in 70% of the cultures but the shoots remained weak and stunted. On the other hand, a single shoot with long internodes was developed from axillary buds in 100% cultures when NAA (1.0 or 5.0 µM) was added to BAP containing medium.

Irrespective of the treatments, excessive adventitious root proliferation was observed either at the base of the explant or all over the surface of the *in vitro* developed shoot. Since the shoot multiplication rate was higher with the addition of BAP, it was further evaluated at a concentration range of (1.0-15.0 µM) to promote shoot proliferation with no adventitious roots. The rate of shoot multiplication and length of shoots varied with the concentration of the BAP (**Graph 3B.2**). At its optimum level (5.0 µM), 20.3-fold shoot multiplication occurred every 5 weeks with no adventitious root formation. On MS + BAP (5.0 µM), bud-break occurred within a week and an average of 2.9 shoots per axillary bud were formed within 4 weeks in 100% cultures. The shoots grew well and attained a length of 4.9 cm with 3.5 nodes (or 7 axillary buds) per shoot after 5 weeks (**Fig. 12A**). Interestingly, at all lower concentration of BAP (1.0-3.0 µM), the incidence of excessive adventitious root proliferation was observed

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from all over the surface of the *in vitro* developed shoots and the explants as well. Moreover, lower concentration of BAP supported multiple shoot formation with long internodes (**Fig. 12B**). There was a marked decline in the growth of shoots and the rate of shoot multiplication with the increasing concentration of BAP; the node was crowned with several newly formed small shoots difficult to count. BAP at 15.0 µM, resulted in stunted shoots with pale leaves (**Fig. 12C**).

The hand sections of the nodal region of the shoots in culture revealed the presence of a ring of lignified xylem cells which fluoresce bright yellow when stained with acridine orange. Multiple (2-3 numbers) adventitious roots (arrow marked) were originated from these lignified tissues (**Fig. 12D, E**); in figure **12D**, an axillary bud can also be viewed at one side.

Thus, MS + BAP (5.0 µM) proved optimum for shoot multiplication. At the end of the passage each shoot was cut into single node segments and planted on the fresh medium of the same composition. Each node again produced multinodal, multiple shoots after 5 weeks. Shoot multiplication rate in various subculture (S) passages, S_1=20.2, S_2=20.3, S_3=20.4, S_4=20.6, S_5 =20.6, S_6=20.5, S_7=20.5, S_8=10.5, S_9=20.6, S_{10}=20.6, was >20 fold from S_1 to S_{10} on MS + BAP (5.0 µM). Since every time the explants were taken from freshly formed *in vitro* shoots, therefore, we have not observed any significant ($p<0.05$) difference (variation) in the results.

v. <u>Rooting and Transplantation</u>

Terminal 3-4 cm long portions of shoots from 5-week-old cultures on MS + BAP (5.0 µM) were used for rooting. The remaining portions of the shoots were cut into single node segments and utilized for further multiplication. MS basal medium was tested at full- and half (½MS)-strengths of the major inorganic salts. All the media were supplemented with 0, 10, 30 and 50 g/l sucrose. Although MS medium supported direct rooting at the base of shoot, it did not promote the growth of the shoot at all tested concentrations of sucrose. Half MS was distinctly better than full MS basal medium, in terms of length of the shoot, percent rooting, number of roots per shoot and number of laterals present on the roots. The rooting was positively correlated with the sucrose concentration in the medium. There were significant increase ($p<0.05$) in the percent rooting and the number of roots per shoot with increase in sucrose

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concentration (**Table 3B.3**). On ½ MS + sucrose (50 g/l), which proved to be the best rooting medium, 100% shoots formed more than 35 roots directly from the basal cut end of the shoots (**Fig. 13A).** On this medium roots appeared after 2 weeks and maximum response was observed after 4 weeks. Some of the roots had developed laterals.

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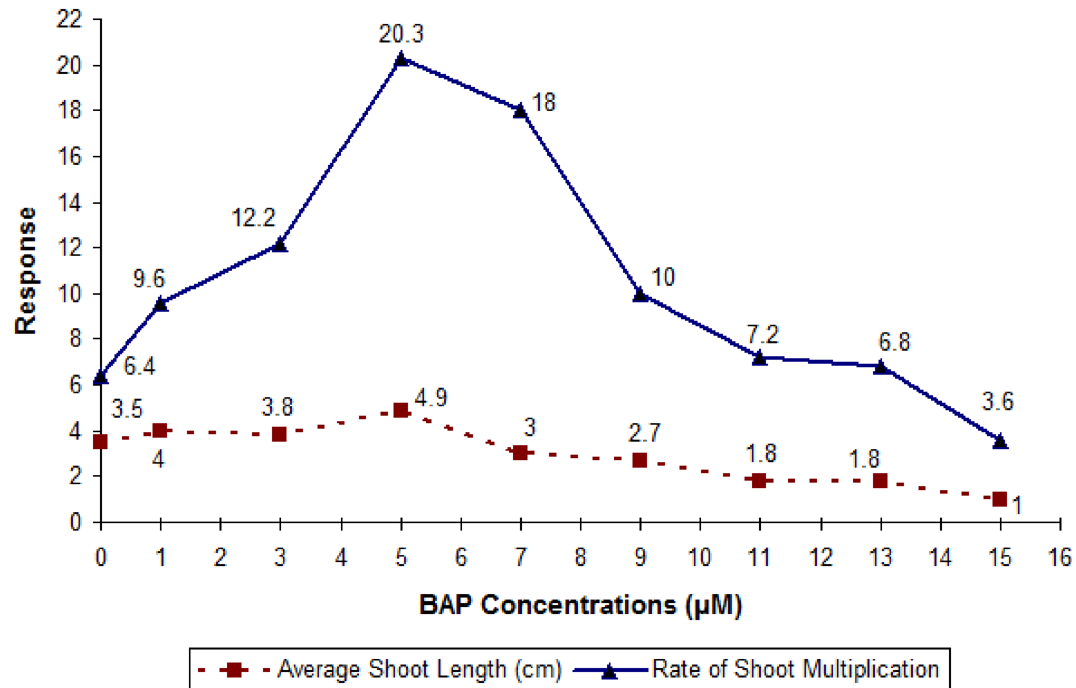
Graph 3B.2. Effect of various concentrations of BAP on shoot proliferation from nodal segments of *Spilanthes acmella* Murr. Growth period: 5 weeks; Control: MS basal medium. Values are mean of three independent experiments.



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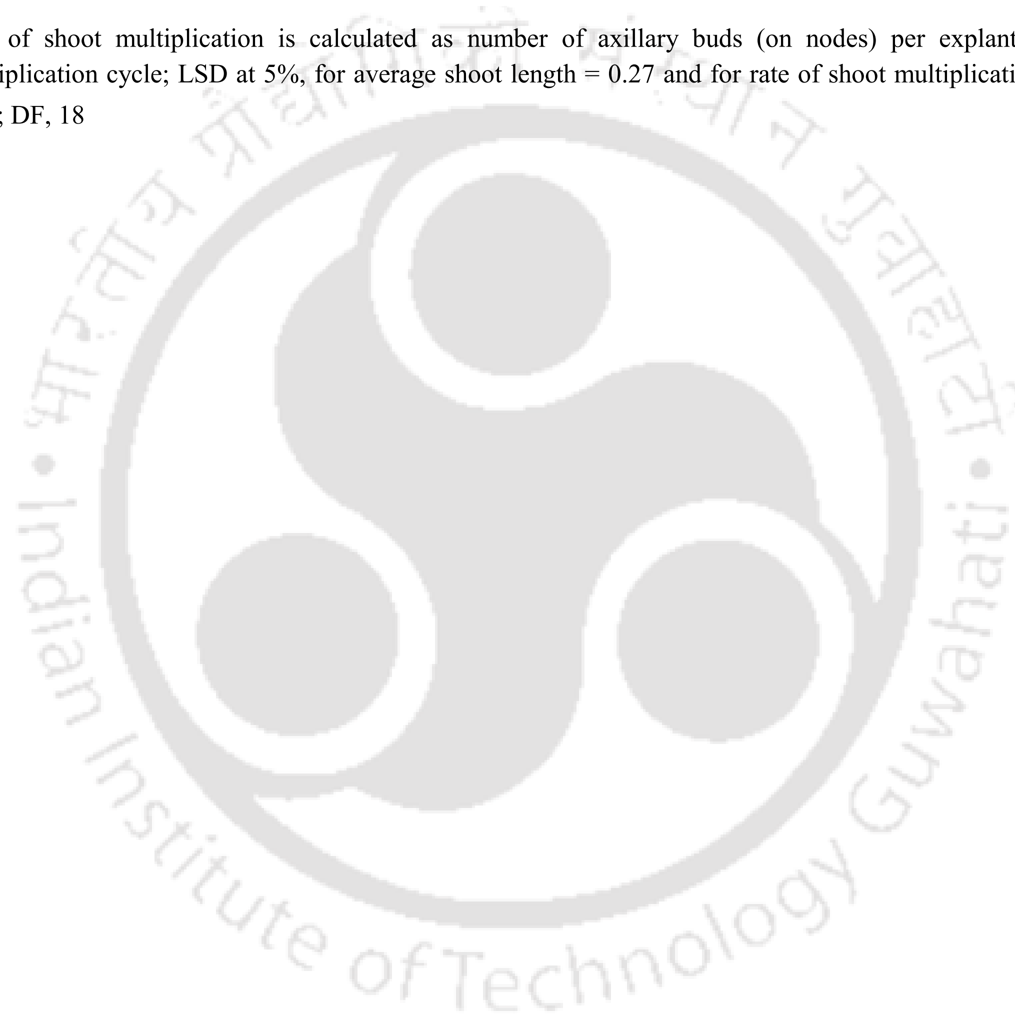
Rate of shoot multiplication is calculated as number of axillary buds (on nodes) per explant per multiplication cycle; LSD at 5%, for average shoot length = 0.27 and for rate of shoot multiplication = 0.68; DF, 18



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Following the protocol described under materials and methods, rooted shoots from ½ MS + sucrose (50 g/l) were transferred out of culture. The plantlets were acclimatized successfully with 88.9% survival rate (**Fig. 13B**). During *in vitro* hardening, shoots elongated, leaves turned greener, and their lamina expanded. Consequently, the plants seemed much healthier and grew more vigorously after *in vitro* hardening.

Table 3B.3. Effect of different sucrose concentrations on *in vitro* rooting of the excised shoots cultured on ½ MS basal medium. Growth period: 4 weeks.

Sucrose (g/l)	Percent rooting response	Number of roots per shoot
0	20.9	3.1
10	76.3	14.1
30	88.9	23.3
50	100	35.1
5% LSD 8 DF	2.11	1.59

Values are mean of three independent experiments. Least significant difference (LSD) was used to compare means. DF, Degree of freedom

3B.1.2. Leaf-Disc Culture

Leaf-disc explants of 5 mm size were cultured on MS basal medium or basal medium consisting of varying concentrations and combinations of cytokinins and auxins like, BAP, TDZ, Kinetin, NAA, IAA, 2,4-D and pCPA (**Table 3B.4).** Leaf-discs cultured on MS basal medium failed to show any response. The morphogenic response of leaf-discs was greatly influenced by the type of growth regulator used in the medium. On most of the media combinations, leaf-discs either callused or differentiated into shoots. Two types of response, observed from leaf-disc cultures, are described below:

i. Shoot Regeneration

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Incorporation of BAP at 5.0 µM to MS basal medium had induced direct adventitious shoot organogenesis. In contrast to this, no shoot proliferation observed from leaf-disc explants cultured on MS medium containing either other cytokinins, TDZ, Kinetin or any of the auxins, 2,4-D, NAA or pCPA, alone at tested concentration of 5.0 µM.

The frequency of shoot proliferation declined at lower concentrations of BAP; BAP at 3.0 µM concentration induced shoot regeneration in 80.3% cultures with only one shoot per explant while no response was seen at BAP 1.0 µM. The higher concentration of BAP (7.0 µM) induced callusing in 100% explants and the callus remained non-regenerative. At its optimal concentration of 5.0 µM, BAP showed highest shoot organogenesis directly from the explants without an intervening callus phase. The first visible change on the cultured leaf-discs was observed within the first week of culture. The leaf-discs enlarged, and adventitious shoot-buds appeared to arise directly from the cut ends (**Fig. 14A-D**). Shoot-buds continued to emerge from the explants till the end of the 4th week of culture. On MS + BAP (5.0 µM), 100% cultures responded for shoot regeneration with an average number of 3.5 adventitious shoots per responsive explant (**Fig. 14E**).

A combined effect of cytokinin with auxin was also evaluated for multiple shoot induction. Addition of IAA along with BAP to the basal medium enhanced the number of shoots per explant significantly ($p<0.05$) (**Table 3B.4**). MS basal medium supplemented with 5.0 µM each of BAP and IAA supported highest number of shoot-buds per explant directly from the leaf-disc explants (**Fig. 15A**). However, only a few shoot-buds (5-6) developed into distinct shoots of 1.0–3.5 cm length over a period of 5 weeks and growth of the rest of the adventitious shoot-buds remained arrested. To overcome this problem, small shoots in bunches of 4-5 were transferred, after 5 weeks, to 250 ml capacity flasks containing fresh medium of parental composition. With this arrangement, on MS + BAP (5.0 µM) + IAA (5.0 µM) an average of 51 shoots were differentiated per explant in 5 weeks (**Fig. 15B**) in 82.9% cultures. This is in comparison to the response obtained when the medium contained BAP alone at 5.0 µM concentration. The developmental ontogeny of shoot organogenesis from leaf-disc explants were recorded by scanning electron microscopy

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which confirmed the direct differentiation of shoot-buds from the explants (**Fig. 16A-D**).

In contrast to IAA, inclusion of NAA (1.0 and 5.0 µM) in BAP containing medium did not show significant effect ($p<0.05$) on differentiation of number of shoot-buds per explant. Although 100% cultures showed regeneration, only one shoot-bud proliferated per explant (**Table 3B.4**).

The long shoots of 3-4 cm size, from 5-week-old cultures on MS + BAP (5.0 µM) and MS + BAP (5.0 µM) + IAA (5.0 µM) were used for rooting. On ½ MS + sucrose (50 g/l), which proved to be the best rooting medium for *Spilanthes* (*see* **3B.1.1.v**.), 100% shoots formed numerous (around 35 in number) roots directly from the basal cut end of the shoots**.** On this medium, roots appeared after 2 weeks and maximum response was observed after 4 weeks. The plantlets were acclimatized successfully with 90% survival rate.

ii. Callusing

On few combinations, like, MS + NAA (5.0 µM), MS + pCPA (5.0 µM), MS + BAP (7.0 µM) and MS + 2,4-D (5.0 µM), cultures responded initially for callusing but the calli did not survive after the first subculture. The best treatment in terms of sustained growth of calli, number of explants showing callus and the degree of callusing, was the combination of MS + BAP (5.0 µM) + NAA (1.0 µM) + 2,4-D (1.0 µM). On this medium, after two weeks, friable, light brown calli began to develop from 100% leaf-discs. Although, callus proliferation increased with subsequent subcultures but remained brown in colour throughout two passages, at 5-week intervals. By 11th week, vigorously growing light green and healthy callus was obtained (**Fig. 17A-D**), which remained in unorganized (dedifferentiated) state. The calli were multiplied and maintained on the fresh medium of same parental composition, at 5-week intervals.

3B.1.3. Ploidy Analysis

i. Flow Cytometric Analyses

The ploidy stability of *in vitro* regenerants was determined by flow cytometry. **Figure 18A-C** shows representative histograms of field grown parent plant (control), micropropagated plants derived from axillary buds in nodal segment cultures, and

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from leaf-disc explants. Presence of single peak at around 200 channel position in histograms of micropropagated plants corresponded to those of control plant suggested that *in vitro* regenerants maintained genetic stability and no changes in ploidy levels occurred during regeneration process.

ii. Cytological Analysis

By following the procedure as described in materials and methods, cytological analysis of root-tip cells from parent plant (control), nodal segment and leaf-disc explants derived *in vitro* plants were analysed to check ploidy stability. Counting revealed that the cells had diploid number of chromosomes as 2n=x=52 (**Fig. 19A-C**). No polyploid or aneuploid cells were found.

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Table 3B.4. Effects of different growth regulator on callusing and shoot regeneration response from leaf-disc culture of *Spilanthes*. Growth period: 5 weeks. Control: MS basal medium.

Media	Percentage of explants forming callus	Percentage of explants forming shoot	Number of shoots per culture
MS basal medium	0.0g	0.0d	0.0e
MS + NAA (5.0 µM)	55.1c	0.0d	0.0e
MS + 2, 4-D (5.0 µM)	77.7b	0.0d	0.0e
MS + pCPA (5.0 µM)	10.0f	0.0d	0.0e
MS + Kinetin (5.0 µM)	0.0g	0.0d	0.0e
MS + TDZ (5.0 µM)	0.0g	0.0d	0.0e
MS + BAP (1.0 µM)	0.0g	0.0d	0.0e
MS + BAP (3.0 µM)	0.0g	80.3b	1.0d
MS + BAP (5.0 µM)	0.0g	100a	3.5c
MS + BAP (7.0 µM)	100a	0.0d	0.0c
MS + BAP (5.0 µM) + IAA (1.0 µM)	33.0d	67.0c	4.0b
MS + BAP (5.0 µM) + IAA (5.0 µM)	17.1e	82.9b	5.3a
MS + BAP (5.0 µM) + NAA (1.0 µM)	0.0g	100a	1.0d
MS + BAP (5.0 µM) + NAA (5.0 µM)	0.0g	100a	1.0d
MS + BAP (5.0 µM) + NAA (1.0 µM) + 2, 4-D (1.0 µM)	100a	0.0d	0.0e

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Values are mean of three independent experiments. Mean values sharing the same letter do not differ significantly ($p<0.05$) according to Duncan's multiple range test.

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3B.2. Analysis of Metabolites in Cultures

Spilanthes acmella is reported to contain a wide array of compounds with diverse range of bioactivities. Of these, two main constituents, scopoletin (a coumarin) and spilanthol (an alkylamide) possess immense pharmacological properties. Since leaves are the main reservoir of bioactive compounds in this plant, in the present work, leaves from field grown and *in vitro* plants, and calli established from leaves of field grown plants were utilized for screening and quantification of above two main constituents of *S. acmella.*

3B.2.1. Detection and Estimation of Scopoletin

i. Calibration Curve Analysis

Following the protocol as described in materials and methods, scopoletin eluted at retention time of 5.1 ± 0.4 min (**Fig. 20A**). Calibration curve for scopoletin showed good linearity with high reproducibility and accuracy at all five tested concentrations (0.006 µg/ml to 0.110 µg/ml). Regression analysis of the calibration curve data points showed admirable correlation coefficient (R^2) of 0.9792. The linear regression equation for standard scopoletin was: y = 2.0964 x + 0.0153, where x is the concentration of standard and y is the total peak area. The equation, thus, generated from the curve by external standard method was used to calculate the amount of scopoletin present in crude sample. The precision of the developed method, as mentioned in materials and methods, was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard samples, at same concentration, was analysed at least 3 times within the same day and the RSD value obtained was 0.7%. Similarly, for inter-day variability, same concentration of standard was run at least twice at one day interval and RSD value for the same was 1.3%.

ii. Analysis and Quantification of Scopoletin

By following the protocol as described in materials and methods, the methanol extracts of leaves and calli were analyzed quantitatively by HPLC, for the presence of scopoletin. Samples were monitored by UV and FL detectors connected succeedingly. Scopoletin could be detected by both UV and FL detectors but the sensitivity of scopoletin by FL detector was much higher than that of UV detector. Thus, in all samples, scopoletin was examined by HPLC-FL detector. A very distinct and clear

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separation of compound was observed in all leaf samples (**Fig. 20B-D**), while it was entirely absent in callus samples. In all samples scopoletin was identified by comparing its retention time with that of authentic standard. Presence of scopoletin in crude leaf samples was further confirmed by spiking (addition) of *in vitro* leaf extract with that of the standard (**Fig. 20E**). The standard equation, generated through the calibration curve, demonstrated that scopoletin content in the leaves of nodal segment derived *in vitro* plants (NP) was comparable to those from field grown mother plants. Leaves of NP accumulated 0.104 ± 0.03 µg/g DW of scopoletin while those of field grown plant contained 0.101 ± 0.04 µg/g DW of scopoletin. Statistical analysis confirmed that these values are not significantly ($P<0.05$) different. In comparison to this, only 0.03 µg/g DW of scopoletin was present in the leaves of *in vitro* plants proliferated on leaf-discs.

iii. Analysis of Scopoletin by Mass Spectroscopy

The fraction of crude extract eluted from HPLC at retention time of 5.1 ± 0.4 min, was collected, analysed by mass spectrometry and the fragment characteristics were compared with that of standard scopoletin procured from Sigma, Aldrich. Spectra were obtained in full scan mode. Base peak of m/z 193 resulted due to proton adduct formation $[M+H]^+$ and m/z 215 correspond to the formation of sodium adduct $[M+Na]^+$. Similar m/z fragments in standard compound and HPLC fraction confirmed the presence of scopoletin **(Fig. 21A,B)**.

3B.2.2. Detection and Estimation of Spilanthol

i. Identification of Spilanthol

Since spilanthol standard was commercially not available, HPLC and then mass spectroscopic analysis were performed for the identification of spilanthol peak in samples. Acetonitrile and water at the ratio of 93:7 as the mobile phase was found to be appropriate for satisfactory separation of compounds at a flow rate of 0.5 ml/min. Typical HPLC profiles of *Spilanthes in vivo* and *in vitro* extracts are shown in **Figure 22A-D.** For spilanthol characterization, all peaks eluted from HPLC, were collected, concentrated and analyzed by mass spectrometry. Samples were analyzed in both positive and negative electrospray ionization mode but, the sensitivity and

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reproducibility of the dominant ions in the positive electrospray ionization mode was better than in negative ionization mode. Therefore, all HPLC peaks were analyzed in positive mode. Spilanthol was identified by its fragmentation profile which was further confirmed with literature data. It has been observed that the peak eluted at 7.34 ± 0.12 min in HPLC, has characteristic fragmentation pattern of spilanthol. **Figure 23** shows the mass spectrum of spilanthol compound. The spectrum has a base peak at m/z 222 corresponding to the protonated $[M+H]^+$ molecular ion. The ion at m/z 244 was generated due to potassium ion adduct formation $[M+K]^+$.The characteristic fragment at m/z 149 was formed due to the dissociation of the C-N bond. It is an acyllium ion fragment, which indicates the amount of carbon atoms in the alkyl chain. This fragment is formed by the loss of isobutyl amine group $[MH-C_4H_{11}N]^+$. Mechanism involved in the acyllium ion formation is charge-remote homolytic cleavage that yields a resonant distonic radical cation, which subsequently undergoes hydrogen rearrangement. Another fragment seen at the m/z 123 can be attributed to the $[MH-C_5H_9NO]^+$. The loss of a fragment with specific m/z 99 from the protonated $[M+H]^+$ molecular ion (m/z 222) confirmed that spilanthol contains isobutylamide group. Analysis of samples, each having a peak at approximately 7.34 ± 0.12 min, were all found to contain the fragments diagnostic for spilanthol. In samples, spilanthol was tentatively quantified on the basis of another alkylamide, dodeca-2(E), 4(E)-dienoic acid isobutylamide. Like spilanthol, this compound also has isobutyl amide group and long carbon chain. In mass spectra, this amide form acyllium ion at the m/z 179 (**Table 3B.5**).

Table 3B.5. Fragments generated in MS analysis of spilanthol and dodeca-2(E), 4(E)-dienoic acid isobutylamide.

S. No.	Compound	MH +	Fragments	References
1.	Spilanthol	222	166, 149*, 123, 121, 81	Boonen *et al.* 2010
2.	Dodeca-2(E),4(E)-dienoic acid isobutylamide	252	196, 179*, 161, 119, 95	Cech *et al.* 2006

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*Acyllium fragment formed by dissociation of the C–N bond to lose the entire amine functional group (MH^{+} −73).

ii. Calibration Curve Analysis

In order to quantify the amount of spilanthol in the methanol extracts of *Spilanthes,* calibration curve was prepared with the available standard dodeca-2(E), 4(E) dienoic acid isobutylamide. Standard showed high linearity at tested concentrations (250 µg/ml to 15 µg/ml) with correlation coefficient (R^2) of 0.987. The linear regression equation for standard was: $y = 1.375\ x + 36.43$, where x is the concentration of standard (µg/ml) and y is the total peak area. The equation, thus, generated from the curve by external standard method was used to calculate the amount of compound present in crude samples.

The precision of the developed method was evaluated by measuring intra- and inter-day variability in terms of relative standard deviation. The standard samples, at same concentration, were analyzed at least 3 times within the same day and the RSD value obtained was 1.8%. Similarly, for inter-day variability, same concentration of the standard was run at least twice at one day interval and the values for the same figured out to be 2.4%.

iii. Quantification of Spilanthol

By following the protocol as described in materials and methods, the methanol extracts of leaves and calli were analyzed by HPLC, for the quantification of spilanthol. HPLC analysis of *in vitro* callus and regenerated plants revealed that the peak for spilanthol was identical to those of field-grown mother plants **(Fig. 22A-D)**. From the external standard equation, the amount of spilanthol in different samples was calculated**.** Spilanthol content in the methanol extracts of callus was 998.03 ± 15.6 µg/g DW and in the leaves of *in vitro* plants derived from leaf-discs was 3294.36 ± 12.4 µg/g DW. Leaves of field grown mother plants and nodal segment derived *in vitro* plants contained 2703.66 ± 9.6 µg/g DW and 2702.33 ± 11.6 µg/g DW of spilanthol. Statistical analysis showed that spilanthol content in leaves of field grown parent plants and nodal segment derived *in vitro* plants are not significantly ($P<0.05$) different. Whereas, leaves of *in vitro* plants derived from leaf-discs contained

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significantly ($P<0.05$) higher amount of spilanthol. Among all *in vitro* and *in vivo* samples, callus accumulated least amount of spilanthol.

3B.2.3. Batch Culture Studies

i. Establishment of Suspension Culture in Shake Flask

Suspension cultures were initiated once the stable, fresh cell lines were obtained on semi-solid medium. The same growth regulator combination, MS + BAP (5.0 µM) + NAA (1.0 µM) + 2,4-D (1.0 µM), which was used for callus culture, worked for cell suspension cultures as well. However, the cell growth was faster in liquid medium than in semi solid medium. It took only 18 days for the cell cultures to complete the growth cycle in comparison to calli on semisolid medium which needed at least 5 weeks to attain maximum growth.

ii. Kinetics of Cell Growth and Metabolite Production

The kinetic profiles of cell growth represented by fresh and dry cell weight, and medium pH in *Spilanthes* suspension cultures are presented in **Graph 3B.3.** It was observed that *Spilanthes* suspension culture moved onto the exponential growth phase after 6 days of lag phase. The biomass increased till 18th day following this the death phase started (**Graph 3B.3)** which may be due to consumption of the nutrients and lack of oxygen in the medium. During death phase fresh and dry cell weight decreased dramatically but the pH of the medium continued to increase. There is an increase in the pH from 6th day till 21st day and after which the medium pH was stable till the last day of cultivation (24th day). The increase in the pH may be attributed to the release of intracellular substances into the medium. Under the experimental conditions, with the formula mentioned in materials and methods, the maximum specific growth rate (µ) was 0.279 d^{-1} during the exponential phase.

The two compounds, scopoletin (a coumarin) and spilanthol (an alkylamide) were screened and quantified as described in the previous section, from callus cultures, in the present study. In an extensive study, in this section, where the cells harvested at every 3rd day from liquid suspension were extracted and analysed by HPLC. The HPLC analysis showed that only spilanthol was produced by suspension culture and scopoletin was altogether absent. The spilanthol production started at the end of the

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lag phase (6th day). During exponential phase spilanthol content increased and was found to be growth associated and showed an increase with the increase in biomass until 15th day. Thereafter, spilanthol production declined rapidly and dramatically due to nutrient depletion and cell death. The same set of experiment was repeated three times and, every time, the similar trend on spilanthol production was observed. Extracellular spilanthol, in the medium, was not detected at any stage during the course of cultivation and kinetic studies.

iii. Nutrient Uptake Kinetics

Typical profiles of the consumption of substrates (phosphate, nitrate and sucrose) during suspension culture are shown in **Graph 3B.4.** Sucrose uptake occurred at a relatively slower rate and it was consumed completely from the medium by the cells in 21 days of culture. In contrast to sucrose, it was invariably observed that the medium phosphate was almost completely consumed by the 15th day of culture. Thereafter, its concentration in the medium increased from 18th day onwards till the end of cultivation which might be due to significant cell death and lysis under stress conditions that resulted into leakage of phosphate from the cells into the medium. In comparison to phosphate, uptake of nitrate was observed at a slower rate, in the present study. It was consumed by cells till 18th day after which its concentration has increased. Therefore, it may be speculated that complete disappearance of phosphate from the medium resulted in the onset of decline phase in *spilanthes* suspension culture and this was the major limiting nutrients for cell growth.

iv. Effect of Different Carbon Sources on Biomass Accumulation and Spilanthol Production

In the present study, the effect of glucose, fructose and sucrose were evaluated on cell biomass growth and metabolite production. The type of carbon source bears profound effect on quantity of metabolite produced. Sucrose was found to be the ideal carbohydrate source for the cell growth, which yielded the biomass of 8.2 g/l DW, followed by glucose which accumulated the biomass 7.2 g/l DW. The lowest accumulation of biomass was recorded in the medium supplemented with fructose which yielded about 1.0 g/l DW. The highest production of spilanthol as 91.4 µg/g

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DW was recorded in the medium supplemented with sucrose followed by glucose, which produced 56.8 μg/g DW. Spilanthol could not be detected in fructose containing medium **(Graph 3B.5; Fig. 24A-C).**

v. Effect of Agitation Speed on Cell Survival and Viability

Speed of agitation on growth and viability of cells in shake flask suspension cultures were assessed after 18 days. The viability profile of *Spilanthes* cells at different agitation speeds is shown in **Fig. 25A-D**. Similar to many other plant species, *Spilanthes* cells were also found to be highly sensitive to increase in agitation speed. The maximum fresh weight (163.63 g/l) (**Graph 3B.6**) and maximum viability (**Fig. 25C)** was observed at 120 rpm. At higher rpm (150-180), the biomass and viability profile was highly unsatisfactory. At lower rpm (60-90), the cells died due to aggregation and clumping. At 60 rpm, cells were aggregated into hard clump while at

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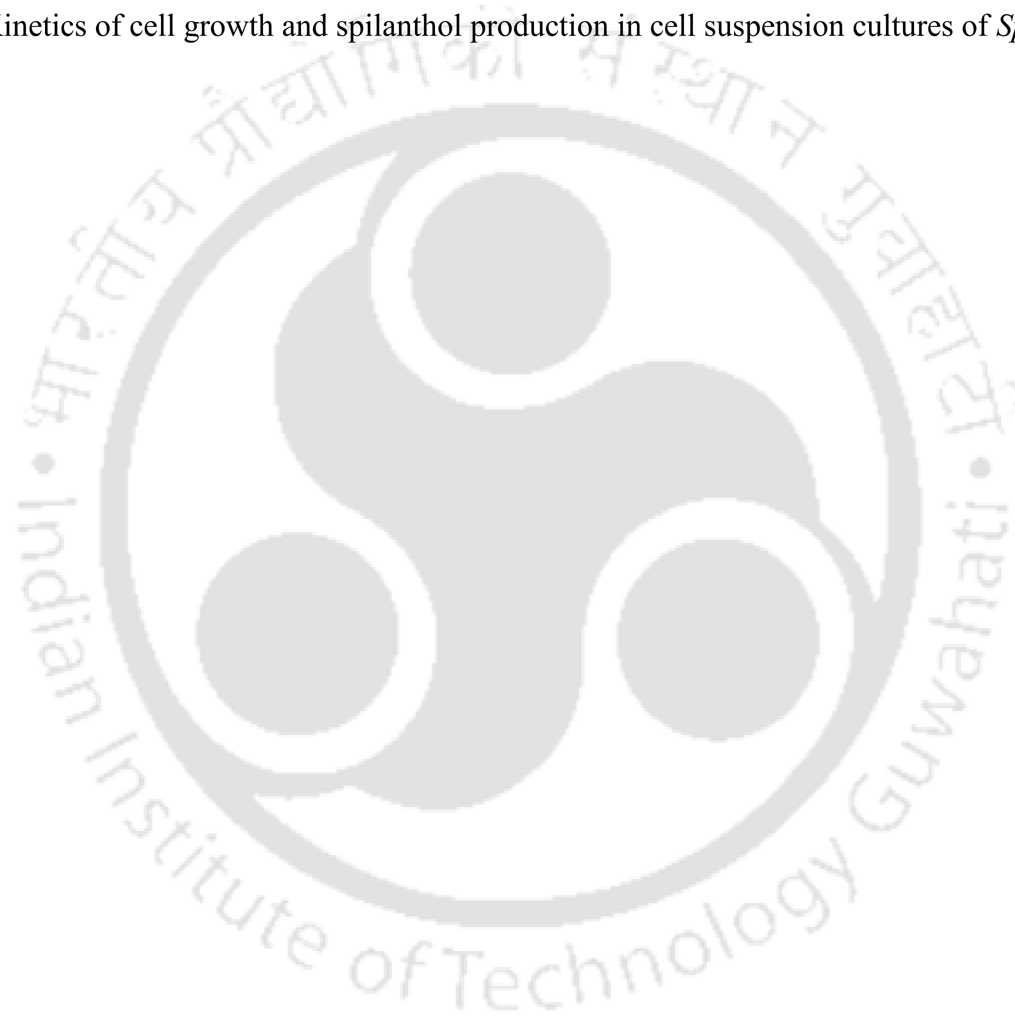
Graph 3B.3: Kinetics of cell growth and spilanthol production in cell suspension cultures of *Spilanthes acmella*.



