

**MICROPROPAGATION OF *STEVIA REBAUDIANA* AND *TYLOPHORA*
INDICA AND EXTRACTION OF SECONDARY METABOLITES**

A Thesis

submitted in fulfillment of the
requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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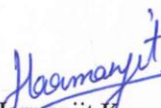
June, 2012

CANDIDATE'S DECLARATION

I hereby declare that the work presented in this thesis entitled “**Micropropagation of *Stevia rebaudiana* and *Tylophora indica* and extraction of secondary metabolites**” in fulfillment of the requirement for the award of **Degree of Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period from March, 2007 to May, 2012 under the supervision of Dr. Manju Anand, (Assistant Professor) and Dr. Dinesh Goyal (Professor and Executive Director, STEP) Department of Biotechnology & Environmental Sciences, Thapar University, Patiala, Punjab. This report has not been submitted for the award of any other degree or certificate in this or any other university.

Date: June, 2012

Place: Patiala


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CERTIFICATE

Certified that the thesis entitled “**Micropropagation of *Stevia rebaudiana* and *Tylophora indica* and extraction of secondary metabolites**” submitted by Ms. Harmanjit Kaur, in fulfillment of the requirement for the award of **Degree of Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is a record of candidate’s own independent and original research work carried out by her under our supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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ACKNOWLEDGEMENT

The day has finally arrived, when I can record my acknowledgements to a great many people who have been instrumental and contributed their time and efforts to assist me in the completion of this work. Words are often less to reveal one's deep regards.

First and foremost, I wish to express my heartfelt thanks to my supervisor Dr. Manju Anand, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala for her valuable guidance, continuous encouragement and precious attention during the progress of the work. I express my deep sense of reverence for her unstinted supervision, affectionate attitude, thoughtful discussions and for editing the script meticulously offering valuable suggestions and giving it a technical perfection. Her valuable insight at all stages of work immensely helped me to improve the quality of work. Her presence was always there whenever I needed and I am highly obliged to her for every such moment when she stood for me like a wise counsel in both personal and professional life. She has been hard at times but surely it made me to learn and achieve better in my life. It is indeed difficult to enunciate in words the deep sense of gratitude and indebtedness I have for her.

I wish to place on record my profound sense of gratitude to my co-supervisor Dr. Dinesh Goyal, Professor and Executive Director, STEP for his constructive suggestions and critical remarks towards amelioration of this work. I thank him for providing flexibility while working and making all the facilities available to me. His systematic approach, unconditional cooperation, experience and knowledge greatly helped me to achieve my goal. Apart from being the integral part of my research venture, he helped me develop my all round personality and provided me with various opportunities to explore myself. His association with me will always remain a beacon light in my life.

I wish to express my sincere thanks and gratitude to Dr. Abhijeet Mukherjee, Director, Thapar University, Patiala for providing support and facilitation. I deem it a pleasure to thank members of doctoral committee Dr. Sanjay Saxena, Associate Professor, DBTES and Dr. Amjad Ali, Assistant Professor, School of Chemistry and Biochemistry for their valuable and constructive suggestions throughout my work. I am deeply privileged to evince a word of sincere thanks to Dr

P. K Bajpai, Dean, Research and Sponsored Projects and Dr. M. S. Reddy, Head, Department of Biotechnology, Thapar University, Patiala for their help and suggestions.

I extend my thanks to all the faculty members, staff and research scholars of the Department of Biotechnology & Environmental Sciences for their kind cooperation and motivation. It would be a remiss if I fail to mention the staff members of Science and Technology Entrepreneurs Park for their friendly assistance and making the path very joyful and easy. The moments I shared with them will always remain as beautiful reminiscences in my life.

Financial assistance in the form of Project Manager and infrastructural support at Science and Technology Entrepreneurs Park (STEP) at Thapar University is gratefully acknowledged.

My heart brims with gratitude for my family for their perpetual blessings, love, faith, immense support and encouragement even in the hard and difficult times along the years. I am ever grateful to them and without their sacrifices I would not have been able to achieve goals in my life. Their unwavering faith in me has been a source of constant inspiration for me. I feel lacuna of words to express my love and deep affection for them.

Above all, I would like to offer thanks to almighty my Guru “Braham Gyani Sant Baba Ajit Singh Jee” for blessing me to complete this work successfully.

To God be the glory. Great things He has done!

(HARMANJIT KAUR)

ABSTRACT

The present study was conducted on two medicinal plant species namely *Stevia rebaudiana* (Bertoni), a member of family Asteraceae and *Tylophora indica* (Burm. f) Merrill belonging to family Asclepiadaceae with an aim to establish an efficient and reproducible *in vitro* regeneration protocol for their mass production and extraction of major secondary metabolites.

The vegetative parts e.g. leaf, stem, root, shoot apices and nodal explants were excised from elite field grown mature plants and thereafter planted on variously supplemented Murashige and Skoog's (MS) medium for callus induction, organogenesis and multiple shoot proliferation. Both the plants exhibited high degree of propensity for *de novo* adventitious shoot formation directly from the explants. In *Stevia rebaudiana*, nodular meristemoids differentiated from the cut ends of leaf lamina when cultured on MS medium supplemented with 9.74 μM 2, 4-dichlorophenoxy acetic acid and 8.8 μM benzyladenine, where uncountable numbers of green healthy shoots developed from these meristemoids on subsequent subculturing. In *Tylophora indica*, 6-benzyladenine (8.8 μM) either singly or in combination with adenine sulphate (1.35 μM) was most effective in inducing *de novo* adventitious shoots through the formation of meristemoids from leaf, stem and root explants.

Leaf explants of *Stevia rebaudiana* cultured on MS supplemented with 19.48 μM 2, 4-dichlorophenoxy acetic acid and 4.65 μM kinetin induced greenish white, soft and friable callus in 98% of the cultures. Synergistic action of α -naphthalene acetic acid (29.4 μM) with kinetin (4.65 μM) was effective in the initiation and sustained growth of calli from stem and shoot apices. Histogenetic differentiation in the form of tracheids which occurred either singly or grouped together as nodules with various thickening patterns, was observed from all the calli of *S. rebaudiana*. High frequency of shoot differentiation with innumerable shoots (too numerous to count) were obtained from the calli cultured and subcultured on MS supplemented with 18.6 μM kinetin or 8.8 μM 6-benzyladenine either singly or in combination. No root differentiation was observed from any of the calli under observation.

In *Tylophora indica*, callus induction from cut ends and entire surface of leaf and stem explants occurred on MS medium supplemented with different concentrations of α -naphthalene acetic acid (9.0- 29.4 μM) and kinetin (4.65 μM) and the callus formed was green and proliferated

further on subsequent subculturings. For root explants, MS medium supplemented with indole 3-butyric acid (24.6 μM) + 6-benzyladenine (13.2 μM) turned out to be optimal for the initiation and sustained growth of calli. The spectrum of induced differentiation from calli was wide and included xylogenesis, rhizogenesis, caulogenesis and embryogenesis. Root differentiation from leaf and stem calli occurred on MS medium supplemented with α -naphthalene acetic acid (29.4 μM) + kinetin (4.65 μM) where the roots formed were thick, white having dense root hairs. High frequency of shoot differentiation from all the calli was observed on both solid and liquid MS media supplemented with either 6-benzyladenine or kinetin alone. Leaf callus when transferred onto MS containing 8.8 μM - 9.84 μM 6-benzyladenine resulted in excellent shoot induction (40.0 ± 1.45 shoots per culture) in 86% of the cultures while liquid MS in combination with 8.8 μM 6-benzyladenine formed 48 ± 1.15 shoots/flask in 80% of the cultures. Prolific shoot differentiation also occurred from stem and root calli when cultured on 8.8 μM 6-benzyladenine supplemented medium.

Stevia rebaudiana and *Tylophora indica* exhibited high degree of multiple shoot proliferation from nodal segments and shoot apices taken from *in vivo* plants. Bud break and axillary shoot proliferation from nodal segments and shoot apices of *Stevia rebaudiana* occurred on MS medium containing 6-benzyladenine with α - naphthalene acetic acid or adenine sulphate. In *T. indica*, multiple shoot induction from the nodal explants occurred on MS supplemented with 8.8 μM 6- benzyladenine forming nearly 20-25 shoots/explant after 6 weeks. However, prolific multiple shoot induction from nodal explants occurred on MS medium supplemented with 13.2 μM 6- benzyladenine in conjunction with α - naphthalene acetic acid (3.67 μM) and L-ascorbic acid (8.4 μM), where 45-50 shoots regenerated from single axillary bud after 5-6 weeks of culturing.

The green compact callus obtained from the leaf explants of *T. indica* on α - naphthalene acetic acid and kinetin medium remained non- embryogenic even after repeated subculturing. For the induction of embryogenesis, the callus was transferred to different concentrations of 2, 4-dichlorophenoxy acetic acid (4.87 – 38.96 μM) and sucrose (1-5%) either singly or in combination with thidiazuron. 2, 4- dichlorophenoxy acetic acid at a concentration of 19.48 μM and 3 % sucrose proved very effective in inducing somatic embryos in 95 % of the cultures. Detailed histological and stereozoom microscopic studies revealed the occurrence of various

stages of development of embryos like globular, heart shaped, torpedo and cotyledonary. Among the numerous globular shaped embryos formed, only 70% developed into cotyledonary embryos after 6-7 weeks of culturing which further developed into tiny plantlets with distinct root and shoot axis. In order to increase the efficacy of plantlet formation, the somatic embryos were transferred to basal MS medium with 2% sucrose only and the formation of complete plantlets with green leafy shoots and roots were observed in 80-90 % of the cultures.

Synthetic seeds were produced in *T. indica*, by encapsulating somatic embryos and shoot buds formed directly from the leaf explants in sodium alginate (2%) followed by dropping them in 2% calcium chloride. Synthetic seeds were stored at 4°C for 90 days; however, there was gradual reduction in the conversion rates and plantlet regeneration upon storage. Despite our repeated efforts, somatic embryogenesis could not be induced in *Stevia rebaudiana*. Artificial seeds were produced from shoot tips as vegetative propagules by encapsulation in 2% sodium alginate in double distilled water or in full strength liquid MS medium and 2% CaCl₂. Synthetic seeds were stored upto 120 days and gradual reduction in the conversion percentage and shoot regeneration upto 15% was observed.

Individual regenerated healthy shoots rescued from the culture vessels were transferred onto half strength MS medium, full strength MS medium and MS medium supplemented with different concentrations of IAA, NAA and IBA for root induction. The best rooting response (90%) was observed on auxin free half strength MS medium whereas addition of IBA to full strength MS medium also showed equally good results in both the plants. Rooted plantlets of *S. rebaudiana* and *T. indica* were transferred successfully to the field conditions through successive hardening stages showing 80% and 90% survival rates respectively with no phenotypic variations.

Extraction of major secondary metabolite stevioside from leaf samples of *in vitro* plants of *Stevia rebaudiana* collected at different time intervals (3, 4, 5, 18, 30 months) were achieved following solvent extraction with petroleum ether, methanol, diethyl ether and butanol. The crude extract was initially purified on glass TLC followed by fine purification on pre coated silica gel 60 F₂₅₄ plates by using HPTLC involving solvent system comprising of chloroform: methanol: water (7:3:1) and scanned at 210 nm. Three months old plant sample yielded 68 µg/ml of stevioside, whereas 4 and 5 month old plant samples contained 52.7 µg/ml and 44.1 µg/ml of stevioside

respectively. Stevioside content in thirty months old *in vitro* and *in vivo* plants was 94.9 and 52.6 µg/ml, while eighteen months old *in vitro* and *in vivo* plants yielded 86.8 and 48.0 µg/ml of stevioside respectively.

Extraction of major secondary metabolite-tylophorine from the leaves and roots of *Tylophora indica* was achieved following cold extraction in methanol: acetic acid followed by extraction in ethyl acetate, chloroform and methanol. Quantitative analysis of tylophorine in the leaf samples revealed maximum amount (80µg/ml) of tylophorine in the plants raised through leaf callus while root callus regenerated plants yielded minimum amount (35µg/ml) of tylophorine. Comparison of crude extract obtained from roots of twelve and twenty four months old *in vitro* and *in vivo* plants when chromatographed using toluene: ethyl acetate: diethyl amine (7:2:1) at 258 nm showed maximum amount of tylophorine 90.75 and 88.34 µg/ml in twenty four and twelve months old *in vitro* plants respectively, while twenty four and twelve months old *in vivo* plants yielded 59.40 and 57.47 µg/ml of tylophorine respectively.

Mass extraction of secondary metabolites was achieved from leaf and stem calli of *Stevia rebaudiana* and leaf and root calli of *Tylophora indica*. Leaf and stem callus cultures of *Stevia rebaudiana* yielded 44.37 and 26.9 µg/ml of stevioside respectively when chromatographs were developed using ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm. Suspension cultures of leaf and stem calli harvested at stationary phase gave 69.40 and 57.69 µg/ml of stevioside which was higher as compared to that of callus cultures. Quantitative analysis of tylophorine from callus cultures of *T. indica* on HPTLC using toluene: ethyl acetate: diethyl amine (7:2:1) gave highest amount of tylophorine i.e. 26.42 µg/ml in root callus cultures whereas leaf callus cultures yielded 24.46 µg/ml of tylophorine. Suspension cultures of leaf and root calli under similar set of conditions yielded 34.71µg/ml and 28.30 µg/ml of tylophorine respectively.

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
AdSO ₄	Adenine sulphate
BA	6-benzyladenine
β	Beta
BMS	Basal Murashige & Skoog's Medium
BSA	Bovine Serum albumin
°C	Degree Celsius
CAMP	Conservation Assessment and Management Plan
CH	Casein hydrolysate
Conc.	Concentration
CO ₂	Carbon dioxide
2, 4-D	2, 4 -dichloro phenoxy acetic acid
2, 4, 5-T	2, 4, 5-trichlorophenoxy acetic acid
FRLHT	Foundation for Revitalization of Local Health Traditions
2-ip	2 -isopentyl adenine
GC	Gas Chromatography
g/l	Gram/Litre
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
IAA	Indole -3- acetic acid
IBA	Indole-3- butyric acid
ICMR	Indian Council of Medical Research
IUCN	International Union for the Conservation of Nature
K	Kinetin
Kg	Kilogram
LAH	Laminar flow hood
LCMS	Liquid chromatography Mass Spectrophotometry
MAPs	Medicinal and Aromatic Plants
MS	Mass spectrometry
ml	Millilitre

NAA	Naphthalene acetic acid
NABARD	National Bank for Agriculture and Rural Development
NIRS	Near Infra Red Reflectance Spectroscopy
NMPB	National Medicinal Plants Board
NMR	Nuclear magnetic resonance
pH	Hydrogen ion concentration
O.D	Optical density (nm)
R _f	Retention factor
SCFE	Supercritical fluid extraction
SE	Standard Error
SGs	Steviol glycosides
<i>S.rebaudiana</i>	<i>Stevia rebaudiana</i>
STS	Sweet tasting stevioside
Sp	Species
subFE	Subcritical fluid extraction
TCP	Tissue culture plants
TDZ	Thidiazuron
TERI	The Energy Research Institute
<i>T.indica</i>	<i>Tylophora indica</i>
TLC	Thin layer chromatography
TMS	Tetramethyl silane
TPP	Tripolyphosphate solution
VIS	Visible spectrometry
μM	Micro molar
UNESCO	United National Educational Scientific and Cultural Organization
UV	Ultra-violet
USA	United States of America
US \$	United States Dollar
W	Watt
Wt	Weight
WHO	World Health Organization

***“To My Revered
Parents”***

INTRODUCTION

1.1 Medicinal Plants: An overview

Plants, since time immemorial have been used to fulfill three fundamental needs of man including food, shelter and clothing and also provide a valuable source of medicine for basic health care needs. Herbal medicine is the oldest form of health care known to man and over 9000 herbs have known medicinal applications in various cultures and countries (Farnsworth and Soejarto, 1985). Medicinal plants synthesize and accumulate a variety of compounds to combat diseases and form primary line of defense for human beings. The use and Knowledge of medicinal plants is evidenced through ancient records of all major systems of medicine such as Ayurveda, Unani, Chinese medicine and Japanese Kampo. According to World Health Organization (WHO), over 4.3 billion people or 80% of the world's population, primarily those of developing countries depend directly on medicinal plants for basic preventive and curative healthcare.

Medicinal plants form the resource base for rapidly growing pharmaceutical industry with 25% of drugs derived directly from the plants and many other compounds isolated as synthetic analogues. These herbal drugs are used in pharmaceuticals, nutraceuticals, cosmetics and as food supplements. Allopathic medicine too owes a tremendous debt to medicinal plants, as one in four prescriptions, filled in a country like United States is either a synthesized form or derived from plant material (Srivastava *et al.*, 1995). Over the past few years, herbal remedies have regained a wide recognition and are making a comeback as the drugs obtained from plants are cheaper, exhibit a remarkable efficacy in the treatment of various ailments and are much safer with least side effects as compared to allopathic medicines (Siddiqui *et al.*, 1995). As a result, a growing awareness for extracting bioactive constituents from medicinal plants by industrialized society has brought global renaissance in the trade of these herbal medicines (Kaido *et al.*, 1997 and UNESCO, 1998).

1.2 Global diversity and trade of medicinal plants

There is no accurate data or reliable figure available to assess the total number of medicinally active plants on earth and the extent of use of plant-derived drugs used in human health care

systems of different countries. However as estimated, the number of medicinally important species worldwide are 35,000-70,000 (Schippmann *et al.*, 2002), out of which 2237 are in Mexico (Toledo, 1995), 7500 in India (Shiva, 1996), 2572 in North America (Moerman, 1998) and 10,000-11,250 in China (Pei, 2002). The highest account (63%) of world herbal product market is mainly in Europe and North America. In North America itself, the sale of medicinal plants has climbed to about \$3 billion (Glaser, 1999) whereas in European market, herbal remedies stand at US \$ 7.5 billion as of 1997. As far as the herbal import is concerned, China stands first with 45% herbal import for drug preparation, followed by 15.6 % for USA, 10.5 % for Australia, 8.1 % for Indonesia and 3.7 % for India (Samy and Gopalakrishnakone, 2007).

According to “The International Council for Medicinal and Aromatic Plants”, the world market for medicinal and aromatic plants (MAPs) is enormous and as estimated world growth for MAPs during 2001 and 2002 was approximately 8–10% a year (Srivastava, 2000). India is a major exporter of raw MAPs and processed plant-based drugs (Lambert *et al.*, 1997). Japan has the highest per capita consumption of botanical medicines in the world (Laird, 1999). In 1992, Australia established Asian-Australian centre for the study of Bioactive Medicinal Plant Constituents at La Trobe University in collaboration with Chulalongkom and Chiang-Mai Universities in Thailand and Bangladesh for conducting research on bioactive constituents in medicinally active plants like turmeric, secondary metabolites in crop plants like cotton and yellow *Sinapis alba*, Islamic medicinal plants and marine toxins. In Guatemala, *Farmaya* Laboratory screened 700 different plants and developed 15 pharmaceutical products using traditional knowledge of indigenous and rural groups engaged in organic cultivation of medicinal plants. In South Africa, there are up to 100 million traditional-remedy consumers and up to 700,000 tonnes of plant material are consumed annually with an estimated value of as much as 150 million US dollars per annum (Mander and Le Breton, 2005). Medicinal plants, thus, offer remarkable opportunities to generate income and employment and to boost country’s economy through foreign exchange.

1.3 Status of medicinal plants in India

India is a gene rich country and accounts for 8% of the total global diversity. Being the largest repository of medicinal herbs, India is called the “Botanical Garden” of the world. India is among twelve most biodiverse countries of world having 16 agro climatic zones, 10 vegetative zones and 15 biotic provinces (Samy and Gopalakrishnakone, 2007). There are about 45,000 plant species with continental hotspots in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar islands. India has a very widespread, safe and traditional usage record of herbal plants recognized through Ayurvedic, Unani, Siddha, Homeopathy and Naturopathy systems of health (Vaidya and Devasagayam, 2007 and Chaturvedi *et al.*, 2007). More than 70% Indians or 1.1 billion population use these herbal-based formulations regularly as spices, home remedies and health foods, as these are non-narcotic and almost without any side effects. The northern part of the country harbors a great variety of medicinal plants because of the majestic Himalayan range. So far, about 8000 species of angiosperms, 44 species of gymnosperms and 600 species of pteridophytes have been reported in the Indian Himalayas, of which 1748 species are known to have medicinal value. Major families highlighted with medicinally important plants include Asteraceae, Liliaceae, Apocynaceae, Solanaceae, Rutaceae, Piperaceae and Sapotaceae (Dhar *et al.*, 2002). A number of medicinal plants are propagated on large scale under private or commercial sector required by drug industry in various formulations to treat dreaded diseases. Some of these are *Rauvolfia serpentina* (wonder drug of India) yielding about 50 alkaloids, major one as Reserpine used in the treatment of hypertension, *Aloe vera* (Ghrita Kumari) having anti-allergic, anti-ageing and anti-histaminic properties, *Withania somnifera* (Indian ginseng) having anti-inflammatory, anti-arthritic, immunomodulatory and anti-tumor properties, *Bacopa monnieri* (Brahmi), *Centella asiatica* (Madukparni), *Catharanthus roseus* (Periwinkle), *Dioscorea deltoidea*, *Nothapodytes nimmoniana*, *Hemidesmus indicus* (Indian sarsaparilla), *Origanum vulgare*, *Saussurea obvallata*, *Ocimum sanctum*, *Cedrus deodara*, *Cynodon dactylon*, *Aegle marmelos*, *Juniperus communis*, *Musa paradissica*, *Nardostachys grandiflora*, *Zanthoxylum armatum*, *Phyllanthus emblica*, *Podophyllum emodi*, *Mucuna pruriens*, *Berberis aristata* and *Azadirachta indica* (Chaturvedi *et al.*, 2007).

Large number of medicinal plant based industries have emerged with over 800 species used in production, which have an annual turnover of about Rs.42,000 million per year and is estimated

to grow at the rate of about 20 per cent per year. Though India has a rich biodiversity, about 90% of the medicinal plants used by industries are collected from the wild with 70% of the plant collection involving destructive harvesting because of the use of plant parts like root, bark, wood, stem and the whole plant. The growing demand is putting a heavy strain on existing resources, which pose a definite threat to the genetic stock and biodiversity of medicinal plants, causing a number of plants to be either threatened or included in the endangered category. The assessments done so far for the prioritized native medicinal species, have resulted in assignment of IUCN red list status to nearly 250 plant species with 44 species being critically endangered, 113 endangered and 87 vulnerable (Ved and Tandon, 1998 and Ved and Goraya, 2007).

1.4 Medicinal plants and Plant Tissue Culture

In view of the growing world population, increasing anthropogenic activities and rapidly eroding natural ecosystems, the natural habitats for a number of medicinal plant species are dwindling. The rising demand of plant-based drugs is creating heavy pressure on selected, high valued medicinal plants populations due to over harvesting. To cope up with this alarming situation, the recent advances in Biotechnology especially Plant Tissue Culture have come as a boon.

Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soil. Thus, mass propagation of disease-free planting material is naturally difficult. Moreover, sexually propagated plants demonstrate a high degree of heterozygosity since their seed progenies are not true to type unless they have been derived from inbred lines. As a result, plants raised through seeds shows tremendous variations in growth, habit, composition and overall yield, so they have to be discarded due to lack of quality for commercial release. Likewise majority of the medicinal plants are not amenable to conventional vegetative propagation methods as they have often proved cumbersome and are more liable to be infected with systemic infections which deteriorates their quality and genetic vigor, thus limiting the multiplication of desired cultivars. Due to the shortage of high quality planting material, cultivation and domestication of medicinal plants is facing great problem. It is therefore, imperative to adopt alternative methods of propagation, having high multiplication rates to produce large number of plants of improved quality and shortened rotation. In this regard, *in vitro* propagation or micropropagation holds the significant promise for true to type, rapid and

mass production of selected elite varieties and also to conserve endangered and threatened species.

1.5 Advantages of Micropropagation

1. The plants raised through micropropagation are of uniform quality and produce superior seeds.
2. Plants are disease free and show improved vigor and quality.
3. They can be produced much more rapidly and throughout the year irrespective of season.
4. It facilitates international exchange of germplasm without the inherent risk of spreading diseases and pathogens.
5. Stock of germplasm can be maintained for many years.

Techniques of Micropropagation:

Three main techniques used for plant propagation under *in vitro* conditions are:

1) *Enhanced axillary shoot proliferation:* Micropropagation through apical and axillary shoot proliferation is the most common method for commercial production. The cells of meristems are uniformly diploid and are least susceptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of clones.

2) *De novo formation of adventitious shoots*

New adventitious shoots can develop either

- Directly from the explants like root, stem, petiole, leaf lamina, flower parts **or**
- Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

3) *Somatic or non-zygotic embryogenesis*

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to the reproducible regeneration of non-zygotic embryos, capable of germination to form complete plantlets. These embryo like structures are bipolar units

containing root and shoot axis and can develop into fully functional plants under appropriate conditions.

Major steps in micropropagation:

Stage 0

Selection of elite mother plant



Stage I

Explant isolation

Trimming

Surface Sterilization

Detergent

Sterilizing chemicals

Antioxidants

Washing



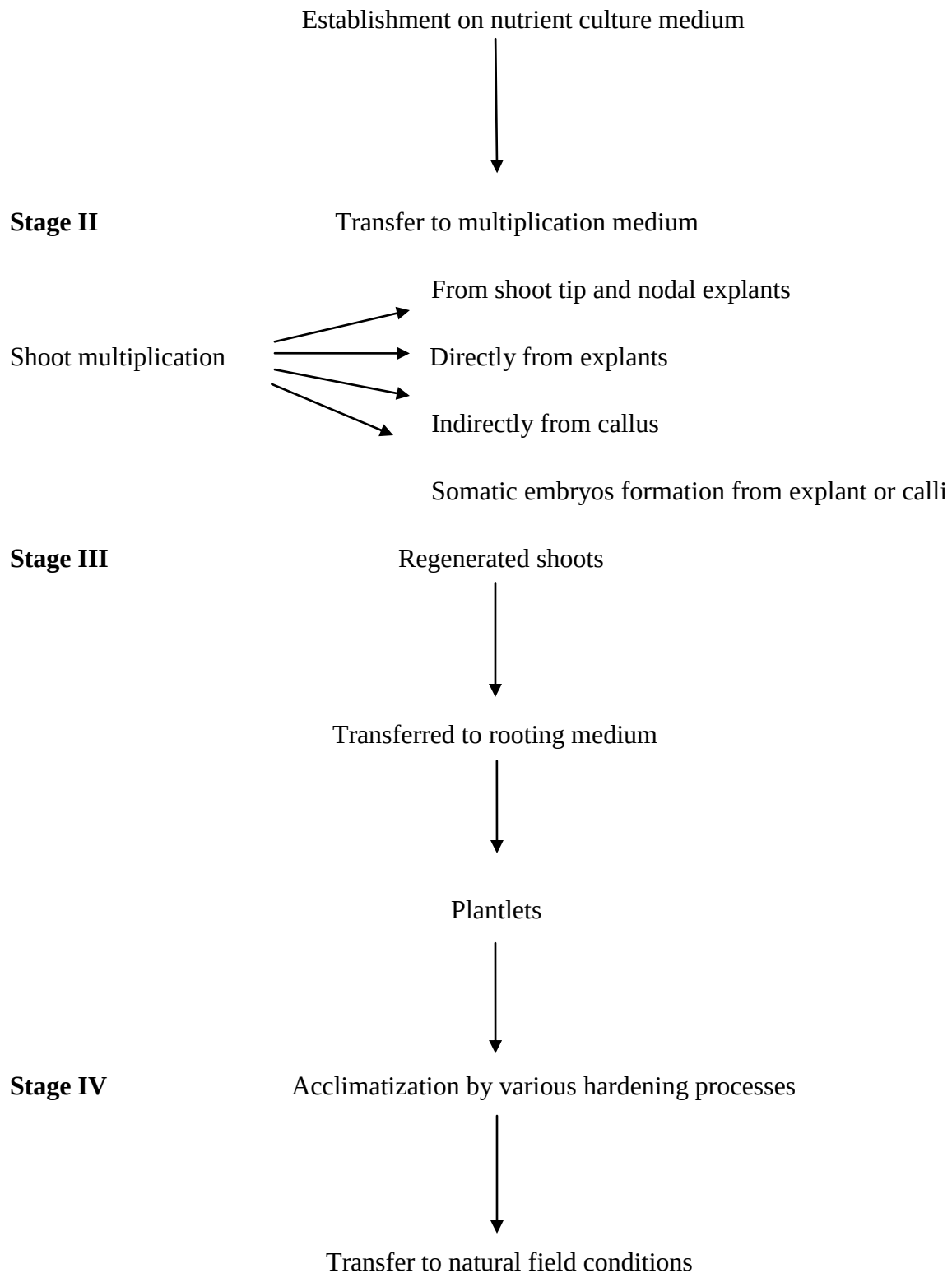


Fig. 1 Schematic representation of various stages of micropropagation

1.6 Secondary metabolite production

Since centuries, plants have an outstanding role in medicine. The beneficial effects of medicinal plants are mainly contributed by the secondary metabolites, which exert a strong physiological effect on the mammalian system and are known as active principles of plant. The extent of therapeutic use of metabolites led to the harvesting of these medicinal species from the wild or natural populations, which subsequently resulted in the loss of their existing genetic stocks. Production of plant secondary metabolites is mainly achieved through the field-cultivation of plants. However, cultivating plants of particular biotopes outside their natural ecosystem is a hard task as these fail to grow properly and are unable to withstand pathogen attacks. Therefore, this led researchers to consider plant cell, tissue or organ culture as an alternative way of producing corresponding secondary metabolites (Bourgau *et al.*, 2001). The technique of plant cell culture technology facilitates rapid production of secondary metabolites achieved by optimizing the cultural conditions, selecting high-producing strains and employing transformation and immobilization techniques to enhance the production of these metabolites (Dicosmo and Misawa, 1995 and Karuppusamy, 2009).