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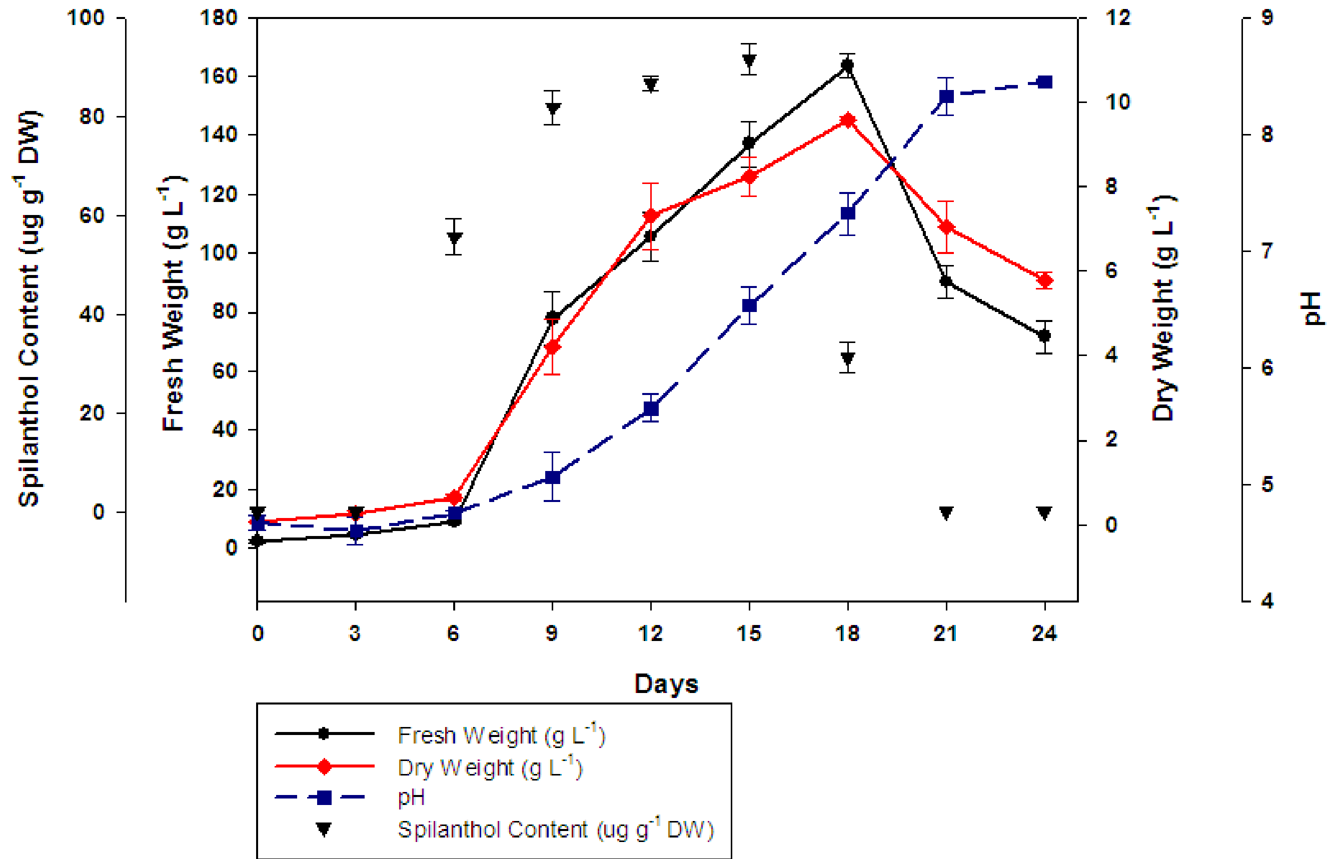

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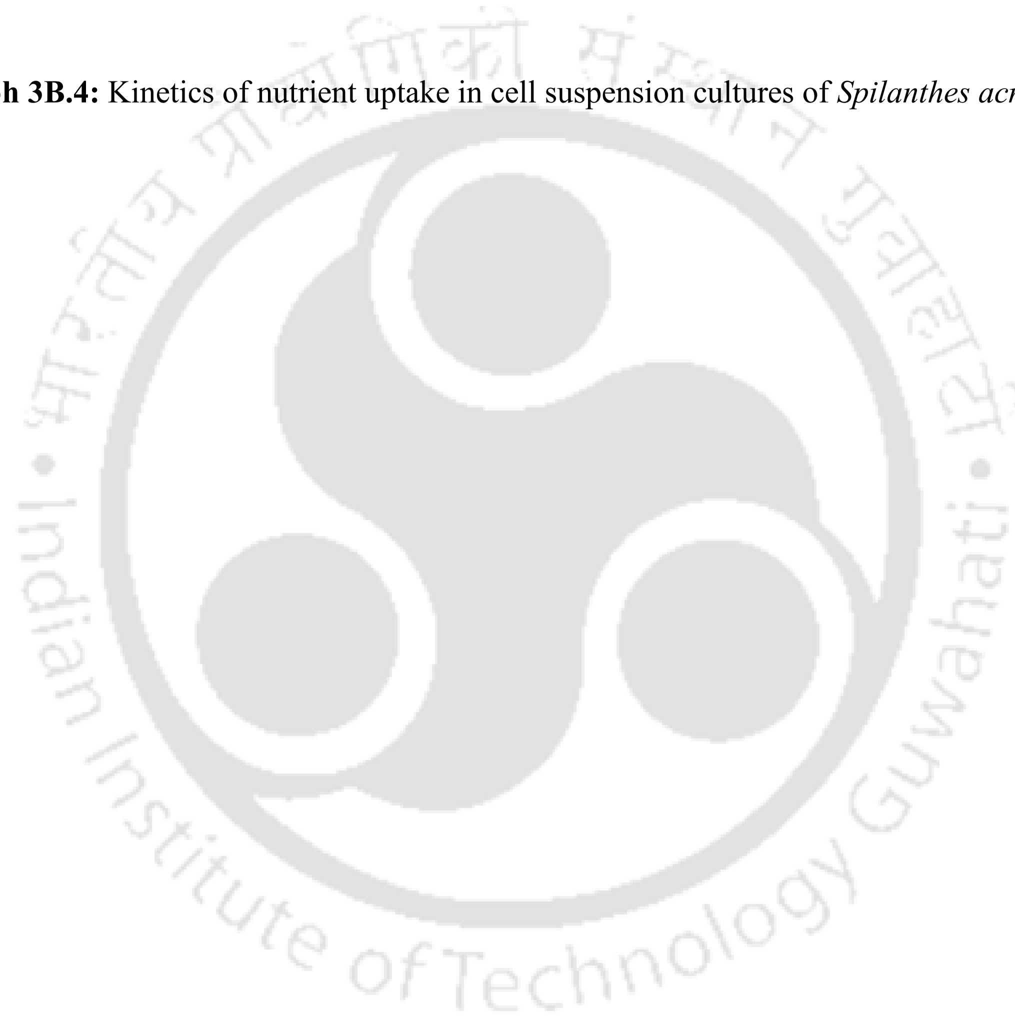
Graph 3B.4: Kinetics of nutrient uptake in cell suspension cultures of *Spilanthes acmella*.



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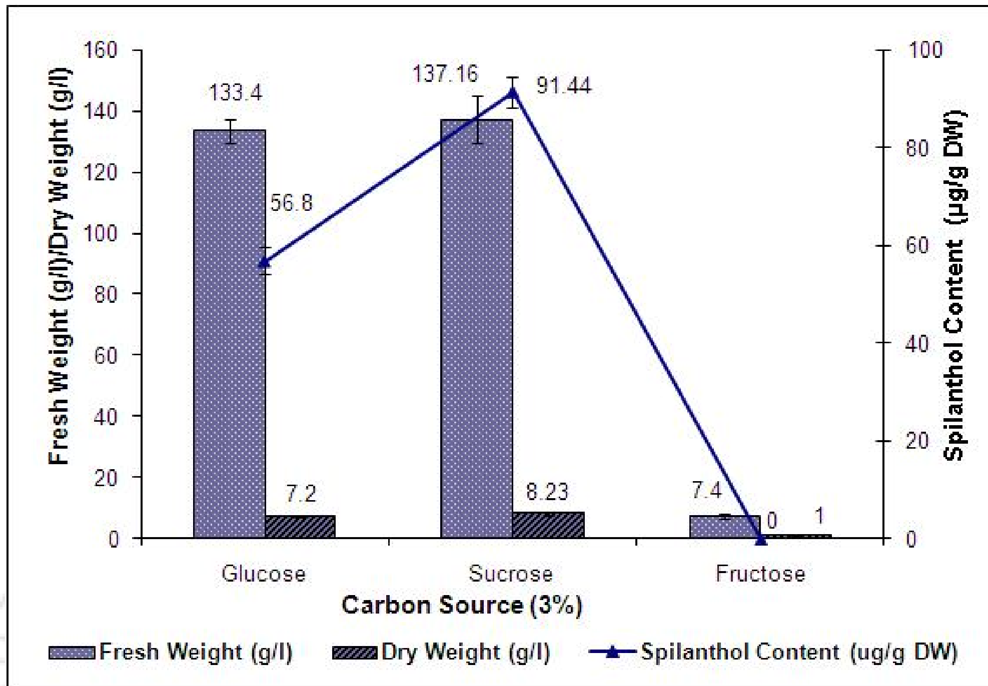
Graph 3B.5: Effect of different carbon sources on cell growth and production profile of spilanthol, in cell suspension cultures of *Spilanthes acmella,* after 15 days.



Graph 3B.6: Effect of different agitation speeds on fresh and dry weights of cells in suspension cultures of *Spilanthes acmella,* after 18 days.

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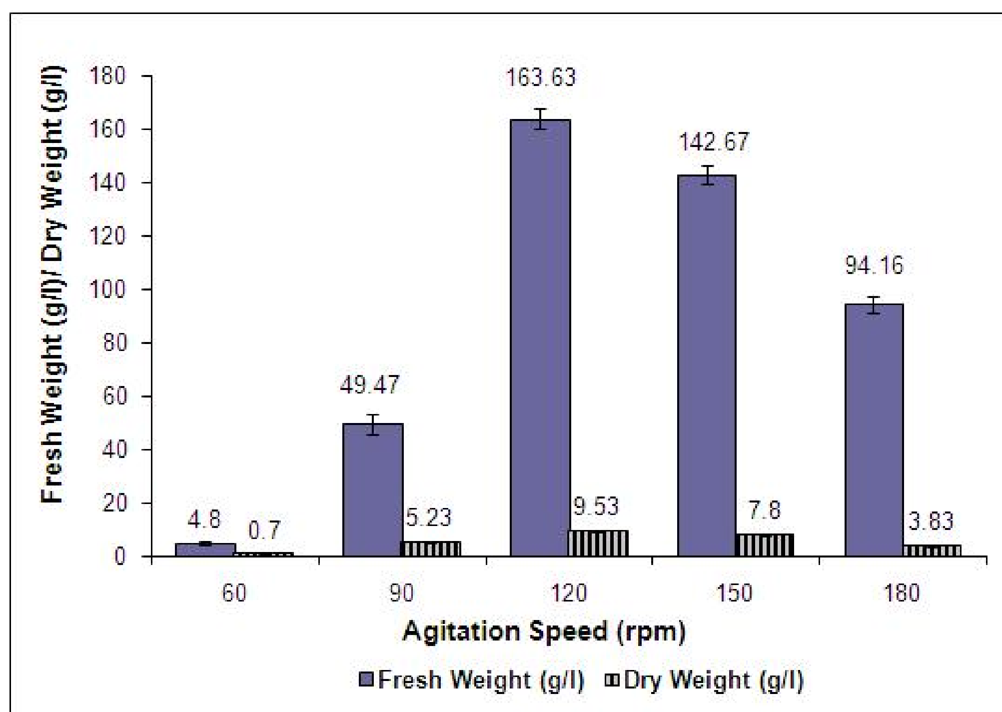

90 rpm cells were loosely attached in the clump. Only the cells at the outermost layer of the aggregate were alive and fluoresce green when stained with Fluorescein diacetate (FDA). At higher rpm (150-180) cells died due to rupturing and shear effect. FDA is a cell permeable dye. Within the living cells, the molecule is cleaved by esterase activity to fluorescein which is unable to pass through the cell membrane of live cells while it leaches out from the dead cells. Hence, only the live, intact cells take up the stain and fluoresce green.

3B.3. Bioassays

3B.3.1. DPPH Radical Scavenging Activity of Various Extracts

Selection of solvent is an important step for obtaining extracts with acceptable yields and strong antioxidant activity. The results on extraction yield of *in vivo* leaves and calli, from various organic solvents are presented in **Graph 3B.7**. The yields of

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extracts from different solvents were obtained in the order: water > methanol > ethyl acetate > hexane ($p<0.05$). It varied from 2.4 to 33.4% for leaf extracts and from 0.5 to 12.8% for callus extracts. The highest and the lowest yields were obtained from water and hexane extracts ($p<0.05$), respectively, in both *in vivo* leaves and *in vitro* callus cultures (**Graph 3B.7**).

Results of the antioxidant assay revealed that *in vivo* leaf and callus extracts possessed a significant variability in their inhibitory activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The radical scavenging activity of each extract was indicated as the percentage of reduction of the initial DPPH absorbance (**Table 3B.6**).

EC_{50} values for the extracts studied, were also calculated and represented in

Table 3B.6. A noticeable effect of the extract on radical scavenging activity was observed at concentration range of 250 µg/ml to 2000 µg/ml and the effect was dose dependent. The highest radical scavenging activity was detected in *in vivo* leaf methanolic extract, followed by dedifferentiated callus methanolic extract. At 1000 µg/ml concentration, methanolic extracts of calli and leaf exhibited 46.2% and 52.2% DPPH inhibition and their EC_{50} values were 1085.1 µg/ml and 1342.9 µg/ml, respectively. The water, ethyl acetate and hexane extracts of *in vivo* leaf and callus showed no radical scavenging activity with DPPH.

In order to understand the free radical scavenging capacities of sample extracts, it is important to compare their activities with the relative activities of a standard antioxidant compounds. For the present study, ascorbic acid and vanillin were used as the standard antioxidant compounds. Ascorbic acid showed excellent radical scavenging activity with EC_{50} value of 14.36 µg/ml. On the contrary, vanillin showed very low radical activity with EC_{50} as 3394.43 µg/ml. Thus, the sample extracts exhibited relatively appreciable antioxidant activities with reasonable EC_{50} values.

The content of total phenolic compounds in the *Spilanthes in vivo* leaf and *in vitro* callus extracts were determined through a linear gallic acid standard curve $y = 0.0022x + 0.0679$; $R^2 = 0.9673$ and the results for the extracts are presented in **Graph 3B.8**. The total phenolic content varied from 0.3 to 6.3 mg GAE/g DW for leaf extract and from 0.1 to 2.8 mg GAE/g DW for callus extract. The highest total phenolic

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compounds were detected in the methanolic extracts of both leaf and callus whereas the lowest content was obtained in the hexane extracts ($p<0.05$) of leaf and callus.

Table 3B.6. DPPH radical scavenging activity of *S. acmella in vivo* leaf and dedifferentiated callus methanolic extracts after 30 min.

S. No.	Sample	Concentration (µg/ml)	% DPPH inhibition	EC_{50}
1.	*In vivo* leaf methanolic extracts	250.0	18.6 ± 1.2	1342.9
		500.0	30.6 ± 2.4	
		1000.0	52.2 ± 4.1	
		1500.0	67.6 ± 3.5	
		2000.0	84.9 ± 1.0	
2	Dedifferentiated callus methanolic extracts	250.0	10.6 ± 0.3	1085.1
		500.0	28.0 ± 2.7	
		1000.0	46.2 ± 2.5	
		1500.0	61.3 ± 2.9	
		2000.0	64.9 ± 3.1	

The values are mean ± SD

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Graph 3B.7. Extraction yield of callus and *in vivo* leaf extracts.

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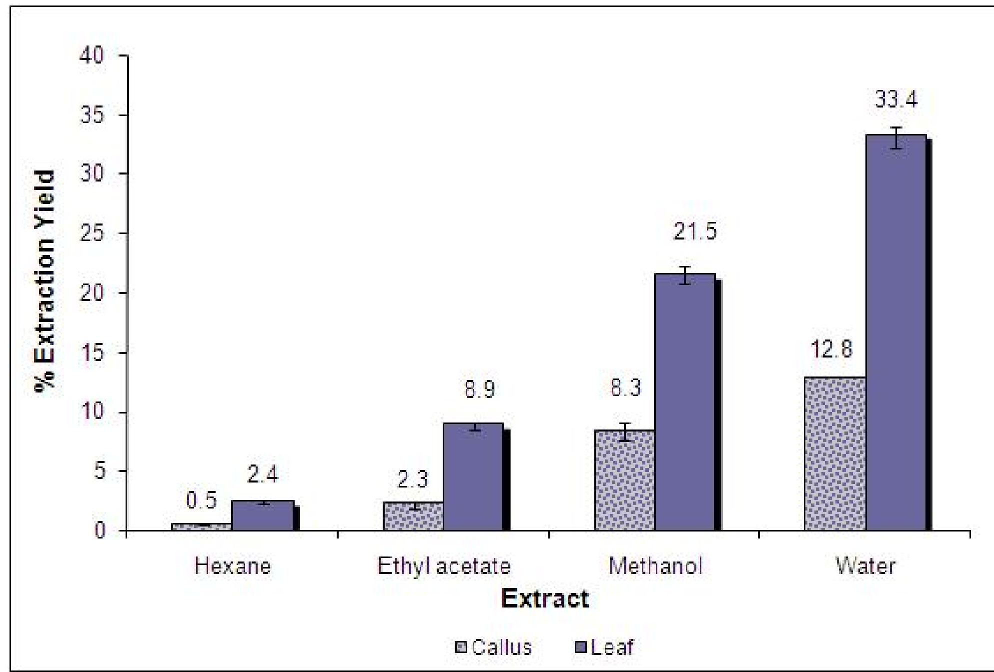

Values are mean of three independent experiments.

Graph 3B.8. Total phenolic content in callus and *in vivo* leaf extracts.

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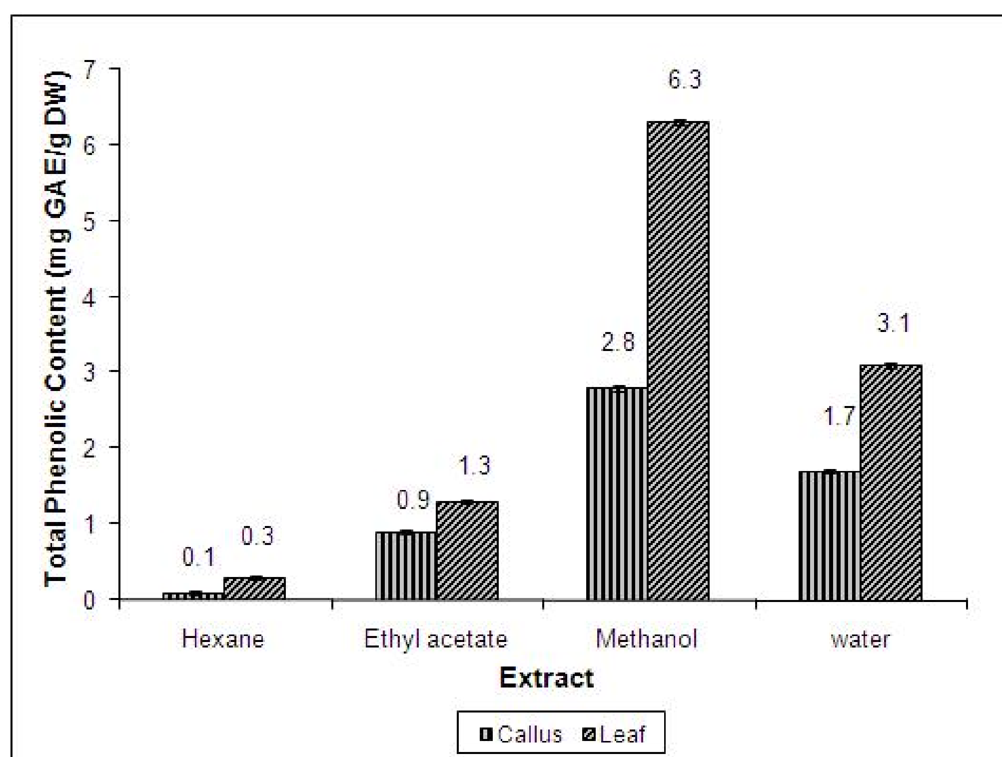

Values are mean of three independent experiments.

3B.3.2. Antimicrobial Assays

The antibacterial activity of *Spilanthes* extracts against certain pathogenic strains of bacteria was tested by using disc diffusion method. Results displayed that *in vivo* leaf methanolic extract has moderate antibacterial activity against *Streptococcus mutans, Escherichia coli* and *Staphylococcus aureus,* and the zone of inhibition ranged from 12.3, 8.6 and 8.0 mm, respectively **(Table 3B.7).** When the growth of the micro-organisms against the solvents (without extract) was evaluated, no marked inhibition of growth was observed. Extract derived from *in vitro* raised dedifferentiated callus lines did not show any activity in the disc-diffusion assay.

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Table 3B.7. Antimicrobial activity of *Spilanthes* methanolic extracts against tested bacterial strains.

S. No.	Tested microorganism	Diameter of disc diffusion (mm)
1.	*Streptococcus mutans* (MTCC 890)	12.3 ± 1.5
2.	*Escherichia coli* (MTCC 443)	8.6 ± 1.2
3.	*Staphylococcus aureus* (MTCC 7443)	8.0 ± 0.5

Values are mean of three independent experiments.

3B.3.3. Anthelmintic Assays

The results of anthelmintic assay revealed that water and methanolic extracts exhibited varying degree of activity against amphistome parasites and caused paralysis followed by death at all tested concentrations. The extracts have a dose and time dependent activity (**Graph 3B.9-10**). However, water extracts showed more potent activity than methanolic extracts at lower concentrations (5 mg/ml and 10 mg/ml). At 5 mg/ml concentration, water extract of callus caused paralysis in 45.7 min and death in 87 min while methanolic extract of callus showed paralysis and death in 83 and 126 min, respectively, against the apmhistomes **(Graph 3B.9)**. Results indicate that higher concentration of each extract produced paralytic effect much earlier and the time to death was shorter. Extract derived from *in vivo* leaves also showed similar activity against amphistomes (**Graph 3B. 10**) where water extract showed best activity at all tested concentration of 5-30 mg/ml.

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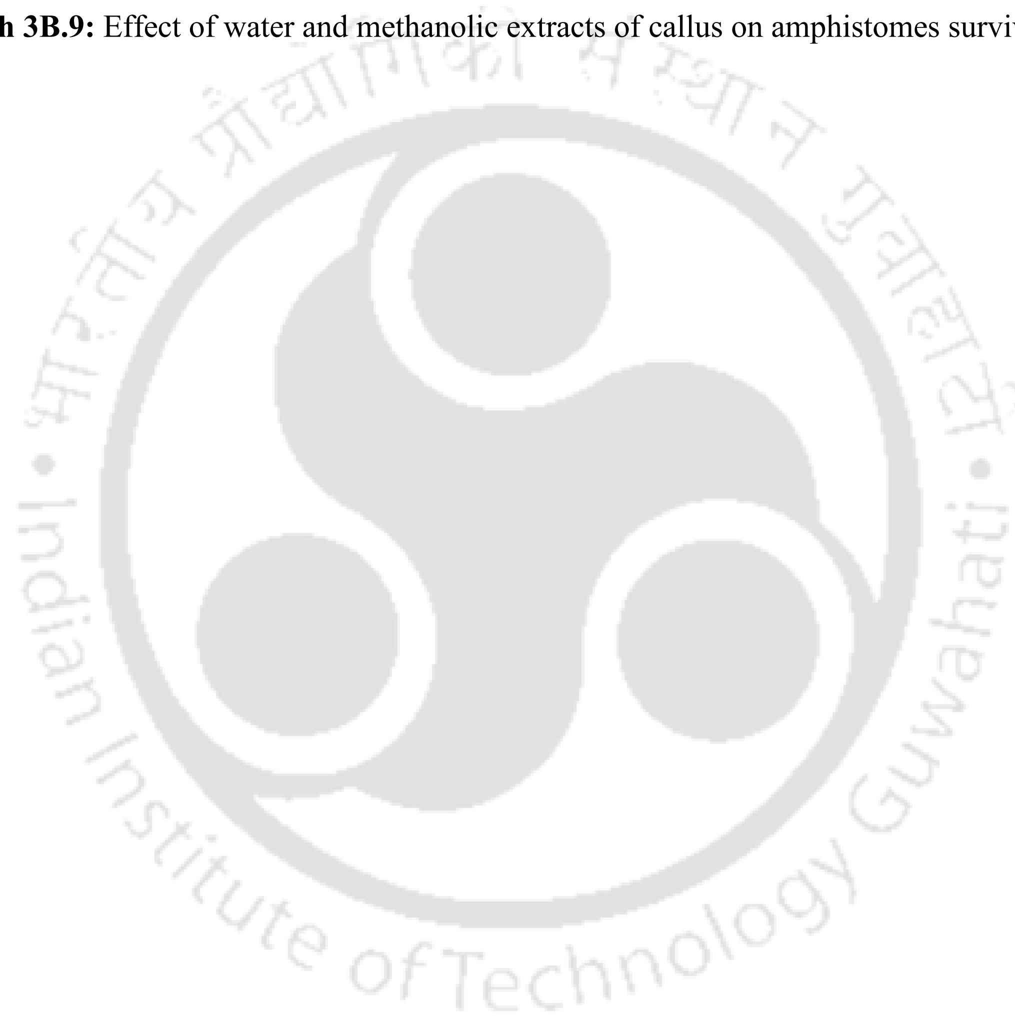
Graph 3B.9: Effect of water and methanolic extracts of callus on amphistomes survivality.



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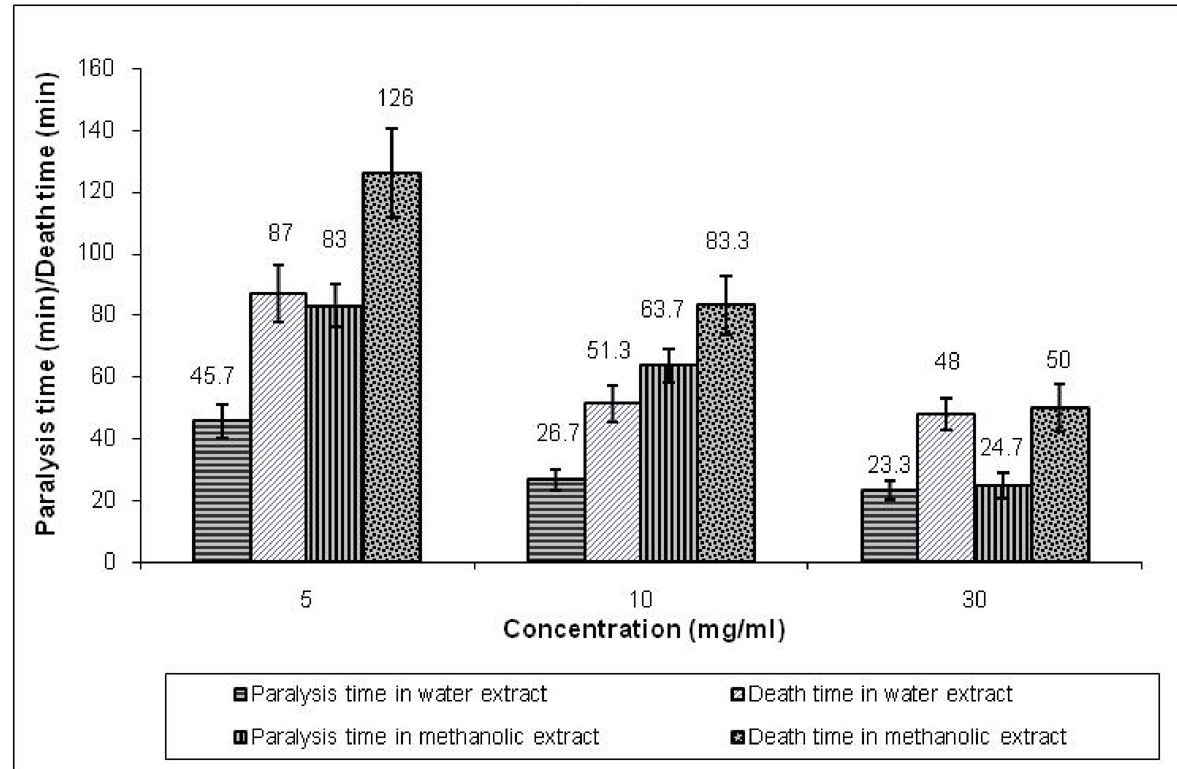

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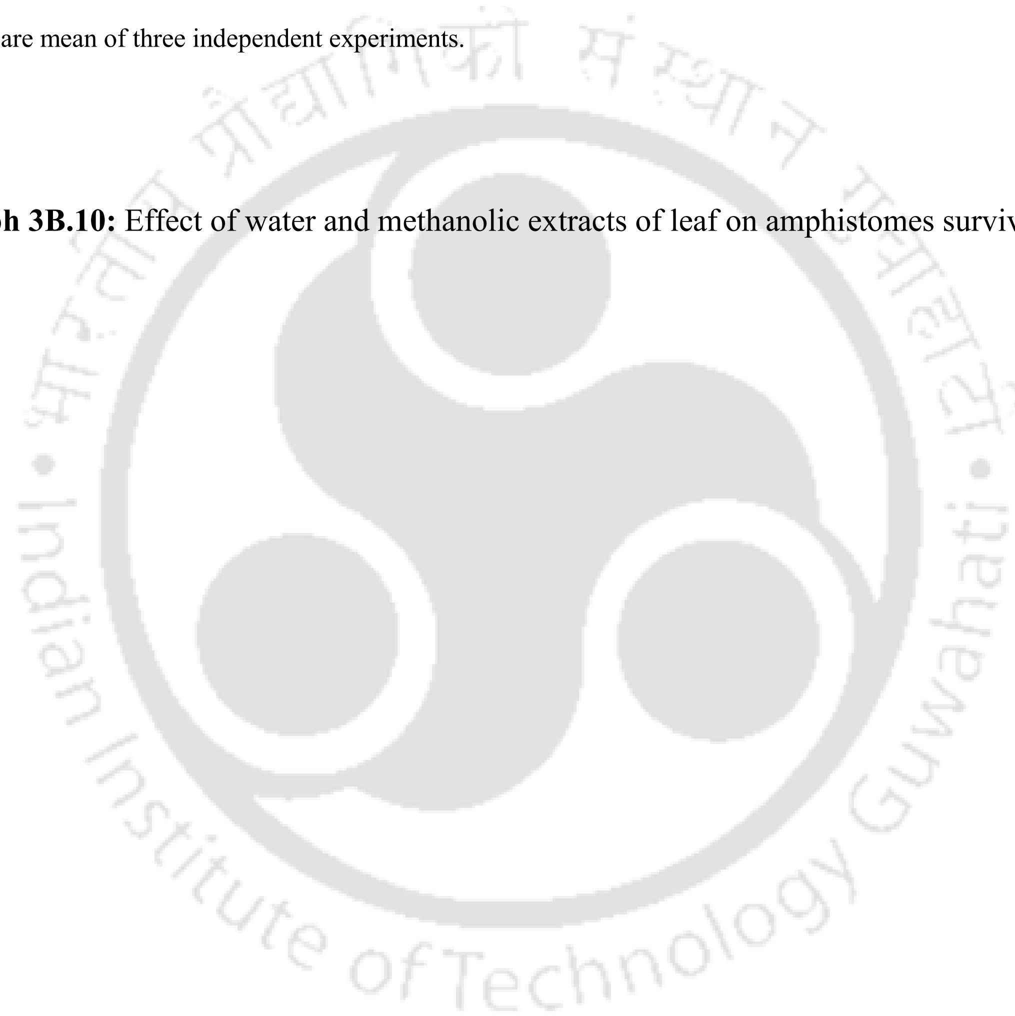
Values are mean of three independent experiments.