Tissue culture is an attractive alternative that offers continuous supply of biochemical or useful compounds produced under controlled environmental conditions independent of climatic changes and plant availability. Moreover, automated regulation of cell growth helps in monitoring the processes and production of secondary metabolites (Briskin, 2000 and Vanisree *et al.*, 2004). Different strategies using *in vitro* systems have been extensively studied with the objective of improving the production of secondary plant compounds. There are numerous reports describing the production of diverse secondary metabolites through tissue culture such as berberine from *Coptis japonica* (Sato and Yamada, 1984), ginsenosides from the roots of *Panax ginseng* (Tang and Eisenbrand, 1992), morphine and codeine from *Papaver somniferum* (Yu *et al.*, 2002), diterpenoids in *Torreya nucifera* (Orihara *et al.*, 2002), production of taxol by various *Taxus* species (Oksman-Caldentey and Inze, 2004), *Leonurus heterophyllus* (Yang *et al.*, 2008), cerpegin in *Ceropegia juncea* (Nikam and Savant, 2009) and several others. Advancement in the technique of plant cell cultures provides new means for the cost effective, commercial production of rare, exotic varieties and yet unknown plant chemicals.

1.7 Rationale, Objectives and Research approach

The present investigation was carried out on two important medicinal plant species namely *Stevia rebaudiana* and *Tylophora indica* which are used as traditional medicine across the globe for their ethno pharmacological values to cure variety of diseases. *Stevia*, a non-calorific sweetening herb is considered to be hypoglycemic, hypotensive, diuretic and cardiogenic (Jeppesen *et al.*, 2000). It nourishes the pancreas and does not raise blood glucose levels, making it not only safe for diabetics but also beneficial. Similarly, *Tylophora indica* has strong medicinal value as a remedy for asthma, bronchitis, allergies, rheumatism and dermatitis.

The importance of these medicinal plants brings in various challenges and opportunities associated with large-scale production of these plants under disease free conditions. Generally, *Stevia rebaudiana* is propagated through stem cuttings but it is a slow, cumbersome and highly labor-intensive procedure and is inefficient because of less number of propagules obtained from single plant (Sakaguchi and Kan, 1982). Severe incidences of attack of diseases and pests are seen where cuttings are used as planting material. Seeds are generally infertile and poor seed germination becomes another major limiting factor for large-scale cultivation of this plant (Soejarto *et al.*, 1982; Nakamura and Tamura, 1985; Shaffert and Chetobar, 1994 and Goettemoeller and Ching, 1999). Moreover, propagation by seeds does not allow the homogeneity in plant populations, resulting in great variability in sweetening levels and composition. In *Tylophora indica*, conventional propagation of the plant occurs through seeds but seeds are too small and have low seed viability and germination. Being cross-pollinated, the seed progenies show great heterozygosity and may not be suitable for large-scale propagation of this plant species. Likewise, the plant is not amenable to vegetative propagation through cuttings or grafting, thus, limiting multiplication of desired cultivars. Apart from these, there are a number of constraints like variations in edaphic and climatic factors and seasonal dormancy, which limit the propagation and conservation of this important plant through conventional methods (Chandrasekhar *et al.*, 2006).

In light of the difficulties in the propagation of these medicinal plants and keeping in view the medicinal importance of glycosides as sugar substitutes and alkaloids as medicinal agents, the present study was conducted with the following objectives:

1. To develop and standardize mass propagation of *Stevia rebaudiana* and *Tylophora indica* under *in vitro* conditions.
2. Extraction and characterization of major secondary metabolites from *in-vivo* and *in-vitro* cultures.
3. To develop protocol for mass extraction of secondary metabolites.

LITERATURE REVIEW

The advent of *in vitro* tissue culture technique has offered a new approach to the morphogenetic investigations. Its advantages over other techniques stem from the fact that it allows a living system to be studied under controlled environmental conditions, thus enabling the study of a complex biological phenomenon in its parts. The technique of plant tissue culture is based on the concept of totipotency i.e. every cell of the plant body is totipotent and capable of giving rise to a new plant under proper nurture conditions. In 1902, Gottlieb Haberlandt gave the idea of totipotency and suggested that “One could successfully cultivate artificial embryos from vegetative cells”. Haberlandt first cultured and isolated fully differentiated cells in an artificial medium, in which the cells increased in size, remained viable for a month but failed to divide. Although his attempt to grow vegetative cells in an artificial medium did not succeed due to the choice of highly specialized tissue and non-discovery of growth regulators at that time, but it did open up new vistas in morphogenesis. Haberlandt’s hypothesis has now flowered into a vigorous discipline- “Tissue Culture” which has emerged as a potential technique for plant improvement and forms the backbone of Plant biotechnology.

The present review is intended to consolidate significant advances made to date in the propagation of medicinal plants particularly *Stevia rebaudiana* and *Tylophora indica* and extraction of useful secondary metabolites under *in vitro* conditions.

2.1 *In vitro* propagation of medicinal plants

The clonal propagation of selected phenotypes is an essential step in most of the plant breeding programmes. Micropropagation has emerged as a promising technique to obtain genetically pure elites rather than having indifferent populations. In order to meet the growing demands of medicinal plants, micropropagation can be effectively used for large-scale multiplication and conservation of endangered, rare and threatened plant species including *Saussurea lappa*, *Picorrhiza kurroa*, *Ginkgo biloba*, *Swertia chirata*, *Gymnema sylvestre*, *Tinospora cardifolia*, *Salaca oblonga*, *Celastrus paniculata*, *Oroxylum indicum*, *Glycyrrhiza glabra*, *Tylophora indica*, *Bacopa mooniera*, *Rauwolfia serpentina*, *Withania somnifera*, *Aloe vera*, *Allium sativum*,

Zingiber officinale, *Dioscorea deltoidea*, *Costus speciosus*, *Solanum khasianum* etc. (Chaturvedi *et al.*, 2007 and Sharma *et al.*, 2010).

For micropropagation of medicinal plants, all the three techniques of *in vitro* propagation viz. forced axillary branching, *de novo* adventitious shoot formation and somatic embryogenesis have been exploited.

2.1.1 Multiplication by enhanced axillary shoot proliferation

It is the most rapid and reliable method for *in vitro* mass multiplication. A shoot tip or an axillary bud has a preformed meristem, which develops axillary shoots on a high cytokinin concentration. These axillary shoots can be subdivided into smaller clumps of shoots, which in turn can develop similar clusters after subculturing on fresh media. This process can go on indefinitely and can be maintained throughout the year and a large number of plants can be raised starting from a single shoot tip or an axillary bud. This method ensures genetic stability of the clones as cells of shoot meristem are uniformly diploid and are least susceptible to genotypic changes (Pattnaik and Cland, 1997).

The role of cytokinins in inducing bud break is well documented and supported by many researchers. Among different cytokinins, BA and K when used either alone or in combination with lower concentration of auxins have been very effective in inducing sprouting of axillary buds in several medicinal plant species namely *Lippia alba* (Gupta *et al.*, 2001), *Phyllanthus urinaria* (Catapan *et al.*, 2002), *Mentha arvensis* (Shahzad *et al.*, 2002), *Rotula aquatica* (Martin, 2003), *Rauwolfia serpentina* (Baksha *et al.*, 2007), *Scrophularia takesimensis* (Sivanesan and Murugesan, 2008), *Withania somnifera* (Fatima and Anis, 2010) and *Cocculus hirsutus* (Meena *et al.*, 2012). In *Stevia rebaudiana*, Sivaram and Mukundan, 2003 established a micropropagation protocol through forced axillary branching on MS medium supplemented with 8.87 μM BA with 5.71 μM IAA. Likewise Debnath, 2008; Kalpana *et al.*, 2009; Naz, 2009 and Anbazhagan *et al.*, 2010 also demonstrated the essentiality of BA along with IAA for initiating multiple shoot proliferation from shoot apices and nodal segments of *S.rebaudiana*. Synergistic effect of cytokinin BA with auxin NAA for inducing bud break and multiple shoot proliferation from nodal segments of *S.rebaudiana* was reported by Himanshu *et al.*, 2006. Verma *et al.*, 2011

observed bud break (80 %) and multiple shoot formation at the rate of 17.5 shoots/nodal explant when cultured on 2.2 μ M BA along with 2.3 μ M K.

Ibrahim *et al.*, 2008 studied the effect of MS salt strength ($\frac{1}{2}$, $\frac{1}{4}$, $\frac{3}{4}$ and full MS) on the number of shoots proliferated from shoot apices of *Stevia rebaudiana* and reported the best results (6.46 shoots/explant) on MS at full salt strength and recorded lowest number of shoots (2.28 shoots/explant) on quarter MS salt strength. Kalpana *et al.*, 2010 reported an enhanced rate of shoot proliferation from nodal explants in *Stevia rebaudiana* when the concentration of CuSO_4 in the induction medium was raised five times the MS level. Combinations of auxins and cytokinins have previously been used to induce multiple shoots in a number of plants of family Compositae (Nikam and Shitole, 1999 and Radhika *et al.*, 2006). Like in *Vernonia amygdalina*, nodal explants when cultured on 2.2 μ M BA with 3.6 μ M NAA supplemented MS medium formed maximum number of 7.2 shoots per explant in almost all the cultures (Khalafalla *et al.*, 2007). Axillary buds from juvenile plants of *Spilanthes mauritiana* resulted in maximum shoot response with an average of 5.6 shoots/explant in 96.7% of the cultures when explants were inoculated on MS medium containing 1mM BA and 0.1mM NAA (Bais *et al.*, 2002).

Sharma and Chandel, 1992 developed protocol for rapid *in vitro* multiplication of *Tylophora indica* from axillary buds on MS supplemented with BA (22 μ M) with NAA (3.67 μ M) and ascorbic acid. Similar set of combination was used by Faisal *et al.*, 2007 to achieve plant regeneration via enhanced axillary shoot proliferation in *Tylophora indica* with highest number of 8.6 ± 0.71 shoots/nodal explant. Synergistic action of cytokinin with an auxin has been demonstrated in many plants of family Asclepiadaceae such as in *Hemidesmus indicus* where 7.35 μ M NAA with 1.15 μ M K produced a maximum number of 8.2 ± 0.4 shoots/explant in 95% of the cultures (Patnaik and Debata, 1996) whereas in *Ceropegia candelabrum*, 8.87 μ M BA with 2.46 μ M IBA formed an average of eight shoots/ node (Beena *et al.*, 2003). Similarly, in another endangered member of this family *Leptadenia reticulata*, synergistic effect of 2.2 μ M BA with 0.6 μ M IAA was most effective in inducing maximum number of multiple shoots from the nodal explants (Arya *et al.*, 2003). In *Psoralea corylifolia*, combination of 5 μ M BA with 0.5 μ M NAA produced 12 ± 0.5 shoots/nodal segments in nearly 90% of the cultures (Anis and Faisal, 2005).

2.1.2 *De novo* formation of adventitious shoots directly from explants

De novo formation of adventitious shoots through direct regeneration is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. The direct regeneration method has the advantage of omitting the callus and embryoids phases and significantly reducing the total number of stages in culture. New adventitious shoots can develop directly from the explants like leaf, stem, petiole and flower parts. *De novo* formation of adventitious shoots employing various explants has been reported in a number of medicinal plant species like *Withania somnifera* (Kulkarni *et al.*, 2000), *Tanacetum cinerariifolium* (Hedayat *et al.*, 2009), *Psorelea corylifolia* (Baskaran and Jayabalan, 2010), *Embelia ribes* (Annapurna and Rathore, 2010) and *Phellodendron amurense* (Yang *et al.*, 2011).

Sivaram and Mukundan, 2003 reported adventitious shoot formation (10 in number) from leaf explants of *Stevia rebaudiana* on MS containing 8.87 μM BA and 5.71 μM IAA. Sreedhar *et al.*, 2008 induced organogenesis from leaf explants of *Stevia rebaudiana* with direct shoot induction occurring on either side of the midrib from adaxial surface of immature leaves after 5 weeks of culture on MS medium supplemented with 8.88 μM of N⁶-benzylaminopurine and 4.65 – 6.98 μM kinetin. Preethi *et al.*, 2011 standardized protocol for direct shoot organogenesis from leaf explants of *S.rebaudiana* with maximum number of 10.4 ± 0.21 shoots obtained after culturing explants on MS supplemented with different concentrations of BA, K and IAA. The combined effect of BA or K with IAA for direct shoot regeneration was also documented in *Piper longum* (Sarasani *et al.*, 1993), *Cajanus cajan* (Misra, 2002) and *Lillium* (Bacchetta *et al.*, 2003) and *Hibiscus cannabinus* (Chen *et al.*, 2010).

Bera and Roy, 1993 established a rapid *in vitro* multiplication system by the formation of multiple adventitious shoot buds from mature leaf explants of *Tylophora indica* when cultured on MS supplemented with 6-benzylaminopurine (22 μM) and adenine sulphate (0.65 μM). Chaudhuri *et al.*, 2004 reported the formation of organogenic nodular meristemoids from the cut ends of root segments of *T. indica* when cultured on BA or 2 ip. These nodular meristemoids showed two types of organogenic responses when maintained on induction medium leading to direct shoot bud formation in 42 % cultures and somatic embryogenesis in 39 % of explants. Kaur *et al.*, 2011 b, c reported high frequency *de novo* adventitious shoot formation from stem

and root explants of *T. indica* when cultured on 8.8 μM BA, whereas leaf explants gave better results when 9.84 μM BA was used in conjunction with 1.35 μM adenine sulphate (Kaur *et al.*, 2011 a). The promotory effect of cytokinins on multiple shoot regeneration has been reported in a number of medicinal plant species employing various kinds of explants (Hiregoudar *et al.*, 2003; 2006 and Loc *et al.*, 2005). In *Hypericum spectabile*, direct plant regeneration from the leaf explants was observed on different concentrations of BA, however, MS supplemented with 4.4 μM BA showed maximum number of 29.6 shoots/explant with an average shoot length of 3 cm/explant in nearly 90% of the cultures (Akbas *et al.*, 2011). Similarly, Pretto and Santarem, 2000 also found BA to be most effective in promoting shoot regeneration from leaf explants of *Hypericum perforatum*. In *Plectranthus barbatus*, K at a concentration of 6.9 μM was most effective in inducing an average number of 19.7 ± 2.08 shoots per explants whereas combination of 8.8 μM BAP with 7.35 μM NAA produced 15.0 ± 2.20 shoots per explants (Thangavel *et al.*, 2011). Among different cytokinins used in *Filipendula ulmaria*, extensive shoot differentiation from leaf, root and petiole explants was observed when TDZ was used in combination with IAA and GA₃ (Yildrum and Turker, 2009) whereas in *Gaultheria fragrantissima*, thidiazuron (TDZ) alone was most effective in inducing direct shoot regeneration from leaf and internode explants (Ranyaphia *et al.*, 2011).

2.1.3 Adventitious Shoot formation through callus

Callus mediated shoot morphogenesis has been accomplished in several medicinal plants including *Stevia rebaudiana* and *Tylophora indica*. Swanson *et al.*, 1992 obtained friable callus from leaf explants of *Stevia rebaudiana* on MS medium containing 3.6 μM NAA and 2.2 μM BA, 0.9% agar and 30 g/l sucrose. Differentiation of callus tissue was achieved by eliminating the agar and modulating the medium hormone composition. Smitha *et al.*, 2005 reported callus formation from shoot buds of *Stevia rebaudiana* on 10.7 μM NAA and 8.8 μM BA medium and induction of multiple shoots occurred on transfer of callus to MS medium supplemented with 4.4 μM BA. Further incorporation of 2.3 μM K enhanced the production of dark green healthy shoots. Uddin *et al.*, 2006 reported callus induction from leaf, nodal and internodal segments of *Stevia rebaudiana* on MS medium supplemented with different concentrations of 2, 4-D (4.87-19.48 μM). The highest amount of callus was found on MS medium with 9.74 μM 2, 4 -D and higher concentrations of 2, 4- D (24.35 μM) proved inhibitory for callus growth. Similarly, the

essentiality of 2, 4-D for callus induction from all the three explants namely leaf, internodal and nodal segments of *Stevia rebaudiana* was also demonstrated by Sadeak *et al.*, 2009 and Tiwari, 2010. Another study by Das *et al.*, 2006 showed that K in combination with NAA and 2, 4-D exhibited better results for callus initiation whereas the combined application of BA and NAA showed most satisfactory performance in maintaining callus when half strength of MS medium was used. Naz, 2009 obtained best callus growth from leaf explants on 12.17 μM 2, 4- D supplemented medium. The callus formed was further transferred to 8.8 μM BA and 4.84 μM IAA supplemented medium for shoot differentiation. Gupta *et al.*, 2010 reported 100% callusing from the leaf explants of *S.rebaudiana* when cultured on combination of NAA and 2, 4- D after 3 weeks of culturing whereas only 10% callusing was observed with 2, 4- D alone. Ahmad *et al.*, 2011 obtained best callogenic response from flowers of *S.rebaudiana* on 8.8 μM BA with 9.74 μM 2, 4- D supplemented medium. Subsequent transfer of callus on to 8.8 μM BA alone formed a maximum of 21.6 shoots/explant in 85% of the cultures.

In *Tylophora indica*, callusing has been observed from different explants when cultured on varied media combinations. Faisal and Anis, 2003, 2005 induced optimal callus growth from leaf and stem explants on 10 μM 2, 4, 5-T supplemented medium. They further reported highest shoot regeneration frequency (80-85%) from the callus when subcultured onto MS medium containing 5 μM K alone. Thomas and Philip, 2005 induced calli from leaf explants on 7 μM 2, 4- D with 1.5 μM BA medium and 92% of the callus cultures differentiated to produce green leafy shoots (66.7 shoots per explant) on transfer to 8 μM TDZ supplemented medium. Likewise, profuse callus growth was reported from petiole explants on MS supplemented with 10 μM 2, 4- D and 2.5 μM TDZ and further differentiation of shoots from the callus was achieved on 2.5 μM TDZ alone (Faisal *et al.*, 2006). Thomas, 2009 reported callus induction from isolated leaf mesophyll protoplasts of *T.indica* on MS supplemented with 2, 4-D (4 μM), 0.4 M mannitol and 3% sucrose. Calli when transferred to MS containing TDZ (1-7 μM) and NAA (0.2-0.4 μM) exhibited differentiation of 12 shoots/callus in 44% of the cultures. Sahai *et al.*, 2010 reported high frequency plant production by shoot organogenesis from leaf callus of *T. indica* on BA (5 μM) supplemented MS medium. Verma *et al.*, 2010 obtained organogenic calli from leaf explants on BA and IBA supplemented medium and further elongation of developed shoots (12 ± 1.50 cm) occurred on MS medium containing 0.45 μM TDZ. Kaur *et al.*, 2011a, b reported

induction of green callus from leaf and stem explants of *T. indica* on MS supplemented with NAA and kinetin which exhibited prolific shoot differentiation when transferred to (8.8 μ M) BA. Callus mediated shoot organogenesis has been reported in several medicinal plants including *Gymnema sylvestre* (Gopi, 2002), *Eurycoma longifolia* (Siregar *et al.*, 2003), *Saussurea obvallata* (Dhar and Joshi, 2005), *Euphorbia nivulia* (Sunandakumari *et al.*, 2005) *Cassia angustifolia* (Agarwal and Sardar, 2006), *Arctium lappa* (He *et al.*, 2006), *Mucuna pruriens* (Faisal *et al.*, 2006), *Sarcostemma brevistigma* (Thomas and Shankar, 2009), *Cassia angustifolia* (Siddique *et al.*, 2010), *Curcuma kwangsiensis* (Zhang *et al.*, 2011) and *Rauwolfia serpentina* (Panwar *et al.*, 2011).

Plants raised through calli may not be true elites because of various morphological, physiological and genetic variations found in callus cells, resulting in high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it. Moreover, shoot multiplication through callus phase is not applicable to many important crop species and wherever applicable, the initial plant regeneration capacity of the tissues may decline with the passage of time and is eventually lost. Still callus constitutes one of the unique materials for rapid multiplication of plants, since large number of plants can be obtained from a small tissue.

2.1.4 Somatic embryogenesis

Somatic embryogenesis is another alternative to traditional vegetative propagation method as it offers a rapid and large-scale propagation system. Somatic embryogenesis is a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue. The somatic embryos are bipolar structures having a radical and plumule and are similar to zygotic embryos in development and can develop into fully functional plants under aseptic conditions. Since somatic embryos carry a pre-formed radical, there is no need of rooting as is required in organogenesis.

There are only a few reports regarding somatic embryogenesis in *Stevia rebaudiana*. Beshpalhok *et al.*, 1993 established embryogenic cultures from the leaf explants of *Stevia rebaudiana* on MS supplemented with 2, 4- D and BA in high sucrose medium. Banerjee and Sarkar, 2010 reported

somatic embryogenesis in *Stevia rebaudiana* from the calli obtained from nodal and leaf explants on reduced concentration of 2, 4- D (4.87 μ M).

Plant regeneration through direct and indirect embryogenesis has been reported in a number of medicinal plants of family Asclepiadaceae including *Hemidesmus indicus* (Sarasan *et al.*, 1994), *Ceropegia* sp. (Patil, 1998), *Tylophora indica* (Manjula *et al.*, 2000), *Holostemma ada-kodien* (Martin, 2003) *Hemidesmus indicus* (George *et al.*, 2008) and *Sarcostemma brevistigma* (Thomas and Shankar, 2009). Manjula *et al.*, 2000 reported somatic embryogenesis from leaf derived callus of *Tylophora indica* on MS medium supplemented with 8.8 μ M BA which also provided the maximum conversion rate (90%) of embryoids to plantlets. Jayanthi and Mandal, 2001 induced embryogenic callus from leaf explants of *T. indica* on MS medium supplemented with 2, 4- D (9.0 μ M) and K (0.05 μ M) and further development and maturation of embryos was reported on medium supplemented with different concentrations of BA or 2 ip. Chaudhari *et al.*, 2004 reported the formation of friable embryogenic calli and somatic embryos from root explants of *Tylophora indica* on BA or 2 ip, out of which 10.72 μ M BA was most effective in inducing somatic embryos. Sahai *et al.*, 2010 highlighted the role of BA and TDZ on the induction of embryogenic calli from leaf explants of *T. indica*. Although induction of somatic embryogenesis by cytokinin alone is very unusual, but has been reported in a number of plants like *Medicago* sp. (Iantcheva *et al.*, 1999), *Quercus robur* (Cuenca *et al.*, 1999), *Arachis pintoii* (Rey *et al.*, 2000), *Pinus roxburghii* (Arya *et al.*, 2000) and *Quassia amara* (Martin and Madassery, 2005).

Thomas, 2006 studied the effects of sugars, gibberellic acid and abscisic acid on somatic embryogenesis from internodal explants of *Tylophora indica*. MS containing 6 μ M/L K with 200 mM/L sucrose showed 71% embryogenesis with an average of 49 embryos per explant. Addition of 10 μ M/L GA₃ into 200 mM/l sucrose resulted in 98% embryogenesis whereas 2 μ M/L ABA with 200 mM/L sucrose showed 95% embryogenesis. Chandrasekhar *et al.*, 2006, reported direct embryogenesis from mature leaf explants of *Tylophora indica* on Murashige and Skoog's (MS) medium supplemented with 2, 4-D (1.5 μ M) and TDZ (0.5 μ M) with an average of 18.2 globular embryos/culture. The importance of 2, 4-D for inducing somatic embryogenesis is well documented in a number of other medicinal plants such as *Pennisetum americanum* (Vasil and

Vasil, 1982), *Vicia narbonensis* (Pickardt *et al.*, 1989), *Cymbopogon martini* (Pattnaik and Cland, 1997), *Manihot glaziovii* (Joseph *et al.*, 2000), *Syngonium podophyllum* (Zhang *et al.*, 2006), *Centella asiatica* (Joshee *et al.*, 2007) and *Withania somnifera* (Sharma *et al.*, 2010).

2.1.5 Synthesis of artificial seeds

Artificial seeds are the living seed like structures derived from somatic embryos or other propagules like shoot buds, cell aggregates or other plant tissues under *in vitro* culture conditions after encapsulation by a hydrogel. They have the ability to grow into complete plants under *in vitro* or *ex vitro* conditions and retain their regeneration potential even after different periods of storage. It is a high volume, low cost production technology which could open new vistas for clonal propagation in several commercially important crop species.

Encapsulation can be done in two different ways: Dropping method and Molding. Redenbaugh *et al.*, 1987 used dropping method to encapsulate somatic embryos of alfalfa, celery, carrot, lettuce, *Brassica* species, cotton and corn. In recent years, encapsulation technology has drawn much attention for the production of artificial seeds as it helps in minimizing the cost of micropropagated plants, ensures high volume of propagation and can be useful for the conservation of germplasm of elite and endangered plants and also facilitates exchange and distribution of germplasm across different laboratories (Rai *et al.*, 2009 and Verma *et al.*, 2010).

Mostly somatic embryos have been used in the encapsulation technology for the production of synthetic seeds. Some of the important medicinal plants where somatic embryos have been used for synthetic seed production include *Paulownia elongata* (Ipekci and Gozukirmizi, 2004), *Arnebia euchroma* (Manjkhola *et al.*, 2005), *Hyoscyamus muticus* (Pandey and Chand, 2005) and ***Catharanthus roseus*** (Maqsood *et al.*, 2012). In *Tylophora indica*, synthetic seed production has been achieved by encapsulating somatic embryos in different concentrations of sodium alginate. Somatic embryos encapsulated with 2% sodium alginate resulted in maximum of 65% germination whereas 1% and 3% sodium alginate coated embryos exhibited less percent germination (Chandrasekhar *et al.*, 2006).