Graph 3B.10: Effect of water and methanolic extracts of leaf on amphistomes survivality.

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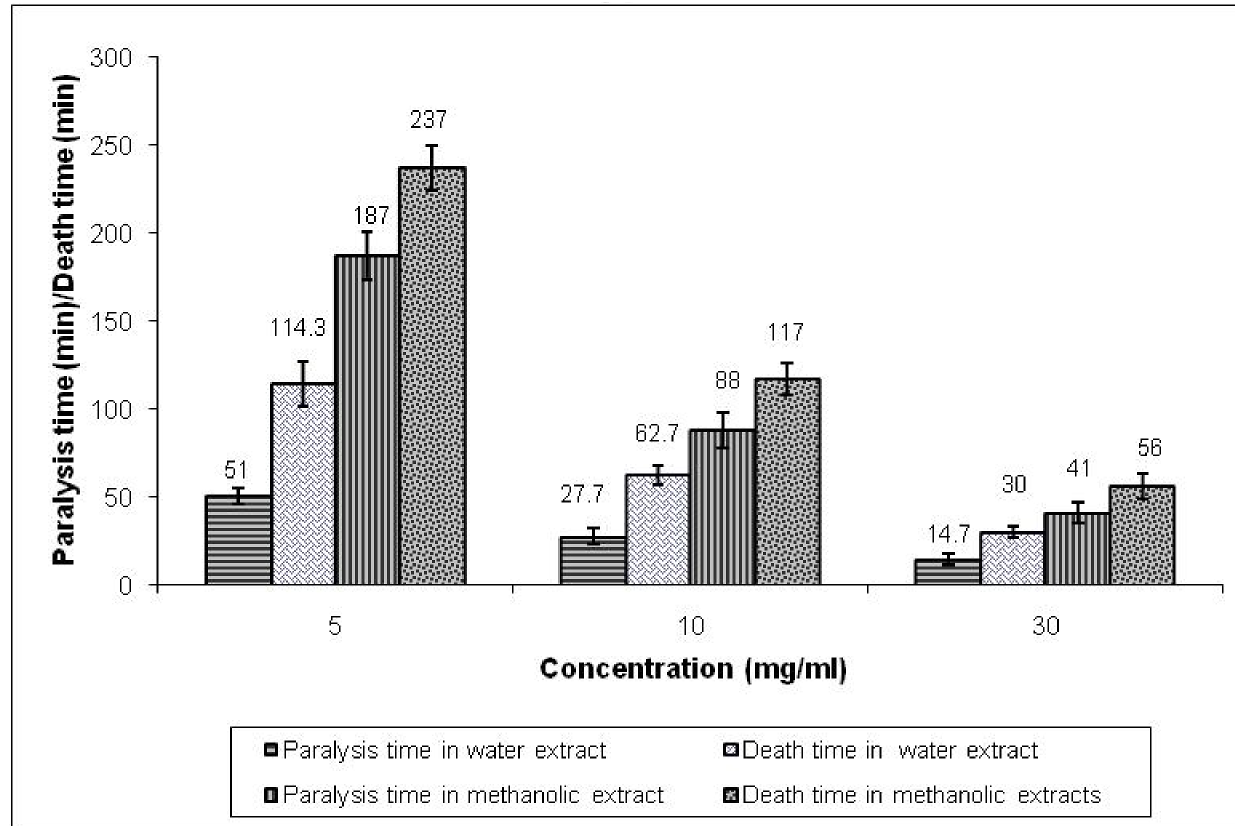

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Values are mean of three independent experiments.

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Chapter 4

Discussion

Medicinal plants have always had an important place in the therapeutic arsenal of mankind. At present, 80% of the populations in developing countries are completely dependent on plants for their primary health care (Ogbadoyi et al. 2011). In spite of enormous progress in medicinal chemistry, over one-fourth of all prescribed pharmaceuticals in developed countries originate directly or indirectly from plants (Newman *et al*. 2000). Furthermore, out of 252 drugs considered as indispensable by World Health Organisation (WHO), 11% are mainly derived from flowering plants and 28% of synthetic drugs are obtained from natural precursors (Namdeo 2007). At present, only 10% of medicinal plant species are cultivated, and the remaining 90% of the species are harvested from wild populations (Julsing *et al*. 2007). Many pharmaceuticals are produced solely from massive quantities of whole plant parts. Excessive harvesting diminishes local plant populations and erode genetic diversity. In this perspective, domestication and acceptance of good agricultural practices are necessary steps towards commercial production. However, the conventional methods of plant propagation are very lengthy and time consuming. The long cultivation periods between planting and harvesting make selection of high yielding strains difficult which in turn results into high cost of the drugs. Moreover, the wild populations are circumvented with the problems of disease, drought, environmental fluctuations, low rate of fruit set, poor seed yield/germination/viability, and in most cases the clonal uniformity cannot be maintained through seeds due to high heterozygosity in populations. Clearly, there is an urgent need of alternative and complimentary methods for conservation of elite germplasms and for constant and uniform production of secondary metabolites. In this context, advantages of plant cell, tissue and organ culture technique has been widely recognized for preservation of elite germplasm. The technique is complimentary and may provide competitive metabolite production systems when compared to whole plant extraction. However, the plant cell culture technology has received limited commercial success because of low and variable product yield. This might be due to the irrational way of culture selection and

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the lack of understanding of biosynthetic pathways of secondary metabolites. Many biotechnological strategies such as, embryogenesis, organogenesis, screening of cell lines, media optimization and elicitation can be carried out for enhanced production of secondary metabolites from medicinal plants. The selection of appropriate technique depends on the results that one wants to retrieve. In plants, where molecules of interest are localized in the specialized cells, dedifferentiated cultures are not desirable. Therefore, establishment of organogenic cultures would be advantageous. In plants, where sought after metabolites are present in leaves, establishing *in vitro* cultures from leaves and using them for the extraction of compounds would be an ideal alternative. In the present study, different *in vitro* strategies have been studied extensively in the two important medicinal plants, *Azadirachta indica* A. Juss. and *Spilanthes acmella* Murr. with the objective of enhanced production of secondary plant compounds. Undifferentiated cell cultures have been mainly studied, but a large interest has also been shown in differentiated cells and organ cultures. Redifferentiated and dedifferentiated cultures, established from various explants of neem, attach to themselves a lot of importance given to the fact that at the same time they can be utilized for the production of important metabolites as well as quality plants. No such detailed studies were available on this aspect, earlier. Presence of azadirachtin, an ecofriendly, non-toxic biopesticidal compound, in various *in vitro* cultures, in the present study, further strengthens its position. As for *Spilanthes*, the present study is an attempt to realize the medicinal potential of this plant, using tissue culture. Organized and unorganized cultures from nodal segment and leaf-disc explants of *Spilanthes* were established and examined, which demonstrate for the first time, the production of pharmacologically important compounds: scopoletin (a coumarin) and spilanthol (an alkylamide), in *in vitro* cultures. Moreover, anthelmintic and antioxidant properties of these cell lines were also reported for the first time, in the present study.

4A. *AZADIRACHTA INDICA*

4A.1 Tissue Culture Studies

During the past decades, concerted efforts have been made to establish *in vitro* cultures of neem and success has been achieved merely in the field of establishment of

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large scale plantation. But, the full potential of neem tissue culture as a means of producing metabolite is limited owing to the fact that in these studies established cultures were not analyzed for metabolite production. Since the plant is highly heterozygous due to cross pollination, neem trees have been shown to exhibit large variability with regard to azadirachtin content of their seeds, irrespective of the habitat (Ermel *et al*. 1984, 1987; Benge 1989; Schmutterer 1990; Ketkar and ketkar 1993; Ermel 1995; Wewetzer 1998; Sidhu et al. 2003). Considerable gains in consistent and homogenous production of azadirachtin can be achieved by utilizing *in vitro* cultures. In the present study, *in vitro* cultures of neem were established using various explants, *viz*. zygotic embryo, leaf and ovary, with the objective of improving the production of secondary plant compounds. As different parts of the plant are at different physiological and biochemical stages, they showed varied responses in *in vitro* conditions. Moreover, growth regulator concentrations and combinations required for *in vitro* responses also varied from explant to explant.

So far, there are two reports on zygotic embryo cultures of neem dealing with the development of plant propagation protocol (Chaturvedi *et al.* 2004b; Rout 2005). In both the reports, the cultures were not evaluated for azadirachtin acculumation and estimation. Chaturvedi *et al.* (2004b) established zygotic embryo cultures by using immature embryos at various stages of development. In their study, zygotic embryos showed different morphogenic responses such as shoot differentiation, somatic embryogenesis and neomorph formation. Maximum shoot-bud differentiation and somatic embryogenesis occurred on MS medium supplemented with BAP (5.0 µM), and most responsive stage of embryo was early dicot followed by torpedo stage. Rout (2005) reported somatic embryogenesis from immature zygotic embryos that were cultured 40 days after anthesis. Embryogenic callus was proliferated on MS medium supplemented with BAP (1.11 µM) and 2,4-D (4.52 µM). When these calli were transferred to same medium containing reduced auxin, 2,4-D at 0.45 µM, numerous embryos proliferated from the surface of the callus.

The factors that decide the growth and development of plant cells remained largely undefined. However, it is apparent from recent studies that the interactions of nutritional, biochemical and environmental factors can determine the fate of competent plant cells. This might be the rationale for different responses of zygotic

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embryos in different studies. In the present investigation, embryos either callused or differentiated into two kinds of organized structures directly from the explants, *viz.* shoots and somatic embryos. Callusing from the explants occurred in the combined presence of auxin and cytokinin. Dedifferentiated (unorganized) callus was proliferated on MS + BAP (5.0 µM) + 2,4-D (1.0 µM) + NAA (1.0 µM), whereas redifferentiated callus was obtained on MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l). Apart from callusing, direct somatic embryogenesis occurred from zygotic embryo explants when TDZ was used alone or in combination with ABA/GA_3. These results corroborate the findings of earlier reports (Murthy and Saxena 1998; Gairi and Rashid 2004) which suggest that TDZ can be used to induce somatic embryogenesis in neem. This is probably due to the participation of TDZ in the modulation of endogenous growth regulators especially auxins and cytokinins (Murthy *et al.* 1996). Additionally, TDZ also promotes the conversion of cytokinin molecule to more biologically active molecule (Capelle *et al.* 1983) and/or the inhibition of cytokine oxidase activity (Kaminek and Armstrong 1990), which further increases TDZ effectiveness for somatic embryogenesis.

Regeneration from neem leaf explants, derived from adult trees was first reported by Narayan and Jaiswal (1985). Their results are in agreement with the present findings where BAP amended medium supported shoot organogenesis. Further, they also noticed that addition of NAA (0.27 µM) in conjunction with BAP enhanced the frequency of shoot differentiation from 54% to 62.5%. However, in the current study, no such increment in the number of shoot proliferation was noticed. However, a significantly better shoot regeneration frequency (76%) has been achieved in a single induction medium, MS + BAP (5.0 µM), from leaf-discs of a mature 35-year-old neem tree. In accordance with this result, Arora *et al.* (2009) have also emphasized the superiority of BAP over other cytokinins on shoot organogenesis from leaf-disc cultures of an adult neem tree. Shoot regeneration from leaf cultures were also established by Ramesh and padhya (1990), Eeswara *et al.* (1998) and Salvi *et al.* (2001), but in these reports either the origin of the explant was seedling and/or age of the source plant was not mentioned.

The first report on shoot regeneration from unfertilized ovary culture came from Srivastava *et al.* (2009). The authors reported an efficient protocol for plant

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propagation from a 50-year-old neem tree but no studies related to biochemical characterization of the ovary cultures were taken up. They achieved shoot organogenesis in unfertilized ovary cultures by a three-step procedure; the calli induced on MS (9% sucrose) + 2,4-D (1.0 µM) + BAP (5.0 µM), was later multiplied on MS medium containing either 2,4-D (0.5 µM) alone or in combination with Kinetin (4.5 µM). The shoot regeneration was observed on MS + BAP (5.0 µM). In the present study, a two-step procedure was developed to obtain organized cultures from unfertilized ovary explants collected from a 35-year-old neem tree. MS medium containing 2,4-D (0.5 µM) and Kinetin (4.5 µM) was suitable for both callus induction and multiplication. A right combination of one auxin and one cytokinin is also essential for shoot induction from calli and the best combination was MS + BAP + IAA/CH. The maximum shoot regeneration frequency observed was 50% on MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l), with cultures forming an average of 5.5 shoots per explant, followed by shoot regeneration on MS + BAP (5.0 µM) + IAA (0.5 µM) where 25.5% cultures formed an average of 2.5 shoots per explants. Besides, in the current study, callus induction was achieved in one week in comparison to that of Srivastava *et al*. (2009), where it took 4 weeks to obtain calli from explants. Of the various factors, age of the donar plant and interactions between the genotype and environment has great influence on the nutritional requirements of explants in culture (Pattnaik *et al*. 2006; Srivastava and Chaturvedi 2011). This might be the reason for different nutritional requirements of ovary explants at culture in the two reports.

Adventitious shoot proliferation in cell and tissue cultures, in response to hormonal manipulation of the culture medium, require *de novo* differentiation of meristematic region, and, thus, it may cause a change in ploidy due to chimera formation (Bhojwani and Razdan 1996) which in turn can influence the metabolite content in *in vitro* cultures. This necessitated the assessment of ploidy stability of the *in vitro* cultures by flow cytometry which showed that all *in vitro* regenerated shoots were at same ploidy level as that of the parent plant. Similarly, Libiakova *et al.* (1995) found that shoots regenerated *in vitro* from mature embryos and cotyledons of seedlings, as well as long term callus cultures of the hybrid *Abies concolor* x *A. grandis* were stable, remaining at the diploid level. Thiem and Śliwińska (2003) also showed that there is no variation in the ploidy level among different types of tissue cultures: axillary shoots from the

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3rd and 20th passage, shoots from cold storage, and shoots developed from alginate-encapsulated buds. Micropropagated cultures of *Juniperus phoenicea* (Loureiro *et al*. 2007), and, more recently, of *Rubus fruticosus* (Vujović *et al*. 2010) also showed ploidy stability.

4A.2 Secondary Metabolite Analysis

Plant tissue culture technique has been considered as an attractive alternative method for bioactive metabolite production. However, it was very soon realized that the range of compounds produced by plant tissue cultures can differ significantly, both quantitatively and qualitatively, from that found in the field grown plant (Verpoorte *et al*. 1998). Moreover, cultured cells produce such compounds in very low concentrations compared to those present in the specialized tissues of the corresponding plants. Several approaches have, therefore, been proposed in order to increase the accumulation of secondary metabolites in the cultures.

In the present study, strategies, such as screening of *in vitro* cell lines and optimization of media constituents, have been adopted to improve azadirachtin production from plant cell cultures. Redifferentiated and dedifferentiated *in vitro* cell lines of neem, obtained from different explant sources, *viz*. zygotic embryo, leaf and ovary, have been analyzed to find out the elite cell lines for azadirachtin biosynthesis. Analysis of *in vitro* cell lines showed the presence of azadirachtin in all samples tested, although, the content is influenced by explant source and cell differentiation response. This is in agreement with the report of Govindachari (1992), where the author reported higher amount of azadirachtin in zygotic embryo cultures of neem than those of leaf and ovary cultures. Furthermore, the results of this study showed that organized *in vitro* callus cultures (redifferentiated) induced higher azadirachtin biosynthesis, while unorganized callus cultures (dedifferentiated) the least. It has been observed that BAP alone or with IAA and CH, promoted shoot organogenesis in all cultured explants that stimulated an increase in azadirachtin production. In *in vitro* conditions, redifferentiation is generally associated with an improved synthesis of secondary metabolites (Collin 2001). This is most probably due to appearance of complex cells and tissues which are metabolically more proficient. In all

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redifferentiated cell lines along with the shoot-forming nodules, non-morphogenic cell masses were also present, which though non-morphogenic might have certain degree of differentiation at the cellular stage, and due to co-evolution, it imitate the biochemistry of redifferentiated cells (Brown *et al*. 1986). Further, the results of the present study are in agreement with the report on *Artemisia annua,* where artemisinin production was very poor in dedifferentiated callus cultures and a certain degree of redifferentiation was obligatory for artemisinin production (Liu *et al*. 1997). Organogenesis was also found to be an essential prerequisite for steroidal saponin production in *Ruscus aculeatus* (Palazón *et al*. 2006). Similar observations were made for the biosynthesis of picroside in *Picrorhiza kurroa,* wherein the metabolite did not accumulate in the dedifferentiated callus cultures but occured specifically in the redifferentiated cultures (Sood and Chauhan 2009). Berkov *et al*. (2010) also demonstrated that alkaloid synthesis in *Pancratium maritimum* is closely related with tissue differentiation.

Till date, the available reports on azadirachtin production, have utilized cultures from different parts of the plant, like leaves (Allan *et al*. 1994; Kearney *et al*. 1994; Sundaram *et al*. 1996; Wewetzer 1998; Kuruvilla *et al*. 1999; Allan *et al*. 2002), bark (Bajagopal and Ramaswamy 1996; Wewetzer 1998), embryos (Srividya *et al*. 1998), seeds (Prakash *et al*. 2005), shoot tips (Schaaf *et al*. 2000) and shoots (Raval *et al*. 2003) and microspore derived calli (Srivastava and Chaturvedi 2011). Whereas in leaf derived callus the amount ranged from 4 µg/g to 64 µg/g DW in different reports; bark, embryo, shoot, shoot tip and microspore derived cultures produced 44-1200 µg/g DW, 4-8 µg/g DW, 250 µg/g DW, 0.5 µg/g DW and 728.41 µg/g DW of azadirachtin, respectively. In the present study, azadirachtin yield (2330 µg /g DW) obtained from redifferentiated zygotic embryo cultures, is significantly higher than the highest azadirachtin yielding dedifferentiated seed-derived cell line (1890 µg /g DW) (Prakash *et al*. 2005).

Apart from organogenesis and explant source, accumulation of secondary products in plant cell cultures depends on the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources (Stafford *et al*. 1986). Realizing the immense potential of azadirachtin, it is worthwhile to optimize these factors to enhance its production. Therefore, attempts

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are made in the present study, by developing processes that are economically viable. The conventional methods for optimization of medium involve varying 'one-factor-at-a-time' and keeping the others constant. Its major disadvantage is that interactions among the factors are not considered so it does not reflect all the potential effects on the process. Moreover, these methods are laborious, time consuming and impractical. To overcome this difficulty, and understand the interactions between different physiological and nutritional parameters, response surface methodology (RSM) has been widely used (Houng *et al*. 1989; Yalimaki *et al*. 1991; Sunita *et al*. 1998). It brings about the effect of interaction of various parameters, generally resulting in higher production and limiting the number of experiments. In addition to analysing the effects of independent variables, this methodology generates a mathematical model that accurately describes the overall process.

In the present study, statistical approach called Plackett-Burman (PB) design has been used, which ignores the interactions among factors. Only the most effective factors with high significance levels were selected for further optimization, while others with lower significance levels or with small effects on response value were omitted in further experiments. Following this, RSM was adopted to determine the relationship between factors and responses. Response surface design methods mainly comprise the central composite design (CCD), the box-behnken design (BBD) and the D-optimal design. Of these, the CCD and the BBD are the most commonly used response surface design methods. The CCD is often suggested for sequential experimentation and is appropriate for assessing first and second order term. BBD, on the other hand, can be used for performing non sequential experiments because they do not have an embedded factorial design. The CCD has axial points outside the design periphery. These axial points have significant role toward design precision, still they are not chosen in many cases, where these conditions are away from the safe operating limits. Though in BBD all factors are set within the experimental margin, but this method has lower accuracy than CCD (Myers and Montgomery 2002). The CCD and the BBD have five levels and three levels for each factor, respectively. D-Optimal design is generally used when CCD or BBD cannot be used due to limited resources or factor setting constraints. Hence, in the present study, CCD was used to optimize the levels of significant variables which will be useful for the mathematical model. Similar to

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this study, PB and CCD have earlier been used in *Podophyllum hexandrum* to optimize culture conditions for podophyllotoxin production (Chattopadhyay *et al*. 2002).

For PB design, five media components, BAP, IAA, CH, MS major salts and sucrose, were chosen that promoted the proliferation of organogenic (redifferentiated) calli with maxiumum azadirachtin yield (2330 µg/g DW), in zygotic embryo cultures. For the first time, media components like plant growth regulators and additives were also taken into consideration, which are important determining factors of metabolite production. Among the variables studied, sucrose, MS major salts and BAP were found to significantly affect azadirachtin production. The optimum concentration levels of these three significant variables, as determined by CCD, were 5.68% sucrose, 10.42 µM BAP and full MS major salts. Under these medium conditions, the model predicted maximum azadirachtin content of 5130 µg/g DW (5.13 mg/g DW). Using optimum medium composition, the experimental value of azadirachtin content was 4970 µg/g DW (4.97 mg/g DW) in redifferentiated zygotic embryo cultures which agreed well with the model predicted value of the compound. Our finding that higher sucrose concentrations resulted in an increase in azadirachtin production is consistent with the reports of *Camptotheca acuminata* (Pasqua *et al*. 2005), *Pueraria lobata* (Fang *et al*. 2006) and *Melissa officinalis* (Kim *et al*. 2011) where higher sugar concentration favoured maximum metabolite biosynthesis. Stimulatory effect of BAP on secondary metabolite production is reported in *Vaccinium pahalae* (Fang *et al*. 1998) and *Oxalis linearis* (Rao and Ravishankar 2002).

To date, a few efforts were made in optimizing media components for the production of azadirachtin (Raval *et al*. 2003; Satdive *et al*. 2007; Prakash and Srivastava 2005, 2008). Raval *et al*. (2003) studied the effect of major nutrients on growth and production of azadirachtin-related limonoids in plant cell cultures of neem with an aim to enhance their yield. While maximum biomass was obtained on MS medium, maximum production of azadirachtin related limonoids was observed on White's medium. Their results indicated the non-growth associated production characteristic of azadirachtin related compounds. Two years later, Prakash and Srivastava (2005) established statistically optimized media for cell growth and production of azadirachtin in cell suspension cultures, produced from neem seed kernels. A

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maximum of 2980 µg/g azadirachtin was produced using optimum nutrient concentrations. Satdive *et al*. (2007) examined the effect of different culture media and elicitor on azadirachtin production in hairy root cultures of neem. In their study, 1400 µg/g DW of azadirachtin was produced from hairy root cultures after elicitation. Prakash and Srivastava (2008) investigated the effect of elicitors like salicylic acid, chitosan, jasmonic acid, methyl jasmonate and yeast extract, at different concentrations, in shake flask suspension cultures of *A. indica*. Chitosan, salicylic acid and jasmonic acid stimulated 2-to 3- fold increases in azadirachtin content in comparison to the control. The combined effect of these elicitors on azadirachtin production was studied by RSM which showed 5-fold higher azadirachtin production (15900 µg/g DW) due to synergistic effect of these elicitors.

The azadirachtin production, in the present study is much higher than most previous studies. However, Prakash and Srivastava (2008) reported higher azadirachtin production after elicitation and precursor feeding. It is believed that 4970 µg/g DW (4.97 mg/g DW) of azadirachtin value, obtained in the present study, will be improved significantly on addition of precursors and elicitors. This study, thus, demonstrates the possibility for biosynthesis of azadirachtin on a large scale under proper growth conditions. Literature suggests that reports on systemic selection and screening of *in vitro* cell lines for azadirachtin production are lacking. In this context, the present study will be of significant importance that can be applied to scale up and maximize azadirachtin production in cell, tissue and organ cultures of neem.

4B SPILANTHES ACMELLA

4B.1 Tissue Culture Studies

During the last few years, few attempts have been made for *in vitro* propagation of this important plant through organogenesis (Saritha *et al*. 2002; Haw and Keng 2003; Saritha and Naidu 2008; Pandey and Agrawal 2009; Singh *et al*. 2009b). However, the conditions for micropropagation were not optimized in either of these studies. Furthermore, in majority of these studies, organogenesis occurred through an intervening callus phase, which can cause genetic instability and somaclonal variation. On the contrary, the present investigation deals with the standardization of procedures

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to raise direct plantlets of *Spilanthes acmella* Murr. by nodal segment and leaf-disc cultures.

The most important technique in micropropagation is axillary bud proliferation where the nodal segments bearing pre-existing axillary buds are cultured to proliferate multiple shoots without any intervening callus phase. This would relinquish dependency on seeds whose availability is always doubtful. Earlier, Haw and Keng (2003) attempted clonal propagation of *Spilanthes* by axillary shoot proliferation but the study lack crucial information on percent culture response, the rate of proliferation in recurrent cycles of shoot multiplication, frequency of rooting, etc., and in that study transplantation was not attempted. The present study gives a systematic report on highly reproducible and recurrent method of clonal propagation whereby detailed description on *in vitro* shoot multiplication, rooting and hardening are described.

Contamination of *S. acmella* nodal explants was a major problem during initiation of cultures under *in vitro* conditions. The extent of contamination was strongly influenced by the season during which the material was collected. The cultures initiated during January-April showed significantly ($p<0.05$) higher (64%) bud-break than those raised in other parts of the year. The reason for the difference in response may be due to high meristematic activity and fresh shoot growth in spring season from January till April. In later part of the year, shoots became old and, thus, make it difficult to break the dormant state of the buds. Similar results of seasonal effects on culture establishment have earlier been reported in mulberry (Chitra and Padmaja 2002), neem (Chaturvedi *et al*. 2004a), litchi (Kumar *et al.* 2006) and lotus (Shou *et al*. 2008). The thickness and maturity of the explant with increasing distance from apex of the shoot not only determine their survival in culture but also contribute to their effective proliferation (Arora *et al*. 2010). Since, in the present study, nodal segments were randomly taken, irrespective of their position in parent plant, this could be the reason for a significant difference (23%) between percent aseptic cultures (87.4%) and percent bud-break (64.1%). Arora *et al*. (2010) noticed a gradual increase in percent explants showing bud-break with the increasing distance (maturity) of node up to a certain level from apex of the shoot. Hsia and Korban (1996) concluded that nodal stem segments having larger diameters had a better nutrient translocation from

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the donor mother plant, hence developed larger buds, which sprouted sooner when placed *in vitro* in case of *Rosa* sp.

Several species require moderate salt concentration for their initial survival in cultures (Bhojwani and Razdan 1996; Chaturvedi *et al*. 2004a). Davies (1980) worked on the effect of different nutrient salt concentrations on multiplication of various cultivars of rose and found that unchanged MS salts proved to be the best. Bressan *et al*. (1982) noticed a negative influence of low salt concentration on vigor and viability of shoots. These are in agreement with the present report where normal strength of major salts proved significantly ($p<0.05$) better than half and double strength of major salts for establishment and multiplication of shoots in nodal segment cultures.

In cultured plant tissues, a continuous supply of carbohydrate from the medium is essential which are needed for growth and organized development of the plant and are necessary as a source of energy and carbon skeletons for biosynthetic process. In the present study, sucrose (3%) supported significantly ($p<0.05$) highest rate of shoot multiplication than glucose or maltose in nodal segment cultures. Sucrose is the most commonly used carbohydrate for plant tissue cultures and most culture media have it as the sole carbohydrate source. One explanation for the favourable effect of sucrose is that it is easily recognized and hydrolyzed by the cell wall bound invertase into more efficiently utilizable forms of sugars, glucose and fructose, which are incorporated into the cells. Moreover, glucose derived from sucrose hydrolysis, is more accessible to the cultured tissues than glucose derived by maltose hydrolysis, due to a rapid sucrose hydrolysis but a slow maltose hydrolysis in the media (Ślesak *et al*. 2004). The starvation effect of maltose due to slow hydrolysis and/or a slow uptake may lead to a drop in available carbohydrates within the cell, which may create a signal to reorient the developmental programmes (Blanc *et al*. 2002; Krogstrup *et al*. 2005). The morphogenic differences found in the present study may also be partly due to difference in osmotic potential of the medium resulting from differential degree of hydrolysis of sucrose and maltose. The positive effect of sucrose may be related to its increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, production of energy required for shoot initiation (Bonga and Von Aderkas 1992). Further in the present study, glucose alone itself was not promotory for bud-break and shoot length whereas combined presence of glucose and fructose,

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which is derived through sucrose hydrolysis, is essential for highest percentage of bud-break and shoot length. In the combined presence of glucose and fructose, cells exhibit a preference for glucose whereas fructose is utilized as a substrate only after glucose is depleted from the medium (Kretzschmar *et al*. 2007). Thus, the presence of sucrose facilitated the constant availability of utilizable forms of carbohydrates in the medium throughout the culture period, in this study. Consequently, initial high flux of glucose might have promoted bud-break and shoot development whereas fructose enhanced shoot length later.

In vitro shoot proliferation and multiplication are largely based on media formulation containing cytokinins as a major plant growth regulator. In the present study, maximum promotive effect on percent bud-break and rate of shoot multiplication were observed with BAP as compared to 2-iP or Kinetin. The promotive role of BAP on shoot proliferation has been documented earlier in *Schinopsis balansae* (Sansberro *et al*. 2003*); Holarrhena antidysenterica* (Kumar *et al*. 2005); *Eclipta alba (*Ray and Bhattacharya 2008), *Nelumbo nucifera* (Shou *et al*. 2008), *Centaurea ultreiae* (Mallón *et al*. 2010) and *Musa acuminate* (Jafari *et al*. 2011). In the present work, 6.4 fold shoot multiplication was obtained in 64% cultures on hormone-free medium. Addition of BAP has further enhanced the percent culture response (100%) with highest rate of shoot multiplication (>20 fold) at its optimum concentration of 5.0 µM. While the effect of Kinetin on bud-break was significantly ($p<0.05$) lower, 2-iP proved inhibitory. Differences in the activity of cytokinins can be explained by their various translocation rates to meristematic regions and metabolic processes, in which the cytokinins may be degraded and conjugated with physiologically inert compounds, like sugars or amino acids (Kaminek 1992).

In the present study, the lower concentrations of BAP (1.0-3.0 µM) resulted into shoots with long internodes and with excessive adventitious root proliferation. However, when exposed to a high concentration of BAP (7.0-15.0 µM), the shoots formed were short and stunted. The changes in morphogenic responses could be attributed to the differences in BAP uptake and metabolism that lead to the formation of other compounds with different degrees of hormonal activity. The optimum level of BAP for shoot proliferation from nodal segments was observed at 5.0 µM. At this

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concentration, BAP favoured >20-fold shoot multiplication, in every 5 weeks, with no adventitious root formation.

The hand sections of the nodal region of the shoots, grown at lower concentrations of BAP (1.0-3.0 μM), revealed the presence of a band of vascular bundles with associated 2-3 numbers of adventitious roots. The sections were when stained with acridine orange (a cationic dye) confirmed the occurrence of a ring of lignified xylem tissues which fluoresce bright yellow. Although acridine orange has pronounced affinity to nucleic acid, being a cationic dye, it has strong affinity to acid materials like lignin (Drnovšek and Perdih 2005; Li and Reeve 2005). It can also get accumulated in vacuoles, the acidic compartments of cells, by ion trap mechanism and gives orange fluorescence (Oparka 1991). Acridine orange is a metachromatic fluorescent dye and the amount of metachromasia or spectral shifts, depend on the concentration of dye, ratio of binding sites to dye molecules, pH, staining duration and chemical and physiological properties of plant samples (Bertalanffy 1963; Robbins and Marcus 1963). Deoxyribonucleic acid (DNA) intercalate acridine orange and fluoresces green while ribonucleic acid (RNA) electrostatically bind to it and fluoresces orange red due to spectral shift (Myc *et al*. 1992). RNA being less polymerized, offers more sites for binding acridine orange and, hence, accumulate a higher concentration, but also DNA fluoresces red if depolymerized. Acridine orange can also be utilized to distinguish between quiescent and activated proliferating cells due to quantitative differences in RNA content (Bertalanffy 1963). In the present study, majority of the cells in the section have taken orange colour which may be due to vacuolar accumulation of acridine orange. The lignified xylem cells are fluorescing bright yellow due to weak dye-dye coupling of neighboring dye molecules. Moreover, the differential staining of cambium cells due to higher RNA content cannot be ruled out.

The use of leaf explants to raise *in vitro* cultures would be very convenient for growers and herbal product companies to clone elite individuals of *Spilanthes* for enhancing its cultivation and metabolite production. This is due to the fact that foliar explants are easy to obtain and do not require to sacrifice the mother plant. Till date, only two reports are available on leaf-disc culture of *Spilanthes* (Saritha and Naidu 2008; Pandey and Agrawal 2009). However, in these studies, *in vitro* cultures were not analyzed for ploidy stability and metabolite production.

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The type and concentration of auxin and cytokinin used in the medium and a possible interaction between exogenous and endogenous concentrations of plant growth regulators have a marked effect on the *in vitro* culture responses (Skoog and Miller 1957). In the present study, leaf-disc explants cultured on media containing various concentrations and combinations of plant growth regulators showed two types of responses, *viz*. callusing and direct shoot proliferation. The highest rate of callus formation (100%) was observed on MS + BAP (5.0 μM) + NAA (1.0 μM) + 2, 4-D (1.0 μM). However, establishment of fresh, friable and constantly growing cell lines of *Spilanthes* was a difficult task to accomplish because of the browning of callus as well as the culture media. Browning of the explants and culture media due to phenol exudation has been discussed previously by many workers (Naik *et al*. 1999; Wu and du Toit 2004). It has been recommended that the antioxidants such as ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), and polyvinyl-polypyrrolidone (PVPP) can be used to surmount explants browning and death. Besides this, recurrent subculturing at regular intervals is also an efficient and alternative approach to overcome browning of cultures (Rout *et al*. 1999; Srivastava *et al*. 2010). In the present study, browning problem was exterminated by repeated subculturing of calli. Use of antioxidants was avoided to prevent changes in the secondary metabolite spectrum of cultured cells.

Shoot regeneration from leaf-disc cultures was occurred on MS + BAP, MS + BAP + IAA, and MS + BAP + NAA. The best treatment for shoot regeneration was MS + BAP (5.0 μM) + IAA (5.0 μM) which promoted adventitious shoot proliferation in >82% cultures with an average of 5.3 shoots per explant. The requirement of auxin along with cytokinin for obtaining optimal response of shoot-bud differentiation is well documented in a number of plants including *Aegle marmelos* (Hossain *et al*. 1995), *Pistacia vera* (Tilkat *et al*. 2009), *Emblica officinalis* (Nayak *et al*. 2010); *Musa acuminate* (Jafari *et al*. 2011) and *Arnebia hispidissima* (Shekhawat and Shekhawat 2011). In this study, shoot proliferation from leaf-disc cultures occurred without an intervening callus formation. This point is of major importance because plants produced by direct organogenesis may exhibit greater genetic stability than those produced via callusing (Karam and Al-Majathoub 2000).

Terminal 3 cm long portions of shoots, from 5-week-old cultures of nodal segments and leaf-discs were utilized for rooting. On ½ MS + sucrose (50 g/l), 100% shoots

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formed more than 35 roots directly from the basal cut end of the shoot. Similar to this study, Serres *et al*. (1990) observed that the percentage of rooting and number of roots per shoot is positively correlated with sucrose concentrations in chestnut microcuttings. The positive effect of higher sucrose concentration may be related to its potential to increase the osmotic pressure of the medium, which stimulate the mitochondrial activity and consequently generating more energy to facilitate rooting (Bonga and Von Aderkas 1992).

The ploidy level of regenerated plants, derived from nodal segment and leaf-disc cultures, was assessed by flow cytometry. Flow cytometry is referred as a rapid and efficient method for estimating the DNA content of micropropagated plants which has enormous practical efficacy and commercial implications. Results of ploidy analysis showed that all investigated plants had the same ploidy level as that of the mother plants. Similar to this study, utility of flow cytometry as a means of evaluating the ploidy level and genetic stability of *in vitro* regenerated plants has been well documented by many workers (Sukhumpinij *et al*. 2010; Vujović *et al.* 2010; Berkov *et al*. 2010; Hamama *et al*. 2011).

4B.2 Secondary Metabolite Analysis

Till date, no reports are available on metabolite production from *Spilanthes in vitro* cultures. The list of new chemical compounds from the plant is constantly increasing day by day, but, this alone will not serve the purpose. Identification of unknown compounds only indicates the further utility of the plant, economically and medicinally. In order to use its potential to the fullest, there has to be a constant source from where these compounds can be extracted, on a regular basis. Field grown plant is not a good choice for this purpose because the plants growing in wild face a lot of climatic and other environmental fluxes that lead to change in their chemical profile. In contrast to this, plant cell cultures are an attractive alternative to whole plant extraction for the production of high-value secondary metabolites (Rao and Ravishankar 2002; Verpoorte *et al*. 2002; Suchismita and Ramawat 2009; Cai *et al*. 2011). In the current study, *in vitro* cultures of *Spilanthes,* established from nodal segments and leaf-discs, were evaluated for the presence of two important plant secondary products *viz.* scopoletin (a caumarin) and spilanthol (an alkylamide).

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A novel HPLC method with fluorescence detector was developed for the quantitative estimation of scopoletin in *S. acmella*, which can detect even a low concentration of scopoletin and could be easily implemented in routine practice. The study revealed that scopoletin is a phytoalexin, even though the uninfected leaves of *Spilanthes* could accumulate the scopoletin. The results of the present study showed that the scopoletin content of the nodal segment derived plants was 0.104 ± 0.03 µg/g DW of leaves which was comparable to that of the mother plant (0.101± 0.04 µg/g DW of leaves). In comparision to this, a significantly lower amount of scopoletin (0.03 µg/g DW of leaves) was synthesized in leaf–disc derived *in vitro* plants and it was completely absent in undifferentiated callus cultures. Scopoletin biosynthesis was induced in several plant species upon infection by different pathogens (Matros and Mock 2004; Kai *et al*. 2006; Lerat *et al*. 2009) and is considered as an important compound that play an important role in defense mechanism against bacteria and fungi (Smith 1996). However, no quantification studies were performed in either of these reports. This is the first report on detection and quantification of scopoletin in *S. acmella*. The study revealed that even the uninfected leaves of *Spilanthes* could accumulate the scopoletin.

Another interesting finding of this study was the production of spilanthol from in *vitro* cultures of *Spilanthes*. Results of the HPLC analysis have shown that nodal segment derived *in vitro* plants (NP) and the field grown mother plant (FP) contain equal amount of spilanthol. Leaves of NP and FP accumulated 2702.33 ± 11.6 µg/g DW and 2703.66 ± 9.6 µg/g DW of spilanthol, respectively. Interestingly, leaf-discs derived plants of *Spilanthes*, accumulated maximum amount of spilanthol (3294.36 ± 12.4 µg/g DW) while in undifferentiated callus, significantly ($p<0.05$) lower amount (998.03 ± 15.6 µg/g DW) of spilanthol was synthesized. The study confirms the earlier reports which suggested that differentiated (organized and redifferentiated) cells and specialized organs generally produce most secondary products compared to dedifferentiated (unorganized) cells in cultures. (Rao and Ravishankar 2002; Tang *et al.* 2010). Further, it has been frequently observed that specialized plant organs show different expression pattern of secondary metabolites when inducted into the *in vitro* systems (Łuczkiewicz and Głód 2005; Lucchesini *et al*. 2009). Stafford et al. (1986) reported that the accumulation of secondary products in plant cell cultures depends on

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the composition of the culture medium, including the kind and concentration of plant growth regulators, mineral salts and carbon sources. Effect of growth regulators on secondary metabolite production has also been very well documented by other workers (Di Cosmo and Towers 1984; Moreno *et al*. 1995; Amit *et al*. 2005; Jaleel *et al*. 2009; Nagella and Murthy 2010; Anuradha *et al*. 2010; Lee *et al*. 2011).

Over the past decade, a number of biologically active compounds have been detected from S*pilanthes,* such as spilanthol and scopoletin (Ramsewak *et al*. 1999; Prachayasittikul *et al*. 2009). However, the studies for quantification of these compounds have never been performed in this plant. Moreover, this is the first study on comparing the scopoletin and spilanthol content in the tissue-culture derived plants of *Spilanthes* established from field grown elite mother plants with known metabolite content. These findings have opened up the possibility of producing *Spilanthes* plants with desired metabolite content, throughout the year, consistently, irrespective of the season, which will help the pharmaceutical industry to achieve better yield by using superior quality raw materials.

In the present investigation, *Spilanthes* cell suspension cultures, from undifferentiated superior callus lines containing spilanthol, were also raised and examined for the production and accumulation of spilanthol and scopoletin. In suspension cultures only spilanthol was detected and scopoletin was altogether absent. Moreover, the amount of spilanthol produced was very low (91.4 µg/g DW) compared to that produced by the source callus (998.03 µg/g DW) lines. This is an important feature observed in many cases, which could be due to low degree of cell differentiation and cell to cell interaction in cell suspension cultures of plants (Chawla 2002). As product formation in plants largely occurs in differentiated tissue, it seems reasonable that the undifferentiated state might not favour the formation of secondary products (Tang *et al.* 2010). For example, in callus cultures of *Ammi majus*, the amount of umbelliferone produced was 0.8 mg of DW whereas in the case of cell suspension cultures, it was reduced to 0.1 mg of DW (Staniszewska *et al*. 2003).

In the current study, phosphate utilization from culture medium was found to be faster than nitrate and sucrose. Similar type of kinetic profiles, where phosphate is consumed faster than the nitrate has been observed by many workers in different plant species (Zhang *et al*. 1996; Fett-Neto *et al*. 2004; Prakash and Srivastava 2007;

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Srivastava *et al*. 2011). Similar to the present study, growth-associated synthesis of secondary metabolites has been observed in case of *Lantana camara* (Srivastava *et al*. 2011) and *Panax quinquefolium* (Kochan and Chmiel 2011). Furthermore, it was found that the growth period of cells in liquid medium is reduced by seventeen days as compared to the growth of callus cultures. This may be due to of facilitated nutrient transport in liquid medium (Nigra *et al*. 1990). In the current study, this reduced growth period, however, had an adverse effect on production of spilanthol. It was observed that the cultures remained in the exponential phase (growth phase) for a very short period of 6 to 18 days. As the synthesis of these compounds was found to be growth associated, this shortened growth phase (thus quick attainment of decline phase), resulted in relatively low yield of compounds as compared to the callus cultures maintained in semi-solid media. The exponential phase was much longer for callus in the semi-solid medium with the growth cycle of five weeks. On the basis of these results, it can be recommended that during scale up, fed-batch cultivation is a better approach in such cases and can be used to extend the exponential phase of cultures with the supply of growth limiting nutrients at regular intervals. Fed batch cultivation has been frequently used in plant cell cultures for enhancing the biomass and production of secondary metabolites (Zhang *et al*. 1996; Wang *et al*. 1999; Chattopadhay *et al*. 2004; Wu *et al*. 2007).

It has been previously reported that growth, development and chemical profile of *in vitro* cell cultures were highly dependent on type and concentration of carbohydrates used in the medium (Rolland *et al*. 2006; Wang and Weathers 2007). Among several carbohydrates, sucrose tends to be the most efficacious carbon and energy source for cultured plant cells, especially with respect to secondary metabolite production. Sucrose introduced externally to plant cell suspensions is usually rapidly hydrolyzed to glucose and fructose that are then taken up by a passive or active transport process, depending on the species. This hydrolysis appears to be catalyzed by a wall-bound or extracellular acid invertase (Masuda *et al*. 1988). Beneficial effect of sucrose over other carbon sources for metabolite production has been previously reported in many species (Bondarev *et al*. 2003; Nagella and Murthy 2010). In addition to serving as carbon and energy sources, sugars also affects the osmotic pressure of the medium (Su 1995). In the present study also, the highest amount of

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spilanthol was synthesized in the medium containing sucrose (91.4 μg/g DW), followed by glucose supplemented medium (56.8 μg/g DW). The lowest amount of spilanthol was present in fructose containing medium. The positive effect of sucrose may be related to its increasing the osmotic pressure of the medium, which stimulates mitochondrial activity and, hence, production of energy (Bonga and Von Aderkas 1992) required for metabolite synthesis. In the combined presence of glucose and fructose, on hydrolysis of sucrose, cell exhibit preference for glucose whereas fructose is utilized only after glucose is depleted from the medium (Kretzschmar *et al*. 2007). Thus, presence of sucrose facilitated the constant availability of utilizable forms of carbohydrates. If glucose or fructose applied individually, in the medium, resulted in starvation like situation due to faster consumption of glucose within few days by the cells or a very slow uptake of fructose by the cells in the intial growth phase.

Agitation speed is a very important parameter for establishment of plant cell suspension cultures. It is mainly responsible for mixing the plant cells in the medium and, thus, to facilitate homogeneous nutrient uptake, and also for providing a sufficient O_2 and CO_2 supply. However, agitation and aeration cause hydrodynamic stress on the cells, which can be attributed to the physical characteristics of the suspended cells, such as their size, the presence of thick cellulose based cell wall, and existence of large vacuoles (Chattopadhyay *et al*. 2002). The cells subjected to these shear forces show many physiological and morphological changes, such as aggregate size and shape, cell integrity and viability, and finally biomass accumulation and secondary metabolism. In this perspective, viability test by a dye, fluorscein diacetate (FDA), is important in determining viability of cells in suspension cultures. In the present study, maximum viable cultures were obtained at 120 rpm as confirmed by bright green colour of FDA. Below (90 and 60 rpm) and above this speed (150 rpm), the cells either aggregated or died due to rupturing and, thus, did not take up the stain. FDA is a non-fluorescing, non-polar dye that freely permeates through the plasma membrane. Live cells cleave FDA by esterase activity and produce the polar fluorescent portion, fluorscein, which cannot cross plasma membrane of living cells while in dead and broken cells it is lost. Therefore, only the live, intact cells take up the stain and fluoresce green.

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4B.3. Bioassays

In spite of being a plant of potential medicinal interest there are a very few reports that document the properties of *Spilanthes* scientifically. Therefore, in the present study, cell culture and *in vivo* leaf extracts of *S. acmella* were investigated for antioxidant, antimicrobial and anthelmintic properties. Antioxidant activity of *Spilanthes* extracts were evaluated by DPPH method. The DPPH is a purple-coloured stable free radical, which is reduced to α,α-diphenyl-β-picrylhydrazine (yellow coloured) by accepting an electron or hydrogen radical from antioxidants (Soares *et al*. 1997; Hossain and Shah 2011). So far, there are two reports on the antioxidant activity of *Spilanthes* extracts (Wongsawatkul *et al*. 2008; Prachayasittikul *et al*. 2009). However, in both these reports, crude extracts obtained from field grown plants were used for the assay. In this study, for the first time, *Spilanthes in vitro* cultures were examined for antioxidant property. Results of the antioxidant assay exhibited that methanolic extracts of both callus and leaf have higher antioxidant potential compared to water, ethyl acetate and hexane extracts. Both callus and leaf methanolic extracts showed DPPH scavenging activities in a dose dependent manner and their EC_{50} values were 1085.1 μg/ml and 1342.9 μg/ml, respectively. In accordance with this study, many reports have suggested that relatively higher antioxidant activity was observed from methanolic extracts compared to the other solvents including acetone, diethyl ether, ethyl acetate and water (Zieliński and Kozłowska 2000; Oki *et al*. 2002). It was generally assumed that dedifferentiated cultures proved to be less active as antioxidants (Grzegorczyk *et al*. 2007). However, present results indicated that dedifferentiated callus extracts showed significant antioxidant potential, which could be explored further as a source of natural antioxidants and nutraceuticals to enhance health benefits. Similar to this study, dedifferentiated cell lines of *Rosmarinus officinalis* (Yesil-Celiktas *et al*. 2007); *Harpagophytum procumbens* (Georgiev *et al*. 2010) and *Psoralea corylifolia* (Shinde *et al*. 2010) have shown promising antioxidant activity in DPPH assay.

Several studies have revealed that the phenolic contents in the plants are associated with their antioxidant activities, probably due to their redox properties, that allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Chang *et al.* 2001). Therefore, the content of total phenolic compounds in the

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Spilanthes callus and *in vivo* leaf extracts were determined through a linear gallic acid standard curve. The highest total phenolic compounds were detected in the methanolic extracts of both, callus and leaf, whereas the lowest content was obtained in the hexane extracts of callus and leaf. These findings clearly demonstrate the influence of the solvent on the extractability of phenolics. Finding of this study is in agreement with previous reports which suggested that nature of solvent exert a great influence on phenolic extraction capacity of many species (Akowuah *et al*. 2005; Turkmen *et al*. 2006).

Besides antioxidant activity, antibacterial activity of *Spilanthes* has also been evaluated, in the present study. Extracts from callus cultures and *in vivo* leaves were tested by disc diffusion method against a range of pure strains of bacteria which consisted of gram +ve and gram –ve or moderate pathogenic to severe pathogenic. The results illustrated that the leaf derived methanolic extract possessed effective antibacterial activity against some of the isolates, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus mutans* with zone of inhibition ranging from 12.3, 8.6 and 8.0 mm, respectively. However, dedifferentiated callus extracts did not exhibit any antibacterial activity. Rani and Murthy (2005) investigated antimicrobial activities of *S. acmella* flower head extracts against *Bacillus sphaericus, B. subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella aerogenes* and *Chromobacterium violaceum* and observed that *B. sphaericus, B. subtilis* and *S. aureus* were more sentitive for *Spilanthes* extract than *P. aeruginosa, K. aerogenes* and *C. violaceum.* Prachayasittikul *et al*. (2009) also demonstrated antimicrobial efficacy of different extracts of *Spilanthes,* against 27 strains of microorganisms, by agar dilution method. They observed that the methanol and chloroform extracts inhibited the growth of many tested organisms. Most susceptible microorganism in their study was *Corynebacterium diphtheriae* followed by *Bacillus subtilis, Staphylococcus epidermidis, Micrococcus lutens,* and *Saccharomyces cerevisiae.* The range of minimum inhibitory concentration of tested extracts was 0.064-0.256 mg/ml.

Regarding anthelmintic activity, results illustrated that callus extracts of *Spilanthes* has higher a anthelmintic activity than the *in vivo* leaf extracts. As far as ascertained, this is the first scientific evidence of the anthelminthic property of *Spilanthes.* Moreover, *Spilanthes in vitro* cultures were evaluated for anthelmintic property, for

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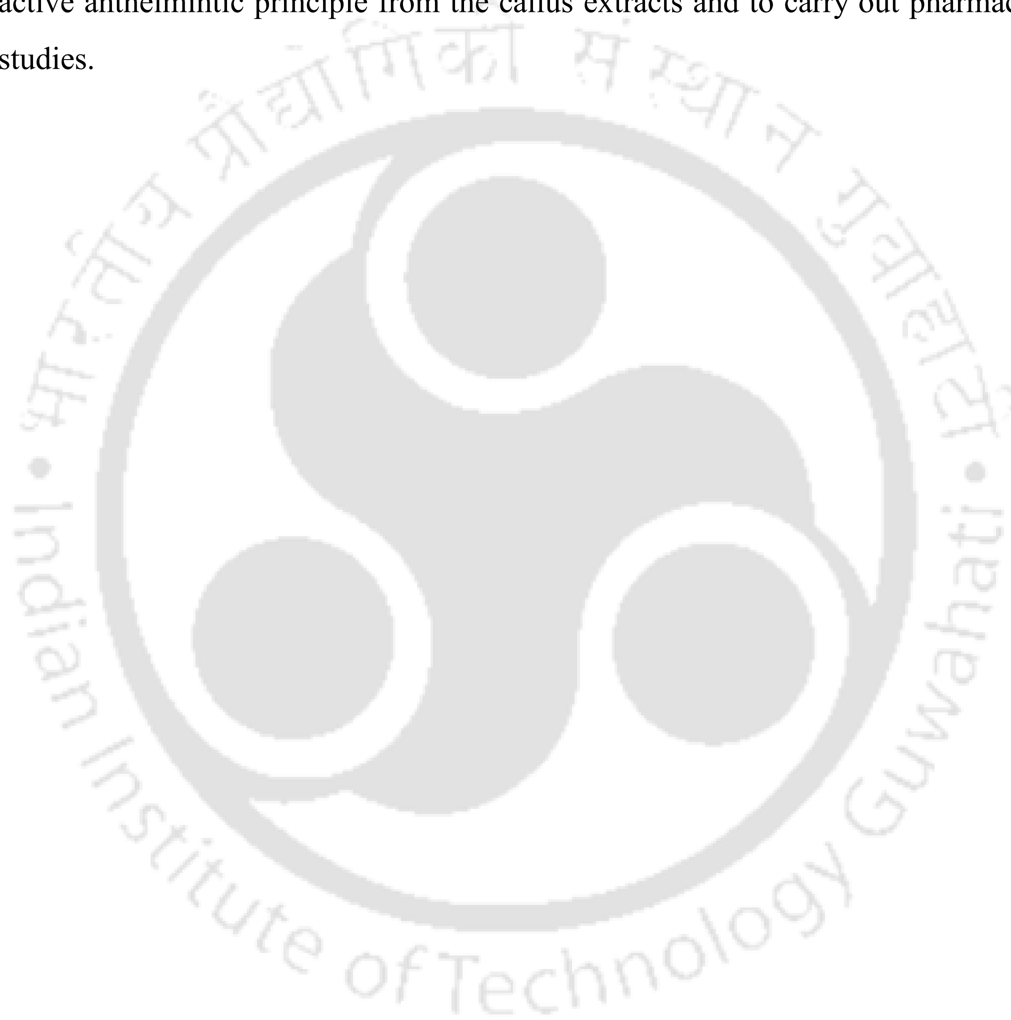
the first time. The higher anthelmintic property of callus extract compared to the field grown plant might be due to enhanced production of some bioactive compounds in callus cultures. Nonetheless, presence and interference of other compounds cannot be ruled out in *in vitro* conditions which might be responsible for the observed anthelmintic activity. The production of effective drugs by *in vitro* cultures may hold a great promise as a source of obtaining effective anthelmintic agents for commercial purpose, particularly in developing countries. Further work is required to find out the active anthelmintic principle from the callus extracts and to carry out pharmaceutical studies.



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Chapter 5

Conclusions and Future Prospects

The present study is an endeavour to recognize the potential of two important medicinal plants, *Azadirachta indica* A. Juss. and *Spilanthes acmella* Murr., through *in vitro* techniques. Biochemical studies demonstrated the potential of cell cultures as source of important secondary metabolites. Bioassays carried out in *Spilanthes* plant further confirmed the utility of these studies. Key points of the entire work are depicted below.

- In *Azadirachta indica* A. Juss. reproducible protocols for regeneration from various explants (embryos, leaves and ovaries) have been established.

- Ploidy analysis of the *in vitro* regenerated shoots strongly indicated that all the samples analyzed have the same ploidy level as that of the parent plant.

- HPLC and MS analysis of six *in vitro* cell lines (dedifferentiated and redifferentiated), established from different explants sources, *viz*. zygotic embryo, leaf and ovary, revealed the presence of azadirachtin in each cell line. Among all, redifferentiated zygotic embryo cultures contained maximum (2.33 mg/g DW) amount of azadirachtin.

- Azadirachtin production from redifferentiated zygotic embryo cultures was optimized by response surface methodology. Among the variables (MS major salts, BAP, IAA, CH and sucrose) studied, sucrose, MS major salts and BAP, were found to significantly affecting azadirachtin production. Under optimal medium compositions (full MS major salts; 5.68% sucrose and 10.42 µM BAP), the experimental value of 4.97 mg/g DW perfectly matched with predicted value

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of 5.13 mg/g DW. The experimental value was 2.16 times higher than the control medium in which 2.33 mg/g DW of azadirachtin was produced.

- In *Spilanthes acmella*, efficient and reproducible protocols for *in vitro* plant regeneration from nodal and leaf explants have been developed. In this study, detailed description on *in vitro* shoot multiplication, rooting and hardening are described.

- Using nodal cultures, 20-fold shoot multiplication was achieved in 5 weeks on MS + BAP (5.0 µM).

- Leaf-disc cultures showed callusing and direct shoot proliferation responses. While the highest rate of callus formation (100%) was observed on media containing combinations of BAP (5.0 µM), NAA (1.0 µM) and 2, 4-D (1.0 µM), maximum direct shoot regeneration (51 shoots) in 250 ml conical flasks occurred on MS + BAP (5.0 µM) + IAA (5.0 µM).

- Shoots obtained from nodal segments and leaf-disc cultures were successfully rooted on half strength MS medium (major salts reduced to half strength) with 50g/l sucrose, with a frequency of 100%. Transplantation survival of micropropagated plants was more than 88%.

- The ploidy level of regenerated plants derived from nodal segment and leaf-disc cultures was assessed. Results of ploidy analysis showed that all investigated plants had the same ploidy level as the mother plants.

- For the first time, HPLC analysis of *Spilanthes in vitro* cell lines has been done for scopoletin estimation. The novelty of the method includes short analysis time (less than 10 minutes) with isocratic elution and low limit of detection by fluorescence detector. The results showed that the scopoletin content of nodal segment derived plants was comparable to the mother plant (0.10 µg/g DW of

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leaves). In leaf-disc derived plants, significantly lower amount of scopoletin (0.03 µg/g DW) was synthesized and it was completely absent in callus.

- Spilanthol detection and quantification in *in vitro* materials is an interesting and new finding in this study. Interestingly, leaf-discs derived plants of the *Spilanthes*, accumulated more amount (3294.36 µg/g DW) of spilanthol compared with their field-grown (2703.66 ± 9.6 µg/g DW) counterparts.

- In cell suspension cultures only spilanthol was detected and scopoletin was altogether absent. Synthesis of spilanthol in suspension culture was found to be growth associated.

- Additionally, positive antioxidant and anthelmintic activities of *in vitro* cultures showed immense importance and application of *Spilanthes in vitro* cultures.

FUTURE PROSPECTS:

- Elicitation and precursor feeding strategies can be carried out for enhanced production of azadirachtin.

- Further purification and characterization of components from aqueous extract of *Spilanthes* and determination of their bioactive potential can be attempted.

- The results of batch kinetics study can serve as a background for further scale-up related aspects in bioreactors.

- Medium constituents can be statistically optimized for enhanced production of spilanthol in cultures.

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APPENDIX

AZADIRACHTA INDICA A. JUSS.

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Order Rutales
Family Meliaceae
Genus *Azadirachta*
Species *indica*

SPILANTHES ACMELLA MURR.

Kingdom Plantae – Plants
Subkingdom Tracheobionta – Vascular plants
Superdivision Spermatophyta – Seed plants
Division Magnoliophyta – Flowering plants
Class Magnoliopsida – Dicotyledonous
Order Asterales
Family Compositae
Genus *Spilanthes*
Species *acmella*

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List of Publications

In National/ International Journals

1. **Mithilesh Singh** and Rakhi Chaturvedi. Statistical media optimization for enhanced azadirachtin production from redifferentiated zygotic embryo cultures of Neem. *In Vitro* Cell. Dev. Biol. **2011.** (Accepted)
2. **Mithilesh Singh** and Rakhi Chaturvedi. Improved clonal propagation of *Spilanthes acmella* Murr. for production of scopoletin. Plant Cell Tiss. Org. 103:243-253, **2010**.
3. **Mithilesh Singh** and Rakhi Chaturvedi. Optimization of *Spilanthes acmella* L. cultivation by *in vitro* nodal segment culture. Acta Hort. 865:109-114, **2010**.
4. Priyanka Srivastava, Rashmi Rekha Hazarika, **Mithilesh Singh** and Rakhi Chaturvedi. Assessment of age and morphometric parameters of seeds on azadirachtin production in Neem seed kernels collected from various ecotypes. Res. J. Chem. Environ. 14:24-28, **2010**.
5. **Mithilesh Singh** and Rakhi Chaturvedi. An efficient protocol for cyclic somatic embryogenesis in Neem (*Azadirachta indica* A Juss). Int. J. Environ. Sci. Eng. 1:49-51, **2009**.
6. Priyanka Srivastava, **Mithilesh Singh**, Prateek Mathur and Rakhi Chaturvedi. *In vitro* organogenesis and plant regeneration from unpollinated ovary: a novel explant of Neem (*Azadirachta indica* A. Juss.). Biol. Plant. 53 (2):360-364, **2009**.
7. Priyanka Srivastava, **Mithilesh Singh** and Rakhi Chaturvedi. Effect of casein hydrolysate and major inorganic salts on clonal propagation from nodal explants of a mature Neem tree, *Azadirachta indica* A. Juss. Res. J. Biotechnol. 4 (4):30-38, **2009**.

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8. Priyanka Srivastava, **Mithilesh Singh** and Rakhi Chaturvedi. Production of azadirachtin in anther cultures of *Azadirachta indica* A. Juss. and its bioactivity against *Aspergillus sydowii*. Icfai Univ. J. Biotechnol. III (3):38-45, **2009**.

Book Chapters

9. **Mithilesh Singh** and Rakhi Chaturvedi. Somatic embryogenesis in neem (*Azadirachta indica* a. Juss.): current status and biotechnological perspectives. (In Press).

10. Priyanka Srivastava, **Mithilesh Singh** and Rakhi Chaturvedi. Biotechnological improvement of neem. In: K. Ashwani (Editor) Advances in Plant Biotechnology. I.K. International Publishing House Pvt. Ltd., New Delhi, India, **2010.** (In Press).

11. **Mithilesh Singh** and Rakhi Chaturvedi. *De novo* shoot and root organogenesis in leaf disc cultures of *Azadirachta indica* A. Juss. In: S.K. Borthakur (Editor) Bioresources of North East India: Industrial Potential and Intellectual Property Right Issues. Bishan Singh and Mahendrapal Singh, Deharadun, India, **2009.** (In Press).

Papers in National/ International Conferences/ Seminars/ Symposia

12. **Mithilesh Singh** and Rakhi Chaturvedi. Antioxidant activity of extracts from *in vitro* cultures of *Spilanthes acmella* Murr. and ferulic acid determination. In: 79th Society of Biological Chemists (india) Meeting, December 13-15, 2010. Indian Institute of Science, Bangalore, Karnataka, India. Page No. 183, **2010.**

13. **Mithilesh Singh** and Rakhi Chaturvedi. Screening of *in vitro* cell lines for enhanced azadirachtin production. In: International Conference on Food Security and Environmental Sustainability, December 17-19, 2009. Agricultural and Food Engineering Department, IIT Kharagpur, India. Page No. 1-7, **2009**.

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14. **Mithilesh Singh** and Rakhi Chaturvedi. An efficient protocol for cyclic somatic embryogenesis in neem (*Azadirachta indica* A Juss) In: International Conference on Energy and Environment, March 19-21, 2009. National Institute of Technology, Kurukshetra, Haryana, India. Page No. 78, **2009**.