In recent years, the possibility of encapsulating vegetative propagules such as axillary buds, shoot tips and nodal segments as an alternative for somatic embryos has also been explored (Machii, 1992; Mondal *et al.*, 2000; Mandal *et al.*, 2000; Chand and Singh, 2004; Rai *et al.*, 2008 a, b; Singh *et al.*, 2009; Verma *et al.*, 2010; Singh and Chand, 2010; Singh *et al.*, 2010; Kumar *et al.*, 2010). In *Stevia rebaudiana*, there is a single report of artificial seed production by encapsulating shoot tips with 3% sodium alginate and 2.5% calcium chloride (Andlib *et al.*, 2011). Faisal *et al.*, 2007 used nodal segments of *Tylophora indica* to synthesize artificial seeds. The best gel complexing was achieved with 3% sodium alginate and 100 mM CaCl₂. 2H₂O and maximum frequency (91%) of conversion of these encapsulated beads into plantlets was achieved on Murashige and Skoog's (MS) medium containing 2.5 µM 6- benzyladenine (BA) and 0.5 µM α-naphthalene acetic acid (NAA) after 6 weeks of culture. Similarly, Makowczynska and Andrzejewska, 2006 encapsulated shoot tips of *Plantago asiatica* to form artificial seeds and studied their regeneration potential to form complete plantlets. Kumar *et al.*, 2010 encapsulated shoot tips excised from *in vitro* proliferated shoots derived from nodal explants of *Simmondsia chinensis* to produce artificial seeds. A gelling matrix of 3% sodium alginate and 100 mM calcium chloride was found to be most suitable for formation of ideal beads.

2.1.6 Rooting of Microshoots

Induction and development of roots at the base of *in vitro* grown shoots is an essential and indispensable step to establish tissue culture derived plantlets in the soil. MS medium supplemented with different auxins is most frequently used for inducing roots at the base of microshoots. However, auxin free basal MS at full strength and half strength concentrations has also proved to be optimum medium for inducing roots in a number of plants including *Crataeva adansonii* (Sharma *et al.*, 2003), *Stevia rebaudiana* (Ibrahim *et al.*, 2008) *Clematis gouriana* (Naika and Krishna, 2008) and *Tylophora indica* (Kaur *et al.*, 2011 a). Out of various auxins, IBA has produced best results for inducing roots in a number of plant species. In *Stevia rebaudiana*, numerous successful reports are available depicting efficient role of IBA supplemented half strength MS medium for root induction (Sivaram and Mukundan, 2003, Kalpana *et al.*, 2010 and Preethi *et al.*, 2011). A number of similar reports highlighting the importance of IBA in root induction are also available in *Tylophora indica* (Thomas and Philip, 2005; Faisal and Anis, 2005; Thomas, 2009; Verma *et al.*, 2010 and Kaur *et al.*, 2011 a, b, c).

Apart from IBA, other auxins such as NAA, IAA and 2, 4- D have also played an important role in inducing roots from the base of microshoots. Slavova *et al.*, 2003 obtained 84-99 % rooting on full strength MS containing NAA whereas Rafiq *et al.*, 2007 observed 81% rooting on same medium in *S.rebaudiana*. Ali *et al.*, 2010 observed 96 % rooting in *Stevia* on 7.35 μ M NAA with 7 roots per plant within 5 days of transfer of microshoots to root inducing medium. In *T. indica*, Murashige and Skoog's medium containing 14.4 μ M IAA formed roots within two weeks of inoculation of microshoots (Bera and Roy, 1993) while Chaudhuri *et al.*, 2004 reported the formation of maximum number of roots (10 per shoot) on 28.54 μ M IAA within 15 days of culture. Effectiveness of auxins viz NAA and IAA on root induction and proliferation has also been observed in *Phyllanthus urinaria* (Catapan *et al.*, 2002), *Ceropegia juncea* (Nikam and Savant, 2009) and *Hypericum spectabile* (Akbas *et al.*, 2011).

2.1.7 Acclimatization of micropropagated plants

The microenvironment of tissue culture raised plants is different in many respects from the *ex vitro* environment. Some of these are high levels of nutrition, low irradiance, limited gas exchange and high relative humidity. When tissue cultured plants are transferred to green house conditions, these experience temperature, nutrition and humidity shocks. In order to overcome this, it is necessary to acclimatize these plants to new environment through various hardening stages.

Different workers have employed different potting mixes towards successful establishment of *in vitro* raised plants under *ex vitro* conditions. Cocopeat was used as a potting mixture with Soilrite for *Carica papaya* (Agnihotri *et al.*, 2004), soaked cotton for *Saccharum officinarum* (Gill *et al.*, 2004) and *Garcinia indica* (Chabukswar *et al.*, 2005), mixture of press mud cake with soil for banana plantlets (Vasane and Kothari, 2006) and vermicompost in *Tylophora indica* (Rani and Rana, 2010). In *Stevia rebaudiana*, plants shifted to potting mixture of soil: vermiculite: cocopeat (1:1:1) showed 60 survival (Sivaram and Mukundan, 2003) whereas Sadeak *et al.*, 2009 reported 70% survival on garden soil: sand (1:1). On the other hand Ali *et al.*, 2010 exhibited 90 % survival of *Stevia* on soil: sand: peat (1:1:1). Preethi *et al.*, 2011 observed maximum survival percentage of hardened plantlets of *Stevia* on soil vermiculite (1:1) mixture. Soil alone proved to be the optimum potting mixture for acclimatization of *Tylophora indica* (Thomas and Philip,

2005; Faisal *et al.*, 2007 and Verma *et al.*, 2010). On the other hand, Sahai *et al.*, 2010 acclimatized plants of *T. indica* on peat moss: perlite (3:1) while Kaur *et al.*, 2011 reported 90% survival of plants of *T. indica* on soil: vermicompost (1:1) potting mixture.

2.2 Medicinal plants as source of bioactive compounds

Medicinal plants are important source of bioactive compounds, which are used as pharmaceuticals, agrochemicals, fragrance ingredients, food additives, medicinal and other dietary supplements (Chaudhuri *et al.*, 2009). These plants species have drawn immense attraction in traditional medicines all over the world as they are safe, cheap and with no side effects. Different ayurvedic formulations made from medicinal plants includes Abana, Amrita bindu, C-phycocyanin, Centalaplus, Chapparal, Geriforte, Jigrine, Liv-52, Maharishi formulations, Muthu marunthu, Ophtacare, P55A, Sandhika, Student rasayana and Tamra bhasma (Vaidya and Devasagayam, 2007). The use of traditional medicines and medicinal plants in most developing countries as therapeutic agents for the maintenance of good health has been widely observed (UNESCO, 1996). Compounds from these plants have potential applications in prevention and therapy of various human ailments which either act directly on the system or through interfering with the metabolism of infecting microbes. In either way, the bioactive compounds from medicinal plants play a pivotal role in regulating host-microbe interaction in favor of the host. There is an ever-increasing inclination towards the identification, isolation, purification and characterization of active ingredients in crude extracts of these medicinal plants by various analytical methods. Some of the important active ingredients isolated from these medicinal plants are listed in Table 1. Biotechnological approaches especially plant tissue culture technology has emerged as a potential alternative source to conventional methods for exploring different plant species for large-scale production of secondary metabolites, irrespective of plant availability (Sajc *et al.*, 2000 and Ramachandra Rao and Ravishankar, 2002).

Table 1: Plant derived ethnotherapeutics used in traditional medicine (Hoareau and Dasilva, 1999 and Samy and Gopalakrishnakone, 2007).

S. No.	Plant species	Active Ingredient
1.	<i>Papaver somniferum</i>	Codeine, Morphine
2.	<i>Atropa belladonna</i>	Atropine
3.	<i>Hyascyamus niger</i>	Hysocyamine
4.	<i>Ephedra sinica</i>	Ephedrine
5.	<i>Cinchona sp</i>	Quinine
6.	<i>Cephaelis sp.</i>	Emetine
7.	<i>Cathranthus rosesus</i>	Vinblastine and Vincristine
8.	<i>Podophyllum emodi</i>	Podophyllataxin
9.	<i>Taxus brevifolius</i>	Taxotere, Paciltaxel
10.	<i>Juniperus communis</i>	Etoposie, Teniposide
11.	<i>Panax quinquefolium</i>	Ginseng
12.	<i>Pausinystalia yohimbe</i>	Yohimbine
13.	<i>Mappia foetida</i>	Comptothechin, Irenoteccan
14.	<i>Lithospermum erythrorhizon</i>	Shikonin

2.2.1 Extraction of secondary metabolites from *Stevia rebaudiana*

Secondary metabolites are the potent bioactive compounds found in medicinal plants and are the precursors for the synthesis of various useful drugs (Sofowora, 1995). These metabolites include

coumarins, glycosides, gums, phenols, tannins, terpenes, terpenoids, various types of acids (caffeic, chlorogenic, etc.), neutral water soluble oligosaccharides, essential oils and trace elements (Komissarenko *et al.*, 1994 and Esmat and Ferial, 2009). Nabeta *et al.*, 1976 isolated three esters of lupenol, triterpenes, β amyrin acetate and sterols from the leaves of *Stevia rebaudiana*. Sholichin *et al.*, 1980 characterized three different metabolites namely jhanol, 6-O acetylaustroinulin and austroinulin from the leaves whereas Darise *et al.*, 1983 obtained all these three metabolites including stevioside and rebaudioside A from the flowers of *Stevia rebaudiana*. Rajbhandari and Roberts, 1983 identified six flavonoids in the aqueous methanolic extract obtained from leaves of *S.rebaudiana*. These identified flavonoids includes apigenin-4'-O-glucoside, luteolin-7-O-glucoside, kaempferol-3-O-rhamnoside, quercitrin, quercetin-3-O-glucoside and quercetin-3-O-arabinoside and centaureidin. Leaves of *Stevia rebaudiana* are the source of diterpene glycosides namely stevioside, dulcoside, steviolbioside, rebaudioside A, B, C, D and E (Bondarev *et al.*, 2001 and Starratt *et al.*, 2002). The content of major metabolite stevioside present in *Stevia rebaudiana* ranges from 4-20 % of the dry weight of leaves depending on variety and growth conditions (Brandle *et al.*, 1998). Apart from these metabolites, plant is also supplemented with other components like β -Sitosterol and stigmasterol (Sholichin *et al.*, 1980), flavonoids (Rajbhandari and Roberts, 1983) and sesquiterpenes based on bisabolane, germacrane (Martelli *et al.*, 1985). The extraction and refining of these principle metabolites from *Stevia* involves use of various organic solvents such as hot and deionised water (Kitada *et al.*, 1989; Bovanova *et al.*, 1998 and Vanek *et al.*, 2001), combination of water, ethanol, ethyl acetate and cyclohexane (Nikolova-Damyanova *et al.*, 1994), chloroform and methanol (Kolb *et al.*, 2001) and a combination of n-butanol, n-hexane and methanol (Choi *et al.*, 2002). Kohda *et al.*, 1976 isolated major glycosides including stevioside, rebaudioside A and B and minor glycoside steviolbioside from the methanolic extract of *Stevia rebaudiana*. Subsequently, it was suggested that rebaudioside A was formed as a result of enzymatic and chemical conversion of stevioside whereas rebaudioside B was an artifact formed from rebaudioside A (Kaneda *et al.*, 1977). Adduci *et al.*, 1987 obtained 80 % pure stevioside from dried leaves of *Stevia rebaudiana* through hot water extraction followed by simultaneous decolorization and demineralization using ion exchange. Phillips, 1987 described a conventional extraction process using different solvents. The *Stevia* leaves were extracted with hot water or alcohols and in certain cases, the leaves were pretreated with nonpolar solvents such as chloroform or hexane to remove the essential oils,

lipids, chlorophyll and other nonpolar substances. The extract was clarified by precipitation with salt or alkaline solution to obtain pure crystals of stevioside. Supercritical fluid extraction (SCFE) with CO₂ and a co-solvent like methanol, ethanol and acetone was used for the production of *Stevia* glycosides (Tan *et al.*, 1988). Tsanava *et al.*, 1989 isolated and identified caryophyllene oxide from the oil of artificially and naturally dried leaves of *Stevia rebaudiana* which had unpleasant aroma, therefore, it did not find much usage in low calorific value food products and drinks (Tsanava *et al.*, 1991).

Liu *et al.*, 1997 used supercritical method for the extraction of stevioside with extraction efficiency of more than 88% using methanol as a modifier. Yoda *et al.*, 2003 used supercritical fluid extraction for the identification of six different compounds such as sesquiterpenes, alcohols, labdanic diterpenes, aliphatic hydrocarbons, sterols and triterpenes. Manish and Rema, 2006 worked on the phytochemical contents of *Stevia rebaudiana* leaves. Gas chromatography analysis of the leaf oil extract showed the presence of palmitic acid, stearic acid, oleic acid, linoleic and linolenic acid. Ibrahim *et al.*, 2007 reported five different labdane diterpenoids from chloroform soluble fractions of methanolic extracts of leaves of *Stevia rebaudiana*. These labdane diterpenoids were identified as austroinulin, iso-austroinulin, sterebin E, sterebin E acetate and sterebin A acetate along with hydrocarbons, aliphatic alcohols, beta amylin, beta sitosterols. Total phenolic and flavonoids content in the leaf explants of *S.rebaudiana* were evaluated by Tadhani *et al.*, 2007. The total phenolic content was found to be 25.18 mg/g whereas flavonoids were 21.73 mg/g of dry weight of leaf powder. Antonoi *et al.*, 2005 identified a new *Stevia* cultivar (UEM-320), which had glycoside almost devoid of any bitter aftertaste. Markovic *et al.*, 2008 analyzed and identified 88 compounds majority being mono and sesquiterpenes from the leaf extracts of *S.rebaudiana*. Hydrodistillation of extract showed the presence of various fatty acids, sesquiterpenes and kaurene type diterpenes.

2.2.2 Characterization of secondary metabolites from *Stevia rebaudiana*

Wide ranges of analytical techniques have been employed to extract sweet diterpenoid glycosides from *Stevia rebaudiana* (Table 2). These includes thin layer chromatography (TLC) (Metivier and Viana, 1979; Kinghorn *et al.*, 1984 and Nikolova- Damyanova *et al.*, 1994), droplet countercurrent chromatography (Kinghorn *et al.*, 1982), over pressure layer

chromatography (Fullas *et al.*, 1989), near infrared reflectance spectroscopy (Nishiyama and Alvarez, 1992), capillary electrophoresis (Liu and Li, 1995), high performance liquid chromatography (HPLC) (Bovanova *et al.*, 1998) and electrospray ionization mass spectrometry (Jackson *et al.*, 2009).

Table 2: Analytical methods for purification and characterization of secondary metabolites from *Stevia rebaudiana*

S. No.	Compounds	Method	Reference
1.	Stevioside	Enzymatic reactions	Mizukami <i>et al.</i> , 1982
2.	Steviolbioside	TLC and Column chromatography	DuBois, 1984
3.	Stevioside	Ion exchange resin	Kumar, 1986
4.	Stevioside	Ion exchange resin	Giovanetto, 1990
5.	Stevioside	Near Infrared Reflectance Spectroscopy	Nishiyama and Alvarez, 1992
6.	Sweet tasting glycosides	Subcritical fluid extraction	Liu <i>et al.</i> , 1997
7.	Sweet tasting glycosides	Solid phase extraction	Bovanova <i>et al.</i> , 1998
8.	Rebaudioside A, Sweet tasting glycosides	TLC and HPLC	Hirata <i>et al.</i> , 2002
9.	Fatty acids (stearic, linoleic acid, palmitic acid, oleic acid)	Gas Chromatography	Manish <i>et al.</i> , 2006
10.	Labdane diterpenoids (austroinulin, iso-austroinulin, sterebin E, sterebin E acetate and sterebin A acetate) along	Thin layer chromatography, Column chromatography Preparative TLC	Ibrahim <i>et al.</i> , 2007

	with hydrocarbons		
11.	Steviolbioside, stevioside, rebaudioside C and dulcoside D	HPLC	Rakasekaran <i>et al.</i> , 2007
12.	α -cadinol (2.98 %), caryophyllene oxide (1.23%), (-)-spathulenol (2.21%) and β -Guaiene (0.32%)	Gas chromatography	Hossain <i>et al.</i> , 2010
13.	Stevioside	TLC and HPLC	Inamake <i>et al.</i> , 2010
14.	13-[(2-O- β -D-glucopyranosyl-3-O- β -D-glucopyranosyl- β -D-glucopyranosyl) oxy]ent-kaur-16-en-19-oic-acid-(2-O- α -Lrhamnopyranosyl- β -D-glucopyranosyl) ester.	HPLC	Chaturvedula and Prakash, 2011
15.	Stevioside	HPLC and HPTLC	Shirwaikar <i>et al.</i> , 2011

Chromatographic separation of glycosides

Among all the analytical methods that have been employed, the most commonly used method is high performance liquid chromatography (HPLC). Separation by means of HPLC has been achieved using silica gel (Nikolova-Damyanova *et al.*, 1994), hydroxyapatite (Kasai *et al.*, 1987), hydrophilic (Hashimoto *et al.*, 1978), size exclusion (Ahmed and Dobberstein, 1982a, 1982b), amino bonded columns (Kinghorn *et al.*, 1984; Liu and Li., 1995; Makapugay *et al.*, 1984 and Striedner *et al.*, 1991) and C₁₈ column (Bovanova *et al.*, 1998 and Kedik *et al.*, 2003). Ahmed *et al.*, 1980 separated and quantified sweet tasting glycosides using HPLC technique with C18 column and acetonitrile: water as mobile phase. Ahmed and Dobberstein, 1982 a, b used high performance liquid chromatography (HPLC) for the determination of eight different types of diterpene glycosides from the chloroform extract of *Stevia rebaudiana* employing two

separate protein columns in series. Makapugay *et al.*, 1984 used NH₂ phase bounded column for HPLC separation of methanolic and chloroform leaf extracts of *S.rebaudiana*. HPLC based separation of stevioside, rebaudioside A, C and dulcoside A from the food samples was achieved using NH₂ column (Kitada *et al.*, 1989). Fullas *et al.*, 1989 used overpressure layer chromatography (OPLC) for the analytical separation of natural sweetening products from the leaves of *S. rebaudiana*. OPLC analysis of the leaf extract carried using pre coated TLC and high performance TLC silica gel 60 F₂₅₄ aluminum plates separated eight sweet diterpene glycosides namely dulcoside A, rebaudioside A-E, stevioside and steviolbioside. Nikolova–Damyanova *et al.*, 1994 used two separate systems one being normal phase HPLC and other being silica gel thin layer chromatography with densitometry for the quantification of sweet tasting glycosides and rebaudioside A from the leaves of *S. rebaudiana*. Zygadlo *et al.*, 1997 identified 41 different components from the leaf extracts of *Stevia achalensis* Hieronymus. GC-MS analysis of the leaf oil showed the presence of high content of sesquiterpene hydrocarbons (74.7%), β -selinene (27.2%), β -caryophyllene (12.8%) and α -murolene (9.5%).

Bovanova *et al.*, 1998 obtained 5 μ g/ml of glycoside using chromatographic separations on HPLC employing C₁₈ column and mobile phase consisting of methanol and water with UV detection at 210 nm. Pasquel *et al.*, 1999 identified non-glycosidic fractions like spathulenol, decanoic acid, 8, 11, 14-ecosatrienoic acid, 2-methyl octadecane, pentacosane, octacosane, stigmasterol, β -sitosterol, α and β -amyrine, lupeol, β -amyrin acetate and pentacyclic triterpene from *Stevia* leaves using supercritical fluid extraction (SCFE).

Pasquel *et al.*, 2000, used liquid extraction with different solvents including supercritical CO₂, hot water or hot alcohols for the determination of stevioside at pilot scale. Stevioside is also determined in *Stevia rebaudiana* by hot water leaching or supercritical fluid extraction followed by purification using liquid chromatography of the extract. Water leaching was performed by mixing leaves in boiling water followed by filtration and cleaning through a solid phase extraction prior to liquid chromatographic analysis (Vanek *et al.*, 2001). Kedik *et al.*, 2003 used thin layer chromatography to identify stevioside from raw plant material. Among the different combinations of solvent systems used, best results were observed with chloroform: methanol: water (60:3:6) and 50% sulfuric acid solution used as developing agent. The amount of

stevioside determined was 0.5 µg/ sample. *Stevia rebaudiana* plants grown under *in vitro* and *ex vitro* conditions were investigated for the variation in the amount of stevioside in different plant parts like leaves, shoots, roots and flowers (Rajasekaran *et al.*, 2007). Stevioside was extracted by hydrolysis followed by esterification and evaporation under reduced pressure. The concentrate was dissolved in methanol for quantitative analysis by HPLC. The HPLC profiles indicated the presence of eight different types of glycosides including steviolbioside, stevioside, rebaudioside C and dulcoside A. The highest glycoside content of 64.8% steviolbioside and 0.099% rebaudioside A on dry weight basis were found *ex vitro* and *in vitro* leaves respectively. Pol *et al.*, 2007 used pressurised fluid extraction using water or methanol for the extraction of stevioside from *Stevia rebaudiana*. The method has become popular alternative for solvent extraction as the consumption is very less. Extracts were analysed by liquid chromatography followed by UV and mass-spectrometric (MS) detection. Methanol showed better extraction ability for isolation of stevioside from *Stevia rebaudiana* leaves than water within a temperature range of 110–160°C. Inamake *et al.*, 2010 isolated and purified stevioside from the leaves of *S.rebaudiana* using TLC and HPLC. The R_f values for stevioside peak on TLC was 0.32 when developed in solvent system consisting of chloroform: methanol: water and HPLC peak was observed at retention time of 1.958 minutes. Hossain *et al.*, 2010 employed gas chromatography (GC) technique to identify sixty-two compounds from the leaf oils of *Stevia*. These includes α-cadinol (2.98 %), caryophyllene oxide (1.23%), (-)-spathulenol (2.21%) and β-Guaiene (0.32%) and some new essential oil compounds, such as ledene oxide-(II), beta.-guaiene, geranyl vinyl ether, tricyclo [5.2.2.0 (1, 6)] undecan-3-ol, indole, aristolene epoxide, 1, 2, 3, 5, 6, 7, 8, 8a-octahydro-1, 4-dione and 2, 6, 6-trimethyl-2-cyclohexene-1, 4-dione. A simple reversed phase high performance chromatography method was used for the isolation of steviol glycosides employing solid phase extraction with C₁₈ cartridges and hydrophilic interaction liquid interface chromatography (HILIC) column (Woelwer-Rieck *et al.*, 2010). HPLC based purification and NMR and Mass Spectroscopy based structural elucidation for the presence of a new diterpene glycoside from the leaves of *S.rebaudiana* was made by Chaturvedula and Prakash, 2011. The new compound was identified as 13-[(2-O-β-D-glucopyranosyl-3-O-β-D-glucopyranosyl-β-D-glucopyranosyl) oxy] ent – kaur – 16 – en – 19 – oic – acid - (2 - O-α-L-rhamnopyranosyl-β-D-glucopyranosyl) ester. Jaitak *et al.*, 2008 used HPTLC technique to quantify three steviol glycosides namely steviolbioside, stevioside and rebaudioside A from the leaves of

S.rebaudiana. For achieving good separation, mobile phase consisting of ethyl acetate: ethanol: water (80: 20:12) on pre coated silica gel 60 F254 HPTLC plate was used. The densitometric evaluation yielded 1-6 µg/spot of stevioside, 0.5-3 µg/spot of rebaudioside and 160-190 µg/spot of steviolbioside. Shirwaikar *et al.*, 2011 identified and estimated stevioside in the leaf powder of *Stevia* using HPLC and HPTLC. HPTLC separation was achieved on pre coated silica gel plates with mobile phase consisting of ethyl acetate: methanol: water (75:15:10) with densitometric evaluation at 510 nm.

Near Infra red Reflectance spectroscopy

Near infrared reflectance spectroscopy is a rapid, low cost method used as a suitable tool in quality control of metabolites from dried plant parts. Nishiyama and Alvarez, 1992 used near infrared reflectance spectroscopy (NIRS) and HPLC for the analysis of stevioside from the leaves of *Stevia rebaudiana*. A C18 column and a mobile phase consisting of methanol and NaOH were used. Hearn and Subedi, 2009 used the application of near infrared reflectance spectroscopy for determining the levels of three steviol glycosides (SGs) namely stevioside, rebaudioside A and rebaudioside C on dry weight basis from the leaves of *Stevia rebaudiana*. Among the different 33 samples analyzed, the most abundant glycoside was stevioside (64.09%) followed by rebaudioside A (25.95%) and rebaudioside C (9.96%).

Electrospray ionization mass spectrometry

Due to high degree of sensitivity and specificity, the technique of mass spectrometry plays an important role in the analysis, detection and structural elucidation of natural products. Although these techniques have proved to be successful but analysis of plant material seems to be a laborious task. Therefore, desorption electrospray ionization mass spectrometry (DESI) when coupled with tandem mass spectrometry helps in rapid screening of plant materials. Choi *et al.*, 2002 used supercritical fluid extraction method along with electrospray mass spectrometry for obtaining better yield and high purity levels of stevioside from the leaves of *S.rebaudiana*. Koyama *et al.*, 2003 used electrospray ionization and negative detection methods for extracting sweet glycosides from *S. rebaudiana*. Jackson *et al.*, 2009 used the technique of DESI to analyze the hexane extracts of leaf samples of *S. rebaudiana*. Characterization and semi-quantitative determination of glycosides was achieved based upon the glycoside profile within the full mass

spectrum. DESI characterization of the plant concerning the location of sweet glycosides clearly showed that the glycosides were mainly concentrated within the leaves and were not observed on the surface of plant stem.

2.2.3 Extraction of secondary metabolites from *Tylophora indica*

Out of nearly 50 members of the family Asclepiadaceae, which are well distributed over Africa, Asia, Australia and the Pacific Islands, only 23 have been reported to contain phenanthroindolizidine alkaloids and among these most of the alkaloids have been obtained from *Tylophora indica* (Ali *et al.*, 1991). The other natural plant sources of tylophorine are listed in Table 3. It was included as an official drug in Bengal Pharmacopoeia of 1884 because of its medicinal properties (Gopalakrishnan *et al.*, 1979). *Tylophora indica* is an important source of phenanthroindolizidine alkaloids like tylophorine (C₂₄H₂₇NO₄), tylophrinidine (C₂₃H₂₅O₄N) and anticancerous tylophorinidine (C₂₂H₂₂O₄N) which exhibit various pharmacological functions such as hepatoprotective, antiallergic, immunomodulatory, diuretic, anti asthmatic and antitumor activities (Gellert, 1982 and Faisal and Anis, 2003).

Table 3: Natural sources of tylophorine (Jha *et al.*, 2005)

S. No.	Plant Source	Reference
1.	<i>Tylophora crebrifolia</i>	Gellert <i>et al.</i> , 1962
2.	<i>Vincetoxicum officinale</i>	Pailer and Streicher, 1965
3.	<i>Cyanchum vincetoxicum</i>	Wiegrebe <i>et al.</i> , 1969
4.	<i>Pergularia pallid</i>	Mulchandani and Venkatachalam, 1976
5.	<i>Tylophora tanakae</i>	Abe <i>et al.</i> , 1995
6.	<i>Ficus septic</i>	Wu <i>et al.</i> , 2002

In 1891, Hooper reported the occurrence of two alkaloids in *Tylophora indica*, however, due to limitations in the methods of characterization, their structures could not be determined properly. The two alkaloids were reisolated and characterized by Ratnagiriswaran and Venkatachalam in 1935 and named as tylophorine (1-1) and tylophorinine (1-2). Chopra *et al.*, 1956 worked on the isolation of alkaloids from *T. indica* but could only identify tylophorine whereas the other

alkaloid remained unidentified. Latter on this unidentified alkaloid, whose base resembled septicine was isolated along with isotylocrebrine by Govindachari *et al.*, 1954. Rao *et al.*, 1971 isolated five new alkaloids viz compound A (identical with tylophorine), B (identical with tylophorinine), C (a desmethyltylophorinine), D, E and a trace of a sixth unidentified alkaloid and tylophorinidine was isolated by Mulchandani *et al.*, 1971. Govindachari *et al.*, 1973 isolated three quaternary alkaloids namely dehydro-tylophorine, anhydrodehydrotylophorinine and anhydrodehydrotylophorinidine (II). Based on structural and stereochemistry study, tylophorine was initially reported as having the (S)-absolute stereochemistry, but a total synthesis and optical rotation measurement led to the revision to (R) stereochemistry (Buckley and Rapoport, 1983). Mulchandani and Venkatachalam, 1984 isolated tylophorinicine, a minor alkaloid from the roots of *T. asthmatica*. Ali and Bhutani, 1989 isolated a set of seven additional alkaloids (known as tyloindicines A-E) from *T. indica*, some of which bore novel structural features. Of the major interest was the presence of angular methyl in indolizidine periphery and varying patterns of substitution in the phenanthrene framework. Ali *et al.*, 1991 isolated another set of tyloindicines from *T. indica* with even more intriguing structural features. Among these tyloindicines F and G having a unique tertiary hydroxy group at 12a position were found to be extremely potent. Tyloindicines F and G showed pronounced activities against certain lung and melanoma cell lines. Huang *et al.*, 1991, carried a similar study for the structure and activity relation of these alkaloids.

Enantioselective synthesis of tylophorine has been accomplished from 3, 4- dimethoxy benzyl alcohol through eight-step procedure by Zeng and Chemler, 2008. Abe *et al.*, 1995 isolated ten phenanthroindolizine alkaloids from fresh leaves of *Tylophora tanakae*. Purification and structural confirmation of alkaloids showed similar molecular formula $C_{24}H_{27}O_4$ which were latter identified as isotylocrebrine (3, 4, 6, 7-tetramethoxyphenanthroindolizidine) and tylophorine (2, 3, 6, 7-tetramethoxyphenanthroindolizidine). Rao *et al.*, 2006 isolated desmethyl - tylophorine and desmethyltylophorinine from the root extracts of *T. indica*. Steam distillation of alcoholic extract of air-dried root powder of *Tylophora* yielded p-methoxysalicylaldehyde and a small amount of oily matter (Ali, 2008). Rare alkaloids like tyloindicines A, B, C, D, E, F, G, H, I and J, desmethyl tylocrebrine, anhydrotylophorinine, 14-hydroxyisotylocrebrine, 4, 6 desmethyloxy-methyltylophorinidine, α and β - amyrins, tetratriacontanol, octacosanyl

octacosanote, sigmasterol, β - sitosetrol, tyloindane, cetyl-alcohol and some non alkaloids such as kaempferol, quercetin, octaosanyl octacosanoate, sigmasterol, cetyl alcohol, quercetin have also been isolated from *T. indica* (Gupta *et al.*, 2010).

2.2.4 Characterization of secondary metabolites from *Tylophora indica*

The types of active metabolites present mainly contribute towards the pharmacological activity of the plant. The methanolic extract of *Tylophora indica* leaves was screened for hepatoprotective activity in albino rats (Gujrati *et al.*, 2007). Arora and Rawat, 2007 showed lysosomal enzyme inhibiting activity in the flavone extract of *T. indica*. Aqueous and alcoholic extracts of *T. indica* leaves were tested for diuretic activity (Meera *et al.*, 2009). Tylophorine not only retards the S-phase progression but also dominantly arrests the cells at G1 phase in HepG2, HONE-1 and NUGC-3 carcinoma cells. Thus, down regulated cyclin A2 plays a vital role in G1 arrest by tylophorine in carcinoma cells (Chia-Mao *et al.*, 2009). Pure and crude extracts of *T. indica* showed anti-fungal activity against *Aspergillus niger*, *A. fumigates* and *Trichoderma viride* (Reddy *et al.*, 2009). The medicinal importance of the plant demands for the screening of various active constituents present. Plant alkaloids have been isolated and characterized using various analytical techniques like infrared spectroscopy (Vishwanathan and Pai, 1985), high performance thin layer chromatography (Chaudhari *et al.*, 2004) and direct-injection electrospray ionization mass spectrometry (Verma *et al.*, 2007). The various types of analytical techniques used for the isolation, purification and characterization of alkaloids from *Tylophora indica* are shown in Table 4.

Table 4: Analytical techniques used for purification of alkaloids from *Tylophora indica*

S.No	Compounds	Method	Reference
1.	Tylophorine, desmethyl tylophorinine	Infra red spectroscopy	Rao and Brook, 1970
2.	Tylophorine	HPLC	Jha <i>et al.</i> , 2005
3.	Alkaloidal and non-alkaloidal constituents	HPTLC	Mayank <i>et al.</i> , 2010
4.	Alkaloidal components	HPTLC	Mujeeb <i>et al.</i> , 2009
5.	Alkaloids, tannin, saponin and terpenoid	TLC and Preparative TLC	Kumar, 2011

Spectroscopic analysis of alkaloids

Ratnagiriswaran and Venkatachalam, 1935 identified tylophorine and tylophorinine from *Tylophora asthmatica* (syn. *Tylophora indica*) by fractional crystallization of the mixed salts. Viswanathan and Pai, 1985 reported chemical examination of *Tylophora mollissima* and yielded caffeine as major alkaloid and tylophorine and tylophorinine as minor alkaloids using techniques like ultra violet, infrared and mass spectroscopy.

Chromatographic analysis of alkaloids

Abe *et al.*, 1995 obtained a mixture of alkaloids from the fresh leaves and stems of *T. indica* by means of column chromatography and preparative TLC. The isolated alkaloids include tylophorine, isotylocrebrine, 3-Demethylisotylocrebrine, 3-Demethyl-14 α -hydroxyisotylocrebrine, isotylocrebrine N-oxide, 6-Demethyltylocrebrine, tylophorinine N-oxide, 7-demethyltylophorine. Structural elucidation was done using NMR and MS study. HPLC (high performance liquid chromatography) based extraction and analysis of tylophorine was achieved from the extract of transformed roots of *Tylophora indica* by modified method of Abe *et al.*, 1995 (Chaudhuri *et al.*, 2004). Variable tylophorine concentrations were found in different root clones of *Tylophora indica* with highest concentration recorded as 1.29 ± 0.02 mg/g DW and lowest concentration recorded as 0.84 ± 0.06 mg/g DW. HPLC based detection of tylophorine was achieved from plants regenerated from transformed root clones (Chaudhari *et al.*, 2006). Presence of tylophorine was detected in all the parts, with highest tylophorine content (20-60 %) obtained from the plants regenerated from transformed root clones. Mujeeb *et al.*, 2009 used HPTLC technique for the phytochemical screening of alkaloids, glycosides, steroids and flavonoids from the leaves of *Tylophora indica*. The chromatogram was developed using solvent system consisting of toluene: chloroform: ethyl acetate (1:5:3) saturated with 10% acetic acid and was scanned in scanner III at 366 nm wavelength using mercury lamp in fluorescence mode. Mayank *et al.*, 2010 used HPTLC fingerprint technique for the identification of different marker compounds from the leaf explants of *T.indica*. Different plant extracts were prepared using chloroform, methanol and petroleum ether separately and each extract was developed using a separate solvent system. For chloroform extract: chloroform (90): methanol (5): ethyl acetate (5) v/v, for methanol extract: toluene (5): chloroform (90), ethyl acetate (5) v/v and for petroleum ether extract: hexane (40): ethyl acetate (60) v/v were used. Kumar *et al.*, 2011

performed quantitative phytochemical screening of bioactive compounds of *T. indica*. Presence of tylophorine, tylophorinidine, tylophorinine, tyloindicine- A, D, F, G, H and I 14-hydroxy tylophorine, stigma sterol, octa cosanyl, skimmianide, tannins, saponins and flavonoids was revealed by thin layer chromatography.

2.2.5 Secondary metabolites from callus and suspension cultures

Plant cell cultures have been successfully used for large-scale production of secondary metabolites such as alkaloids, flavonoids, glucosides, sterols, phenolics, saponins, terpenoids and diterpenoids from rare and exotic varieties (Sierra *et al.*, 1992 and Orihara *et al.*, 2002) (Table 5). Wide variety of pharmaceuticals like Berberine from *Coptus japonica*, Diosgenin from *Dioscorea deltoidea*, Camptothecin from *Camptotheca acuminata*, Vinblastine and Vincristine from *Catharanthus roseus*, Capsicum from *Capsicum* sp. are produced in sufficient quantities through cell culture technique (Vanisree and Tsay, 2004). Production of secondary metabolites from callus or cell suspension culture offers ample advantages as these are more reliable, simpler and efficient and allows selection of cultures with higher yield of metabolites and elimination of negative biological influences which otherwise interferes in *in vivo* (Mulabagal *et al.*, 2004 and Karuppusamy, 2009). In order to obtain high yields of plants, plant tissue culture technology is found to be an attractive alternative approach to traditional methods as it offers a controlled supply of biochemicals independent of plant availability (Sajc *et al.*, 2000). Moreover, cell cultures have higher rates of metabolism than intact differentiated plants because cell growth in cultures leads to fast proliferation of cell mass within a condensed biosynthetic cycle (Dornenburg and Korr, 1995).

Table 5: Bioactive secondary metabolites from callus and cell suspension cultures (Mulabagal and Tsay, 2004 and Karuppusamy, 2009).

Plant Name	Active Ingredient	Culture Type	Reference
<i>Scutellaria columnae</i>	Phenolics	Callus	Stojakwska and Kisiel, 1999
<i>Polygala amarelle</i>	Saponin	Callus	Desbene <i>et al.</i> , 1999
<i>Cassia acutifolia</i>	Anthraquinones	Suspension	Nazif <i>et al.</i> , 2000
<i>Rauvolfia serpentine</i>	Reserpine	Suspension	Gerasimenko <i>et al.</i> , 2001
<i>Aspidosperma ramiflorum</i>	Ramiflorin	Callus	Olivira <i>et al.</i> , 2001
<i>Aspidosperma ramiflorum</i>	Ramiflorin alkaloid	Callus	Olivira <i>et al.</i> , 2001
<i>Taxus spp</i>	Taxol	Suspension	Wu <i>et al.</i> , 2001
<i>Podophyllum hexandrum</i> Royle	Podophyllotoxin	Suspension	Chattopadhyay <i>et al.</i> , 2002

<i>Torreya nucifera</i>	Diterpenoids	Suspension	Orihara <i>et al.</i> , 2002
<i>Salvia officinalis</i>	Terpenoids	Callus	Santos-Gome <i>et al.</i> , 2002
<i>Eribotrya japonica</i>	Triterpenes	Callus	Taniguchi <i>et al.</i> , 2002
<i>Salvia fruticosa</i>	Rosmarinic acid	Callus and Suspension	Karam <i>et al.</i> , 2003
<i>Taxus cuspidate</i>	Taxoids	Suspension	Ketchum <i>et al.</i> , 2003
<i>Plumbago rosea</i>	Plumbagin	Callus	Komaraiah <i>et al.</i> , 2003
<i>Ammi majus</i>	Triterpenoid	Suspension	Staniszewska <i>et al.</i> , 2003
<i>Hyssopus officinalis</i>	Sterols	Suspension	Skrzypek and Wysokinsu, 2003
<i>Nothapodytes foetida</i>	Camptothecin	Callus	Thengane <i>et al.</i> , 2003
<i>Eucommia ulmoides</i>	Chlorogenic acid	Suspension	Wang <i>et al.</i> , 2003
<i>Fabiana imbricate</i>	Rutin	Suspension and callus	Schmeda-Hirschmann <i>et al.</i> , 2004
<i>Camptothecin acuminata</i>	Camptothecin	Suspension and callus	Vanisree <i>et al.</i> , 2004
<i>Hypericum perforatum</i>	Hypericin	Suspension	Hohtola <i>et al.</i> , 2005
<i>Vaccinium myrtillus</i>	Flavonoides	Callus	Hohtola <i>et al.</i> , 2005
<i>Frangula alnus</i>	Anthraquinones	Callus	Kovacevic and Grabisic, 2005
<i>Rauvolfia tetraphylla</i>	Reserpine	Callus	Anitha and Kumari, 2006
<i>Gymnema sylvestre</i>	Gymnemic acid	Callus	Gopi and Vatsala, 2006
<i>Saprosma fragrans</i>	Anthraquinone	Callus	Singh <i>et al.</i> , 2006
<i>Silybum marianum</i>	Silymarin	Callus	Tumova <i>et al.</i> , 2006
<i>Vitis vinifera</i>	Anthocyanin	Suspension	Qu <i>et al.</i> , 2006
<i>Momordica charantia</i>	Flavonoid	Callus	Agarwal and Kamal, 2007
<i>Eleutherococcus senticosus</i>	Eleuthrosides	Suspension	Shohael <i>et al.</i> , 2007
<i>Simmondsia chinensis</i>	Fixed oil	Callus	Aftab <i>et al.</i> , 2008
<i>Pluchea lanceolata</i>	Quercetin	Callus	Arya <i>et al.</i> , 2008
<i>Rauvolfia serpentine</i>	Reserpine	Callus	Nurchgani <i>et al.</i> , 2008
<i>Catharanthus roseus</i>	Catharathine	Suspension	Ramani and Jayabaskaran, 2008
<i>Tinospora cordifolia</i>	Berberin	Suspension	Rama Rao <i>et al.</i> , 2008
<i>Rauvolfia serpentine</i>	Serpentine	Callus	Salma <i>et al.</i> , 2008
<i>Azadirachta indica</i>	Azadirachtin	Suspension	Sujanya <i>et al.</i> , 2008
<i>Corydalis terminalis</i>	Corydalin	Callus	Taha <i>et al.</i> , 2008
<i>Brucea javanica</i>	Cathin	Suspension	Wagiah <i>et al.</i> , 2008
<i>Vitis vinifera</i>	Resveratrol	Callus	Kin and Kunter, 2009
<i>Cayratia trifoliata</i>	Stibenes	Suspension	Roat and Ramawat, 2009
<i>Withania somnifera</i>	Withanoloid A	Callus	Mirjalilli <i>et al.</i> , 2009
<i>Camellia chinensis</i>	Flavones	Callus	Nikolaeva <i>et al.</i> , 2009

Secondary metabolites are often produced in response to stress conditions and these metabolites are either stored intra-cellularly or extra-cellularly. The storage and release is cell line dependent and involves use of various organic solvents (Chang *et al.*, 1994), polymeric resins like non-ionic amberlite (Wang *et al.*, 1999) and chemical inducers like jasmonic acid, salicylate, fungal elicitor protein, cryptogein and killed fungi (Chaudhuri *et al.*, 2009). Genetic methods via functional genomics (Goossens *et al.*, 2003), traditional breeding and screening (Long *et al.*, 2006), gene encoding enzymes (Liu *et al.*, 2007) could also add to increased production and *in situ* recovery of secondary metabolites. The hairy root system based on inoculation with *Agrobacterium*

rhizogenes resulted in higher accumulation of secondary metabolites *in vitro* (Palazon *et al.*, 1997 and Guillon *et al.*, 2006)

Secondary metabolites from callus cultures

Callus tissue is an essential material in plant cell culture system as it retains characteristics of the plants from which they are derived during their biosynthetic and regenerative potential. Production of secondary metabolites mainly occurs at the time of differentiation wherein different factors like specific growth rate, specific product formation and biomass concentration during production plays an important role. While a large number of valuable secondary metabolites are produced in unorganized callus cultures, but in some cases production may require more differentiated microplant or organ culture. A prime example is ginseng (*Panax ginseng*) since saponin and other valuable metabolites are specifically produced in ginseng roots, hence root culture is required *in vitro*. Biosynthesis of lysine to anabasine occurs in tobacco roots (*Nicotiana tabacum*) and callus and shoot cultures of tobacco can produce only trace amounts of nicotine because they lack the organ-specific compound anabasine. Similarly, in some cases, at least some degree of differentiation in a cell culture must occur before a product can be synthesized e.g., vincristine or vinblastine from *Catharanthus roseus* (Karuppusamy, 2009).

Komatsu *et al.*, 1976 reported the production of sweet stevioside in callus cultures of *Stevia rebaudiana* while Handro *et al.*, 1977 reported that the callus cultures did not taste sweet after an extended period of storage. Hsing *et al.*, 1983 compared stevioside content in leaves, flowers and calli of *Stevia rebaudiana*. The study indicated that callus contained maximum stevioside i.e. 16.24% followed by leaves (8.46%) and flowers (3.66%). Sivaram and Mukundan, 2003 compared the level of sweet tasting glycosides among leaves of *in vitro* and *in vivo* plants and callus cultures of *Stevia rebaudiana*. HPTLC analysis using chloroform: methanol: water (65: 25: 4) showed maximum amount of stevioside (5.8 %) in callus cultures raised on 8.87 μM BA with 9.80 μM IBA supplemented medium. Leaves of *in vivo* and *in vitro* plants contained 4.9 and 3.6 % stevioside respectively. Similar results for the presence of higher levels of active metabolites in the cultured cells as compared to those in native plants have been observed in *Colleus blumei* (Ulbrich *et al.*, 1985), *Panax ginseng* (Ushiyama, 1991) and *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991). Jadeja *et al.*, 2005 carried out quantitative studies

for the isolation of stevioside from the callus cultures of *Stevia rebaudiana*. Presence of stevioside was detected when TLC was developed using isopropanol: n-butanol: distilled water in 50:30:20. Rajasekaran *et al.*, 2008 studied callus and suspension cultures of *Stevia* for the analysis of predominant stevioside. Liquid Chromatography Mass Spectrophotometry Electrospray Ionization based detection showed the detection of various glycosides such as dulcoside-A, stevioside, rebaudioside C, A and E except steviolbioside. The contents of the stevioside in leaves of intact *Stevia* leaves appeared to be several times more than that *in vitro*, callus, and suspension cultures. However, according to some earlier reports (Lee *et al.*, 1982 and Hsing *et al.*, 1983), the stevioside content in callus cultures is 16– 24% by plant dry weight and is 2–4 times higher than that in leaves and flowers of the same plant. In contrary to this, Swanson *et al.*, 1992 found that stevioside is exclusively present in the leaves of *Stevia rebaudiana* whereas both callus and *in vitro* shoot cultures lacked stevioside. Janarthanam *et al.*, 2010, produced stevioside from the callus cultures of *Stevia rebaudiana*. TLC and HPLC profiling of the extracts showed that callus cultures contained 17.63 g/kg of stevioside, which was less as compared to 19.25 g/kg in the leaf parts.

Benzamine and Mulchandani, 1973 studied tylophorine productivity from callus cultures of *Tylophora indica*. Four secondary metabolites such as β -amyrin, β -sitosterol, stigmasterol and campesterol have been isolated from the callus tissue raised from stem cuttings as well as from excised root of germinating seedlings of *Tylophora indica*. The tissue lacked the ability to synthesize phenanthroindolizidine alkaloids. Incorporation of phenylalanine, a known precursor of *Tylophora* alkaloids and auxins like 2, 4-D and IAA into the basal medium neither induced alkaloid synthesis nor varied the composition of steroidal and triterpenoidal constituents. Although in a number of plants, presence of various plant growth regulators together in the nutrient medium had synergistic effect on both the callus growth as well as metabolite production. Like in *Ceropegia juncea* Roxb callus, growth and alkaloid content was more or less doubled when supplemented with both auxin and cytokinin together (Nikam and Savant, 2009). Growth regulator concentration is often a crucial factor in secondary metabolite accumulation. The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio dramatically alter both the growth and product formation ability in *in vitro* cultures. Ramawat and Merillon, 2008 reported higher levels of secondary metabolite production in a number of plant species of

Ephedra, *Lithospermum*, *Datura*, *Hyscocyamus*, *Atropa* and *Papaver* when IAA or NAA were used instead of 2, 4- D, BA or K.

About 20 years back, *Ruta graveolens* cultures were initiated via tissue culture for the extraction of secondary metabolites. Callus cultures were maintained for more than 3 years on MS supplemented with 2, 4-D and K (1mg/l) and alkaloids like acridone and furoquinoline were isolated (Baumert *et al.*, 1992). Efforts were made to extract alkaloids from *Tylophora tanakae*, *Calystegia sepium* and *Withania somnifera* by inserting synthetic gene encoding cryptogen into roots of the plants using *Agrobacterium rhizogenes*. In all the species examined, accumulation of secondary metabolite tylophorine was observed in the transformed roots in *T. tanakae*, calystegines in *C. sepium* and withanoferin in *W. somnifera* (Chaudhuri *et al.*, 2009). Alkaloid production through callus and suspension cultures was also observed in *Leonurus heterophyllus* (Yang *et al.*, 2008). Conditions of callus and suspension cultures were optimized using various plant growth regulators and 0.081% alkaloid was extracted from 16-day-old suspension culture, but the concentration increased to 2.1% with the addition of 0.03mg/l L-proline. Hence, cell suspension culture is an effective method for scale up of secondary metabolite production in cells.

Secondary metabolites from suspension cultures

Suspension cultures constitute a good biological material as compared to callus cultures for studying biosynthetic pathways as they allow the recovery of large quantities of secondary metabolites (Dougall, 1981). Moreover, suspension cultures offer ample advantages over stationary cultures, as they permit precise manipulations and investigations into organ differentiation (Ammirato, 1984). Ferreira and Handro, 1988 established a method of cell suspension cultures of *Stevia rebaudiana* (Bertoni). Chaudhari *et al.*, 2005 obtained tylophorine from the suspension and callus cultures of root clones of *Tylophora indica*. The amount of tylophorine obtained from suspension cultures was higher than obtained from callus cultures.

Cell suspensions are maintained by sequential subcultures during early stationary phase and when cell aggregation is maximal, the optimum stage of subculturing is identified. To increase the productivity of metabolite, different physical and chemical parameters such as screening of

cell lines, optimizing culture conditions (temperature, pH, medium composition, aeration, phytohormones and light intensity) needs to be optimized (Verpoorte *et al.*, 1991). Although secondary metabolite production through cell culture technology has revolutionized the pharmaceutical and industrial sector through large-scale metabolite production, still cell viability and economic feasibility of plant cell cultures remains a major bottleneck (Steward *et al.*, 1999). They require high cost bioreactors associated with aseptic conditions that are expensive to maintain (Curtis, 1999). In lieu of these problems, several new routes have been investigated like the design of low cost bioreactor unit or use of plastic bags, which are much cheaper than culture reactors (Borowitzka, 1999).

Many attempts have been made that could lead to major breakthroughs for the production of secondary metabolites. Some of the biotechnological strategies that can be employed to increase the production of secondary metabolites include plant metabolic engineering, precursor addition, elicitation of *in vitro* products, hairy root cultures and endophytic microbes (Karuppusamy, 2009). Although efforts have been made in the field of Plant cell culture technology for the production of phytochemicals, but only a few industrial processes involving a limited number of secondary products such as shikonin, berberine, ginsenosides and paclitaxel have been successfully developed (Ramachandra and Ravishankar, 2002). In other cases, the production is too low for commercialization. Metabolic engineering provides alternative strategies to enhance the productivity of these metabolites. The technique involves targeted and purposeful alteration of metabolic pathways to achieve better understanding and use of cellular pathways (Lessard, 1996). These manipulated pathways resulted in the generation of transgenic crops in which the range, scope, or nature of a plant's existing natural products are modified to provide beneficial commercial produce (Kinney, 1998). Yun *et al.*, 1992 used hyoscyamine 6 β -hydroxylase gene from *Hyoscyamus niger* in over expressing h6h activity in *Atropa belladonna*. These transgenic *Atropa* plants displayed an enhanced conversion of hyoscyamine into scopolamine, which has more pharmaceutical application. A prime example is modification of carotenoid pathway in golden rice by the introduction of three novel genes to re-route the precursor geranylgeranyl diphosphate to the desired β -carotene (Ye *et al.*, 2000). Engineering of two branching-point enzymes putrescine *N*-methyltransferase (PMT) in transgenic plants of *Atropa belladonna* and *Nicotiana sylvestris* and (*S*)-scoulerine 9-*O*-methyltransferase (SMT) in cultured cells of *Coptis*

japonica and *Eschscholzia californica* increased the nicotine content in *Nicotiana sylvestris* and expression of SMT caused the accumulation of benzyloisoquinoline alkaloids in *E. californica* (Sato *et al.*, 2001).

Gas Chromatography and Gas Chromatography- Mass Spectroscopy analysis of chloroform and methanol extracts indicated a higher accumulation of umbelliferone in the elicited cell suspension and hairy root cultures of *Ammi majus* L. Use of elicitor benzo(1, 2, 3)-thiadiazole-7-carbothionic acid S-methyl ester and autoclaved lysate of cell suspension of bacteria—*Enterobacter sakazaki* enhanced production of secondary metabolite (Staniszewska *et al.*, 2003). Use of elicitor also enhanced the growth and ginseng saponin biosynthesis in the hairy roots of *Panax ginseng* (Jeong and Park, 2006). Similarly, the influence of biotic and abiotic elicitors on cultures was also studied to improve the production and accumulation of taxol from *Taxus* tree (Yukimune *et al.*, 1996), anthraquinone in *Rubia akane* (Jin *et al.*, 1999) berberine from *Tinospora cordifolia* (RamaRao *et al.*, 2008). Medicinal plants have active ingredients, which are too complex to be synthesized in large quantities, so induction of biotic and abiotic stress through cell suspension cultures offers an application for increased accumulation and extraction of secondary metabolites (Karuppusamy, 2009). Moreover, in order to produce high quality of secondary metabolites for commercial use, cell suspension culture offers several advantages over other techniques as it is a continuous and reliable source of plant based drugs due to its rapid growth cycle (Bourgaud *et al.*, 2001).

MATERIAL AND METHODS

3.1 Choice of material

Two medicinal plants namely *Stevia rebaudiana* and *Tylophora indica* were selected for the present study. Explants of *Stevia rebaudiana*, procured from Punjab Agricultural University, Ludhiana were collected and for *Tylophora indica*, explants were collected from field-grown healthy plants maintained at Thapar University campus, Patiala.

3.1.1 Plant Profile

Stevia rebaudiana

Distribution

Stevia rebaudiana Bertoni (family Asteraceae) popularly known as ‘Sweet leaf’, ‘Honey leaf’ or ‘Candy leaf’ is one of the most valuable tropical medicinal plant native to the valley of the Rio Monday in highlands of North-Eastern Paraguay in South America (Madan *et al.*, 2010). The plant grows in sandy soils near streams on the edges of marshland (Soejarto *et al.*, 1983; Katayama *et al.*, 1976 and Lewis, 1992). As the unique properties of the herb have gained renown, its cultivation has spread across the continents to Asia, Europe, Israel, North America and other regions of South America.

Habitat

Stevia is a semi humid crop plant that grows well between 22-24 °C with continual moisture without being waterlogged. *Stevia* often grows well in acidic and infertile soils, with an average of 55 inches of rain per year keeping the ground constantly moist. This slender plant reaches a height of about 2 feet and flowers in late summer or early fall.

Morphology

Stevia is a small, herbaceous, semi-bushy, perennial shrub with sessile, oppositely arranged lanceolate to oblanceolate leaves (Fig. 2). The upper surface of the leaf is slightly glandular, pubescent with serrate margins from the middle to the tip (Shaffert and Chetobar, 1994). Plant can initiate flowering after the formation of minimum of four leaves (Carneiro, 1990). The flowers are small, white, composite and arranged in irregular cymes. The fruit is a five-ribbed

spindle-shaped achene (Blumenthal, 1996). *Stevia* is diploid having 22 chromosomes (Frederico *et al.*, 1996).



***Stevia* plant in vegetative phase**



***Stevia* plant in flowering phase**

Fig. 2. *Stevia rebaudiana*

Propagation of *Stevia*

Stevia is normally propagated through cuttings, because seed germination rates are very less or poor. However, severe incidences of diseases and pests like leaf spot (*Sclerotinia* sp.) (Chang *et al.*, 1997), black spot infection (*Alternaria* sp.) (Skaria *et al.*, 2004) are seen where cuttings are used as planting material. Average economic life of plant is 4-5 years (Das *et al.*, 2010) and is mainly harvested after a gap of 4 month i.e. three times in a year (Mishra *et al.*, 2010). The plant gives highest foliage yield in 3rd year after plantation, when grown under long day conditions (Metiver and Viana, 1979).