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FIGURE 1

NEEM

ZYGOTIC EMBRYO CULTURE

Callus Induction and Shoot Regeneration

A. Early-late dicotyledonary stage of embryos at the time of culture (bar = 0.05 cm)

B. A 4-week-old dedifferentiated callus proliferated from zygotic embryo explants on MS + BAP (5.0 µM) + 2,4-D (1.0 µM + NAA (1.0 µM) medium (bar = 0.4 cm)

C. Histological section of a nodulated 5-week-old callus culture on MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l), showing vascular strands within the calli (bar = 25 µm)

D. A 5-week-old callus subculture of **C**, showing shoot proliferation and well developed shoots (bar = 0.59 cm)

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FIGURE 2

NEEM

ZYGOTIC EMBRYO CULTURE

Somatic Embryogenesis

A. 4-week-old culture showing somatic embryogenesis at hypocotyl (hyp) and plumular (plu) regions of zygotic embryo on MS + TDZ (1.0 µM) + GA_3 (1.0 µM). There was no differentiation at radicular (rad) region (bar = 0.32 cm)

B. Same as **A**, showing somatic embryos as well as shoots at plumular end (bar = 0.18 cm)

C-D. 4-week-old cultures showing somatic embryogenesis from cotyledons of zygotic embryo on MS + TDZ (1.0 µM) + ABA (1.0 µM). Note the presence of somatic embryos at various stages of development from globular, heart, torpedo and dicot stages (**C,** bar = 0.07 cm; **D**, bar = 0.14 cm)

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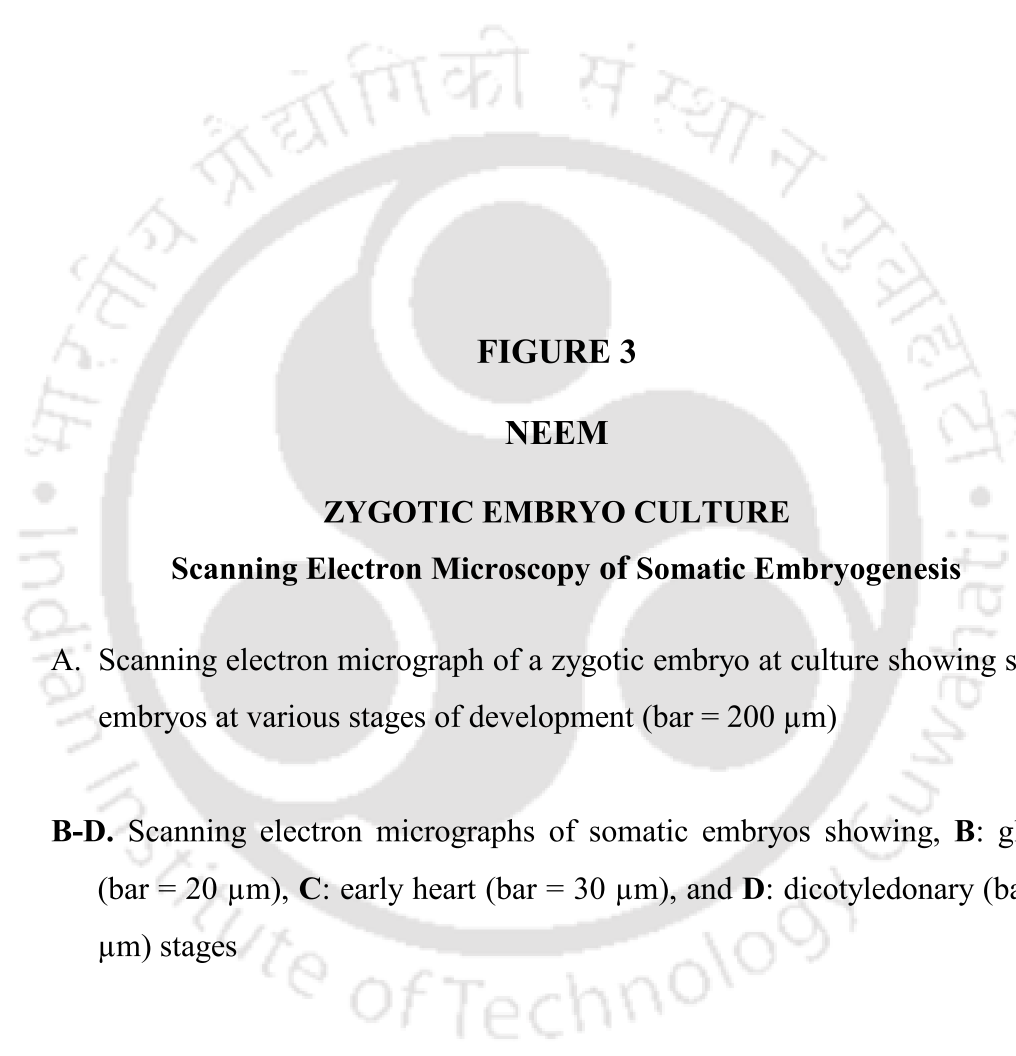

FIGURE 3

NEEM

ZYGOTIC EMBRYO CULTURE

Scanning Electron Microscopy of Somatic Embryogenesis

A. Scanning electron micrograph of a zygotic embryo at culture showing somatic embryos at various stages of development (bar = 200 µm)

B-D. Scanning electron micrographs of somatic embryos showing, **B**: globular (bar = 20 µm), **C**: early heart (bar = 30 µm), and **D**: dicotyledonary (bar = 30 µm) stages

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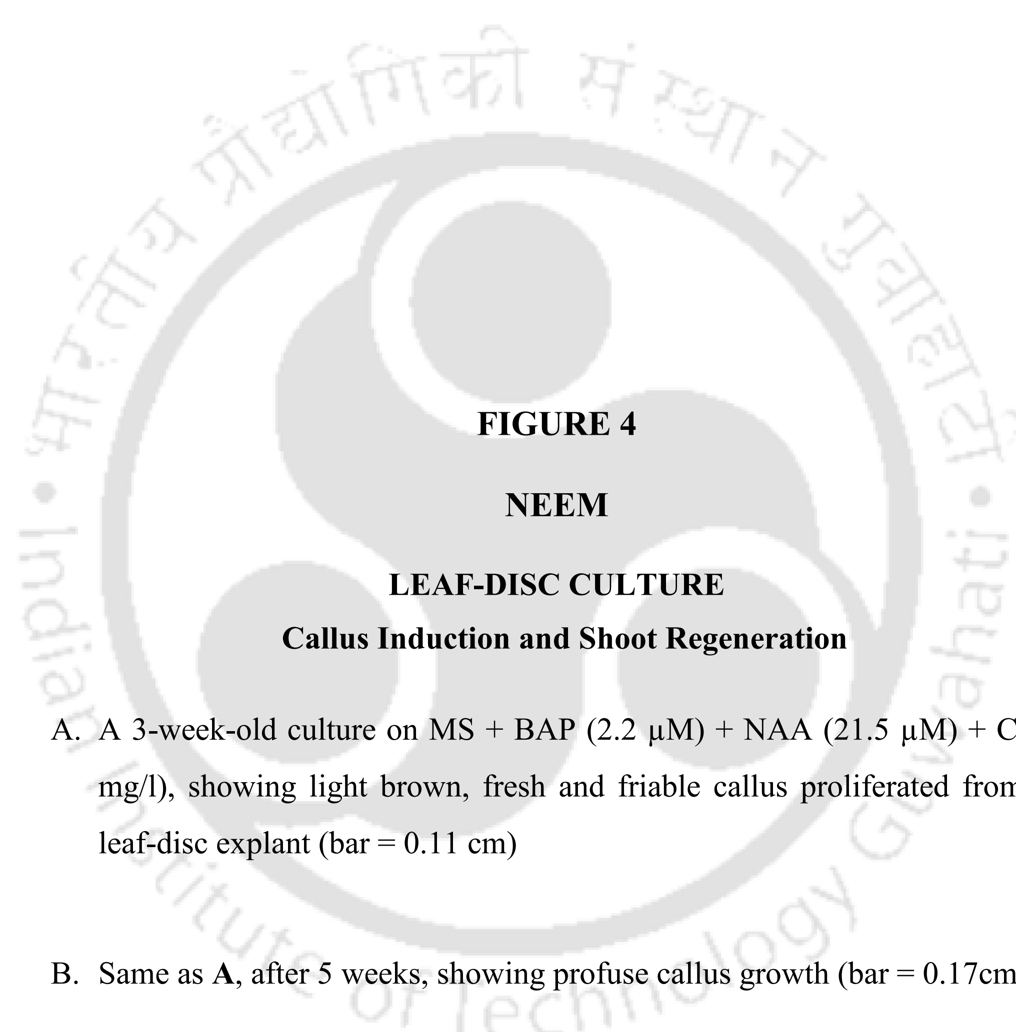

FIGURE 4

NEEM

LEAF-DISC CULTURE

Callus Induction and Shoot Regeneration

A. A 3-week-old culture on MS + BAP (2.2 µM) + NAA (21.5 µM) + CH (500 mg/l), showing light brown, fresh and friable callus proliferated from entire leaf-disc explant (bar = 0.11 cm)

B. Same as **A**, after 5 weeks, showing profuse callus growth (bar = 0.17cm)

C. A 3-week-old culture of leaf-disc on MS + BAP (5.0 µM), showing shoot differentiation from brown and dark-green, nodulated, compact callus developed at the cut end of the explant (bar = 0.11 cm)

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D. Histological analysis of a regenerating culture from **C**, showing compactly arranged cells and tracheids (33 µm)

FIGURE 5

NEEM

OVARY CULTURE

Callus Induction and Shoot Regeneration

A. Flower buds of 4 mm size used for ovary culture (bar = 0.7 cm)

B. An excised ovary from 4 mm sized flower bud (bar = 0.05 cm)

C. A 2-week-old ovary slice culture on MS + 2,4-D (0.5 µM) + Kinetin (4.5 µM), showing emergence of cream, fresh and friable callus from cut regions (bar = 0.2 cm)

D. Same as **C**, after 4-weeks, where the entire explant is covered with the cream, friable and fast growing callus (bar = 0.37 cm)

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E. A 4-week-old callus subculture on MS + BAP (5.0 μM) + IAA (0.5 μM), showing shoot proliferation (bar = 0.35 cm)

F. A 4-week-old bright green, compact callus on MS + BAP (9.0 μM) + IAA (5.0 μM) + CH (500 mg/l) (bar = 0.4 cm)

G. Same as **F**, 4 weeks after subculture to the same medium, showing differentiation of shoots from dark green compact nodular regions (bar = 0.52 cm)

H. Histological section of a regenerating ovary callus, showing well developed tracheids (bar = 30 μm)

FIGURE 6

NEEM

SHOOT ELONGATION, MULTIPLICATION AND ROOTING

A. A shoot on elongation medium, MS + BAP (0.5 μM), after 5 weeks (bar = 1cm)

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B. Single nodal segment from **A** cultured on MS + BAP (1.0 µM) + CH (250 mg/l). The axillary bud has developed into single, long multinodal shoot, after 6 weeks (bar = 1 cm)

C. An *in vitro* shoot on ¼ MS + IBA (0.5 µM), showing healthy, thick, cream coloured, branched roots developed directly at the basal cut end (bar = 1.1 cm)

FIGURE 7

NEEM

FLOW CYTOMETRIC ANALYSIS

Flow-cytometric histograms of nuclei stained with propidium iodide and isolated from:

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A. Leaf tissues of neem parent plant (control)

B. Leaf tissues of micropropagated shoots derived from zygotic embryo cultures of neem

C. Leaf tissues of micropropagated shoots derived from leaf-disc cultures of neem

D. Leaf tissues of micropropagated shoots derived from ovary cultures of neem

FIGURE 8

NEEM

AZADIRACHTIN ANALYSIS

Chromatograms of Standard and Control Samples

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A. Chromatographic profile of standard azadirachtin (arrow marked)

B. Chromatographic profile of *in vivo* seed extract showing azadirachtin peak (control I)

C. Chromatographic profile of *in vivo* leaf extract showing azadirachtin peak (control II)

D. Chromatographic profile of *in vivo* ovary extract showing azadirachtin peak (control III)

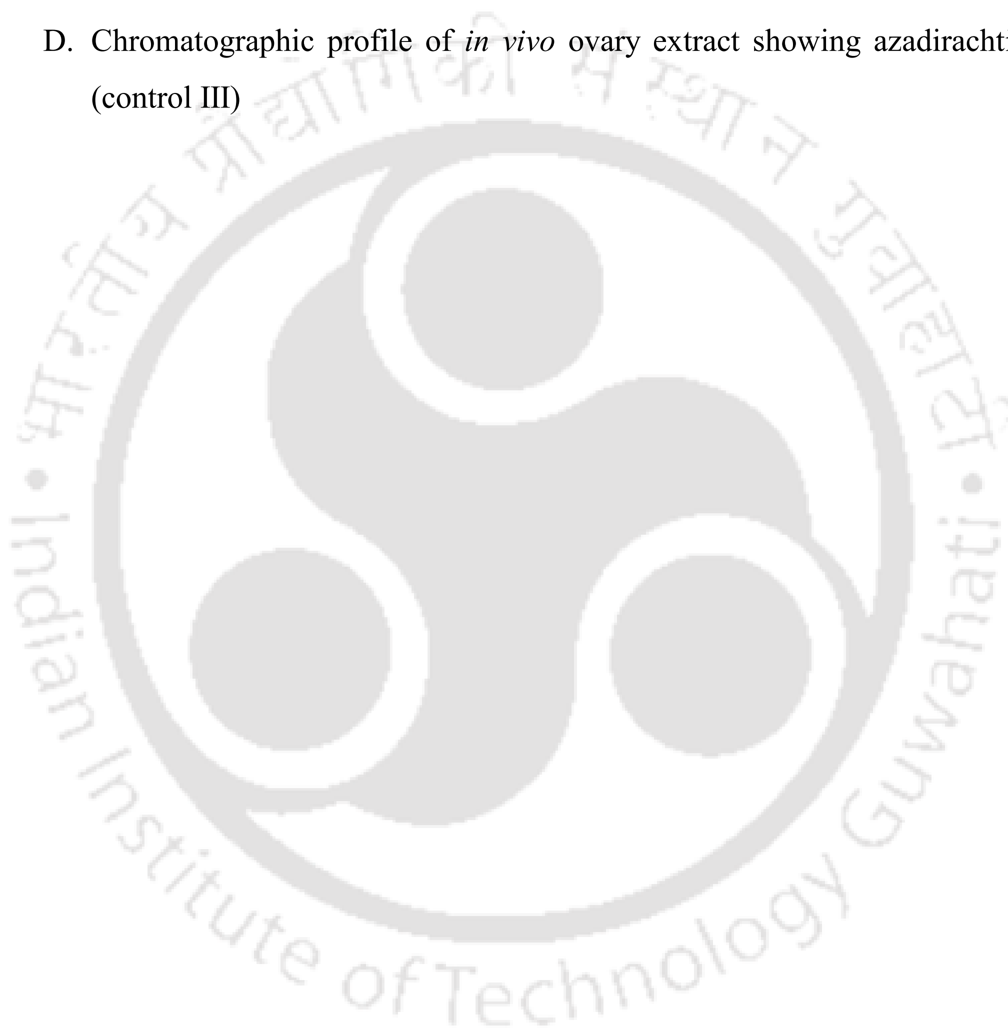

FIGURE 9

NEEM

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AZADIRACHTIN ANALYSIS

Chromatograms of Callus Lines

A. A chromatogram of *in vitro* redifferentiated zygotic embryo callus showing azadirachtin peak (arrow marked)

B. A chromatogram of *in vitro* dedifferentiated zygotic embryo callus showing azadirachtin peak (arrow marked)

C. A chromatogram of *in vitro* redifferentiated leaf callus showing azadirachtin peak (arrow marked)

D. A chromatogram of *in vitro* dedifferentiated leaf callus showing azadirachtin peak (arrow marked)

E. A chromatogram of *in vitro* redifferentiated ovary callus showing azadirachtin peak (arrow marked)

F. A chromatogram of *in vitro* dedifferentiated ovary callus showing azadirachtin peak (arrow marked)

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FIGURE 10

NEEM

AZADIRACHTIN ANALYSIS

Mass Spectroscopy

A. Mass spectrometric profile of standard azadirachtin

B. Mass spectrometric profile of HPLC eluted fraction of crude extract obtained from one of the best callus lines on MS + BAP (9.0 µM) + IAA (5.0 µM) + CH (500 mg/l) medium

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FIGURE 11

NEEM

STATISTICAL OPTIMIZATION

Response Surface Curves

Response surface curves of azadirachtin production showing interaction between:

A. Sucrose and BAP, fixed level: Major Salts

B. Major Salts and BAP, fixed level: Sucrose

C. Sucrose and Major Salts, fixed level: BAP

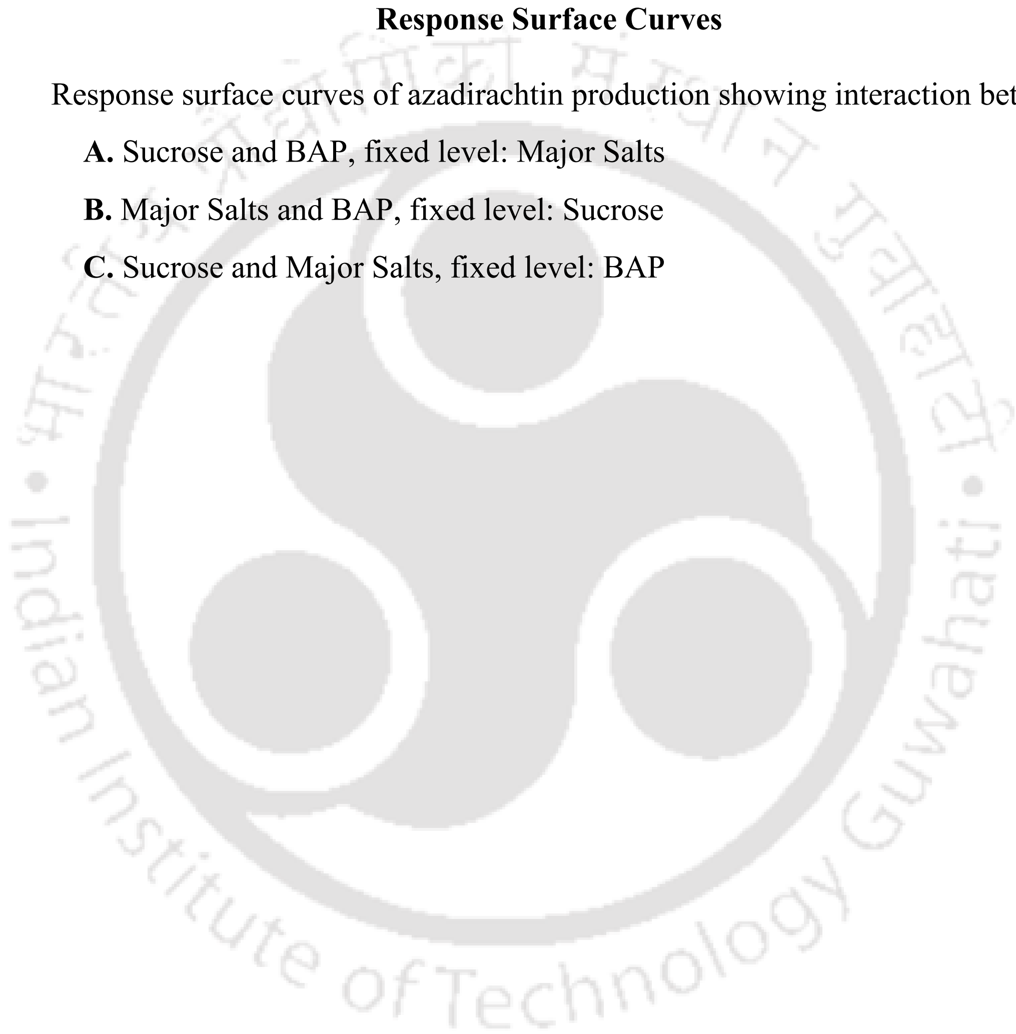

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FIGURE 12

SPILANTHES ACMELLA

NODAL SEGMENT CULTURE

Axillary Shoots Proliferation

A. A nodal segment on MS + BAP (5.0 µM), showing long, multinodal shoots, after 5 weeks of culture initiation (bar = 0.5 cm)

B. A nodal segment on MS + BAP (3.0 µM), after 8 weeks of culture initiation, showing a shoot with long internodes and with adventitious roots all over its surface (bar = 1.1 cm)

C. A nodal segment on MS + BAP (15.0 µM), after 8 weeks of culture initiation, showing stunted shoots with pale colored leaves (bar = 0.33cm)

D. An anatomical section of nodal segment from **B**, stained with acridine orange, showing differentiation of adventitious root (AdR) and position of axillary bud (AxB) (bar = 0.5 mm)

E. Same as **D**, showing origin of adventitious root differentiation (AdR) at different points (bar = 0.5 mm)

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FIGURE 13

SPILANTHES ACMELLA

NODAL SEGMENT CULTURE

Rooting and Hardening

A. A micropropagated shoot from MS + BAP (5.0 µM), rooted on ½ MS + Sucrose (50 g/l). Roots have developed directly from the basal cut end of the shoot (bar = 1.0 cm)

B. A hardened micropropagated plant, 1 month after transfer to soil (bar = 2.0 cm)

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FIGURE 14

SPILANTHES ACMELLA

LEAF DISC CULTURE

Shoot Proliferation

A-B. Leaf-disc cultures on MS + BAP (5.0 µM), showing shoot bud initiation; **A**. After one week (bar = 0.12 cm); **B**. After two week (bar = 0.18 cm)

C. Same, after 3 weeks, showing development of foliar structures (bar = 0.23 cm)

D. Four-week-old leaf-disc culture showing well developed shoots (bar = 0.9 cm)

E. Five-week-old leaf-disc culture, showing multiple number of shoots some of them have elongated (bar = 0.9 cm)

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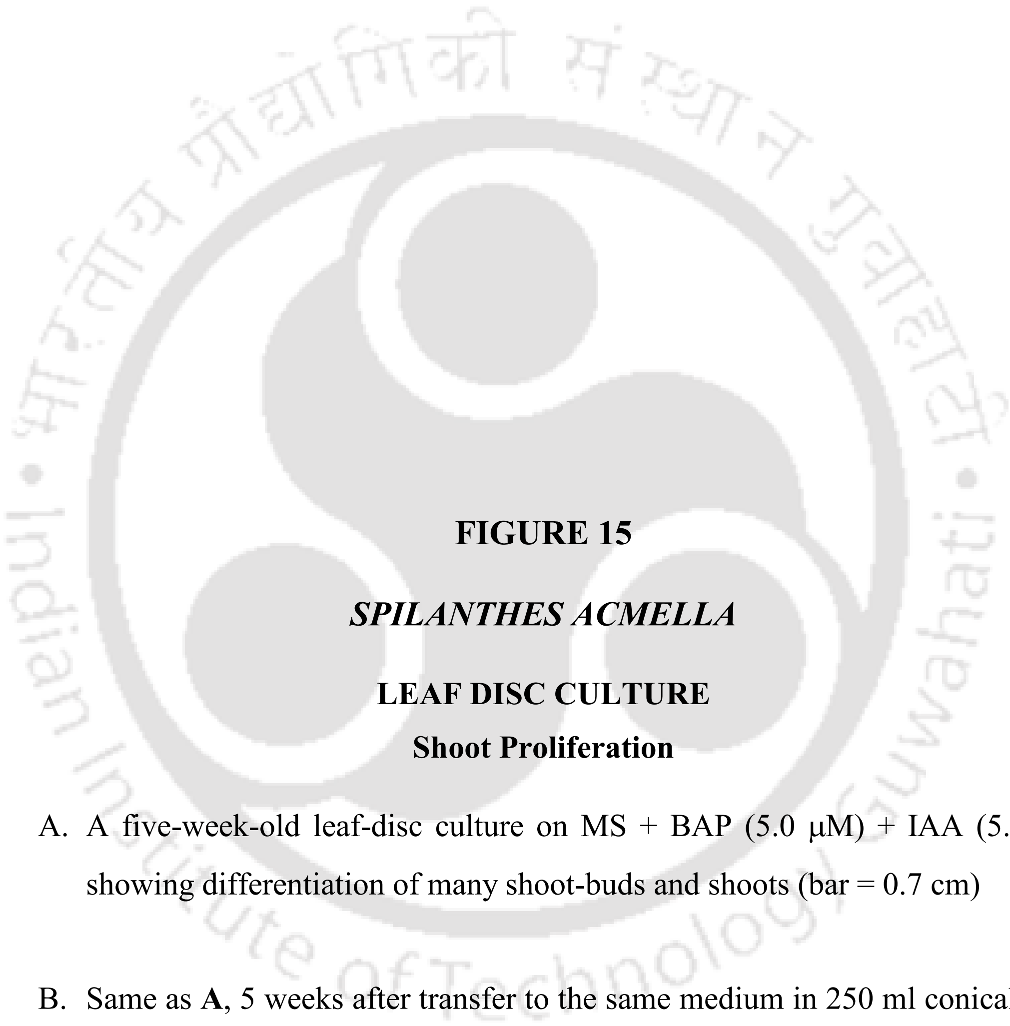

FIGURE 15

SPILANTHES ACMELLA

LEAF DISC CULTURE

Shoot Proliferation

A. A five-week-old leaf-disc culture on MS + BAP (5.0 µM) + IAA (5.0 µM) showing differentiation of many shoot-buds and shoots (bar = 0.7 cm)

B. Same as **A**, 5 weeks after transfer to the same medium in 250 ml conical flask, showing proliferation of many well developed shoots (bar = 1.49 cm)

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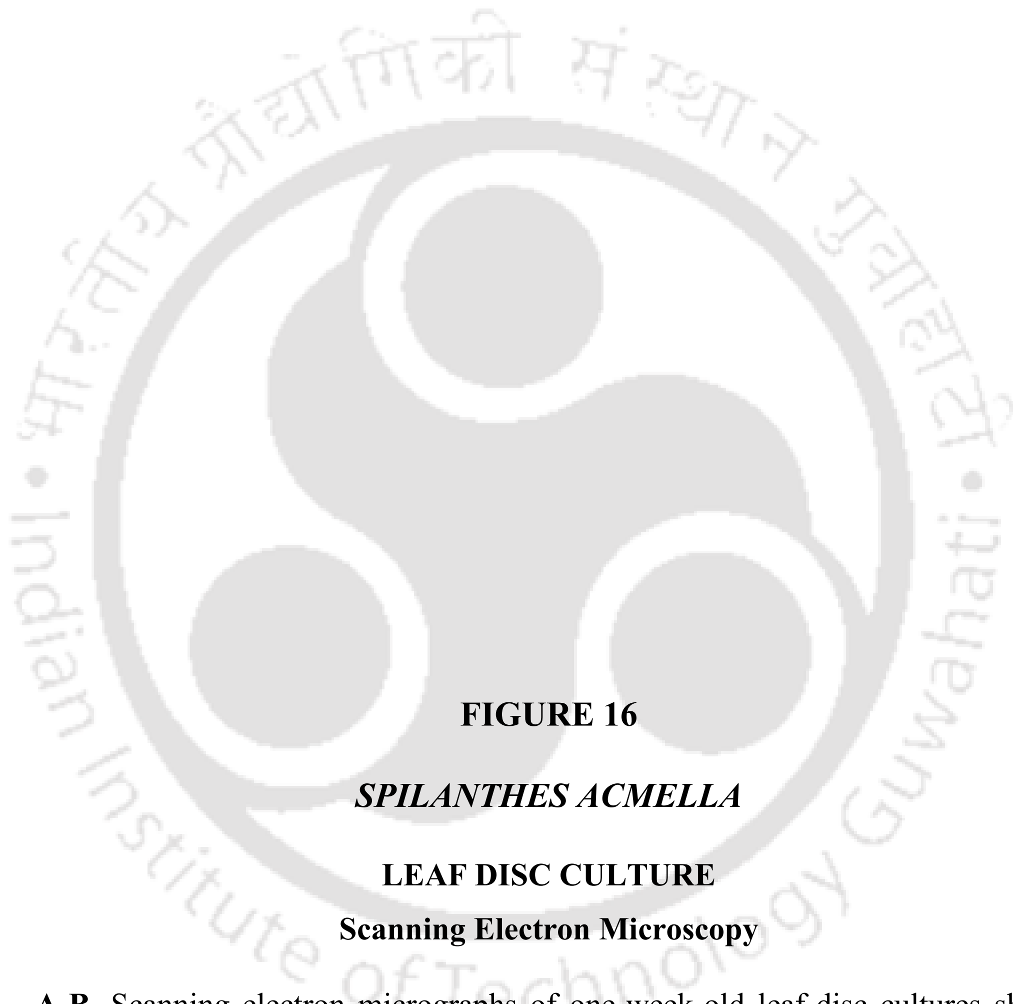

FIGURE 16

SPILANTHES ACMELLA

LEAF DISC CULTURE

Scanning Electron Microscopy

A-B. Scanning electron micrographs of one-week-old leaf-disc cultures showing initiation of shoot bud (A, bar = 246 µm; B, bar = 100 µm)

C. Scanning electron micrograph of 2-week-old leaf-disc cultures showing cluster of leaf primordial (bar = 300 µm)

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D. Enlarged view of same (**C**) with distinct leaf primordial (bar = 200 µm)

FIGURE 17

SPILANTHES ACMELLA

LEAF DISC CULTURE

Establishment of Callus Cultures

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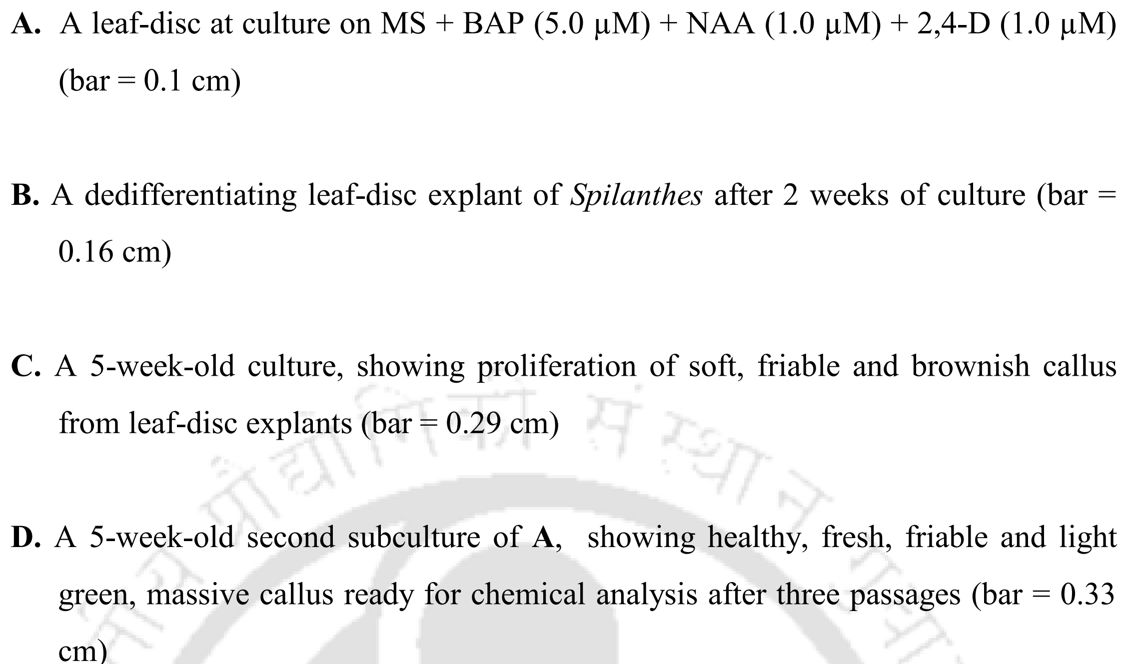

A. A leaf-disc at culture on MS + BAP (5.0 µM) + NAA (1.0 µM) + 2,4-D (1.0 µM) (bar = 0.1 cm)

B. A dedifferentiating leaf-disc explant of *Spilanthes* after 2 weeks of culture (bar = 0.16 cm)

C. A 5-week-old culture, showing proliferation of soft, friable and brownish callus from leaf-disc explants (bar = 0.29 cm)

D. A 5-week-old second subculture of **A**, showing healthy, fresh, friable and light green, massive callus ready for chemical analysis after three passages (bar = 0.33 cm)

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FIGURE 18

SPILANTHES ACMELLA

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PLOIDY ANALYSIS

Flow Cytometric Analysis

A. Representative flow-cytometric histogram of nuclei stained with propidium iodide and isolated from leaf tissues of field grown parent plant of *Spilanthes* (control)

B. Same, from micropropagated shoots obtained from nodal segment cultures of *Spilanthes*

C. Same, from micropropagated shoots derived from leaf-disc cultures of *Spilanthes*

FIGURE 19

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SPILANTHES ACMELLA

PLOIDY ANALYSIS

Cytology

A. A root-tip cell of parent plant (control), stained with 1% aceto-orcein, showing diploid number of chromosomes, 2n=2x=52 (x 3000X)

B. A root-tip cell of an *in vitro* plant, obtained from nodal segment culture, stained with 1% aceto-orcein, showing diploid number of chromosomes, 2n=x=52 (x 4000X)

C. A root-tip cell of an *in vitro* plant, obtained from leaf disc culture, stained with 1% aceto-orcein, showing diploid number of chromosomes, 2n=x=52 (x 3000X)

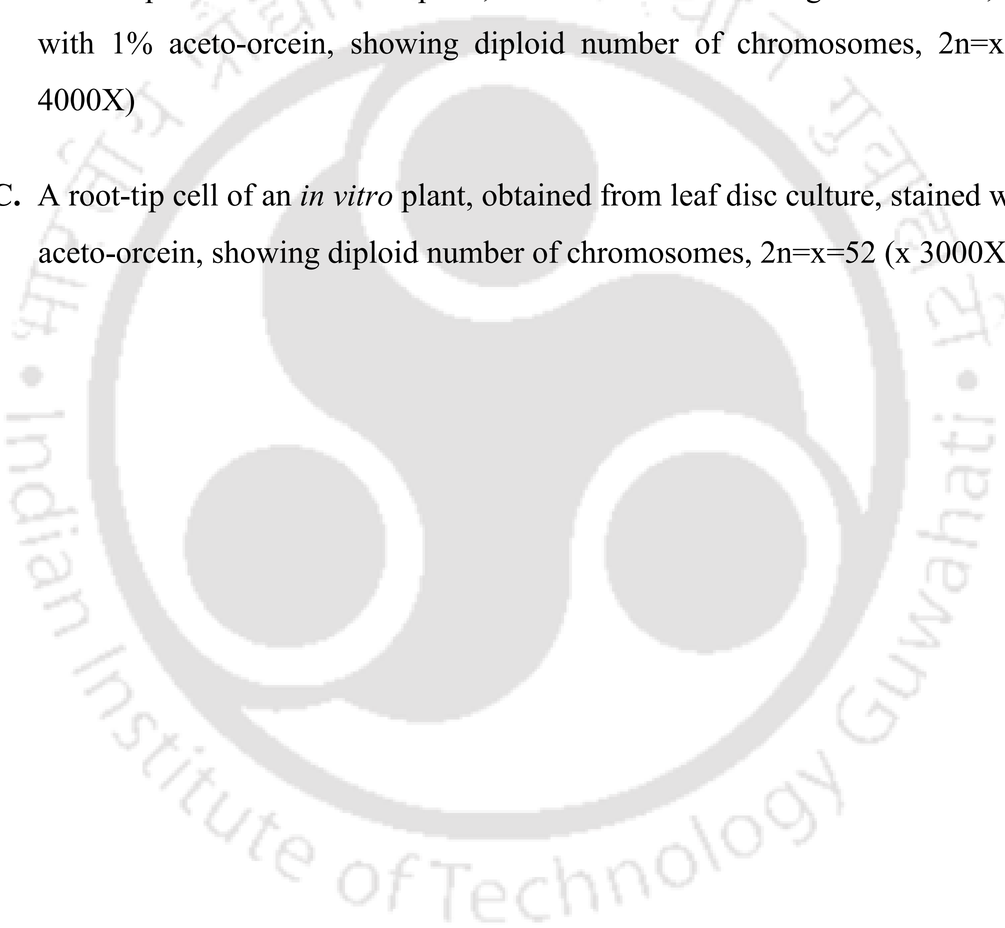

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FIGURE 20

SPILANTHES ACMELLA

SCOPOLETIN ANALYSIS

High Performance Liquid Chromatography

A. A chromatogram of standard scopoletin (arrow marked) showing single peak at retention time of 5.1 min

B. A chromatogram showing presence of scopoletin (arrow marked) in leaf extract of field grown plants (control)

C. A chromatogram showing presence of scopoletin (arrow marked) in leaf extract of *in vitro* plants proliferated on leaf-disc explants