Commercial Cultivation and Status

The unique medicinal properties of *Stevia rebaudiana* attracted Bertoni in 1887 to highlight the importance of this important medicinal plant species, however, its first report of commercial cultivation in Paraguay was in 1964 (Lewis, 1992). Since then, it has been added as a crop plant in a number of countries like Korea, Mexico, United States, Indonesia, Philippines, Canada and Brazil for food and pharmaceutical products (Goenadi, 1983; Saxena and Ming, 1988 and Fors,

1995). In Japan itself, *Stevia* holds an annual market value of 220 million Canadian dollars for 50 tonnes annual consumption of stevioside (Brandle and Rosa, 1992). In India, *Stevia* was introduced both in cash crop market and export market and as per Agriculture and Industry Survey 2005, the annual *Stevia* production in the country was near 600 tons. The average market price for the sale of *Stevia* leaves in India for the last two years is Rs 200 per kg with a cost benefit ratio of 1.89 (Megeji *et al.*, 2005). However, *Stevia* is new to the Indian market and hence, there is lot of scope and potential with regard to its cultivation and propagation (Kumar and Kaul, 2005).

Chemical constituents

The leaves of *Stevia* are the source of diterpene glycosides namely stevioside, dulcoside, steviolbioside, rebaudioside A, B, C, D and E (Bondarev *et al.*, 2001 and Starratt *et al.*, 2002). Among these, stevioside is a high intensity, non caloric, high potency sweetener being 300 times sweeter than sucrose and is non-fermentable, non- discoloring and heat stable at 95°C (Crammer and Kan, 1986, Chalapathi and Thimmegowda, 1997, Bhosle, 2004 and Uddin *et al.*, 2006). Its contents vary between 4-20 % of the dry weight of leaves depending on variety and growth conditions (Brandle *et al.*, 1998). Beside these glycosides, plant also contains phenylpropanoids, caffeic acid, chlorogenic acid, scopoletin, umbelliferone, quercetin, avicularian, polystachoside and isoquercitrin extracted from leaves (Komissarenko *et al.*, 1994). Six new labdane types, non-glycosidic diterpenes, sterebins I-N were isolated from the leaves of *S. rebaudiana* which are structurally analogous to those of the previously described sterebins A-H (Madan *et al.*, 2010). Phytochemical analysis of leaves shows the presence of typical components like proteins (6.2%), lipids (5.6%) and total carbohydrates (53%) (Geuns, 2000).

Medicinal importance

Stevia is used for obesity, heart diseases and dental caries (Kinghorn and Soejarto, 1985), as contraceptive (Melis, 1999), anti-hypersensitive agent (Jeppensen *et al.*, 2002), anticancerous agent (Jeppensen *et al.*, 2002, 2003), also has hypotensive (Liu *et al.*, 2003) and hypoglycaemic activities (Jeppesen *et al.*, 2003 and Lailerd *et al.*, 2004). Ikan, 1993 reported the use of *Stevia* products and extracts as weight watcher diets, diabetics diets such as in diabetic mayonnaise, as flavours, colour and odour enhancer. *Stevia* is also used medicinally in toothpastes and mouthwash as a plaque retardant and caries preventer.

Tylophora indica

Distribution

Tylophora indica (Burm. f.) Merrill (family Asclepiadaceae) commonly known as 'Antmool' is indigenous to India, found in the plains and hill forests of Eastern, Southern and Central India invading up to an altitude of 900 m (Gupta, 2003). Plant is also seen growing in states of Uttar Pradesh, Bengal, Assam, Orissa and Himalayan and sub-Himalayan India (Ali, 2008). Patalkot is a place in Madhya Pradesh commonly known as a treasure of *T. indica* (Acharaya *et al.*, 2010). Apart from harboring plains of India, it is also found in Ceylon, Malay island and Borneo (Kirtikar and Basu, 1975).

Habitat

Plant grows in plains, forests and hilly slopes and outskirts of the forests with optimum temperature range of 5- 35°C and annual rainfall of 500-2500 mm. Plant forms dense patches in well drained soils under moist and humid conditions in open hill slopes, forests and narrow valleys, but shows stunted growth in the areas with lesser rainfall (Nadkarni, 1976). Plant is mainly rain fed and requires slightly acidic to neutral pH (6.3-7.3) with black, red, lateritic and sandy loam soil texture.

Morphology

Tylophora indica is a slender, perennial climber with long, fleshy and Knotty roots. It is semi shrubby with long and twinning stem (Fig. 3). Leaves (3.0-10 cm long x 1.5-7.0 cm wide) are ovate-oblong to elliptic oblong, green in color with smooth surface (Kirtikar and Basu, 1991 and Gupta, 2003). Leaf Mesophyll is differentiated into 2-3 layered palisade tissue and 6-8 spongy parenchyma layers, containing rosettes of calcium oxalate crystals (Gupta *et al.*, 2010). Flowers (1-1.5cm) are hermaphrodite and occur in many colors like green, yellow and purple, arranged in 2 to 3 flowered fascicles in axillary umbellate cymes. Calyx is divided nearly to the base and densely hairy outside. Corolla has oblong acute lobes and is greenish-yellow or greenish purple in colour. Fruit (7 x 1 cm) are ovoid-lanceolate, tapering at apex and occurs in clusters. Seeds (0.6-0.8 x 0.3-0.4 cm) are broadly ovate or ovate-oblong, flat, brown and dark in colour near the centre (Chopra *et al.*, 1956 and Jagtap and Singh, 1999).



***Tylophora* plant in vegetative stage**

***Tylophora* plant in blooming stage**

Fig. 3. *Tylophora indica*

Propagation of *Tylophora*

Tylophora is normally propagated through seeds, but seeds are too small and have low seed viability and germination (Thomas and Philip, 2005). Moreover, propagation by vegetative cuttings like stem cuttings is rather difficult as they failed to produce proper roots (Faisal *et al.*, 2007).

Chemical constituents

Both alkaloid and non-alkaloid constituents have been isolated and characterized from the roots and leaves of *Tylophora indica*. The roots and leaves contain therapeutically important alkaloids such as tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$), tylophrinidine ($C_{22}H_{22}O_4N$) and septidine (Mulchandani, 1971 and Bhutani *et al.*, 1985). Recently, some rare alkaloids namely tyloindicines A, B, C, D, E, F, G, H, I, and J, desmethyltylophorine, desmethyl tylophorinine, isotylocrebrine, 4,6- desmethylisodroxy-o- Methyltylophorinindine have been reported. The non-alkaloidal compounds isolated from *Tylophora indica* are kaempferol, quercetin, α - and β -amyrins, tetratriacontanol, octaosanyl octacosanoate, sigmasterol, β -sitosetrol, tyloindane, cetyl-

alcohol, wax, resin, coutchone, pigments, tannins, glucose, calcium salts, potassium chloride, quercetin and kaempferol (Gupta *et al.*, 2010).

Medicinal importance

Tylophora indica is traditionally used as a folk remedy in treatment of bronchial asthma, bronchitis, dysentery, whooping cough, allergies and inflammation (Kirtikar and Basu, 1975; Bhavan, 1992 and Varrier *et al.*, 1994). The leaves and roots are used medicinally as they have laxative, expectorant, diaphoretic and purgative properties. It has reputation as a blood purifier, often used in rheumatism and syphilitic rheumatism. It is regarded as one of the best indigenous substitute for ipecacuahna (Joshi, 2000).

Phytochemical studies

Major alkaloid tylophorine has various pharmacological activities like anti-leukemia properties (Gellert *et al.*, 1962), anti-tumor and cancer cell inhibition (both *in vivo* and *in vitro*) (Donaldson *et al.* 1968; Li *et al.*, 2001 and Fu *et al.*, 2007), immunosuppressive, anti inflammatory (Gopalakrishnan *et al.* 1979), anti-allergic (Sundana *et al.*, 1979), immunomodulatory and hepatoprotective properties (Raina and Raina., 1980, Faisal and Anis, 2003 and Huang *et al.*, 2004), stimulant of adrenal cortex (Udupa *et al.* 1991), diuretic activity (Meera *et al.*, 2009), anti-amoebic (Bhutani *et al.*, 1985), anti-bacterial and anti-fungal properties (Reddy *et al.*, 2009).

3.2 Experimental Requirements

3.2.1 Chemicals and Reagents

The chemicals used for tissue culture work were from Hi-Media/Sigma/Sd fine brands (AR grade). All the reagents used for biochemical analysis were of either extra pure analytical grade or HPLC grade.

3.2.2 Plasticware/Glassware

Plasticware (Tarsons Products Pvt. Ltd, Kolkata) included autoclavable narrow and wide mouth bottles (500 ml), wash bottle (500 ml), graduated measuring cylinders (100, 500 and 1000 ml), gas bulb (250 ml), utility tray, test tube basket, funnel, retort stand, burette clamp, separatory

funnel holder, Buchner funnel, racked graduated tips (10, 200, 1000 μ l), micro tip box (0.2-10 μ l, 2-200 μ l, 200-1000 μ l), syringe filter (25 mm), micro centrifuge tube (0.5, 1.5, 2 ml), oak ridge centrifuge tube (10, 30, 50, 70ml), float rack (16 places), centrifuge tube box (36 places), measuring scoop, test tube stand, test tube cap (25 mm), carboy with stopcock (20, 50 lt), fixapipette (5, 10, 100, 200, 500, 1000 μ l), acupipet (0.5-2, 10-100, 100-1000 μ l) and biohazardous waste container (5 lts).

All glassware were made up of borosilicate glass (Borosil glass Ltd. Mumbai). The glassware used for experimental work included conical flasks (100 ml, 150 ml, 250 ml, 500 ml, 1000 ml, and 2000 ml), test tubes (25 x 125; 25 x 150 mm), beakers (100, 200, 250, 500 and 1000 ml), glass pipettes (1 ml, 2 ml, 5 ml, and 10 ml), round bottle flask (500 ml and 1000 ml), amber wide mouth bottles (500 ml), aspirator bottles with stopcock (1000 ml), reagent bottles (100, 200 and 500 ml), dessicator vacuum, graduated cylinders, volumetric flask, soxhlet extractor (60, 100, 200 ml), pear shaped flask (500, 1000 ml), two neck flask, centre neck and one angled side neck flask (250, 500 ml), column chromatography (10, 18, 30 mm), separating funnel (500, 1000 ml), stirring rod (16 x 16 mm), Petridish, TLC applicators and secador dessicator cabinet (51 x 34 x 41 cm). Tissue culture bottles (Make Glasil, New Delhi) were also used for routine subculture work. Laboratory consumables included tissue roll, cotton, aluminum foil, surgical blades, pre-coated aluminum plates, glass TLC plates, disinfectants, forceps etc.

Before use, the glassware were thoroughly brushed with alkaline detergent teepol (10 %) and then washed in running tap water. These were then treated with chromic and sulphuric acid mixture (1:3) for 24 hours, followed by thorough washing under running tap water. Distilled water was poured into every culture vessel, which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin cloth. Glassware were steam sterilized in an autoclave at a pressure of 1.1 kg/cm² for 15 - 20 minutes and oven dried prior to use.

3.2.3 Instruments

Included High Performance thin layer chromatography (HPTLC) (CAMAG), High Performance liquid chromatography (Waters), Nuclear magnetic resonance (Brukers spectrophotometer, Model AVANCE DPX), pH meter (Eutech Instruments), weighing balance (Sartorius), Microwave oven (LG), BOD Incubators (NSW), Orbital shakers (Orbitek), Horizontal shakers

(Labcon), Air conditioners (Voltas), Heater (Lexus, USHA), Timers for stands (Legrand), Vortex (SPINIX), Refrigerators (LG), Deep Freezers (Vestfrost), Autoclaves (Equitron), Laminar Flow Chambers (Thermadyne), Microtome (Shandon Finesse), Water bath incubator NSW-133 (Narang Scientific Works), centrifuge (Plastocrafts), magnetic stirrer (Tarsons), Hot air oven (Narang Scientific Works), Rotary Flash evaporator (Superfit), Spectrophotometer (Systronics), Microscope (Olympus), Sonicator (Sonics) and Nitrogen supply cylinders (SGS).

3.3 *In vitro* culture technique

3.3.1 Culture Medium

Murashige and Skoog's (1962) medium (MS) (Annexure I) was used for micropropagation. The media stocks were prepared in distilled water and refrigerated at 4°C. Stock solutions of auxins like naphthalene acetic acid (NAA), 2, 4- dichloro acetic acid (2, 4- D), indole 3-acetic acid (IAA), indole 3- butyric acid (IBA) and cytokinins i.e. kinetin (K), benzyl adenine (BA), thidiazuron (TDZ) and adenine sulphate were prepared and stored at 4° C.

3.3.2 Preparation of working media

The requisite amount of salts, vitamins and growth regulators from respective stock solutions were added in a conical flask with desired quantity of distilled water. 2% sucrose (Hi-Media) was used as a carbon source unless otherwise specified and the final volume was made up to the required level with distilled water. The pH was adjusted to 5.7 with either 0.1N HCl or 0.1 N NaOH and medium was gelled with 0.8 – 1.0% w/v agar Type-I (Hi-media). 75 ml and 25 ml medium was dispensed in culture bottles and test tubes respectively. Bottles were closed with autoclavable plastic caps whereas tubes were plugged with non-absorbent cotton wrapped in muslin cloth and were steam sterilized by autoclaving at 121°C and 1.1 kg/cm² for 20 minutes.

MS medium supplemented with various concentrations and combinations of auxins and cytokinins was used for callus induction, differentiation and somatic embryogenesis. The details of plant growth regulators (PGRs) combinations used are given below:

- Half strength basal MS medium (1/2 BMS)
- Basal MS medium (BMS)

- MS + NAA (7.35 μ M – 58.4 μ M)
- MS + 2, 4 –D (4.87- 38.96 μ M)
- MS + IBA (4.92 – 39.36 μ M)
- MS + IAA (4.8-38.4 μ M)
- MS + NAA (7.35 μ M – 58.4 μ M) + K (4.65 – 18.6 μ M)
- MS + NAA (7.35 μ M – 58.4 μ M) + BA (4.4 -17.6 μ M)
- MS + 2, 4 –D (4.87-38.96 μ M) + K (4.65 – 18.6 μ M)
- MS + 2, 4 –D (4.87-38.96 μ M) + BA (4.4 -17.6 μ M)
- MS + 2, 4 –D (4.87-38.96 μ M) + TDZ (2.25 μ M – 9.0 μ M)
- MS + IBA (4.92 – 39.36 μ M) + K (4.65 – 18.6 μ M)
- MS + IBA (4.92 – 39.36 μ M) + BA (4.4 -17.6 μ M)
- MS + IAA (4.8-38.4 μ M) + K (4.65 – 18.6 μ M)
- MS + IAA (4.8-38.4 μ M) + BA (4.4 -17.6 μ M)
- MS + BA (4.4 -17.6 μ M)
- MS + K (4.65 – 18.6 μ M)
- MS + TDZ (2.25 μ M – 9.0 μ M)
- MS + BA (4.4 -17.6 μ M) + K (4.65 – 18.6 μ M)
- MS + BA (4.4 -17.6 μ M) + Adenine sulphate (1.35 – 10.84 μ M)
- MS + K (4.65 – 18.6 μ M) + Adenine sulphate (1.35 – 10.84 μ M)
- MS + TDZ (2.25 μ M – 9.0 μ M) + K (4.65 – 18.6 μ M)
- MS + TDZ (2.25 μ M – 9.0 μ M) + BA (4.4 -17.6 μ M)
- MS + TDZ (2.25 μ M -9.0 μ M) + L- Ascorbic acid (5.6 – 22.4 μ M)

3.3.3 Surface sterilization of explants

Explants such as leaves, internodal segments, nodal segments, shoot apices and roots were washed under running tap water for 30 minutes to remove all adhering dust particles and microbes on the surface, followed by their immersion in teepol solution 1% (v/v) for 5 minutes and repetitive washings with tap water. Thereafter, the explants were treated with bavistin (0.1% w/v) for 10-12 minutes followed by repeated washings with water. Final sterilization was carried out in laminar flow hood by treating explants with 0.05 - 0.1% (w/v) aqueous solution of

mercuric chloride for 3-6 minutes depending upon the explant followed by thorough washing with sterile distilled water. On both the ends, a few mm portions of explants exposed to the sterilant were removed with the help of a sharp sterile secateur. The explants were then cultured on variously supplemented MS medium.

All operations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal UV tube (15 W, peak emission 2537Å). The floor of chamber was scrubbed thoroughly with cotton dipped in alcohol. The surface of all the vessels and other accessories such as spatula, forceps, glass plate, needles and scalpel, tubes containing absolute alcohol etc were also cleaned with spirit. Alcohol was sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with UV rays kept on for one hour. Hands and arms, were cleaned with alcohol before start of work and inoculation. The rims of test tubes/culture bottles and the sides of the plugs were flame sterilized. Forceps and scalpel were also sterilized by dipping in alcohol and flaming a number of times and cooled before putting into operation.

3.3.4 Growth conditions

Inoculated cultures were incubated in growth room in controlled conditions at a temperature of $25.0 \pm 2^{\circ}\text{C}$ with a photoperiod of 16-hours per day. Illumination was provided by cool white fluorescent tubes (Philips India Limited, Mumbai) at $50\text{ }\mu\text{mol/m}^2/\text{s}^1$.

3.3.5 Acclimatization of plantlets

The micropropagated plants (4-6 cm long, with 3-4 healthy roots) were acclimatized through successive acclimatization stages. The plantlets were rescued carefully from culture tubes and were washed under tap water to remove agar adhering to them. The plantlets were initially transferred to the culture bottles containing moist cotton covered with perforated plastic covers kept under growth room conditions for a period of 15-20 days. Plantlets were then transferred to plastic cups (of capacity 125 ml) containing soil either alone or in combination with various organic supplements (in 1:1 ratio) and were kept inside growth room for another 2 weeks. Soil and vermicompost were taken from Thapar University Campus, Patiala and were air dried before use and sieved through a mesh size of 2 mm. Physicochemical analysis of soil samples was carried out using standard protocols of Jackson, 1967 for measuring both pH and electrical

conductivity, of Black *et al.*, 1965 to measure water holding capacity, of Buoyoucos, 1962 to determine soil texture and method of Walkley and Black, 1934 for estimation of organic carbon. For the hardening of *Stevia rebaudiana* plants, only soil: vermicompost were used, whereas for the plants of *Tylophora indica* different potting mixtures were used (Table 6). The pure cultures of *Pseudomonas striata* and *Azotobacter chroococcum* were procured from Division of Microbiology, IARI, New Delhi and were maintained on Pikovskaya's media (PKV) and Jensen's media plates respectively and were grown in respective broths on shaker (120 rpm) at 30 ± 2 °C for 48 hrs. The cultures were harvested after 48 hrs of growth (when OD of 1.32 for *Azotobacter* and 1.52 for *Pseudomonas* were achieved) and carrier based microbial inoculant (containing 25 ml of grown bacterial culture in 250 gm of carrier material) was inoculated in soil under different treatments before planting plantlets.

Table 6: Different potting mixtures for hardening of *in vitro* raised plants of *T. indica*

S. No.	Potting mixture	S. No	Potting mixture
To	Soil	T3	Soil: <i>Azotobacter</i> : <i>Pseudomonas</i>
T1	Soil: vermicompost	T4	Soil: <i>Azotobacter</i>
T2	Soil: vermicompost: <i>Azotobacter</i> (N ₂ fixer): <i>Pseudomonas</i> (phosphate solubilisers)	T5	Soil: <i>Pseudomonas</i>

Young plantlets were taken out carefully from plastic cups and were shifted to plastic bags containing the same potting mixture kept inside growth room for another 2 weeks. The hardened plants were thereafter transferred to green house at ambient temperature and humidity for another two weeks before their final transfer to outdoor conditions. Plant height, number of photosynthetically active leaves formed and percentage survival of the plants were measured at different intervals.

3.3.6 Histological Studies:

Fixing of material

For ascertaining different morphogenetic stages in *Tylophora indica*, leaves regenerating adventitious shoots and embryogenic calli were fixed in a freshly prepared fixative FAA (Formalin: Acetic acid: 50 % ethanol:: 5: 5: 90) and subsequently in 70% ethanol until further use. After which the tissue was dehydrated in TBA (t-butyl alcohol) as given in Table 7.

Table 7: Dehydration of tissue in different TBA series

Step No.	Ethanol (ml)	TBA (ml)	Water (ml)
1	30	20	50
2	50	20	30
3	50	35	15
4	45	55	-
5	25	75	-
6	-	100	

The plant tissue was kept in each grade for 3-4 hrs except in Step No.3 where it was kept for overnight.

Waxing

For waxing, the tissue in TBA was kept in an oven preset at 60°C and paraffin wax flakes were added after every 15-20 min. The whole process was carried out till there was no smell of TBA left in the samples indicating complete waxing. The blocks were made and 12 µm thick sections were cut using a microtome and stretched on the glass slides. Dewaxing was done in the following grades and sections were stained in safranin and fast green dyes and permanent slides were made using a transparent mountant DPX (Table 8).

Table 8: Dewaxing in different grades and sections stained in safranin

S. No.	Xylol: Ethanol	S. No.	Water: Ethanol
1	75:25	13	25:75
2	50:50	14	Ethanol-1
3	25:75	15	Ethanol-2
4	0:100	16	Clove oil 25% in ethanol
5	Water: Ethanol	17	Clove oil 50% in ethanol

6	25:75	18	Fast green (prepared in 50% clove oil)
7	50:50	19	Clove oil 50% in Xylol
8	75:25	20	Clove oil 25% in Xylol
9	Safranin (6-24 hrs)	21	Xylol-1 (30 min)
10	Water: Ethanol	22	Xylol-2 (30 min)
11	75:25	Mounted the slides in D.P.X. mountant	
12	50:50		

Sections were examined under the light microscope (Nikon-Labphot-2) and photographed by means of an automatic photomicrography system.

3.3.7 Artificial seed production

For artificial seed production, somatic embryos and different vegetative propagules like shoot buds and shoot tips were subjected to encapsulation with sodium alginate or chitosan by dropping method (Redenbaugh *et al.*, 1987).

Encapsulation with sodium alginate:

1. Sodium alginate (in the range of 1-4% w/v) was complexed with 2 % of calcium chloride.
2. Explants mixed with sodium alginate were dropped into pre-chilled calcium chloride solution and were kept as such for 20-30 minutes for polymerization.
3. Embryos and shoot buds/tips were completely gelled by calcium alginate and after hardening, these beads were rinsed with sterile distilled water 3-4 times.
4. The alginate beads about (5 mm in diameter) were collected on a sterile filter paper in a Petridish sealed with parafilm. These were then stored in refrigerator at 4° C.

Encapsulation with chitosan:

1. Somatic embryos and shoot buds/tips were mixed in chitosan solution prepared in the range of 0.1 - 1% in 0.1N acetic acid.
2. Propagules were then dropped into 0.1% Tripolyphosphate solution (TPP). Chitosan being polycationic in acidic medium interacted with negatively charged TPP leading to the formation of biocompatible cross-linked chitosan beads.

3. Encapsulated beads (5mm in diameter) were then collected on a sterile filter paper in a Petridish and were stored at 4° C for further use.

In order to retrieve plantlets from stored encapsulated somatic embryos and shoot buds/tips after different time intervals, they were cultured on growth regulator free 1% agar-solidified full strength MS medium.

3.3.8 Statistical analysis

Twenty replicates were used for each treatment and all the experiments were repeated thrice. The data was analyzed by analysis of variance (ANOVA) to detect significant differences between mean values (Sokal and Rohlf, 1987). Means differing significantly were compared using the multiple range test (DMRT) at 5% probability level (Duncan, 1955).

3.4. Extraction and estimation of primary and secondary metabolites.

3.4.1 Preparation of plant sample

Different vegetative parts (leaf, stem and root) were collected from 2 yrs old healthy *in vitro* raised plants of *Stevia rebaudiana* and *Tylophora indica*. They were washed thoroughly under running tap water to remove impurities and stubborn dust particles. Explants were shade dried at room temperature, powderised to a particle size of 1mm using pestle and mortar, packed in airtight containers and stored at room temperature. Powderised explant samples were then analyzed for various primary (sugars, sucrose, starch, proteins and lipids) as well as secondary metabolites (sterols, phenols and flavonoids).

Primary metabolites (sugars, sucrose, starch, proteins and lipids)

3.4.2 Extraction of sugars (Dubois *et al.*, 1956)

A method of Dubois *et al.*, 1956 was used for the extraction and estimation of sugars. Dried leaf sample (1gm) with 5 ml of 80 % ethanol was taken in a conical flask kept in boiling water bath for 20 minutes. The process was performed twice followed by extraction with 70 % ethanol. Finally, the extract was filtered through Whatmann filter paper No. 40 and centrifuged at 2500 rpm for 5 minutes. The supernatant was evaporated using rotary flash evaporator at 50° C and 24 ml of distilled water was added to the concentrate followed by the addition of 0.25 ml saturated

lead acetate solution to precipitate the proteins. The contents were again filtered through Whatmann filter paper No 40. The excess lead ions in the filtrate were removed by precipitation with crystals of sodium oxalate and the filtrate was preserved at 4°C for the estimation of total sugars, reducing sugars and sucrose.

3.4.3 Estimation of total sugars (Dubois *et al.*, 1956)

Reagents

- a) 95% sulphuric acid (0.1 N):- 95 ml of concentrated sulphuric acid was taken in 100 ml of volumetric flask and final volume was made to 100 ml with distilled water.
- b) 5% phenol (w/v) redistilled: - 5g of phenol was mixed with 60 ml of distilled water in 100 ml conical flask and final volume was made to 100 ml with distilled water.

Procedure:

To 1ml of plant extract in a glass tube, 1ml of 5% phenol was added followed by the addition of 5 ml of 95 % sulphuric acid. The tubes were vortexed and kept at room temperature for 20 minutes to develop brown color. The intensity of the colour was read spectrophotometrically at 490 nm. The concentration of total sugars was determined using standard curve of reagent grade glucose in the working range of 20-100 µg/ml.

3.4.4 Estimation of reducing sugars (Nelson, 1994)

Reagents

- a) Reagent A (alkaline copper tartarate reagent): Was prepared by dissolving 25 g anhydrous sodium carbonate, 20 g of sodium bicarbonate, 25 g of potassium sodium tartarate and 200 g anhydrous sodium sulphate in 800 ml of distilled water and final volume was made to 1000 ml.
- b) Reagent B (copper sulphate reagent): Was prepared by dissolving 15 g copper sulphate in distilled water containing 2-3 drops of concentrated H₂SO₄. Final volume was made to 1000 ml with distilled water.
- c) Reagent C: Was freshly prepared by mixing reagent A and B in the ratio of 25:1(v/v).

- d) Reagent D (Arsenomolybdate reagent): To 25 g of ammonium molybdate dissolved in 450 ml of distilled water, 2 ml of concentrated sulphuric acid was added slowly with continuous stirring. Sodium arsenate (3g) was dissolved separately in 25 ml of distilled water, this solution was added drop wise to ammonium molybdate and volume was made 500 ml with distilled water. The solution so prepared, was incubated in amber colored bottle for incubation at 37° C for 48 hrs.

Procedure

To 1 ml of plant extract in a glass tube fitted with water condenser, 1ml of reagent C was added and kept in boiling water bath for 20 minutes. After cooling the tubes to room temperature, 1ml of reagent D was added and left undisturbed for 1 minute till the effervescence ceases. Then 7 ml of distilled water was added and intensity of blue colour developed was read at 520 nm. The concentration of reducing sugars was determined using standard curve of reagent grade glucose as prepared in section 3.4.3.

3.4.5 Estimation of sucrose (Roe, 1934)

Reagents

- a) Reagent A (Resorcinol solution): 100 mg resorcinol and 250 g thiourea were dissolved in 60 ml of glacial acetic acid and the final volume was made to 100 ml with acetic acid.
- b) Reagent B (6% KOH): 6 g KOH was dissolved in 60 ml distilled water and final volume was made to 100 ml.
- c) Reagent C (30% HCl): 83 ml concentrated HCl was mixed with distilled water to make its final volume to 100 ml.

Procedure

To 0.5 ml of plant extract in a test tube, 0.5 ml of reagent B was added and the tubes were heated in a water bath for 20 minutes to destroy free fructose. After cooling the test tubes to room temperature, 1ml of reagent A and 3 ml of reagent C were added. The tubes were then incubated at 80 °C for 10 minutes to develop pink color and the intensity of colour was measured at 490 nm. The concentration of sucrose was determined using standard curve of reagent grade sucrose in the working range of 20-100 µg/ml.

3.4.6 Extraction of Starch (Yashida *et al*, 1976)

To the sugar free residue (obtained in section 3.4.3) 5 ml distilled water was added followed by the addition of 6.5 ml chilled 52% perchloric acid. The mixture was stirred continuously for 5 minutes and then intermittently for 10 minutes on a magnetic stirrer. After stirring, 5 ml of distilled water was added and tubes were centrifuged at 1500 rpm for 20 minutes. The supernatant was then transferred into a 25 ml volumetric flask and final volume was made 25 ml with distilled water.

3.4.7 Estimation of starch:

Estimation of starch was done by method of Dubois *et al.*, (1956) as used for total sugar estimation and the standard curve was prepared using standard glucose in working range from 10-100 µg/ml prepared from stock solution (100 µg/ml) of glucose in distilled water. The content of starch was calculated by multiplying the value of the concentration of glucose by a factor of 0.9.

3.4.8 Extraction of Proteins (Lowry *et al.*, 1951)

1 g of dried plant sample was extracted twice with 25 ml of 0.1N NaOH, with each extraction lasting for 30 minutes. After each extraction, the extracts were filtered through Whatmann filter paper no. 40 and the combined filtrate was centrifuged at 14,000 rpm for 15 minutes. The supernatant was pooled out, diluted to 50 ml with distilled water. To the 2 ml aliquot of the supernatant, 2ml chilled 20% trichloro acetic acid (TCA) was added and mixed thoroughly. After ageing for 1hr at 4° C, the contents were centrifuged (14,000 rpm) for 15 minutes and the precipitates so obtained were dissolved in 2 ml of 0.1 N NaOH. In the extract, total proteins were estimated as per the method of Lowry *et al.*, (1951).

3.4.9 Estimation of Proteins

Reagent

- a) Lowry A: 2% Sodium carbonate in 0.1N NaOH
- b) Lowry B: 0.5% copper sulphate in 1% sodium citrate.
- c) Lowry C: Mix Lowry A and Lowry B in ratio of 50:1(v/v)
- d) Lowry D: Folin- Ciocalteu reagent was diluted to 50% with distilled water.

Procedure

To 1 ml of the plant extract, 5ml of Lowry C was added and was allowed to stand for 10 minutes at room temperature. Then 0.5 ml of Lowry D was added rapidly and was again left undisturbed for another 30 minutes. The quantity of protein was determined at 520 nm using standard curve prepared by using protein bovine serum albumin (BSA) in the working range of 20 -100µg/ml.

3.4.10 Extraction and Estimation of Total lipids

Extraction of lipids and their quantification was determined by the method of Folch *et al.*, 1957.

Reagents

- a) Chloroform : methanol (2:1,v/v)
- b) 0.9 % NaCl solution: 0.9 g of NaCl was dissolved in distilled water and final volume was made to 100 ml.

Procedure:

4 g of dried plant samples was transferred to an airtight glass stoppered flask containing 80 ml of chloroform: methanol (2:1) mixture and the contents were shaken thoroughly for 2-3 hrs on an orbital shaker. The contents were filtered through a sintered glass funnel to remove the insoluble impurities from the lipids. To this filtrate, 16 ml of 0.9% NaCl was added and fractionated using separatory funnel. The contents were shaken and allowed to stand until two separate layers were formed. Free sugars, short chain amino acids and other insoluble impurities were mixed in the upper layer whereas, pure liquid fraction went to lower chloroform layer. The lower layer was removed and upper layer was again treated with a some volume of chloroform to obtain the residual lipids. Pooled lower fractions were collected in a pre –weighed crucible, which was reweighed to find the lipid contents. After calculating the weight of total lipids, they were dissolved in chloroform and final volume was made to 25 ml with chloroform and kept at 4 °C for estimation of sterols.

Secondary metabolites (sterols, phenols and flavonoids)

3.4.11 Extraction and estimation of free sterols (Zlatkis *et al.*, 1953)

For extraction, same procedure was followed remains the same as described in the lipid extraction (section 3.4.10).

Reagents

- a) 0.05% ferric chloride – acetic acid reagent: 5 g of ferric chloride hexahydrate was dissolved in glacial acetic acid and the final volume was made to 100 ml. 5 ml of stock solution was dissolved in acetic acid and volume was made to 500 ml.

Procedure

40 µl of lipid extracted in chloroform was air dried in test tube and 5 ml of 0.05% ferric chloride acetic acid reagent was added. Further, 2 ml of concentrated sulphuric acid was added and the contents were vortexed and kept at room temperature for 20 minutes to develop bluish green color. The intensity of colour was measured at 540 nm. The concentration of free sterols was calculated from reference curve using standard cholesterol in the range of 100-500 µg/ml.

3.4.12 Extraction and estimation of total phenols (Malik and Singh, 1971)

1g of dried powder was taken in 100 ml of conical flask and to this 10 ml of 0.3 N HCl in methanol was added and kept on shaker at 150 rpm for 1hr. After shaking, the crude extract was filtered through Whatmann filter paper no. 1 and evaporated to dryness in a boiling water bath. To this residue, hot water was added and final volume was adjusted to 25 ml with distilled water.

Reagents

- a) Folin – Ciocalteu reagent (diluted 1:2)
- b) 35 % sodium carbonate

Procedure

To 0.05 ml of the above extract, 1 ml each of Folin – Ciocalteu reagent and sodium carbonate reagent were added. After 10 minutes 2 ml of distilled water was added and intensity of the colour developed was recorded at 620 nm. The concentration of total phenols was calculated from the standard curve using gallic acid in range of 20-100 µg/ml prepared from stock solution (100 µg/ml) of gallic acid dissolved in few drops of ethanol and final volume was made to 100 ml with distilled water.

3.4.13 Extraction of flavonoids (Malik and Singh, 1971)

0.2 ml of phenolic extract (as obtained under section 3.4.12) was taken in a test tube and to this 1ml of 20 % HCl and 0.5 ml of formaldehyde was added and test tube was allowed to stand overnight. After 24 hrs, the content of the tube was centrifuged at 1000 rpm for 20 minutes and supernatant was used for the estimation of flavonoids. 1 ml of the supernatant was taken in a test tube and treated as described for the estimation of phenolic compounds (Section 3.4.12).

3.5 Extraction of major secondary metabolite –Stevioside from *Stevia rebaudiana*

Leaves from *in vivo* and *in vitro* raised healthy plants were excised after different periods of growth (three, four, five, eighteen and thirty months old), washed under running tap water and were shade dried at room temperature. The dried leaves were powderised using pestle and mortar and sieved through mesh size of 1 mm. Extraction of sweet tasting glycoside (stevioside) from dried leaf powder was done using different extraction protocols with modification as follows.

Extraction Protocol I

A method of Kedik *et al.*, 2003 was modified for the extraction of stevioside from raw leaf powder. 50 g of powder was extracted thrice with 200 ml of 95 % ethanol in a soxhlet apparatus for 1 hr each followed by extraction with diethyl ether and methanol again for 1 hr. The extract was cooled, filtered and evaporated using rotary flash evaporator at 50° C. The concentrate was resuspended in methanol and analyzed further using HPTLC.

Extraction Protocol II

A method given by Kumar *et al.*, 1986 was modified for extraction and isolation of stevioside from the leaves of *Stevia rebaudiana*. 200 g dried powder was extracted twice with 1litre hot water for two hrs at 60°C. The extract was filtered through Whatmann filter paper no. 1 and the clear green solution thus obtained was concentrated using rotary flash evaporator at 60°C. The concentrate was filtered and the pH was adjusted to 8-10 with dilute sodium hydroxide. The filtrate was kept as such for 24 hrs and was distilled with n-butanol. The concentrate was recrystallized using methanol to yield stevioside, which was purified using HPTLC.

Extraction Protocol III

The extraction of stevioside from leaf parts was carried following the method described by Hearn and Subedi, 2009. 50 g powdered sample was extracted in 100 ml of 70 % ethanol in hot water bath at 50° C for 2 hrs. The extract was cooled to room temperature, filtered through syringe filter and analyzed further using HPTLC.

Extraction Protocol IV

A method described by Bovanova *et al.*, 1998 was modified for the determination of stevioside from *Stevia rebaudiana*. 50 g dried powder was subjected to threefold extraction with 150 ml deionised water at room temperature. The extract was filtered through Whatmann filter paper no. 1, centrifuged at 10, 000 rpm for 10 minutes. The supernatant obtained after centrifugation was concentrated using flash evaporator at 50° C for further purification using HPTLC.

Extraction Protocol V

A method of Rajasekaran *et al.*, 2008 was modified for the extraction of stevioside from leaves of *Stevia rebaudiana*. 200 g dried powder was stirred three times in 500 ml petroleum ether for 4 hrs each, followed by filtration using fine coarse filter paper. The filtrate was discarded and the extract was treated again with 50 ml of hot methanol for 2 hrs. The process was repeated thrice and the extract was filtered again. The filtrate was concentrated in a rotary flash evaporator at 60°C and the concentrate was redissolved in 50 ml of distilled water. Further extract was fractioned with 50 ml of diethyl ether (to remove green colour of the extract) in separatory funnel. Lower transparent layer was collected and washed thrice with butanol. Upper butanol layer was collected, evaporated using flash evaporator at 60°C up to dryness. The suspension was resuspended in methanol, kept at 4°C for crystallization and mother liquor was further column chromatographed over silica and analyzed by HPTLC.

Extraction protocol VI

50 g dried powder was extracted thrice with 150 ml of water for 1 hr each in water bath at 50°C according to method given by Janarthanam *et al.*, 2010. The extract was cooled to room temperature, filtered through Whatmann filter paper no. 1 and analyzed using HPTLC.

Extraction protocol VII

The sweet tasting glycoside was extracted using method of Rajasekaran *et al.*, 2008. 100 g dried powder was first defatted thrice with 50 ml of petroleum ether using soxhlet apparatus and then extracted with 500 ml of distilled water. The resultant extract was filtered using Whatmann filter paper and the filtrate was refluxed on a steam bath for 90 minutes with 5 ml of potassium hydroxide. The pH of the filtrate was adjusted to 5.0 and the resultant mixture was extracted thrice with chloroform: methanol (2:1). Extract so obtained was concentrated using rotary flash evaporator and was redissolved in methanol for further analysis using HPTLC.

Extraction protocol VIII

A protocol given by Swanson *et al.*, 1992 was modified for the extraction of stevioside from the leaf explants. 50 g dried powder was soaked for overnight in methanol: water (60:40) solution followed by filtration. The filtrate was dried on rotary flash evaporator at 55°C, redissolved in 10 ml of water and was defatted twice with equal volumes of chloroform. Aqueous layers were partitioned using n-butanol and the combined butanol layers were again evaporated in flash evaporator. The concentrate was dissolved in methanol, applied to pre coated silica gel plates for HPTLC analysis.

Extraction protocol IX

Extraction protocol VIII was repeated as such with change in the ratio of methanol: water to 40:60.

3.6 Extraction of major secondary metabolite -Tylophorine from *Tylophora indica*

Vegetative plant parts (leaves and roots) excised from twelve and twenty four months old *in vivo* and *in vitro* raised plants of *T. indica* were surface washed under running tap water and shade dried at room temperature. The dried parts were fine grounded using pestle and mortar to size of 1mm and further used for the extraction of alkaloid tylophorine using different protocols as follows.

Extraction Protocol I

Method of Rao and Brook, 1970 was used for the extraction of tylophorine. 100 g of dried powder was washed twice with 50 ml hexane to remove oily components. The powder was extracted thrice in cold with 100 ml of 1% acetic acid in methanol, with each extraction lasting for a day. The methanol acetic acid fraction was concentrated in rotary flash evaporator at 50° C and the concentrated fraction was extracted with ethyl acetate: HCl (1:1) three times. Two immiscible layers were formed, the acid layers were collected and pH was adjusted to 8.5-9.0. The acid layer was extracted further three times with chloroform (50 ml each). Alkaloid passes through the chloroform layer and yellow pigments remains in the aqueous layer. The chloroform extracts were concentrated using rotary flash evaporator and digested with 50 ml of hot methanol. The mixture was cooled, filtered and further analyzed using TLC/ HPTLC.

Extraction Protocol II

Method of Jain *et al.*, 2007 was used for the extraction of tylophorine from *Tylophora indica*. Powdered plant parts were moistened with distilled water and mixed with lime. This was refluxed three times with chloroform (100 ml each). The chloroform extract was filtered, concentrated using flash evaporator (60° C) and fractioned thrice with 2% aqueous sulphuric acid in methanol in a separatory funnel. Aqueous layer was collected and washed three times with ethyl acetate (50 ml each). Again, aqueous layer was collected and basified to pH 9 with solution of sodium hydroxide. Precipitates formed were separated by centrifugation at 4000 rpm for 15 minutes. Precipitates were washed thrice with distilled water, redissolved in 2% aqueous sulphuric acid and were dried in dessicator to get fine powder. The powder was resuspended in methanol and further purified using HPTLC.

Extraction Protocol III

A method of Reddy *et al.*, 2009 was used for the extraction of tylophorine from *T.indica*. 50 g of powdered plant parts were extracted with 500 ml of methanol in soxhlet apparatus. The residue was filtered, concentrated to dryness in rotary flash evaporator at 50°C. The concentrate was macerated with 25 ml of 2N HCl, allowed to stand for 15 minutes. The acid layer was separated, extracted with ethyl acetate in a separating funnel. The upper ethyl acetate layer was collected

and finally concentrated in flash evaporator. The concentrate was resuspended in methanol for further purification of tylophorine using HPTLC.

3.7 Purification of metabolite

For purification of metabolite, the mother liquor obtained was chromatographed by thin layer chromatography (TLC), column chromatography and high performance thin layer chromatography (HPTLC).

Optimization of standard was the first step towards standardization for herbal analysis. The total extract can only be characterized using fingerprint technique of HPTLC in which large amount of chromatographic data of standard was compared with the data from the sample. Prior to characterization of data, standard parameters like loading volume, optimum wavelength and developing solvent system were optimized.

Standard stock preparation:

A) Stevioside stock preparation

Stevioside standard procured from Sigma Aldrich, Mumbai was prepared by dissolving 0.5 mg stevioside in acetonitrile: water (8:2).

Working concentration (0.02µg/µl) was made from standard stock solution (1µg/µl).

B) Tylophorine stock preparation

Tylophorine standard procured from Alexis Biochemical, New Delhi was prepared in 100% ethanol and working concentration of 0.02 µg/µl was made from the standard stock solution (1µg/µl).

From the working standard concentration, the amount of standard present in different samples was calculated by the given formula (Harris, 2003 and <http://blake.montclair.edu/~olsenk/>).

Concentration of standard in sample (µg/ml) =

$$\frac{\text{Area of standard peak in sample} \times \text{Concentration of standard}}{\text{Area of standard peak}}$$

3.7.1 Thin layer chromatography

Plate preparation: Plates of dimensions (20 x 20 and 5 x 20 cm) were thoroughly cleaned with chromic acid followed by washing with water and were completely dried in the oven. For plate preparation, the adsorbent Silica gel G was mixed with water (2:1) and fine slurry was made. This mixture was spread on an unreactive carrier sheet like glass with the help of TLC applicator. The resultant plate was dried, activated by heating in an oven for thirty minutes at 110 °C. The thickness of the adsorbent layer was kept around 0.1 – 0.25 mm.

Sample loading: A small spot of solution containing the sample was applied to the plate, about 1.5 cm from the bottom edge using fine capillary tube. The plate was dried in vacuum oven to completely evaporate the solvent.

Plate development: An appropriate amount of elutant (solvent system) was poured into a glass beaker (separation chamber) to a depth of less than 1 cm. Chamber was saturated using a strip of filter paper which touches the solvent at the bottom and reaches almost to the top of the container. The container was closed with a glass lid and was left undisturbed for few minutes. The TLC plate was then placed in the chamber so that the spot(s) of the sample do not touch the surface of the elutant in the chamber and the lid was closed. The solvent moved up the plate by capillary action, meets the sample mixture and carried it up the plate (elutes the sample). When the solvent front reaches no higher than the top of the filter paper in the chamber, the plate was removed, solvent front was marked and the plate was dried.

Sample separation: Different compounds travel at variable rates, due to differences in their solubility in the solvent and were viewed as separate components. By changing the solvent system, the separation of components (measured by the R_f value) was adjusted. Plates after development were viewed using iodine pellets kept in iodine chamber. The position of different fractions in TLC was characterized by R_f value which is distance travelled by solute from origin /distance travelled by solvent front (R_f can be from 0 to 1) (Sawhney and Singh, 2005).

$$R_f = \frac{\text{Distance travelled by solute (cm)}}{\text{Distance travelled by solvent (cm)}}$$

3.7.2 Column Chromatography

For column chromatography the stationary phase (Silica Gel) was placed within a vertical glass tube, the mixture to be analyzed was added to the top and as it flows down through the column (by gravity), separation of compounds was achieved.

Column Preparation: Column length was selected based on the amount of material, which needs to be separated. Disposable glass pipettes with broken tops were used for 1g of sample to be loaded. The column was attached to a ring stand, safely fastened in vertical position. A piece of glass wool was gently passed to the bottom of the column with the help of a glass rod. For column packing, slurry method was used where the adsorbent (Silica gel mesh size 60-120) was mixed with the solvent and fine slurry thus made was poured into the vertical glass column. Silica in the beaker was added very gently and slowly with continuous swirling, little at a time until the column was about half filled. The column was then filled with the solvent and the screw clamp was opened so that liquid could drain down. When column was packed, the excess solvent was drained out until it just reaches the top level of the silica. The screw clamp was closed and the column was ready for use.

Sample loading and elution: Drying of mother liquor was done by adding silica gel followed by dichloromethane. The solvent was reduced on rotary (40° C) after thorough mixing and finally the solid extract left behind was loaded in the column. Sample to be loaded was added slowly from the top of the column and pinch clamp was opened to allow the solvent drain down until the mixture was little way into the solvent. As the solvent keeps on flowing from the glass column different fractions of elute were collected from the bottom and more solvent was continuously added from the top so that column does not dry up. The eluting solvent was changed depending upon the polarity as the process proceeds and the process was continued until desired compounds were obtained.

3.7.3 High performance liquid chromatography (HPLC)

High performance liquid chromatography is a closed column system in which samples were introduced in the moving fluid streams and solutes were eluted from the column by the mobile

phase. The isolated extracts were analyzed using Waters detector and C18 column with a particle size of 7 μ M. For optimal detection settings, a separation assay was developed in which different parameters were selected where a clean peak of the known sample was observed from the chromatograph. The identifying peak showed reasonable retention time and was well separated from extraneous peaks at the detection levels.

Sample Application

Samples (10 μ l) were injected into the HPLC via an injection port, which consisted of an injection valve and the sample loop. The sample to be injected was dissolved in the mobile phase before injection into the sample loop. As sample solution flows through a column with the mobile phase, the components of the solution migrate according to the non-covalent interactions of the compound with the column. A rotation of the valve rotor closed the valve and opened the loop in order to inject the sample into the stream of the mobile phase.

3.7.4 High Performance thin layer chromatography (HPTLC)

High performance thin layer chromatography is an enhanced form of thin layer chromatography. HPTLC has come as an improvement over normal TLC in the quality of sorbents, use of optimized techniques and equipments for sample application, plate development, detection reagent application and densitometric scanning.

The different elements of HPTLC includes:

Sample application: The samples were applied onto the plate as bands using Linomat 5 sample applying device. Precision of the applied volume, exact positioning and compactness of application zone determine the quality of the result. For quantitative analysis, samples were applied normally in the form of bands of size 6-8 mm. Samples (6-20 μ l) were applied on to pre coated silica gel 60 F 254 (20 x 10, 10 x10 and 5 x 10 cm plates) using 100 μ l syringe and nitrogen as spray gas. Distance from side was kept 12-15 mm and distance from bottom was 8 mm. Position of first track was normally at 20 mm, distance between two tracks was adjusted to 12 mm and scan position was 5 – 85 mm.

Chromatograph development: For chromatographic development, all the phases including stationary, mobile and vapor phase were activated in chromatographic development chamber. The mobile phase was drawn through the stationary phase by capillary action. Twin trough (TT) chamber was used for better separation of HPTLC plates. TT Chamber offers less consumption of solvent (20 ml of solvent was sufficient for 20 X 20 cm chamber, 10 ml for 20 X 10 cm chamber and 5ml for 10 X 10 cm chamber) with higher degree of separation. Samples separated into different components, which remained in their position after the mobile phase has been evaporated using TLC plate heater which ensures homogenous heating across the plate at a temperature of 80-150°C. The 20 X 20 cm heating surface has a grid to facilitate correct positioning of TLC plate. Programmed and actual temperatures were digitally displayed and the plate was protected from overheating.

Evaluation (Detection): Options range from visual inspection of electronic images to quantitative determinations using scanning densitometry. For chromatograph to qualitative and quantitative results, evaluation was done using Scanner 3 and all functions of scanner were controlled using win CATS software. Densitometry in scanner 3 was done using monochromatic light and a slit of selectable length and width to scan the chromatograph. TLC scanner uses spectrum range from 190 to 900 nm with high spectral selectivity. The spectrum scan speed was normally at 100 nm/sec with data resolution mode per plate at 1nm/step. Lamps were selected within different wavelength ranges (D₂ and UV from 190-400 nm, W for 400-800 nm and Hg for 312 and 366 nm). Auto spectra comparison of test sample with the standard (based on R_f values) identified the standard in the sample whereas auto scan of spectra quantified the standard (either through calibration curve or manually through peak area and concentration of standard) in the sample.

Evaluation (Documentation): For documentation, electronic images were easy to capture and to archive. Electronic image capturing required polychromatic light (white light, UV 254 and 366) to illuminate the entire plate and the image was captured using digital camera for documentation of chromatograph. An integrated powerful 12-bit camera with linear CCP chip and excellent color capture was all together controlled by win CATS.

3.7.5 Preparative TLC

TLC performed on small semi- preparative scale refers to preparative TLC where thickness of plate ranges from 0.2-5 mm. The procedure for preparation of plates was same as done in normal TLC. When the plate was developed with a selective solvent system, compounds separate as horizontal bands and each band was scraped off using UV light. Band was marked with pencil, scraped using knife or edge of spatula. The scraped band was then extracted using suitable solvent and filtered through syringe filter to remove silica gel. The solvent was then evaporated under vacuum in flash evaporator and further HPTLC was done to confirm the banding pattern followed by NMR analysis.

3.7.6 NMR

Nuclear magnetic resonance (NMR) spectrophotometer makes use of a magnet, a radio frequency, a detector and an amplifier. The test sample was placed in glass tube placed between the pole face of the magnet and was measured on a Bruker spectrophotometer (Model AVANCE DPX) with a working frequency of 400 MHz. The spectra was recorded at Central Instrumentation Laboratory, NIPER, Mohali. The chemical shifts were determined relative to the internal standard (TMS) and assigned based on published data.

Solvent selection

A substance free of protons such as dimethyl sulphoxide (DMSO = $(\text{CH}_3)_2\text{SO}$) an organosulphur compound and a polar solvent, which does not give any adsorption of its own in NMR spectrum was selected. This colourless liquid is an important polar aprotic solvent as it dissolves both polar and non-polar compounds, is miscible in a wide range of organic solvents as well as water, chemically inert and devoid of any hydrogen atom. DMSO was able to dissolve the test samples to a reasonable range.

3.8 Plant cell cultures for production of secondary metabolites

3.8.1 Secondary metabolites from callus cultures

Murashige and Skoog's (MS) medium supplemented with different plant growth regulators was selected for the induction, growth and maintenance of callus from different explants of *Stevia*

rebaudiana and *Tylophora indica*. Different callus cultures were screened separately for their growth and the best combination of growth hormones yielding higher biomass of callus in terms of fresh weight (g/l) was selected for establishing callus cultures. Fresh weight of callus was taken after removing the excess moisture on the surface using blotting paper. Best growing callus, thus selected was dried in hot air oven at 50° C for 24 hr and dry wt (g/l) was recorded. Dried callus (40 g) was grounded in mortar and pestle to fine powder using liquid nitrogen. The dried callus of *S. rebaudiana* were extracted using standardized extraction protocol V (section 3.5), whereas those of *T. indica* were extracted using protocol I (discussed in detail in section 3.6). The crude extracts obtained were analyzed using HPTLC.

3.8.2 Secondary metabolites from suspension cultures

1 gm callus (wet weight) was transferred from solid to liquid MS medium in 250 ml conical flasks. The cultures were incubated on rotary shakers at 120 rpm in growth room at 25 ± 2° C. The suspension-culture were harvested using filter paper at regular intervals and biomass yield was recorded in terms of fresh weight (g/l) and dry weight (g/l). To scale up the production of secondary metabolite, inoculum (1- 2%) was transferred from 250 ml conical flask to 5 liter conical flask. Cultures were maintained under similar set of conditions until stationary phase was obtained. After successful optimization of biomass production in large sized conical flasks, cultures were harvested for extraction and purification of major secondary metabolite. After drying to a constant weight at 60° C for 24 hrs and they were grounded to a fine powder. Secondary metabolites were extracted using protocols as discussed in section 3.5 and 3.6 for callus cultures and were analyzed by HPTLC.

Objective 1: To develop and standardize mass propagation of *Stevia rebaudiana* and *Tylophora indica* under *in vitro* conditions

4.1 Micropropagation of *Stevia rebaudiana*:

In order to raise sterile cultures of *Stevia rebaudiana*, different vegetative parts like leaf, stem, nodal segments and shoot apices were excised from healthy field grown mother plant. The explants were disinfected with bavistin (0.1% w/v) for 10-12 minutes followed by surface sterilization with 0.05% (w/v) mercuric chloride for 2 minutes. Fresh cuts were given to the segments after sterilization to remove undesirable or dead portions. These were then planted on variously supplemented Murashige and Skoog's (MS) medium. For all the preliminary experiments, the medium was supplemented with 2% sucrose and various adjuvants depending on the nature of experiment.

4.1.1 Leaf Culture

4.1.1.1 Direct *de novo* adventitious shoot induction

Among the various auxins and cytokinins tried, *de novo* adventitious shoot formation from the leaf explants occurred on different concentrations of 2, 4-D (4.87 – 29.2 μ M) and BA (4.4 -17.6 μ M). Best results were observed on 9.74 μ M 2, 4-D and 8.8 μ M BA supplemented medium where numerous nodular meristemoids were induced from the entire surface of leaf lamina after 6-7 days of culturing (Fig. 4 A). Initially 5-6 adventitious shoots emerged from these meristemoids after 15 days of culturing (Fig. 4 B) and with passage of time many more shoots were formed. These shoots multiplied further (Fig. 4 C) and within 30 days, uncountable number of healthy, green shoots (mean shoot length 9.0 ± 0.05 cm) were formed in nearly 88% of the cultures (Fig. 4 D).