D. A chromatogram showing presence of scopoletin (arrow marked) in leaf extract of *in vitro* plant established from nodal segment explants

E. A chromatogram of *in vitro* leaf extract spiked with standard scopoletin. Samples scopoletin and standard scopoletin co-eluted at the same retention time of 5.1 min (arrow marked)

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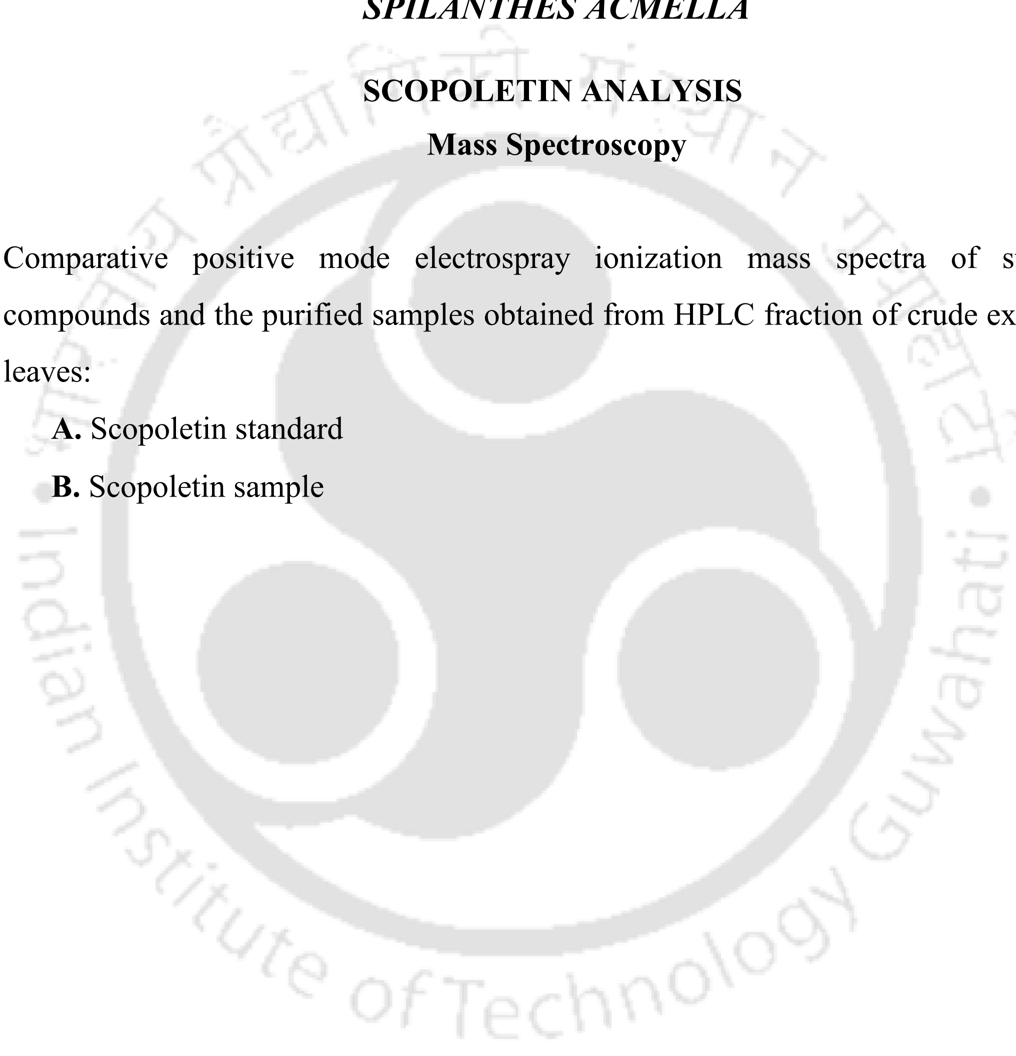
FIGURE 21

SPILANTHES ACMELLA

SCOPOLETIN ANALYSIS

Mass Spectroscopy



Comparative positive mode electrospray ionization mass spectra of standard compounds and the purified samples obtained from HPLC fraction of crude extract of leaves:

- **A.** Scopoletin standard
- **B.** Scopoletin sample

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FIGURE 22

SPILANTHES ACMELLA

SPILANTHOL ANALYSIS

High Performance Liquid Chromatography

A. A chromatogram showing presence of spilanthol (arrow marked) in leaf extract of field grown *Spilanthes* plants (control)

B. A chromatogram showing presence of spilanthol (arrow marked) in leaf extract of *in vitro* plant established from nodal segment explants

C. A chromatogram showing presence of spilanthol (arrow marked) in leaf extract of *in vitro* plants proliferated on leaf disc explants

D. A chromatogram showing presence of spilanthol (arrow marked) in calli established from leaves of field grown plants

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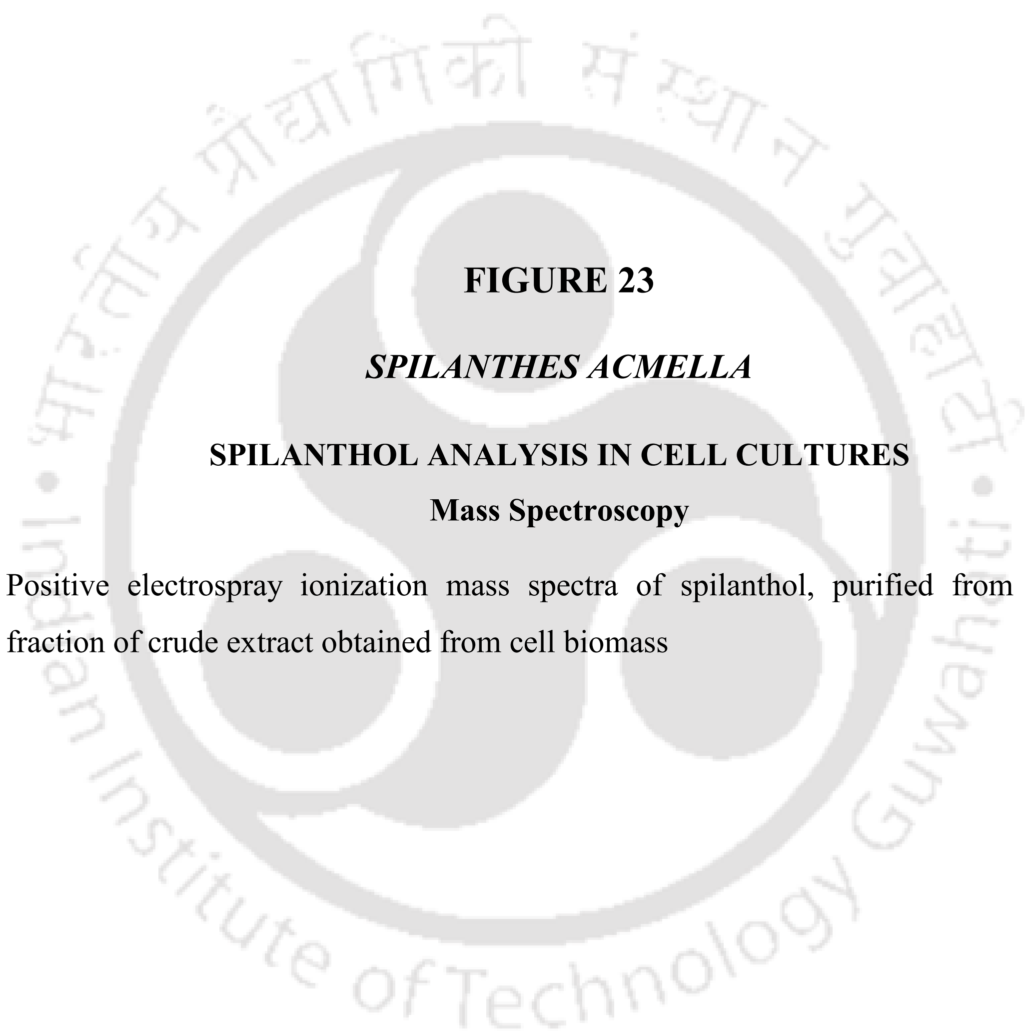

FIGURE 23

SPILANTHES ACMELLA

SPILANTHOL ANALYSIS IN CELL CULTURES

Mass Spectroscopy

Positive electrospray ionization mass spectra of spilanthol, purified from HPLC fraction of crude extract obtained from cell biomass

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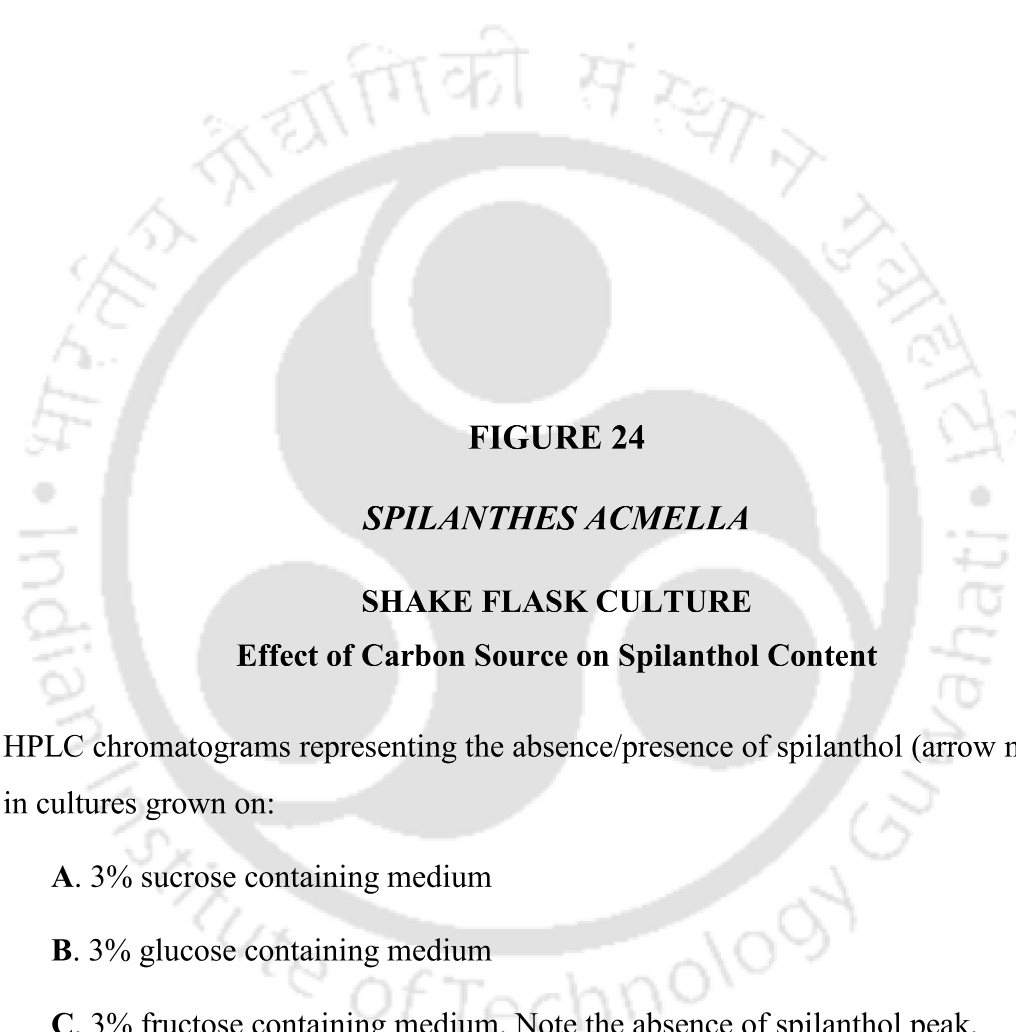

FIGURE 24

SPILANTHES ACMELLA

SHAKE FLASK CULTURE

Effect of Carbon Source on Spilanthol Content

HPLC chromatograms representing the absence/presence of spilanthol (arrow marked) in cultures grown on:

A. 3% sucrose containing medium

B. 3% glucose containing medium

C. 3% fructose containing medium. Note the absence of spilanthol peak.

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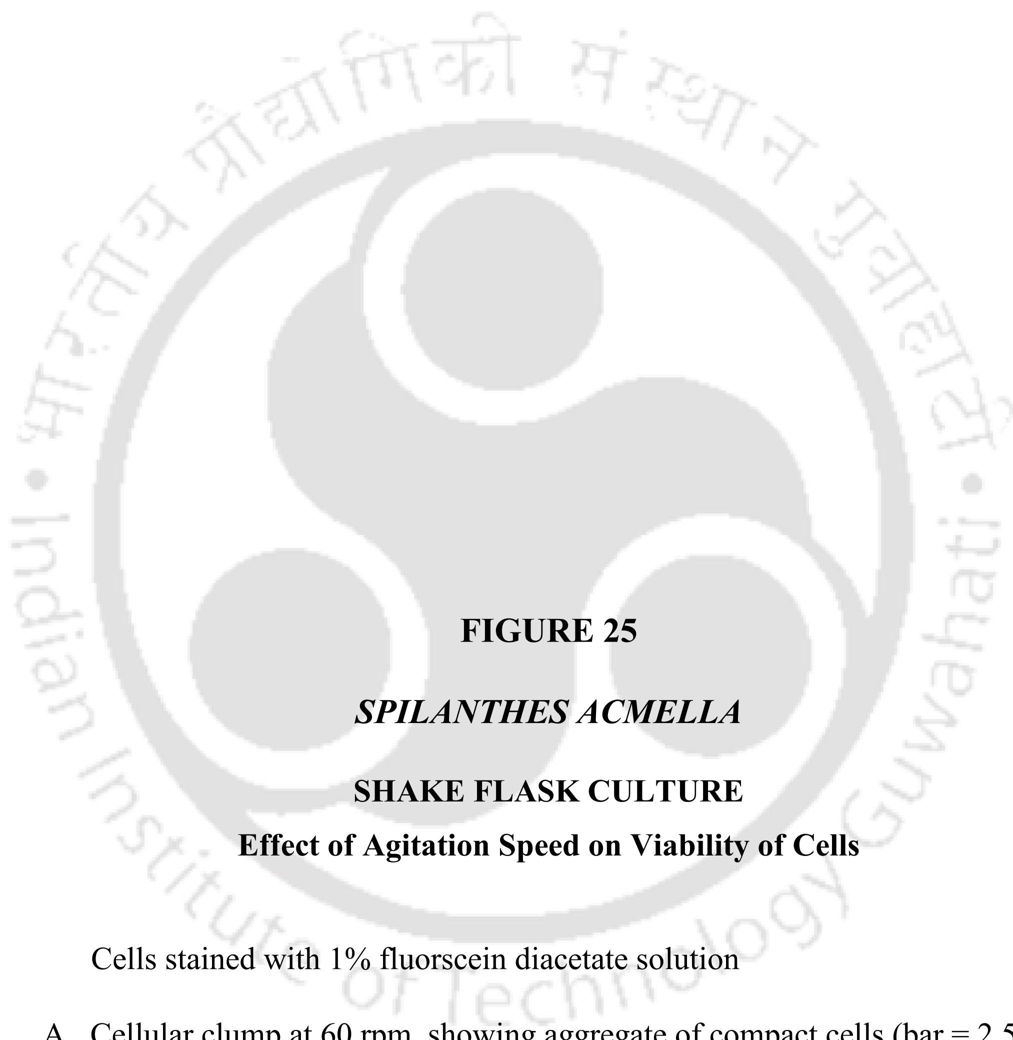

FIGURE 25

SPILANTHES ACMELLA

SHAKE FLASK CULTURE

Effect of Agitation Speed on Viability of Cells

Cells stained with 1% fluorscein diacetate solution

A. Cellular clump at 60 rpm, showing aggregate of compact cells (bar = 2.5 mm)

B. Cellular clump at 90 rpm, showing aggregate with loosely attached cells (bar = 2.5 mm)

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C. The cultures maintained at 120 rpm in the cell suspension, showing individual, live and healthy, fluorescent green stained cells (bar = 9.3 mm)

D. Same at 180 rpm, showing dead (dark bodies) and sheared cells (bar = 9.3 mm)

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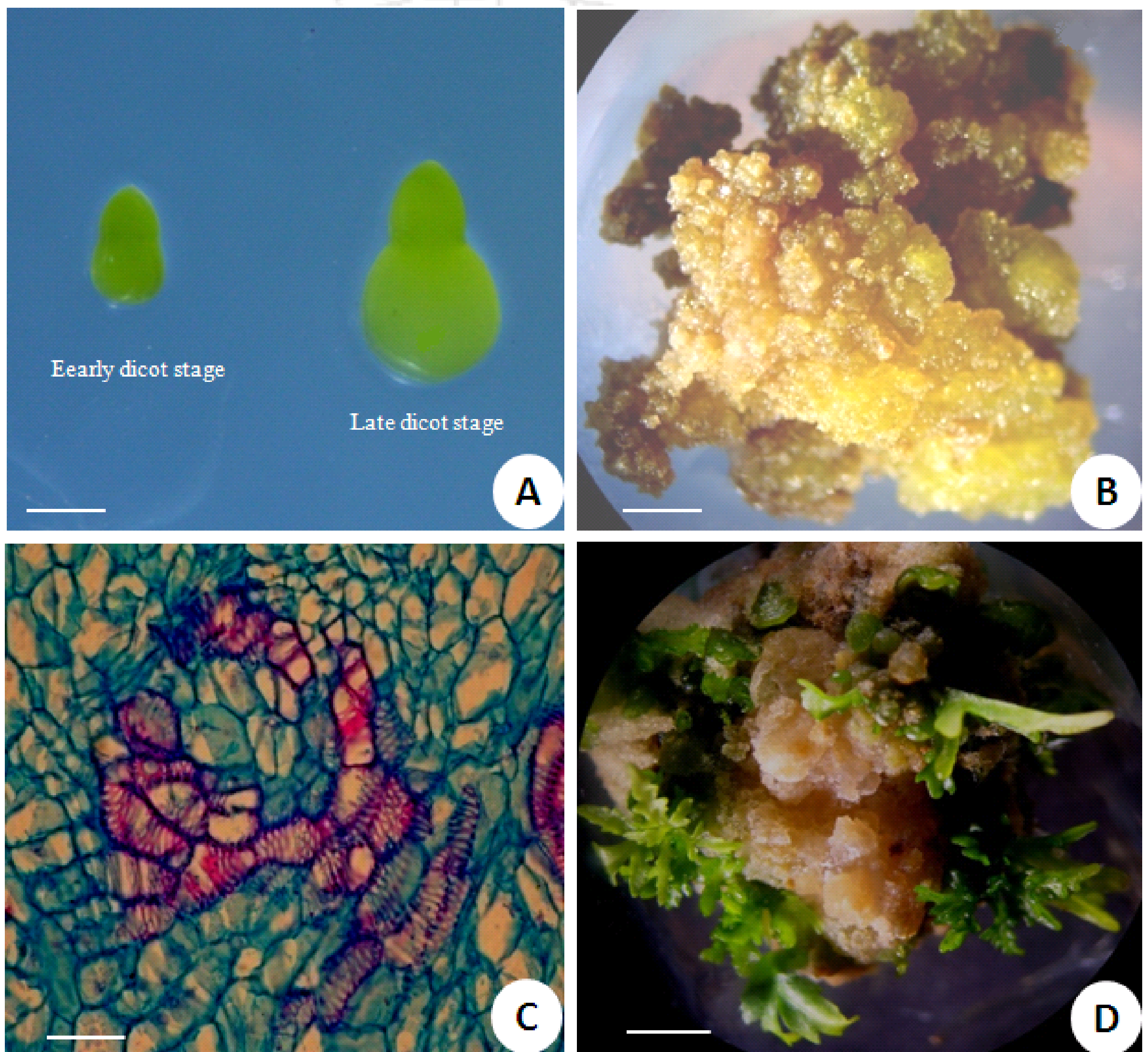

Figure 1

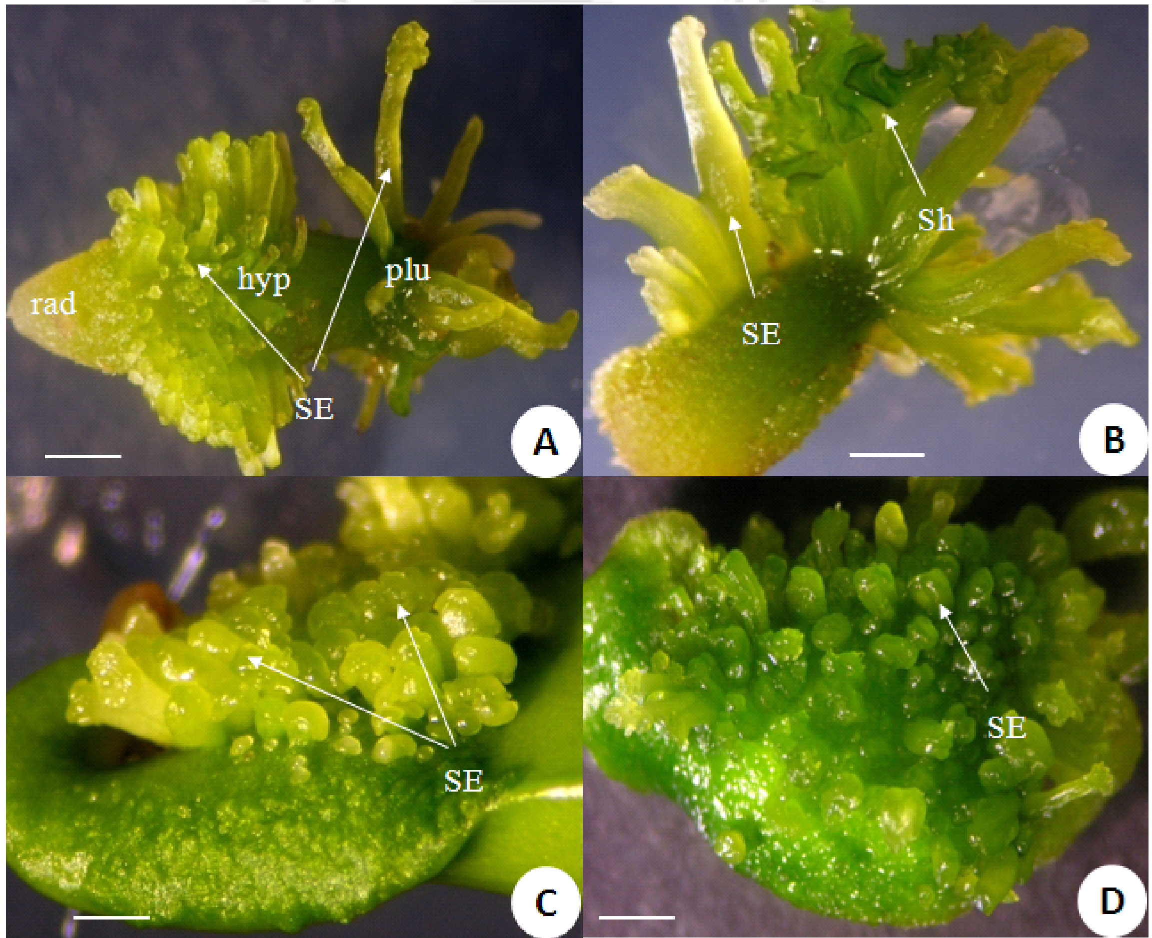

Figure 2

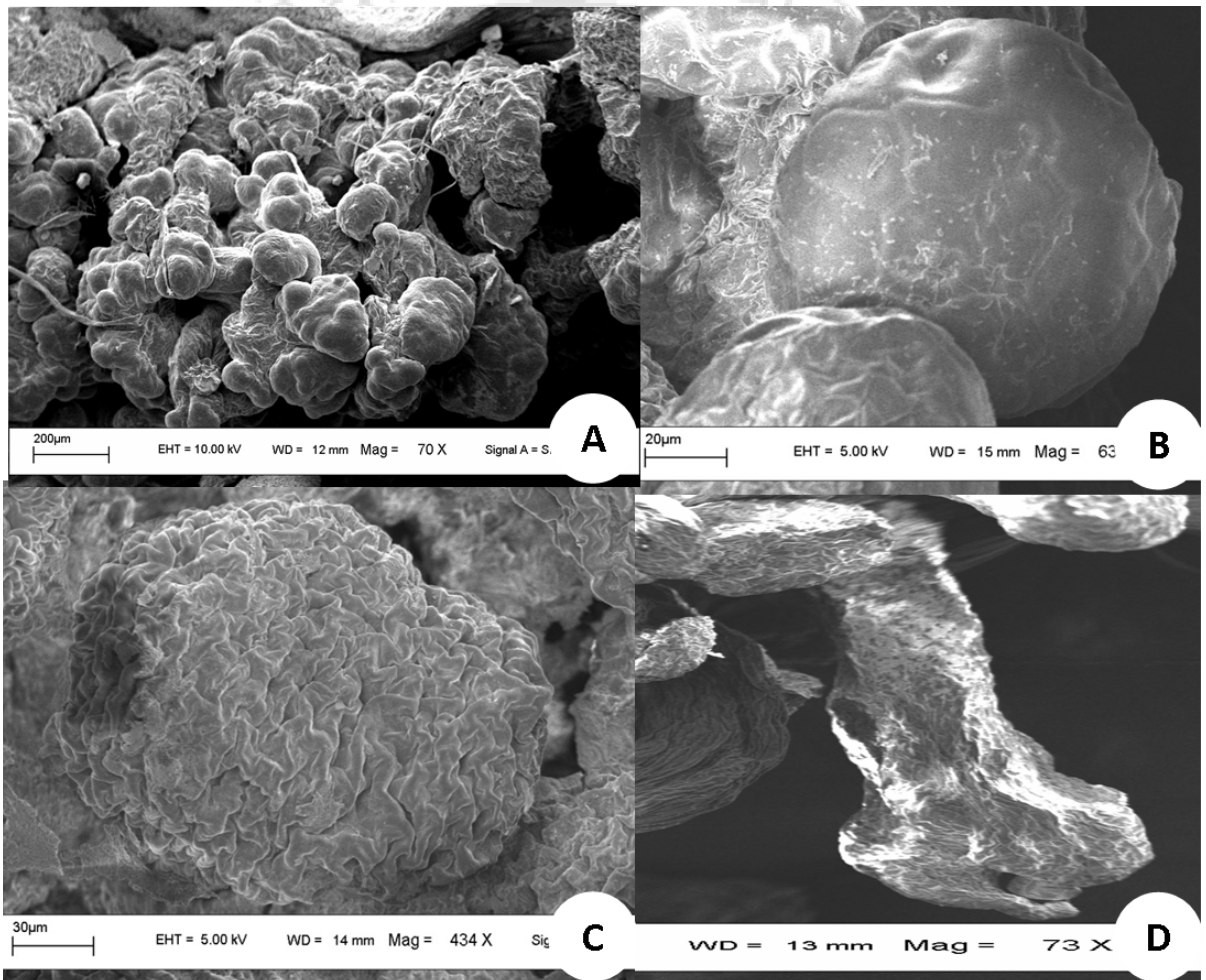

Figure 3

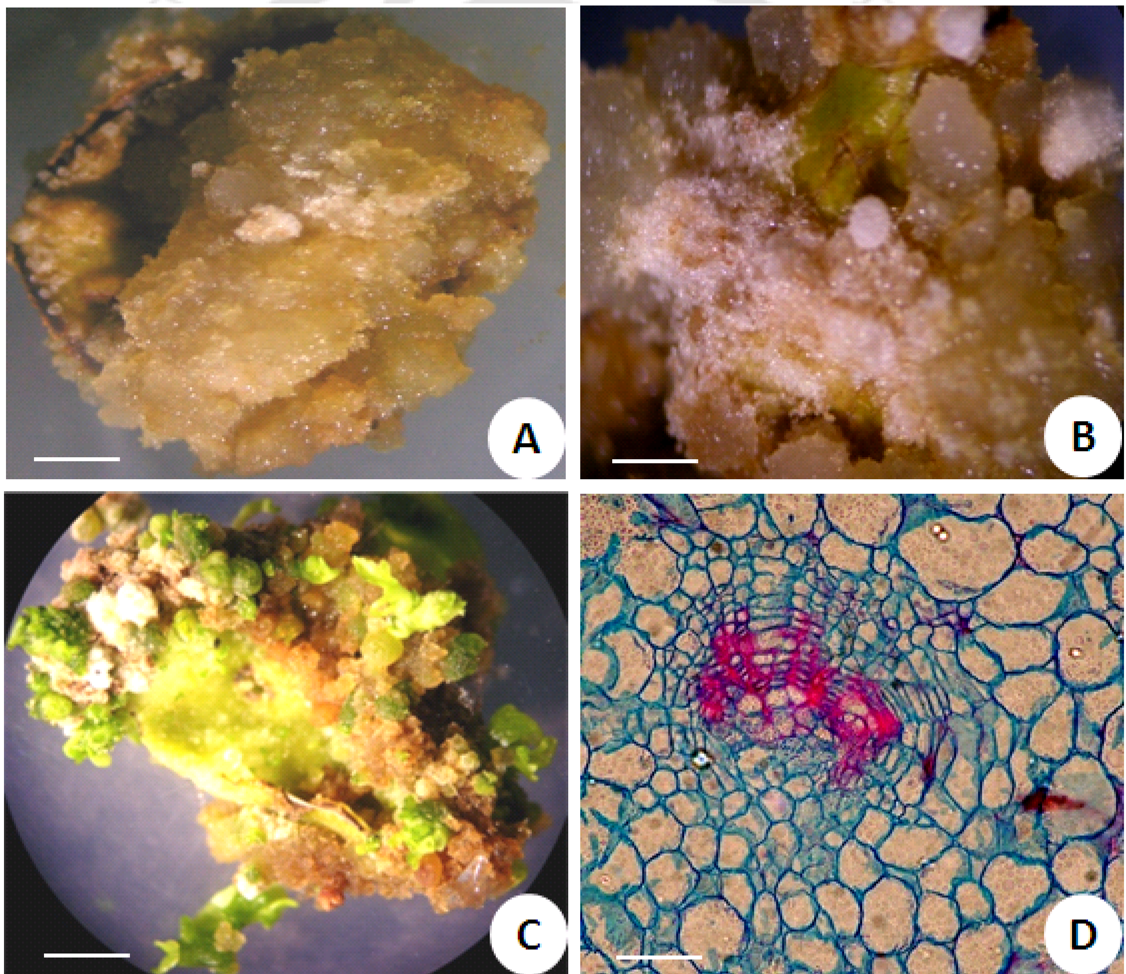

Figure 4

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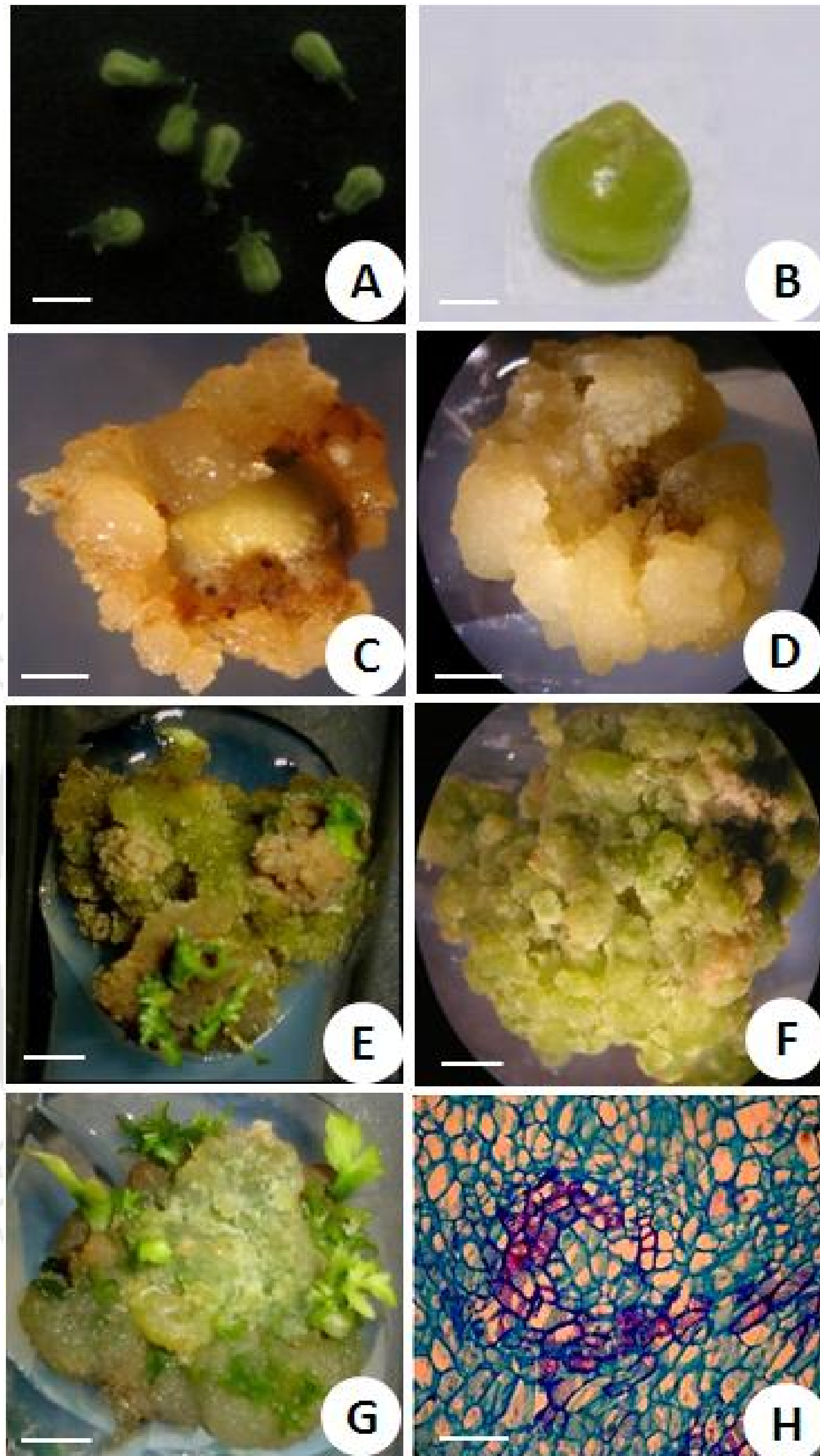