High frequency shoot regeneration was also observed on NAA (7.35 μ M) and BA (4.4 μ M) supplemented medium, where initiation of meristemoids occurred after 10 days of culturing (Fig. 5 A). These meristemoids proliferated further and grew into green leafy shoots (Fig. 5 B) forming many clusters after 3 weeks (Fig. 5 C). When subcultured on the fresh medium, the

shoots multiplied further and increased manifold in number (Fig. 5 D). A moderate rate of shoot regeneration from the leaf explants was also observed when BA (8.8 μ M) was used in conjunction with adenine sulphate (1.35 μ M) forming on an average 35 ± 2.88 shoots/culture after 8 weeks.

A supplementation of different concentrations of TDZ (4.5 – 9.0 μ M) and L-ascorbic acid (5.6 – 22.4 μ M) was also demonstrated for *de novo* adventitious shoot formation. Numerous meristemoids were induced from leaf explants on TDZ (2.25 μ M) and L-ascorbic acid (2.8 μ M) which proliferated into green leafy shoots forming 45.00 ± 1.15 shoots/flask in 60% of the cultures after 5 weeks. The effect of various growth regulators on *de novo* adventitious shoot formation from the leaf explant is depicted in Table 9.

Table 9: Effect of different PGR's on *de novo* adventitious shoot formation from leaf explants (Mean followed by same letter are not significantly different by Duncan's multiple range test at 0.05% probability level)

S. No	Plant growth regulator (PGR)	Concentration (μ M)	Explant forming shoots (% age)	Shoots/culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	2, 4-D + BA	4.87 + 8.8	63	37.66 $\pm$ 1.40 ^c	6.1 $\pm$ 0.08 ^b
		9.74 + 8.8	88	TNTC	9.0 $\pm$ 0.05 ^b
		19.4 + 8.8	74	57.66 $\pm$ 1.45 ^a	6.8 $\pm$ 0.05 ^a
		29.2 + 8.8	69	44.00 $\pm$ 1.15 ^b	6.4 $\pm$ 0.05 ^b
2.	NAA + BA	7.35 + 4.40	80	TNTC	8.5 $\pm$ 0.05 ^b
		14.70 + 4.40	72	37.66 $\pm$ 1.45 ^c	6.1 $\pm$ 0.05 ^b
		21.05 + 4.40	61	32.33 $\pm$ 1.45 ^d	5.7 $\pm$ 0.05 ^c
		29.40 + 4.40	50	21.00 $\pm$ 0.57 ^h	5.0 $\pm$ 0.05 ^{de}
		14.70 + 8.80	60	32.66 $\pm$ 1.45 ^d	5.4 $\pm$ 0.05 ^d
		14.70 + 13.2	55	22.66 $\pm$ 0.88 ^g	4.7 $\pm$ 0.05 ^e
		22.05 + 17.6	50	20.00 $\pm$ 1.15 ⁱ	3.7 $\pm$ 0.08 ^{fg}
3.	BA + Adenine sulphate	4.4 + 1.35	54	27.66 $\pm$ 1.45 ^e	3.8 $\pm$ 0.05 ^{fgh}
		8.8 + 1.35	60	35.00 $\pm$ 2.88 ^d	4.8 $\pm$ 0.05 ^e
		13.2 + 1.35	58	32.33 $\pm$ 1.45 ^d	4.0 $\pm$ 0.05 ^f
4.	TDZ + L-Ascorbic acid	2.2 + 2.8	60	45.00 $\pm$ 1.15 ⁱ	7.4 $\pm$ 0.05 ^b
		4.5 + 2.8	51	26.66 $\pm$ 0.88 ^e	3.2 $\pm$ 0.08 ^{fghi}
		9.0 + 2.8	44	24.00 $\pm$ 0.57 ^f	3.0 $\pm$ 0.05 ^{ghi}

TNTC: Too numerous to count, 2, 4- D: 2, 4- dichlorophenoxy acetic acid, BA:6-benzyl adenine, NAA: α -naphthalene acetic acid, TDZ: thiadiazuron.

4.1.1.2 Induction and Growth of callus

For callus induction, leaf explants (4-5 mm in size) were cultured on MS medium supplemented with different concentrations of 2, 4-D (4.87-38.96 μM), NAA (7.35 μM – 58.4 μM), IBA (4.92 – 38.96 μM), TDZ (4.5 – 36.0 μM), K (4.65 – 18.6 μM), BA (4.4 – 17.6 μM) either alone or in combination with each other. Best growth of callus occurred on MS + 2, 4-D (19.48 μM) and K (4.65 μM) supplemented medium where callusing occurred in 98% of the cultures. The callusing initiated after 7 day of culturing (Fig. 6 A) and within 3 weeks the entire explant turned into a mass of greenish white, soft and friable callus showing sustained growth (Fig. 6 B). Likewise synergistic action of NAA (29.4 μM) with K (4.65 μM), hereby, designated as NK medium was also equally effective in the initiation and sustained growth of callus. Good callus growth was observed at the cut ends as well as from entire surface of explant after 7-8 days of culturing (Fig. 6 C) and within 3-4 weeks large friable light brown calli were formed in nearly 95% of the cultures (Fig. 6 D).

Leaf segment also showed callus induction on MS supplemented with IBA (9.74 μM) and K (4.65 μM) but the callus took longer time to initiate and was comparatively slow growing (Table 10).

Table 10: Effect of different concentrations and combinations of PGR's on callus induction and growth from leaf explants of *Stevia rebaudiana*.

S. No	Plant growth regulator (PGR)	Concentration (μM)	Explants forming callus (% age)	Nature of callus	Color of callus	Growth of callus
1.	2,4-D + K	4.87 + 4.65	50	Soft	Greenish white	Good growth
		14.6 + 4.65	78	Soft	Greenish white	Good growth
		19.4 + 4.65	98	Soft	Greenish white	Very Good growth
		29.2 + 4.65	62	Soft	Greenish white	Good growth
2.	NAA + K	7.35 + 4.65	33	Soft	Light brown	Slow growing
		14.7 + 4.65	48	Soft	Light brown	Slow growing
		22.0 + 4.65	76	Soft	Light brown	Slow growing
		29.4 + 4.65	95	Soft	Light brown	Good growth
		36.7 + 4.65	66	Soft	Light brown	Slow growing
3.	IBA + K	4.90 + 4.65	60	Soft	Green	Slow growing & non-sustainable
		9.74 + 4.65	80	Soft	Green	Slow growing & non-sustainable
		13.2 + 4.65	76	Soft	Green	Slow growing & non-sustainable
		17.6 + 4.65	62	Soft	Green	Slow growing & non-sustainable

2, 4- D: 2, 4- dichlorophenoxy acetic acid, K:kinetin, NAA: α -naphthalene acetic acid, IBA: indole 3-butyric acid

Study of callus: The leaf calli raised on different media were extremely friable breaking up into single cells or cell groups when placed in water. The callus was heterogeneous in nature comprising of cells of different shapes and sizes. The cells were oval, round and elongated (Fig. 7A), each with numerous starch grains scattered throughout the cytoplasm (Fig. 7 B). Microscopic observation of 3-week-old callus revealed differentiation of tracheids, which occurred singly or in groups and possessed reticulate thickenings on their walls (Fig. 7 C). Organized tissues having well-developed vascular strands were also observed in the callus prior to organogenesis (Fig. 7 D and E).

4.1.1.3 Differentiation from leaf callus

Organogenetic differentiation in the form of shoots was observed from the leaf callus raised on NK medium after 6-7 weeks where only a few shoots differentiated in 20-30 % of the cultures. Likewise callus cultures raised on MS + 2, 4-D (19.48 μ M) and K (4.65 μ M) supplemented medium exhibited a low frequency of shoot differentiation. High frequency shoot differentiation from the leaf callus occurred when the latter was transferred to MS medium supplemented with either K (4.65 – 23.25 μ M) or BA (4.4- 17.6 μ M) alone (Table 11). Media containing K as an exclusive PGR showed *de novo* shoot formation at all the concentrations. However, 18.6 μ M of K was most effective for inducing 90% of the cultures to form shoots and induced greatest number of shoots/culture (too numerous to count) with highest average length of the shoot (8.8 $\pm$ 0.05 cm). Various progressive stages of shoot differentiation from friable mass of leaf callus are depicted through Figures 8 A-D.

Excellent shoot differentiation from leaf callus also occurred on BA (8.8 μ M), where initially 5-7 shoots/ callus mass were formed after 15 days of culturing which further developed into shoots (Fig. 8 E & F). The shoots proliferated and elongated forming large clusters consisting of an average of 67.66 ± 1.45 shoots/flask in nearly 85% of the cultures (Fig. 8 G & H). Prolific shoot differentiation also occurred on MS medium augmented with IBA (4.92 μ M) and K (9.3 μ M) where a large number of shoots with an average of 85 ± 2.88 shoots (mean length 6.9 ± 0.05 cm) per culture were formed. Although good rates of shoot differentiation from leaf calli were observed but none of the media combination showed differentiation of roots from the callus.

Table 11: Morphogenetic response of leaf callus on MS supplemented with different growth regulators (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level).

S.No	Plant growth regulator	Concentration (μM)	Explant forming shoots (% age)	Shoots per culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	8.80	85	67.66 $\pm$ 1.45 ^c	9.4 $\pm$ 0.05 ^c
		17.6	72	57.00 $\pm$ 1.45 ^d	5.9 $\pm$ 0.05 ^d
		26.4	66	45.00 $\pm$ 2.88 ^f	5.2 $\pm$ 0.05 ^e
2.	K	4.65	52	35.00 $\pm$ 2.88 ^g	5.0 $\pm$ 0.05 ^{ef}
		9.30	66	45.00 $\pm$ 2.88 ^f	5.5 $\pm$ 0.05 ^e
		13.9	80	85.00 $\pm$ 2.88 ^a	7.1 $\pm$ 0.08 ^a
		18.6	90	TNTC	8.8 $\pm$ 0.05 ^b
		23.2	74	70.00 $\pm$ 2.88 ^b	4.7 $\pm$ 0.12 ^{ab}
3.	IBA + K	4.92 + 4.65	56	25.00 $\pm$ 2.88 ^h	4.7 $\pm$ 0.12 ^{fg}
		4.92 + 9.30	82	85.00 $\pm$ 2.88 ^a	6.9 $\pm$ 0.05 ^b
		4.92 + 13.9	77	75.00 $\pm$ 2.88 ^b	6.3 $\pm$ 0.05 ^c
		4.92 + 18.6	65	55.67 $\pm$ 2.88 ^e	5.0 $\pm$ 0.08 ^{ef}

TNTC: Too numerous to count, BA: 6-benzyl adenine, K: Kinetin, IBA: indole 3-butyric acid

Liquid Culture:

Callus raised on NK medium when transferred to liquid MS containing different concentrations of K (4.65 – 23.25 μM) or BA (4.4 -17.6 μM) exhibited excellent shoot organogenesis. Differentiation of shoots from calli occurred on all the concentrations though the optimum concentration was 18.6 μM of K when used in conjunction with 8.8 μM of BA. Initially emergence of few shoot regenerants was observed (Fig. 8 I) and the entire mass of callus differentiated into small cluster of shoots after 25 days of culturing (Fig. 8 J). Further elongation and multiplication of shoots resulting in the formation of an average of 67 ± 1.45 shoots/flask was observed in 80% of the cultures (Figures 8 K and L).

Fig. 4. *De novo* adventitious shoot formation from leaf explants of *Stevia rebaudiana* on MS medium supplemented with 2, 4-D (9.74 μ M) and BA (8.8 μ M).

- A. Leaf surface showing initiation of nodular meristemoids (arrowheads) after 7 days of inoculation.
- B. Emergence of adventitious shoots from nodular meristemoids after 15 days.
- C. Further elongation of regenerated shoots (arrowhead).
- D. Numerous healthy green leafy shoots formed after 4 weeks.

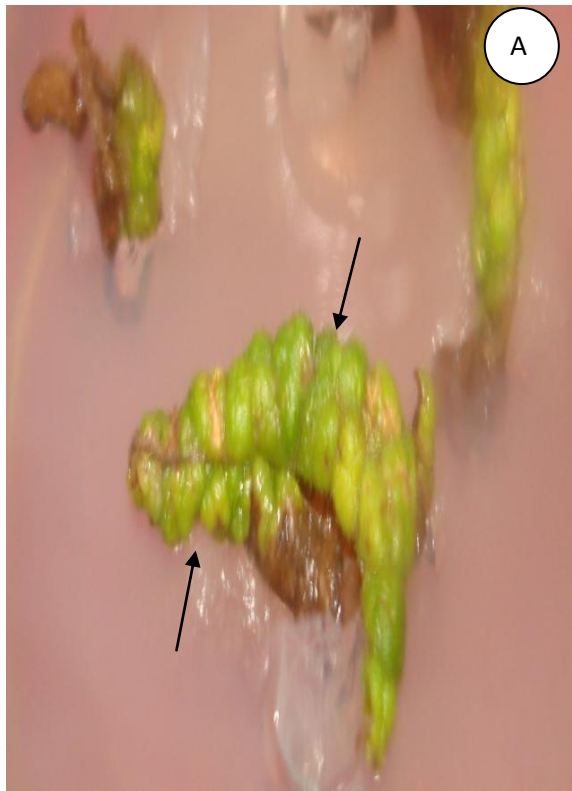


Fig. 4.

Fig. 5. *De novo* adventitious shoot formation from leaf explants of *Stevia rebaudiana* on MS medium augmented with NAA (7.35 μ M) and BA (4.4 μ M).

- A. Nodular meristemoids (arrowheads) induced from leaf explants after 10 days of culturing.
- B. Emergence of shoot initials (arrowheads) from nodular meristemoids.
- C. Clusters of adventitious shoots formed after 3 weeks.
- D. 8-week- old culture showing the formation of numerous shoots.

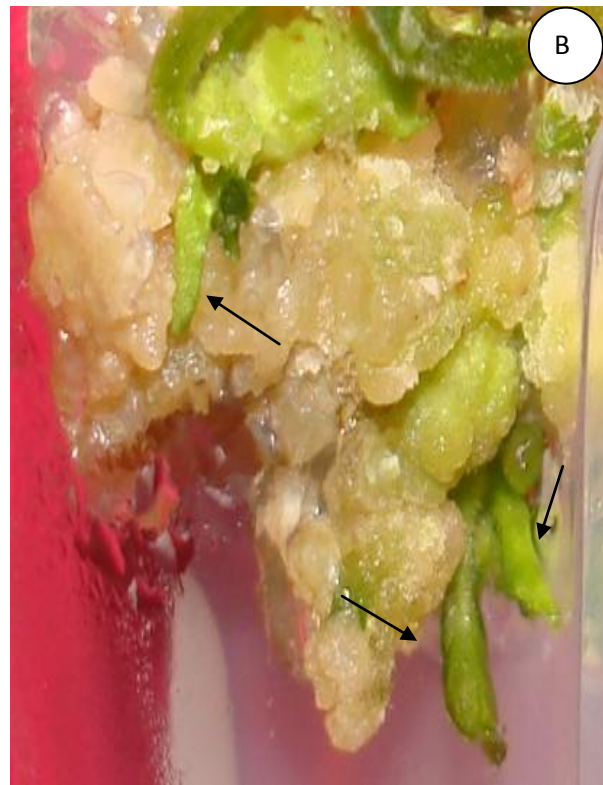
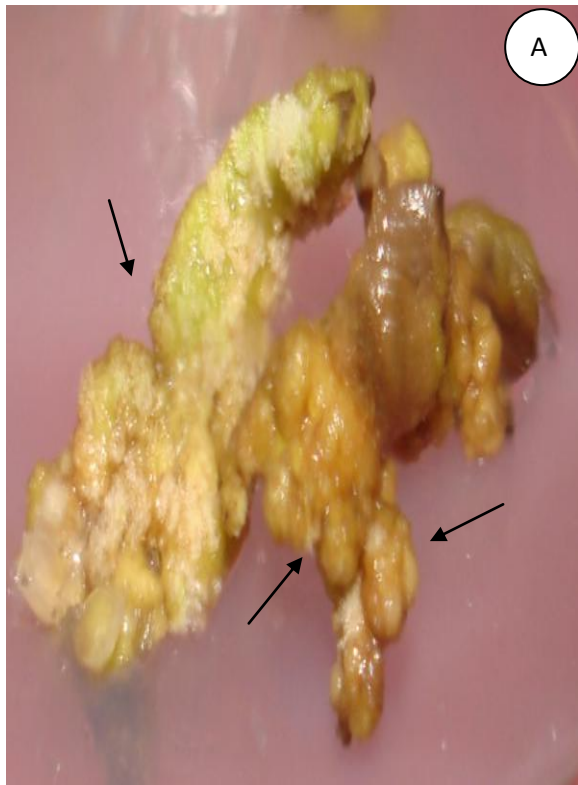


Fig. 5.

Fig. 6. Callus induction from leaf explants of *Stevia rebaudiana* on different sets of media combinations.

- A. Initiation of callus on 2, 4-D (19.48 μ M) and K (4.65 μ M) supplemented medium after 7 days of culturing.
- B. 4-week-old greenish white leaf callus showing prolific growth.
- C. Callus formation on NAA (29.4 μ M) and K (4.65 μ M) or NK medium after 8 days of culturing.
- D. Friable brown callus formed on NK medium after 4 weeks.

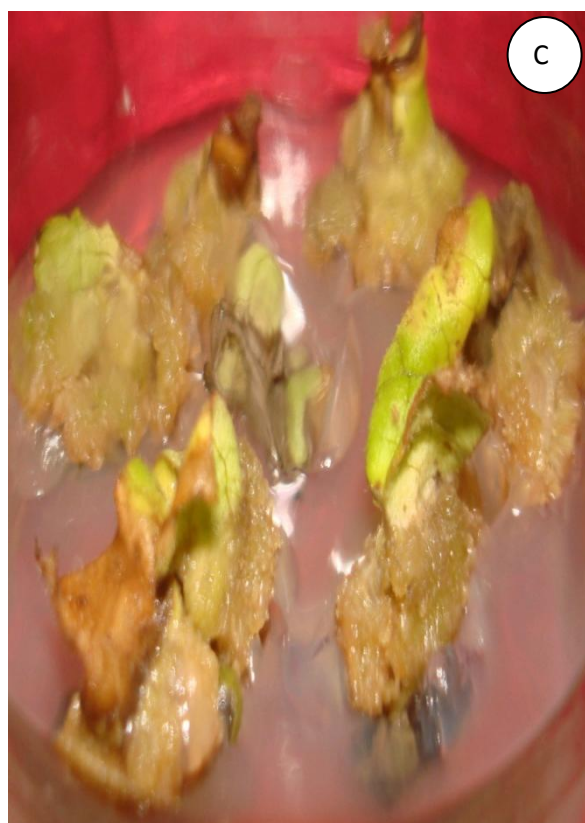


Fig. 6.

Fig.7. Study of leaf callus and histogenetic differentiation of tracheids.

- A. A group of elongated cells isolated from the leaf callus (100 X).
- B. A cell from leaf callus packed with starch grains (400 X).
- C. A Group of tracheids differentiated from the leaf callus showing reticulate thickenings on their walls (400 X).
- D & E. Callus depicting organized tissue and formation of vascular strands before organogenesis (100 X).

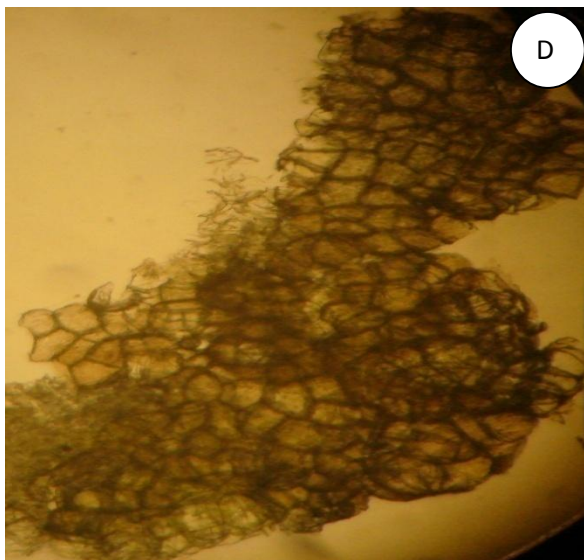
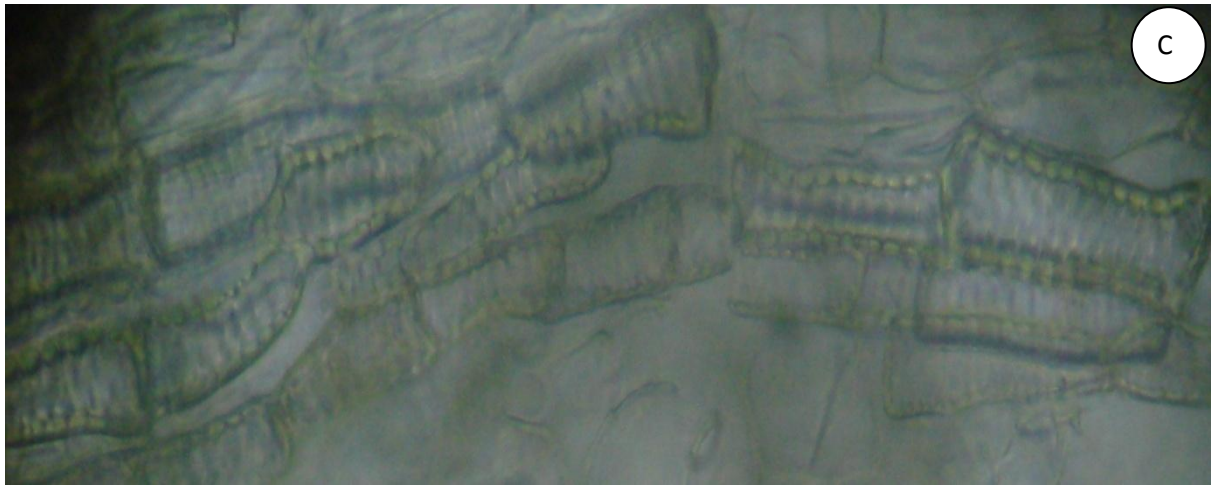
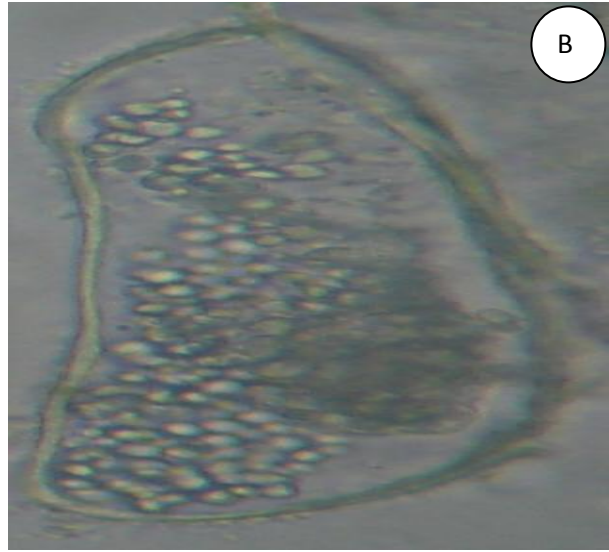
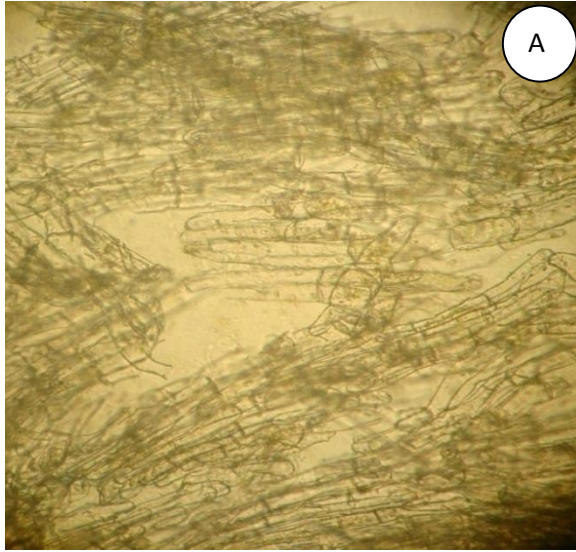


Fig. 7.

Fig. 8. Morphogenetic response of leaf derived callus of *Stevia rebaudiana*.

- A. Differentiation of numerous shoot initials (arrowheads) from leaf callus on K (18.6 μ M) supplemented medium after 10 days of culturing.
- B. Growth of shoot initials into shoots on the same medium after 18 days.
- C & D. Further proliferation and formation of numerous shoots from the callus after 4 & 7 weeks respectively. .
- E & F. Formation of many shoot initials from leaf callus and their development into shoots on BA (8.8 μ M) supplemented medium.
- G & H. Clusters of shoots formed from leaf callus after 5 & 7 weeks of culturing.
- I. Emergence of few shoot regenerants from leaf callus on liquid MS supplemented with K (18.6 μ M) + BA (8.8 μ M) after 12 days of culturing.
- J. 4 weeks old culture showing further shoot multiplication.
- K & L. Formation of several green leafy shoots on same medium after 6 and 8 weeks respectively.