Figure 5

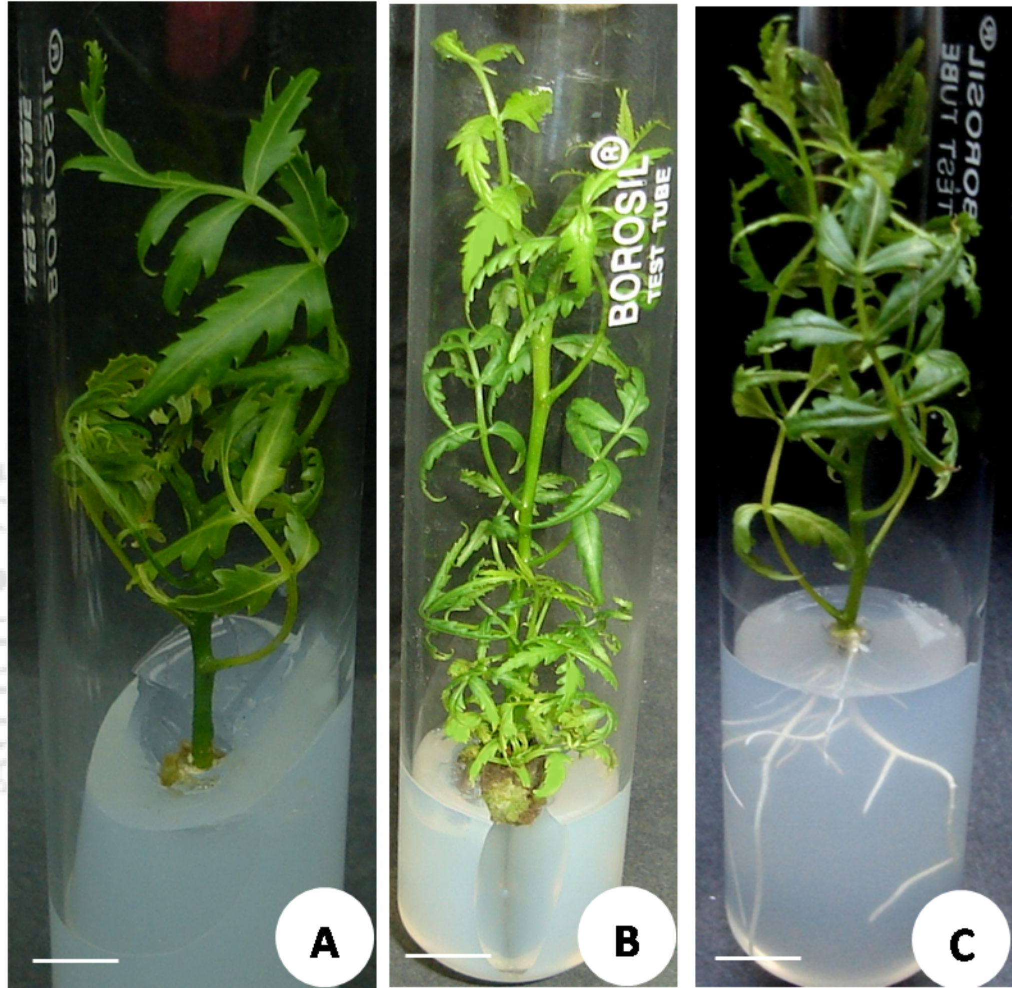

Figure 6

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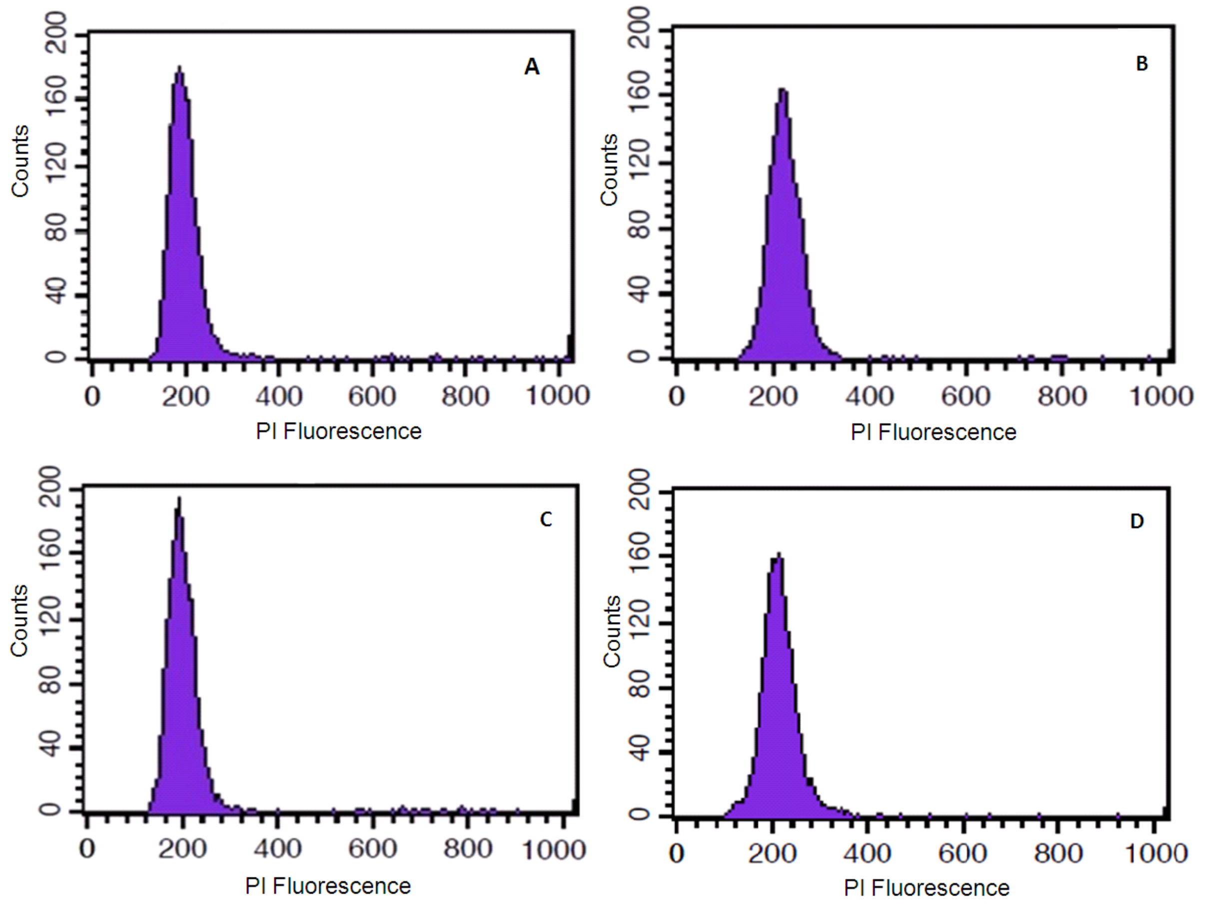

Figure 7

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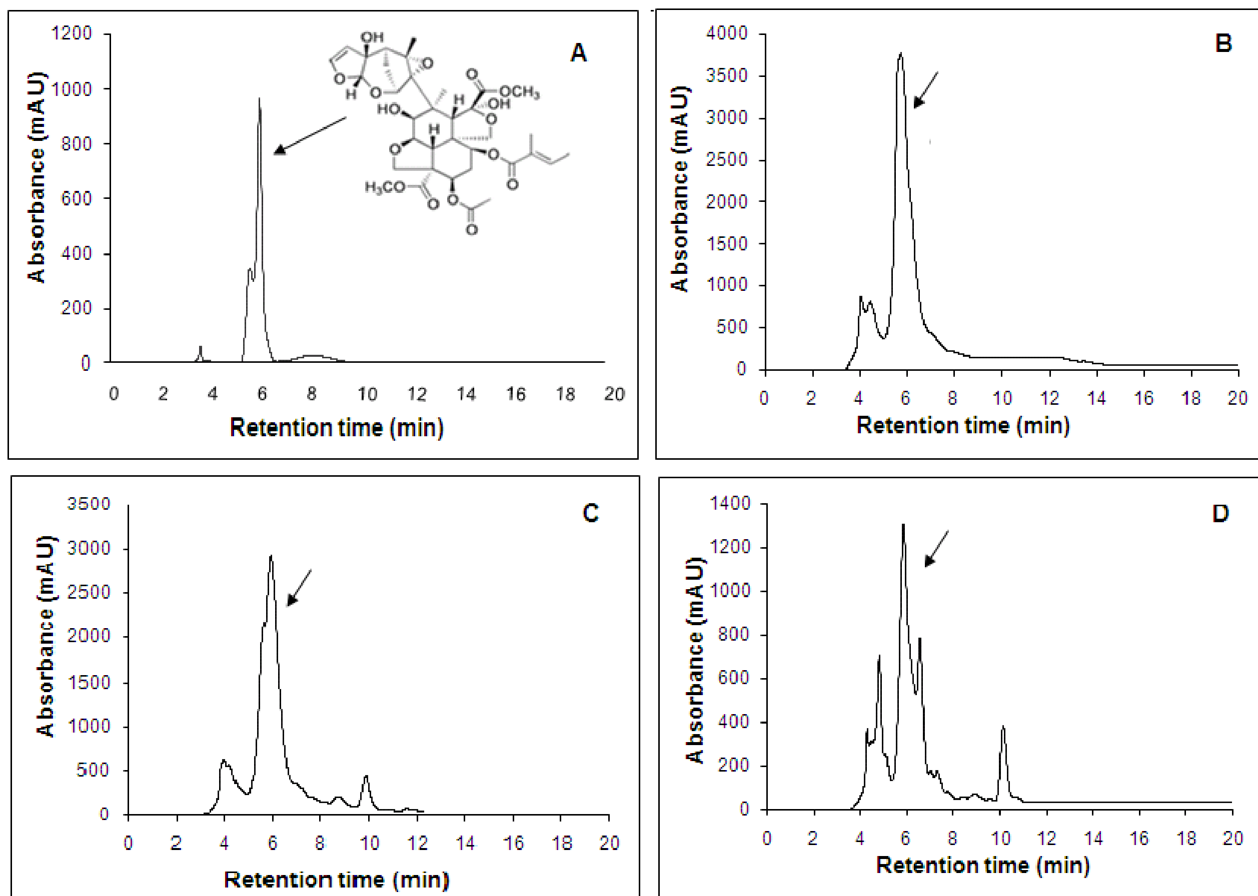

Figure 8

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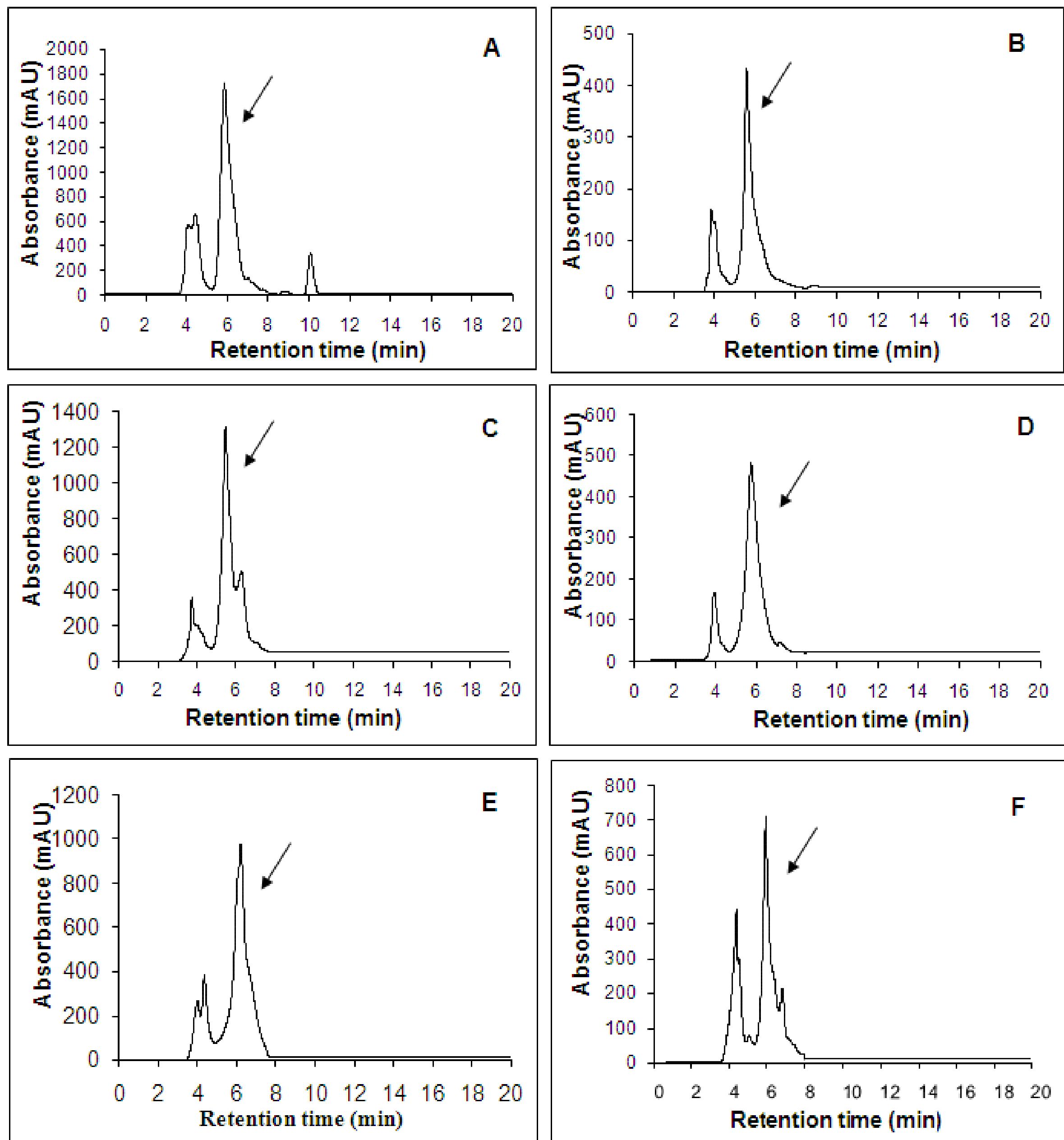

Figure 9

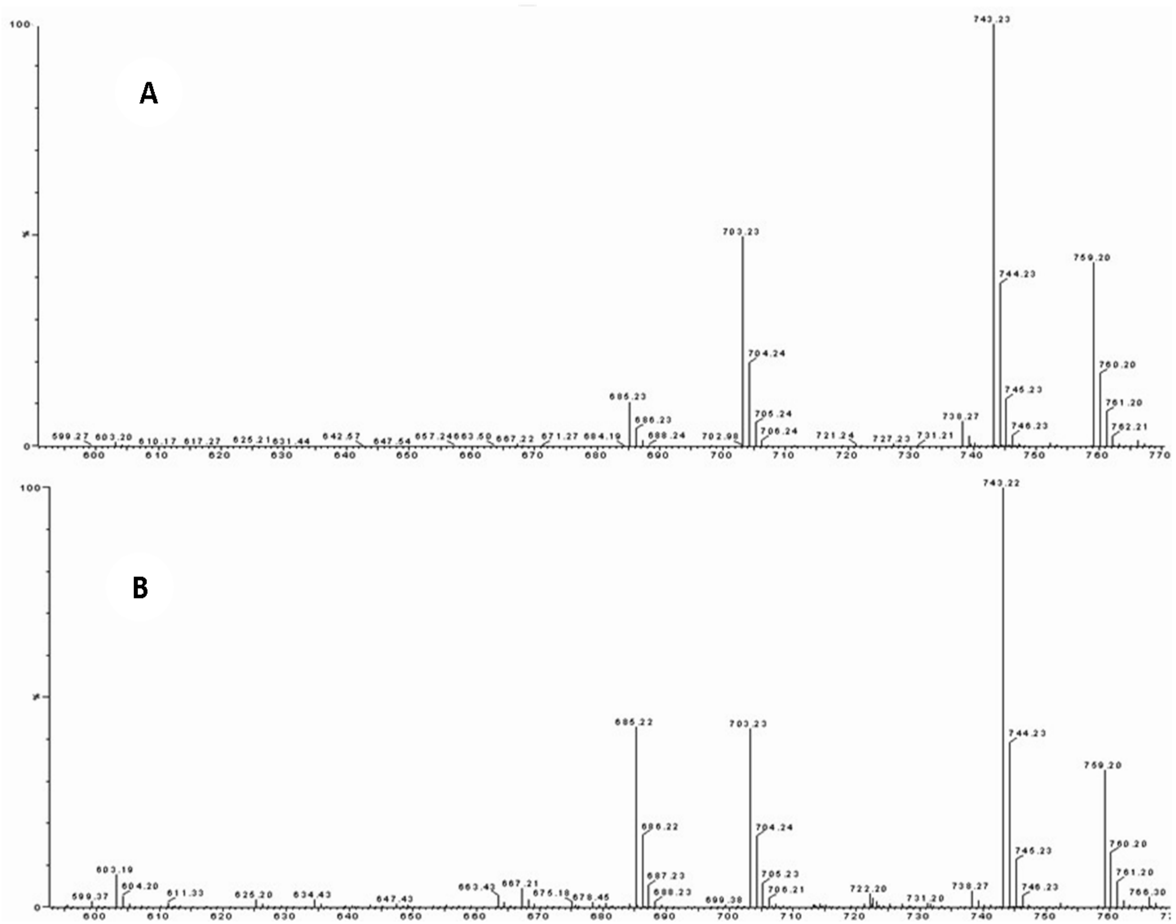

Figure 10

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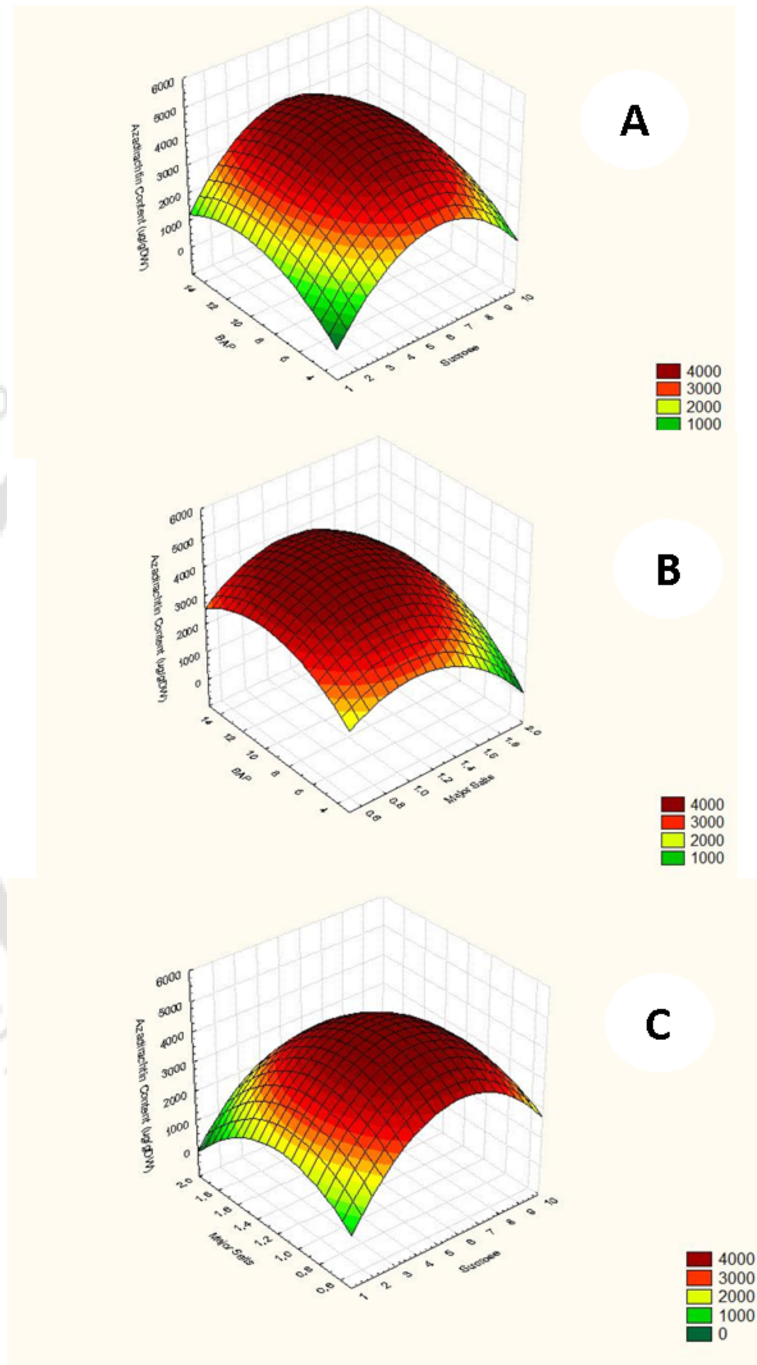

Figure 11

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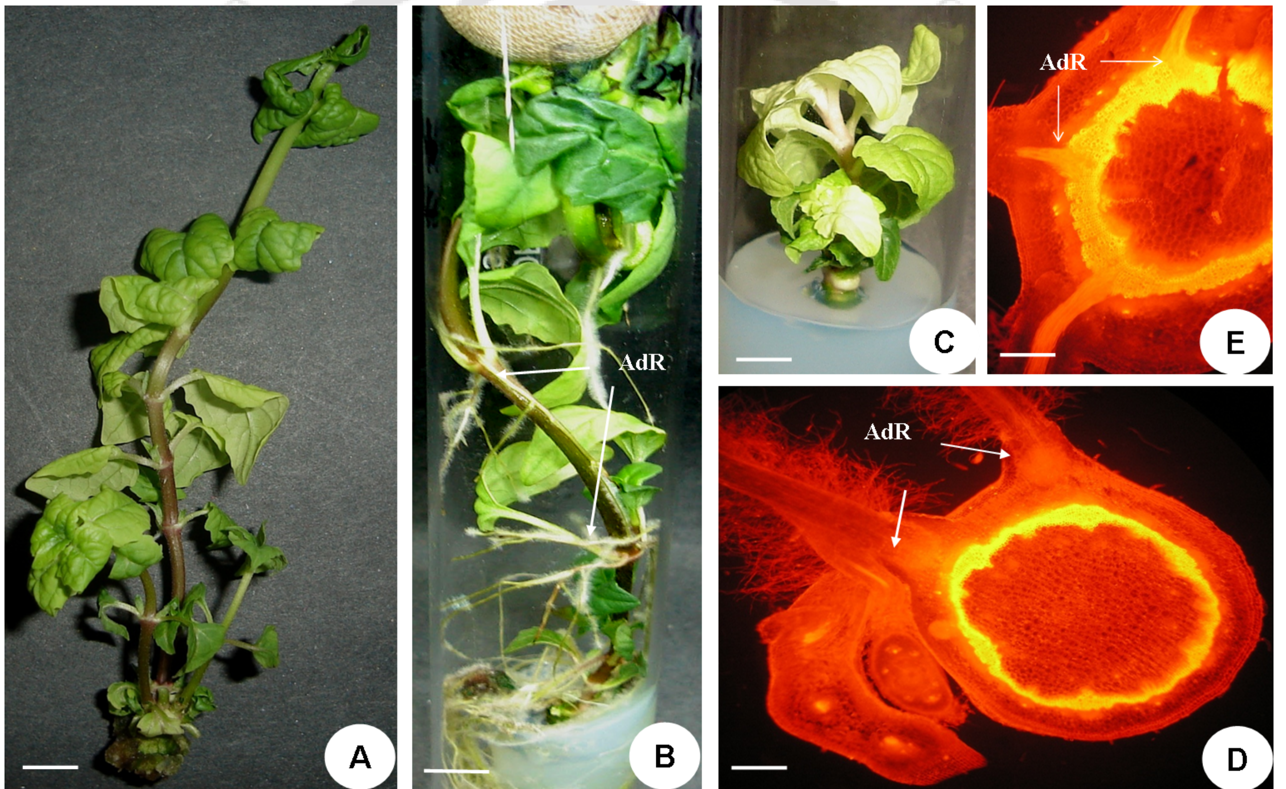

Figure 12

Figure 13

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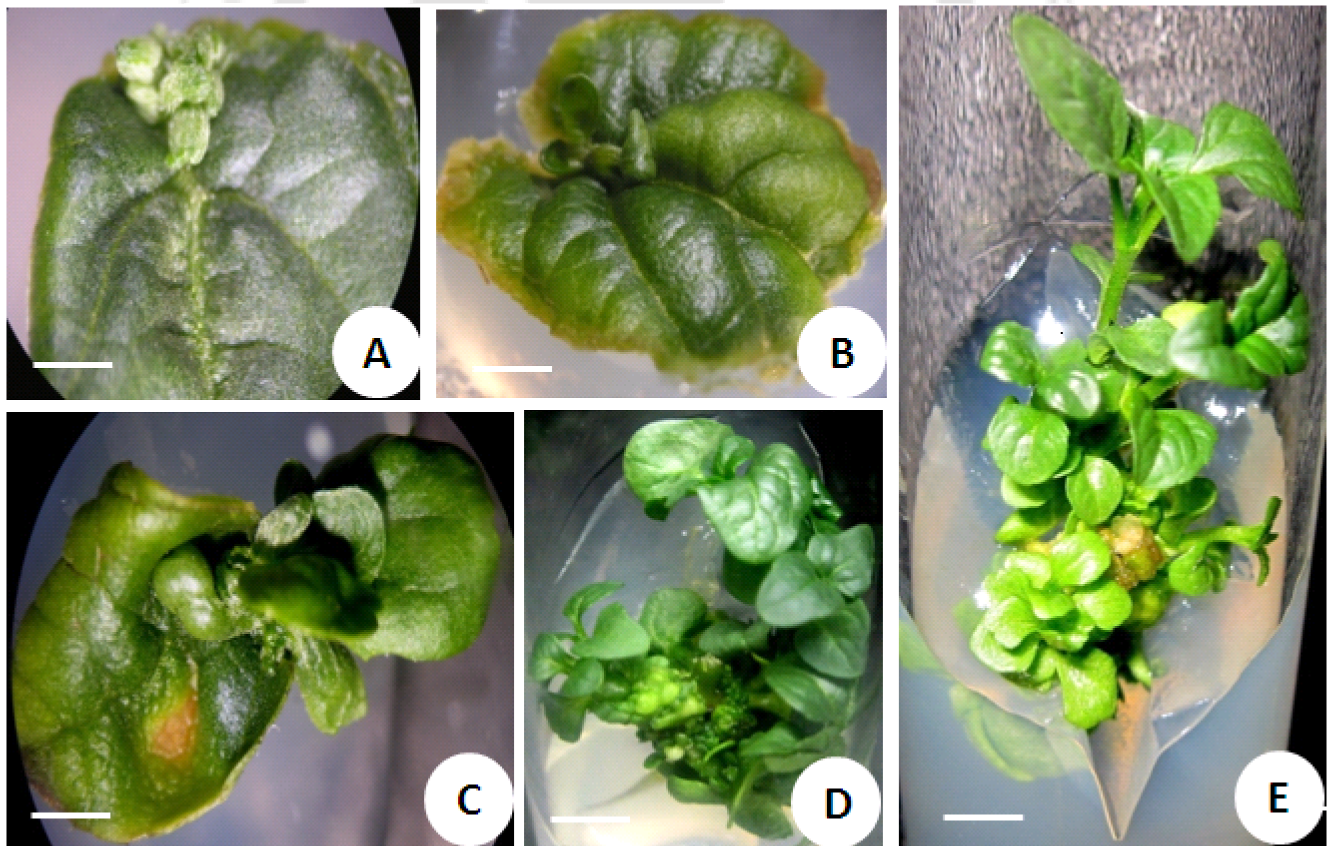

Figure 14

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Figure 15

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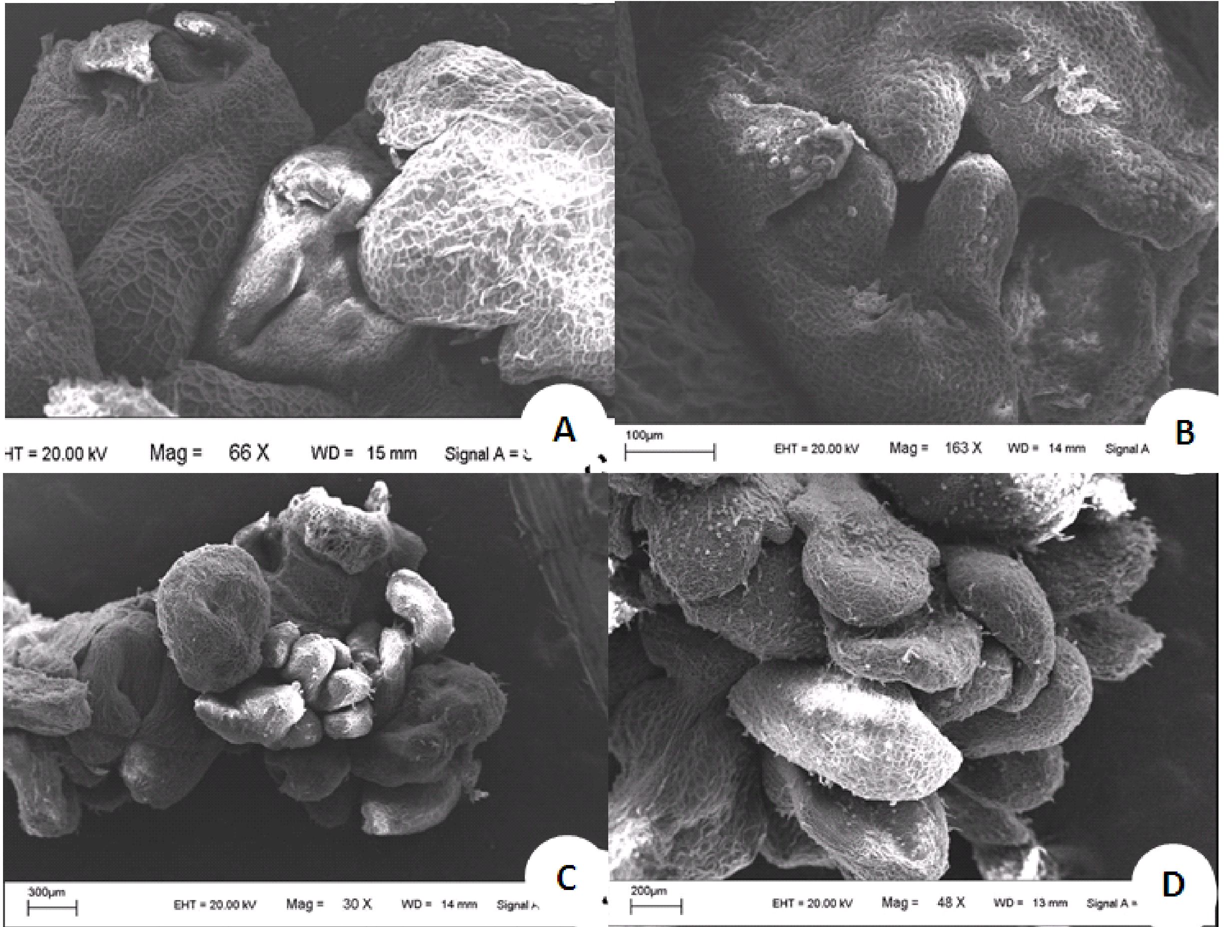

Figure 16

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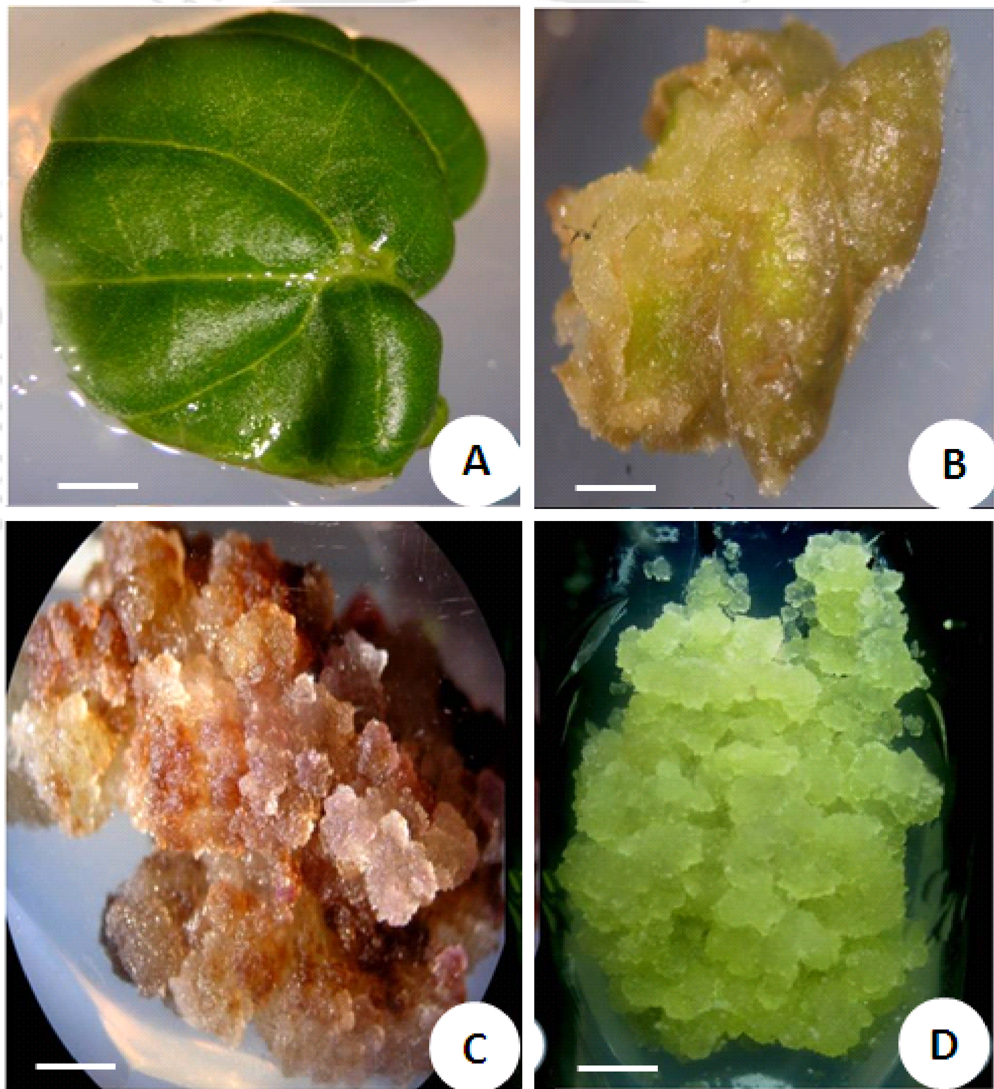

Figure 17

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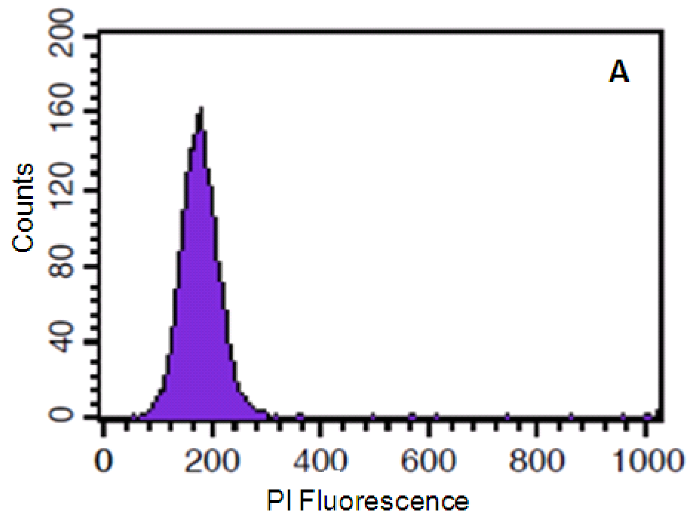

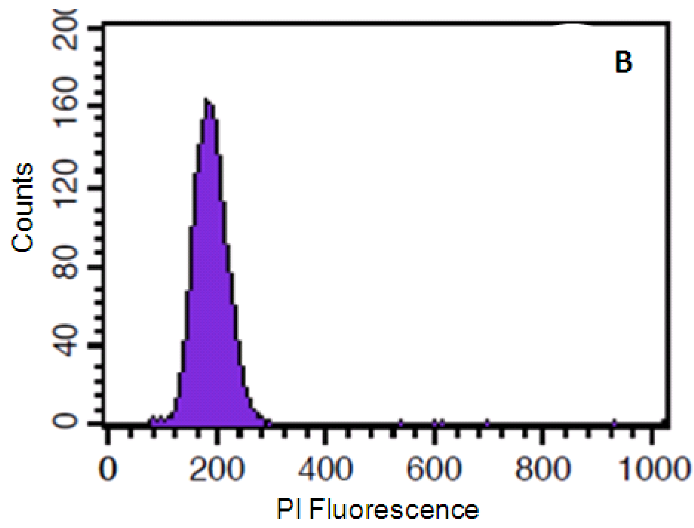

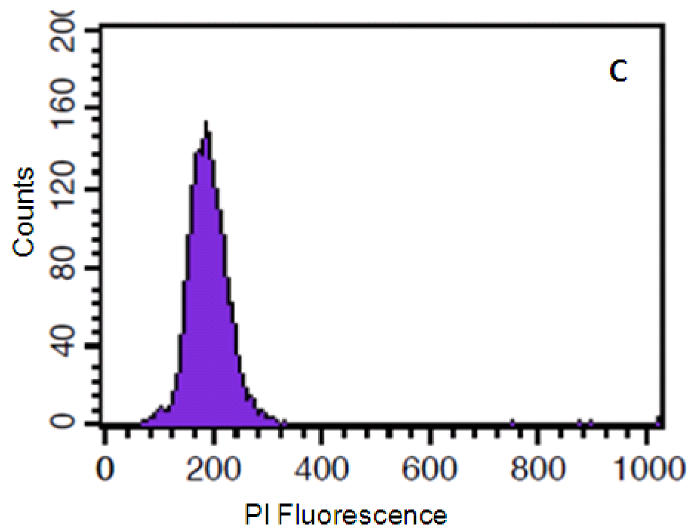

Figure 18

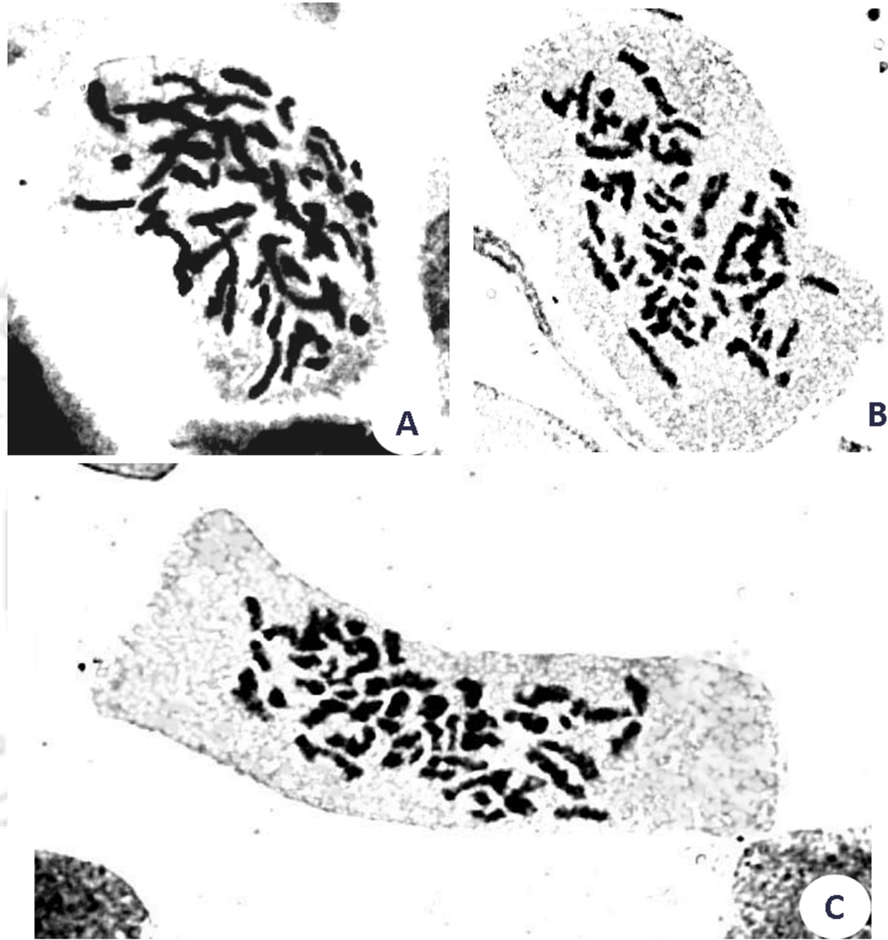

Figure 19

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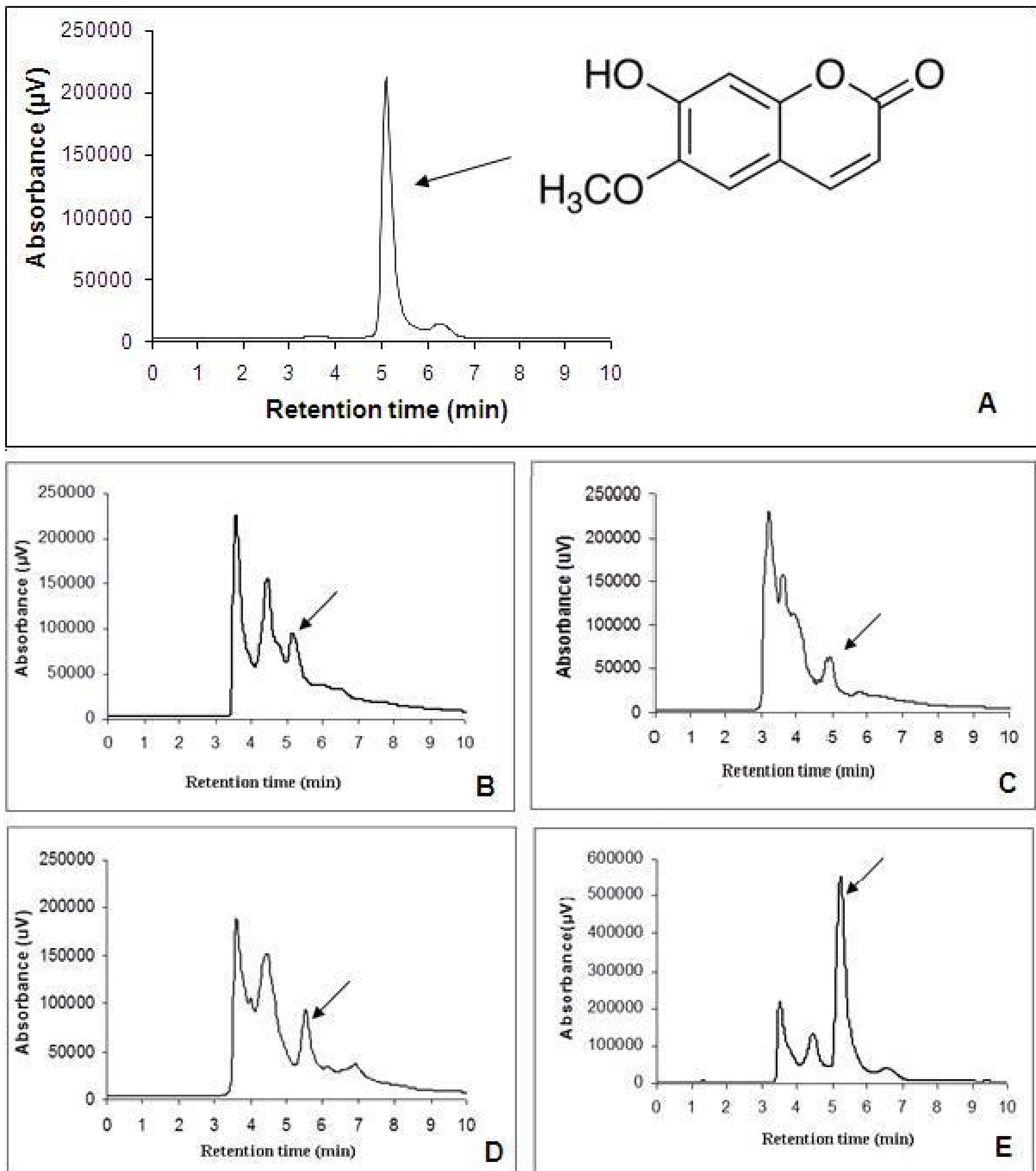
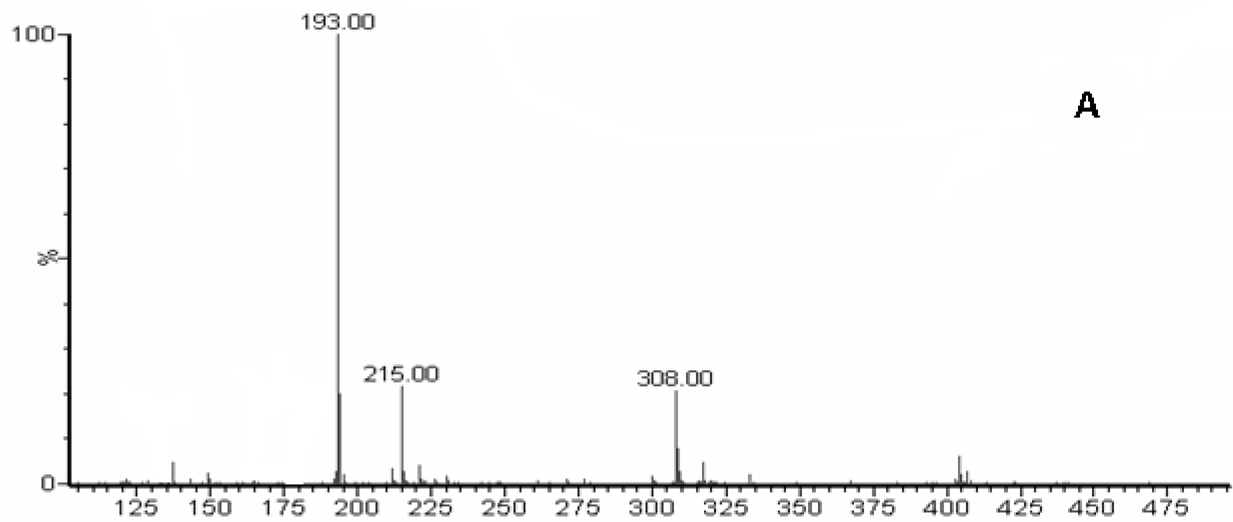


Figure 20



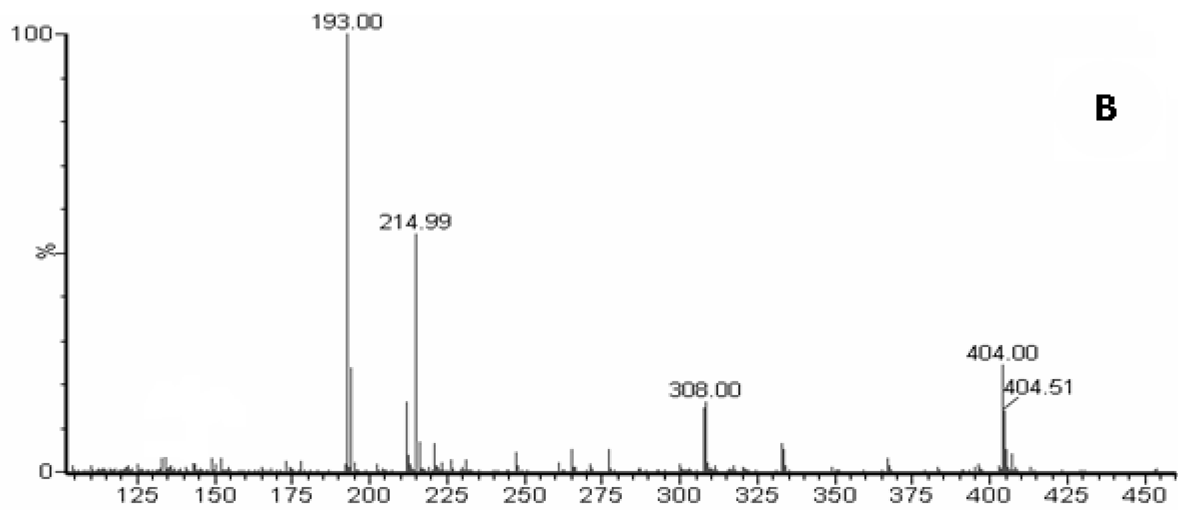


Figure 21

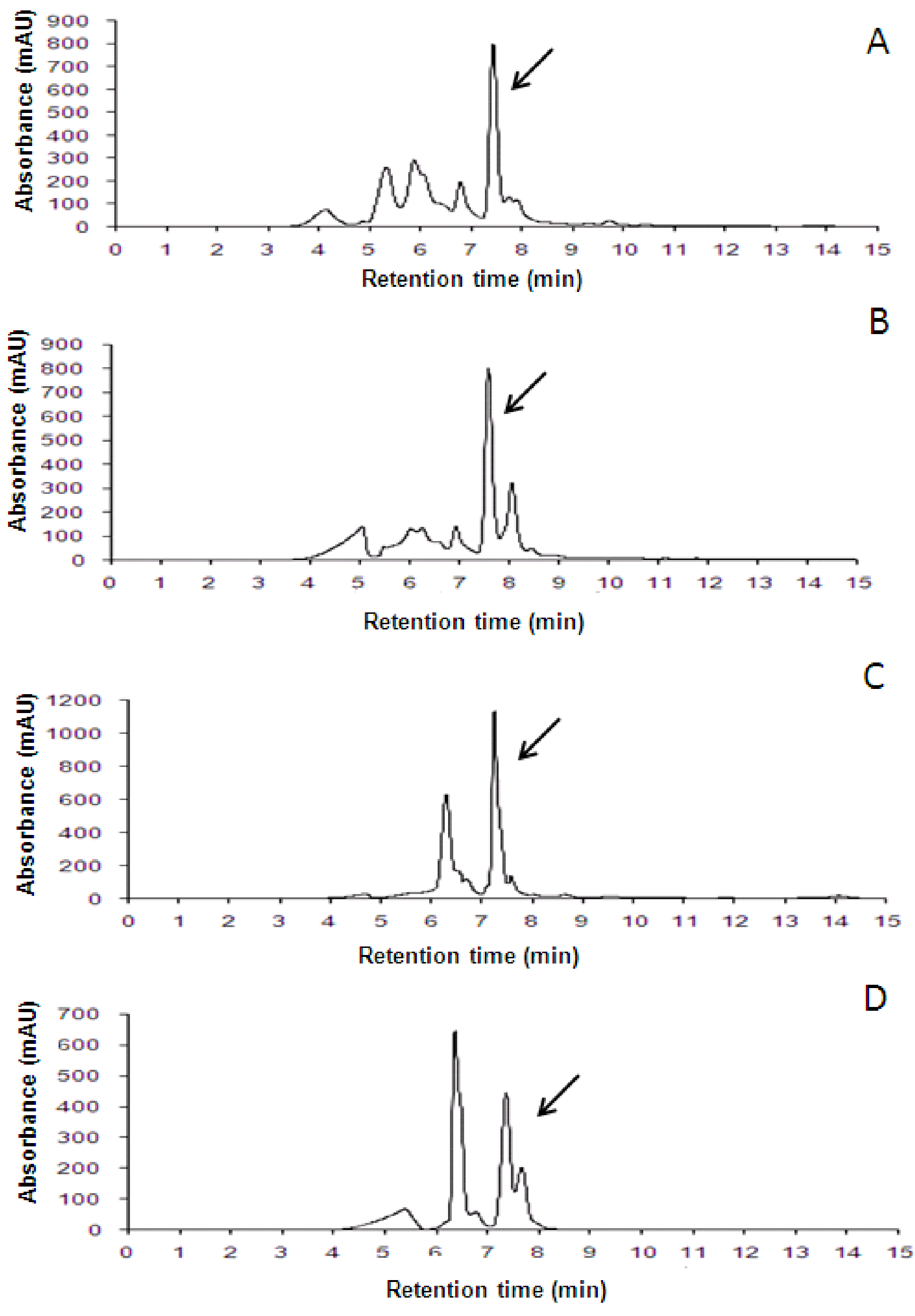


Figure 22

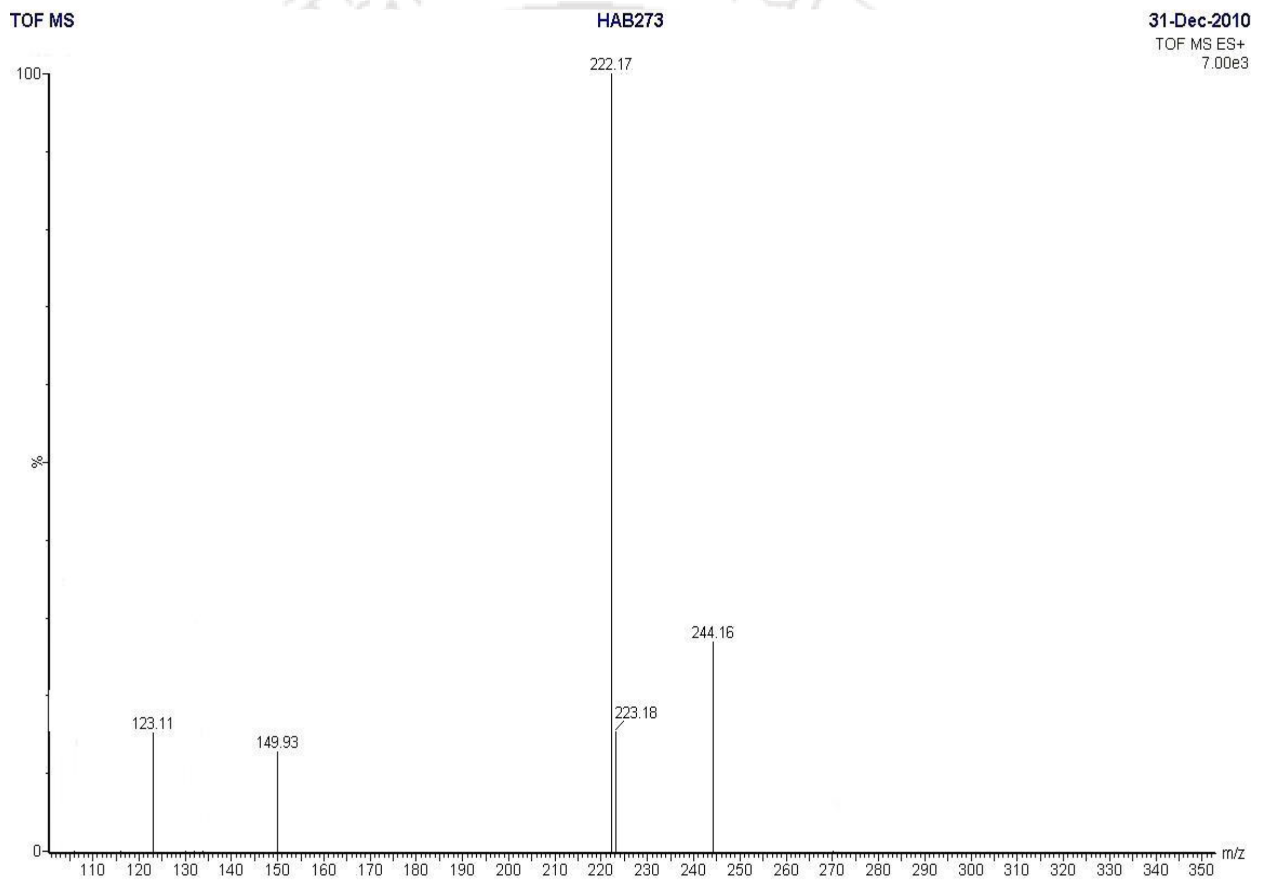
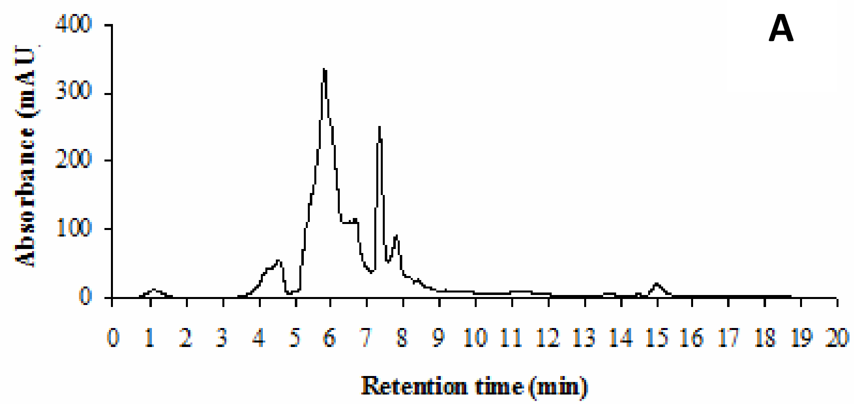
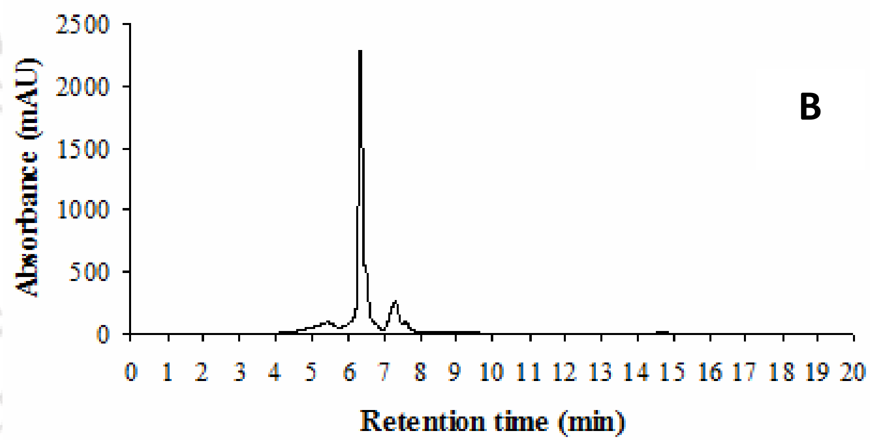


Figure 23





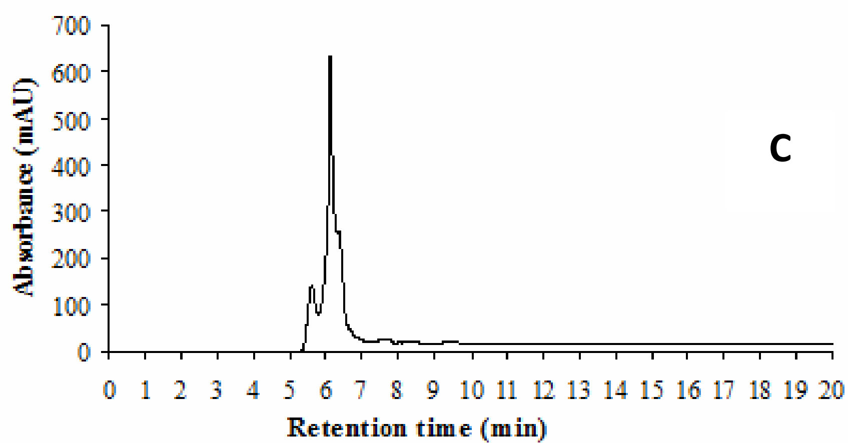


Figure 24

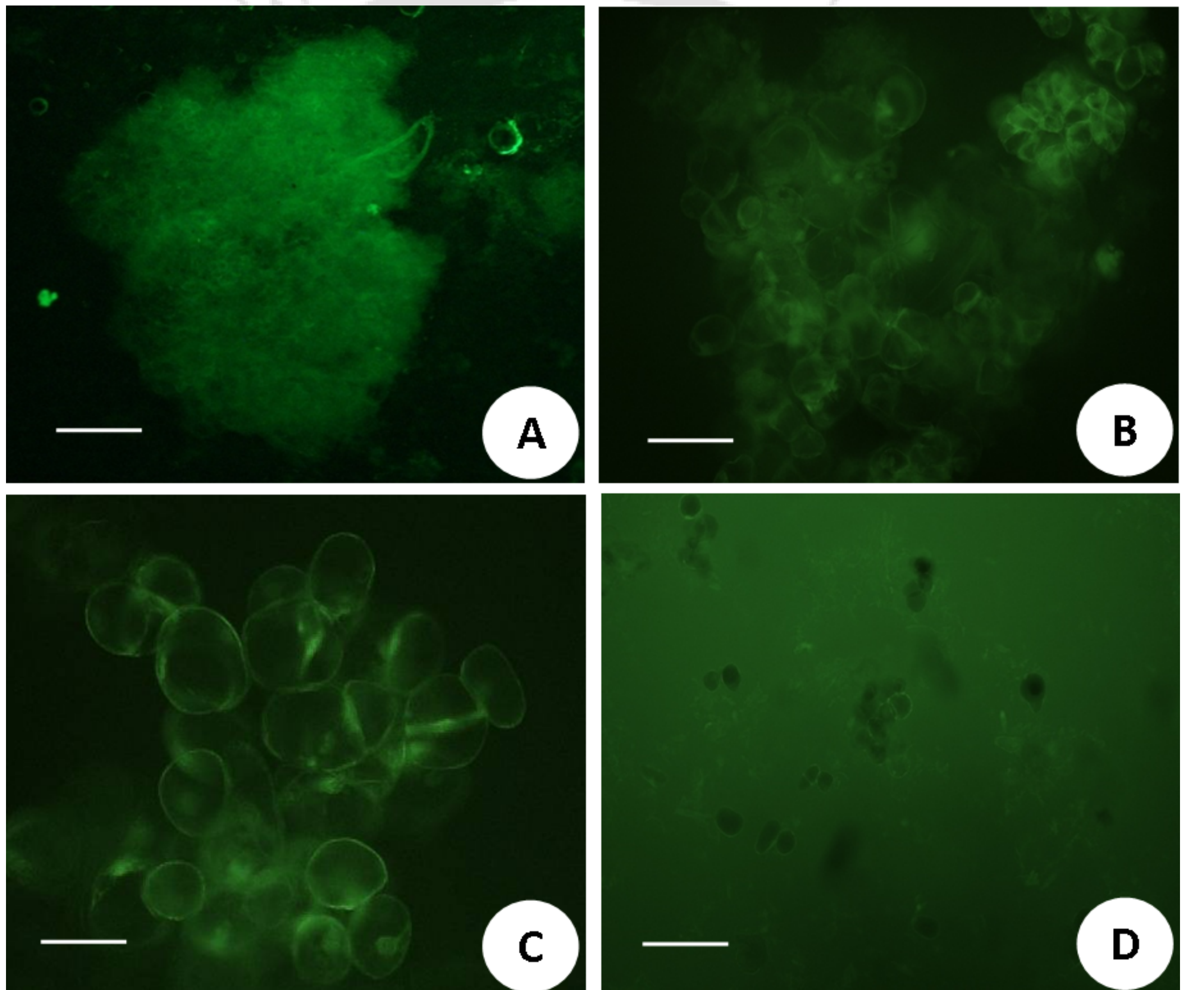


Figure 25

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