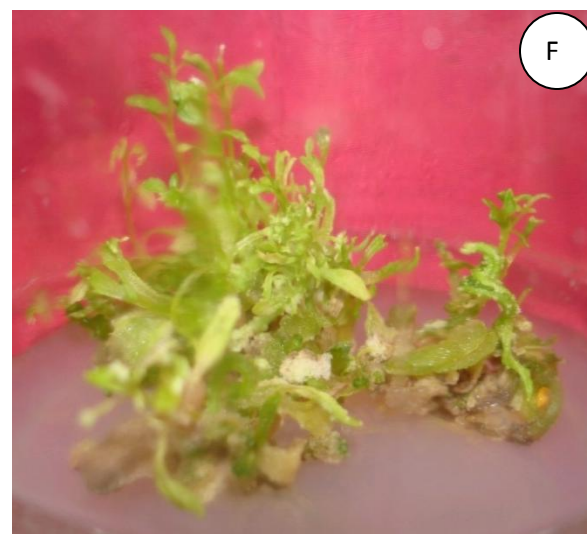
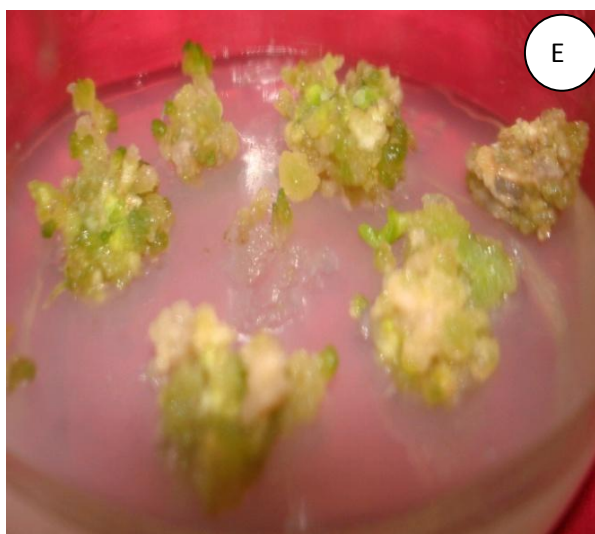
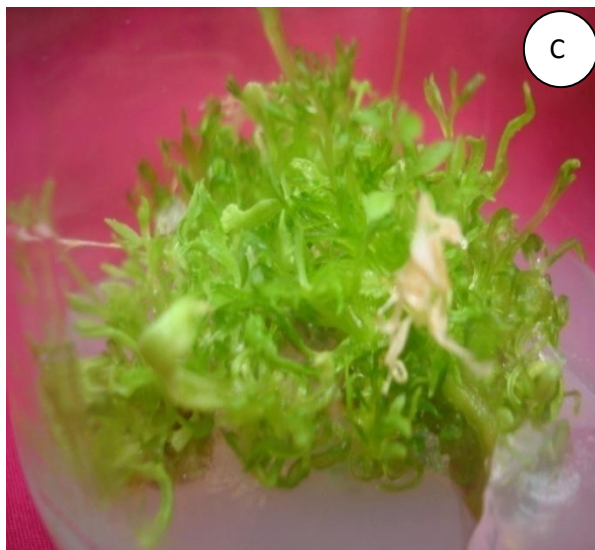
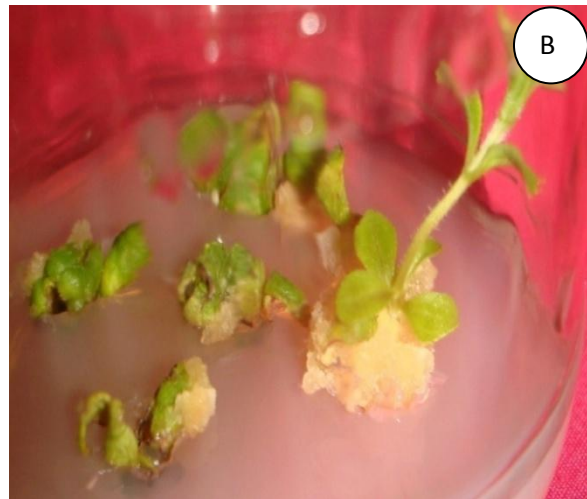
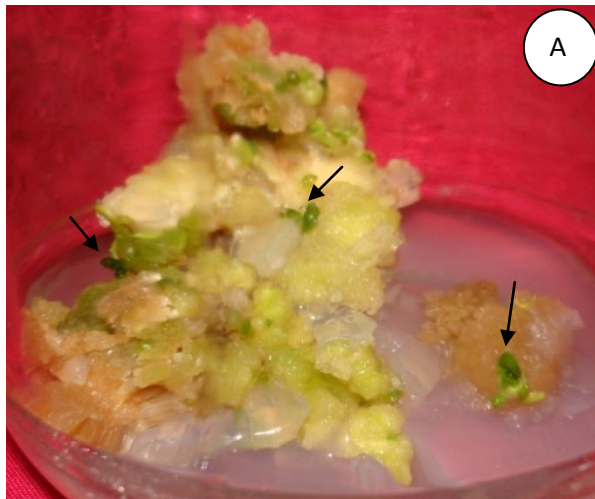


Fig. 8. A-F

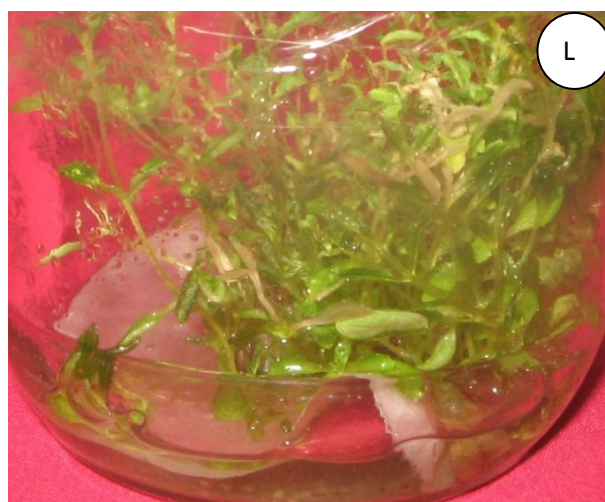
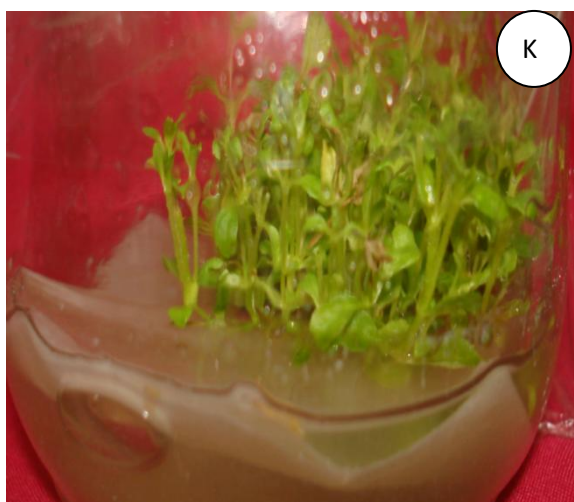
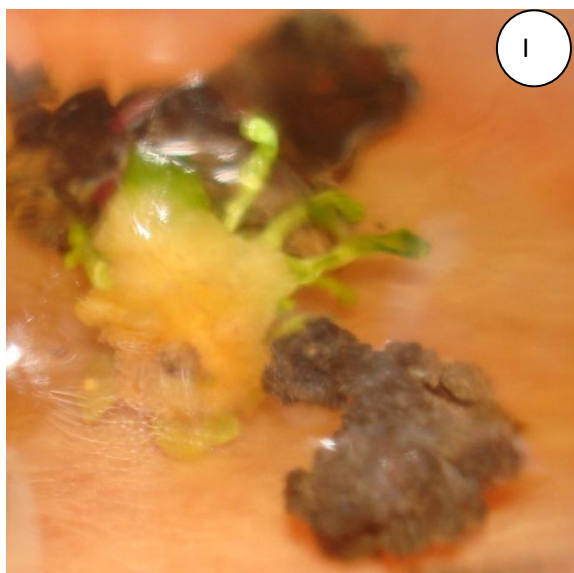


Fig. 8. G-L

4.1.2 Stem Culture

4.1.2.1 Induction and Growth of Callus

Callusing of stem segments (3-5 mm in length) was observed on MS medium containing either of the three auxins i.e. NAA (7.35 – 29.4 μ M), 2, 4 –D (4.87 – 29.2 μ M) and IBA (4.9-19.6 μ M). Good growth of callus occurred on MS supplemented with 29.4 μ M NAA but the growth rate was further enhanced with the addition of K, thereby, establishing a definite synergism between these two growth regulators. Callusing of the explants also observed on MS + 2, 4- D or IBA with or without K but the callus was slow growing and did not show sustained growth. The best callus growth occurred on MS with 29.4 μ M NAA and 4.65 μ M K or NK medium, where callusing initiated at the cut ends of stem segment after 8-10 days of culturing showing rapid growth (Fig. 9 A) and large friable callus masses were formed within 5 weeks (Fig. 9 B). The callus was light green and showed sustained growth on subsequent subculturing. The effect of different combinations of phytohormones on callus induction and its maintenance is given in Table 12.

Table 12: Effect of various growth regulators on callus induction and growth from stem explants of *Stevia rebaudiana*

S. No	Plant growth regulator	Concentration (μ M)	Explant forming callus (% age)	Nature of callus	Colour of callus	Growth of callus
1.	NAA	3.8	50	Friable	Light Green	Good growth
		7.3	60	Friable	Light Green	Good growth
		14.7	65	Friable	Light green	Good growth
		22.0	68	Friable	Light green	Good growth
		29.4	70	Friable	Light green	Good growth
2.	NAA + K	7.35 + 2.30	60	Soft	Light green	Good growth
		22.0+ 2.32	60	Soft	Light green	Good growth
		29.4 + 4.65	78	Soft	Light green	Very Good growth
3.	2,4-D + K	4.87 + 4.6	50	Friable	Light Green	Good growth
		9.7 + 13.9	65	Friable	Light Green	Slow growing and non-sustainable
		14.6 + 4.6	60	Friable	Light green	Slow growing and non-sustainable
		29.2 + 13.9	54	Friable	Light Green	Slow growing and non-sustainable
4.	IBA + K	4.9 + 9.30	60	Friable	Light green	Slow growing and non-sustainable
		9.8 + 18.6	42	Friable	Light green	Slow growing and non-sustainable

NAA: α -naphthalene acetic acid, K: kinetin, 2, 4-D: 2, 4-dichlorophenoxy acetic acid, IBA: indole3-butyric acid.

4.1.2.2 Organogenesis from callus

Friable callus formed on NK medium showed poor regenerative response with only 20 % of the cultures showing shoot differentiation after 7-8 weeks of culturing. Similarly, callus raised on combinations of 2, 4-D or IBA with or without K failed to show good rates of shoot differentiation. To obtain high frequency of shoot differentiation, callus was transferred onto media containing different concentration of BA (4.4- 17.6 μ M) or K (4.65- 18.6 μ M). Murashige and Skoog's medium containing 8.8 μ M BA showed best response of organogenetic differentiation from callus with emergence of 5-6 shoot initials/ callus mass after 8-9 days of culturing (Fig. 9 C). The shoot primordia developed into shoots (Fig. 9 D) which multiplied further forming cluster of shoots after 4 weeks (Fig. 9 E). The shoot formation accelerated on further subculturing resulting in the formation of innumerable shoots in about 90 % of the cultures (Fig. 9 F). Kinetin when used singly also induced shoot organogenesis with the highest frequency (67.6 ± 1.45 shoots/ flask) at 9.3 μ M concentration in about 86 % of the cultures. Effect of different growth regulators on shoot differentiation from stem callus are depicted is shown in Table 13. Differentiation of roots from the callus did not occur with any media supplement.

Table 13: Regenerative response of stem callus on different plant growth regulators (Mean followed by same letter are not significantly different by Duncan's multiple range test at 0.05% probability level)

S. No.	Plant growth regulator	Concentration (μ M)	Explant forming shoots (% age)	Shoots per culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.40	70	42.3 ± 1.45^c	4.9 ± 0.57^{bc}
		8.80	90	TNTC	5.8 ± 0.57^b
		13.2	80	57.6 ± 1.45^b	5.2 ± 0.57^b
		17.6	62	32.3 ± 1.45^f	3.6 ± 0.11^c
2.	K	4.65	72	41.0 ± 0.57^e	4.6 ± 0.57^{bc}
		9.30	86	67.6 ± 1.45^a	4.9 ± 0.57^{cd}
		13.9	84	59.6 ± 1.20^b	6.1 ± 0.08^a
		18.6	75	46.6 ± 0.88^d	5.7 ± 0.05^a
		23.25	68	32.6 ± 1.45^f	4.0 ± 0.57^{de}
3.	NAA + K	7.35 + 4.65	15	19.0 ± 0.55^h	3.4 ± 0.57^{de}
		29.4 + 4.65	20	23.0 ± 1.50^g	3.6 ± 0.57^{de}

TNTC: Too numerous too count, BA: 6-benzyl adenine, K: kinetin, NAA: α -naphthalene acetic acid

Fig. 9. Induction and growth of callus from stem explants and their organogenetic differentiation

- A. Callus induction at the cut ends of stem segment on NAA (29.4 μ M) and K (4.65 μ M) supplemented MS medium after 2 weeks of culturing.
- B. Formation of light green, friable callus after 5 weeks of culturing.
- C. Shoot buds differentiating from callus mass after 8-days of culturing (arrowheads).
- D. Further elongation of shoot primordia and their development into shoots after 15 days.
- E. Clusters of shoots formed after 4 weeks of culturing.
- F. Differentiation of innumerable shoots on subsequent subculturing.

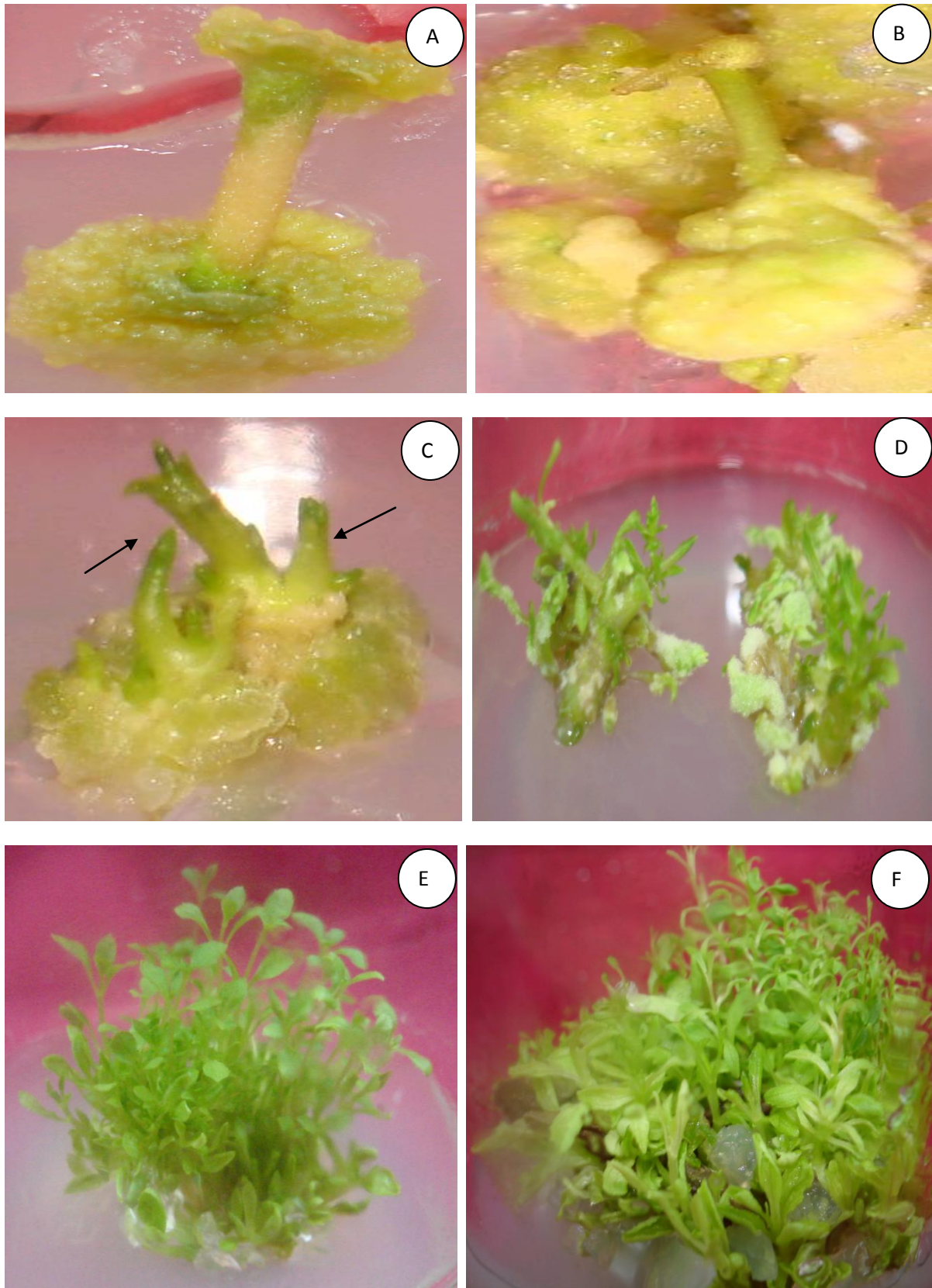


Fig. 9.

4.1.3 Nodal Explant Culture

4.1.3.1 Multiple shoot proliferation

Nodal explants (5mm long) holding one dormant lateral bud each were excised from healthy and field grown mature *Stevia* plant and were cultured on media supplemented with different plant growth regulators, viz BA, K, NAA, IBA and adenine sulphate either alone or in combination for multiple shoot induction.

Bud break from nodal segments was observed on MS medium containing BA (8.8 μ M) and adenine sulphate (2.7 μ M) after 8-10 days of culturing (Fig. 10 A). The multiple shoots were initiated (Fig. 10 B) which formed cluster of shoots within 35 days of culturing in 82% of the cultures. The shoots grew continually thereafter forming innumerable shoots (Fig. 10 C). MS medium supplemented with 8.8 μ M BA in conjunction with either NAA (7.35 μ M) or IBA (9.80 μ M) was also effective in inducing bud break and forming multiple shoots at the rate of 44 ± 1.15 and 36 ± 0.71 shoots per nodal explant respectively after 40 days of culture. Table 14 shows effects of different PGRs on multiple shoot proliferation from nodal explants.

Table 14: Multiple shoot proliferation from nodal explants of *Stevia rebaudiana* (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level)

S.No	Plant growth regulator	Concentration (μ M)	Explant forming shoots (% age)	Shoots per culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA + Adenine Sulphate	8.8 + 2.7	82	TNTC	8.3 $\pm$ 0.14 ^a
		17.6 + 2.7	76	42 $\pm$ 1.15 ^b	7.7 $\pm$ 0.08 ^a
		26.4 + 2.7	62	30 $\pm$ 1.15 ^f	7.3 $\pm$ 0.05 ^a
2.	NAA + BA	7.35 + 8.8	77	44 $\pm$ 1.15 ^a	7.7 $\pm$ 0.05 ^a
		14.7 + 8.8	74	35 $\pm$ 1.45 ^d	7.3 $\pm$ 0.05 ^a
		17.6 + 8.8	68	26 $\pm$ 1.15 ^f	6.3 $\pm$ 0.08 ^a
3.	IBA + BA	4.65 + 8.8	62	27 $\pm$ 0.88 ^f	7.7 $\pm$ 0.11 ^a
		9.80 + 8.8	74	38 $\pm$ 1.15 ^c	7.2 $\pm$ 0.05 ^a
		29.5 + 8.8	68	32 $\pm$ 1.15 ^e	7.0 $\pm$ 0.05 ^a

TNTC: Too numerous to count, BA: 6-benzyl adenine, NAA: α -naphthalene acetic acid, IBA: indole 3- butyric acid

4.1.4 Shoot apex culture

4.1.4.1 Multiple shoot proliferation

Young healthy shoot apices were collected from field grown mature plants of *Stevia rebaudiana* and cultured on MS medium supplemented with different concentrations of BA (4.4 – 17.6 μM) or K (4.65 -18.6 μM) either alone or in combination with NAA (7.35 – 14.7 μM) or adenine sulphate (2.7-10.84 μM). Best shoot proliferation from the shoot apex occurred on MS supplemented with BA (8.8 μM) and NAA (7.35 μM) forming 6-8 shoots in 70% of cultures after 3 weeks (Fig. 10 D). The shoots elongated and multiplied thereafter with an uncountable increase in number (Fig. 10 E and F).

Synergistic role of BA (8.8 μM) with adenine sulphate (2.71 μM) was also demonstrated for shoot proliferation from shoot apex forming multiple shoots in 66 % of the cultures. A change in the concentration level of BA above and below the optimum value (8.8 μM) resulted in decline in the number of shoots formed.

4.1.4.2 Induction and growth of callus

Callus induction from shoot apices was observed when cultured on MS supplemented with higher concentration of NAA (29.4 μM) in conjunction with K (4.65 μM). The callusing started after 10- 12 days of culturing (Fig. 11 A), the callus grew vigorously (Fig. 11 B) and within 4 weeks, the entire explant turned into a mass of greenish white and highly friable callus (Fig. 11 C). The growth rate enhanced during subsequent subculturing and the callus has now been maintained for more than 13 months.

Good callus growth also occurred on MS supplemented with 2, 4-D (14.6 μM) in combination with K (4.65 μM). The callus formed was light green, highly friable and proliferative on subsequent subculturing. IBA was found to be a poor substitute for 2, 4- D with or without K resulting in the formation of slow growing callus incapable of sustained growth.

4.1.4.3 Caulogenesis from callus

Differentiation of shoots from the calli was effected when the latter was transferred to MS medium containing different concentrations of BA (4.4 – 22 μ M) either alone or in conjunction with K (4.65- 18.6 μ M). The optimum response (82%) was observed on 17.6 μ M BA where an average of 78.3 ± 0.88 shoots per culture were formed (Table 15). Addition of K at a concentration of 4.65 μ M with 17.6 μ M BA further enhanced the shoot differentiating frequency of the callus forming shoot primordia after 10 days of culturing (Fig. 11 D). In the beginning, fewer shoots differentiated (Fig. 11 E) but the number increased with passage of time forming 90 ± 1.15 shoots per culture (Fig. 11 F) after 5 weeks. Rhizogenesis did not occur on any media combination tried.

Table 15: Morphogenetic response of callus on different growth regulators (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level)

S.No	Plant growth regulator	Concentration (μ M)	Explant forming shoots (% age)	Shoots per culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.4	60	46.0 ± 1.15^g	3.9 ± 0.05^a
		8.8	66	49.6 ± 0.88^f	3.9 ± 0.17^a
		17.6	80	78.3 ± 0.88^b	4.8 ± 0.17^a
		22.0	74	57.0 ± 1.73^e	4.4 ± 0.05^a
2.	BA + K	4.4 + 4.65	63	47.0 ± 1.52^g	3.7 ± 0.12^a
		13.2 + 4.65	74	58.3 ± 0.88^d	4.4 ± 0.17^a
		17.6 + 4.65	88	90.0 ± 1.15^a	4.9 ± 0.17^a
3.	K	4.65	62	49.6 ± 1.20^f	3.5 ± 0.17^a
		9.3	68	57.0 ± 1.70^e	4.4 ± 0.14^a
		13.9	70	64.0 ± 1.15^c	4.9 ± 0.05^a
		23.25	68	59.6 ± 0.88^d	4.3 ± 0.05^a

BA: 6-benzyl adenine, K: kinetin

Fig. 10. Multiple shoot proliferation from nodal segments and shoot tips of *Stevia* on different medium compositions

- A. Axillary bud break from nodal segment on BA (8.8 μM) and adenine sulphate (2.7 μM) 8-10 days after culturing.
- B. Initiation of multiple shoots from nodal segment after 20 days of culturing.
- C. 8 weeks old culture showing profuse development of adventitious shoots.
- D. Initial development of axillary shoots from the shoot tip on NAA (7.35 μM) and BA (8.8 μM) after 3 weeks.
- E & F. Numerous shoots formed from shoot apex after 7 and 9 weeks respectively.



Fig. 10.

Fig. 11. Induction and growth of callus from the shoot apices and their organogenetic differentiation.

- A & B. Induction and formation of callus from the shoot apex on NK medium after 12 and 21 days of culturing.
- C. Production of greenish white friable callus on NK medium after 4 weeks.
- D. Emergence of shoot initials (arrowhead) from the callus on BA (17.6 μ M) supplemented medium.
- E. Development of shoot initials into green healthy shoots after 3 weeks.
- F. 5 weeks old callus culture showing differentiation of several shoots on same medium.

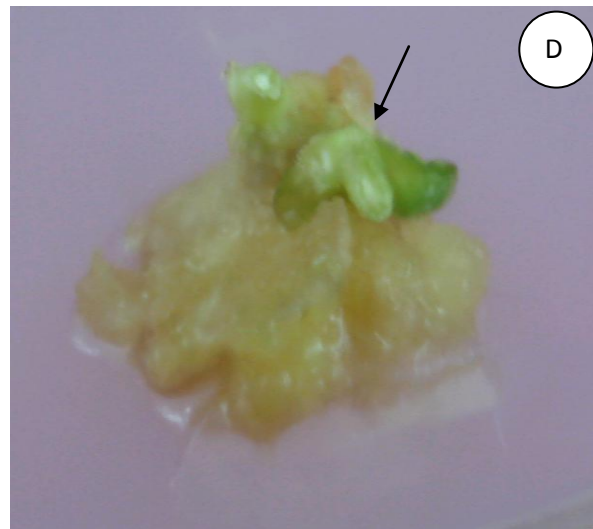
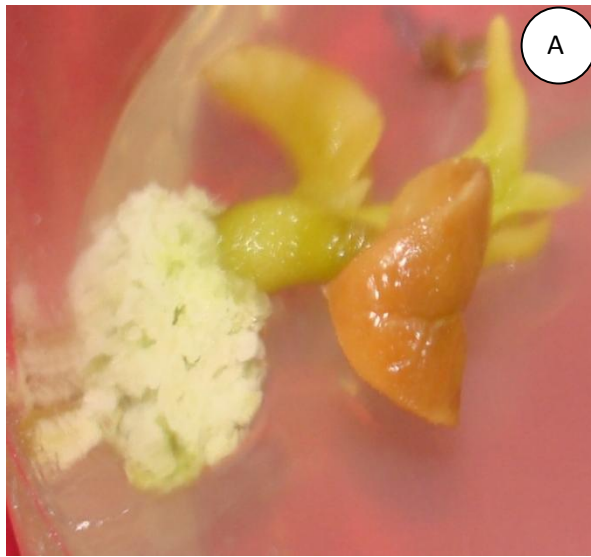


Fig. 11.

4.1.5 Artificial Seed production from shoot tips

Shoot tips obtained from *in vitro* regenerated shoots were used for synthetic seed production since somatic embryogenesis could not be effected in the present study. For the encapsulation of shoot tips, sodium alginate (1%, 2%, 3% and 4%) was used as the gelling agent and 2% of CaCl_2 was used as complexing agent. The best gel complexation was achieved using 2% sodium alginate prepared either in double distilled water or in full strength liquid MS medium and 2% of CaCl_2 which resulted in the formation of firm, clear and isodiametric beads (Fig.12 A). The encapsulated beads (5 mm in diameter) thus formed were collected on a sterile Petriplate, sealed with parafilm and stored in refrigerator at 4° C. For retrieving shoots, the encapsulated shoot tips were cultured on basal MS medium supplemented with 2% sucrose. Shoots emerged from the encapsulated beads breaking the capsule wall after 6-7 days of culturing (Figures 12 B and C). Sodium alginate prepared in full strength MS nutrient medium demonstrated significant superiority over the double distilled water with respect to shoot conversion and shoot development. Clusters of shoots emerged from the shoot tips encapsulated with alginate prepared in MS nutrient ingredients after 15-20 days (Fig. 12 D) which proliferated further forming a number of shoots after 4 weeks (Fig. 12 E). Encapsulated shoot tips of *Stevia* could be stored at 4 ° C for 120 days but there was a gradual reduction in the conversion percentage and shoot regeneration which decreased to 15% after 120 days. The percentage conversion of these stored synthetic seeds monitored up to 120 days is depicted in Figure 13.

4.1.6 Rooting of Microshoots

Regenerated shoots formed from different vegetative parts were carefully rescued from the culture vessels and were inoculated upright on rooting medium which comprised of half or full strength MS media or MS medium supplemented with various auxins namely IBA, IAA, 2, 4-D and NAA. Among the various growth regulators tested, root initiation occurred on IBA (9.84 μM) supplemented medium after 10 days of culturing in nearly 90% of the cultures (Fig. 14 A). Further elongation of roots occurred but the roots formed were very thin and weak. Best root initiation occurred on half strength MS medium where 4-6 healthy roots emerged in 90% of the cultures (Fig. 14 B). The roots grew further, were light green in colour and devoid of root hairs. Development of roots from microshoots also occurred on basal MS medium, where 1-2 very thin roots appeared after 15 days of culturing in 78% of the cultures.

Induction of roots was also observed on half strength liquid basal MS medium where thick-branched roots having profuse root hairs were formed after 12 days in about 80% of the cultures (Fig. 14 C) which elongated further (Fig. 14 D).

4.1.7 Acclimatization of plantlets and their transfer to the field conditions

For acclimatization, plantlets were carefully rescued from the culture bottles, washed under running tap water to remove traces of agar sticking to it. They were initially transferred to the culture bottles containing moist cotton covered with perforated plastic covers and were kept for a period of 20 days under growth room conditions (Fig. 15 A). The plantlets were further shifted to plastic cups containing potting mixture of soil: vermicompost (1:1) and were kept inside the growth room for another 2 weeks (Fig. 15 B). The plants were monitored and watered and hardened plantlets were then shifted to the green house for 2 weeks before transferring them to full sunlight outdoor (Fig. 15 C). By this time, plants have become sturdy, developed an efficient root system and formed new leaves (Fig. 15 D). The acclimatized plants showed 80% survival rate and thrived well in the field conditions (Fig. 15 E).

Fig. 12. Encapsulation of shoot tips in sodium alginate (2%) and calcium chloride (2%).

- A. Encapsulated shoot tips.
- B & C. Emergence of shoots from encapsulated shoot tip after 6-7 days of culturing and
 their further growth on basal MS medium supplemented with 2% sucrose.
- D. Cluster of shoots emerging from encapsulated shoot tips in alginate prepared in
 MS nutrient medium.
- E. Elongation and further growth of shoots after 4 weeks of culturing.

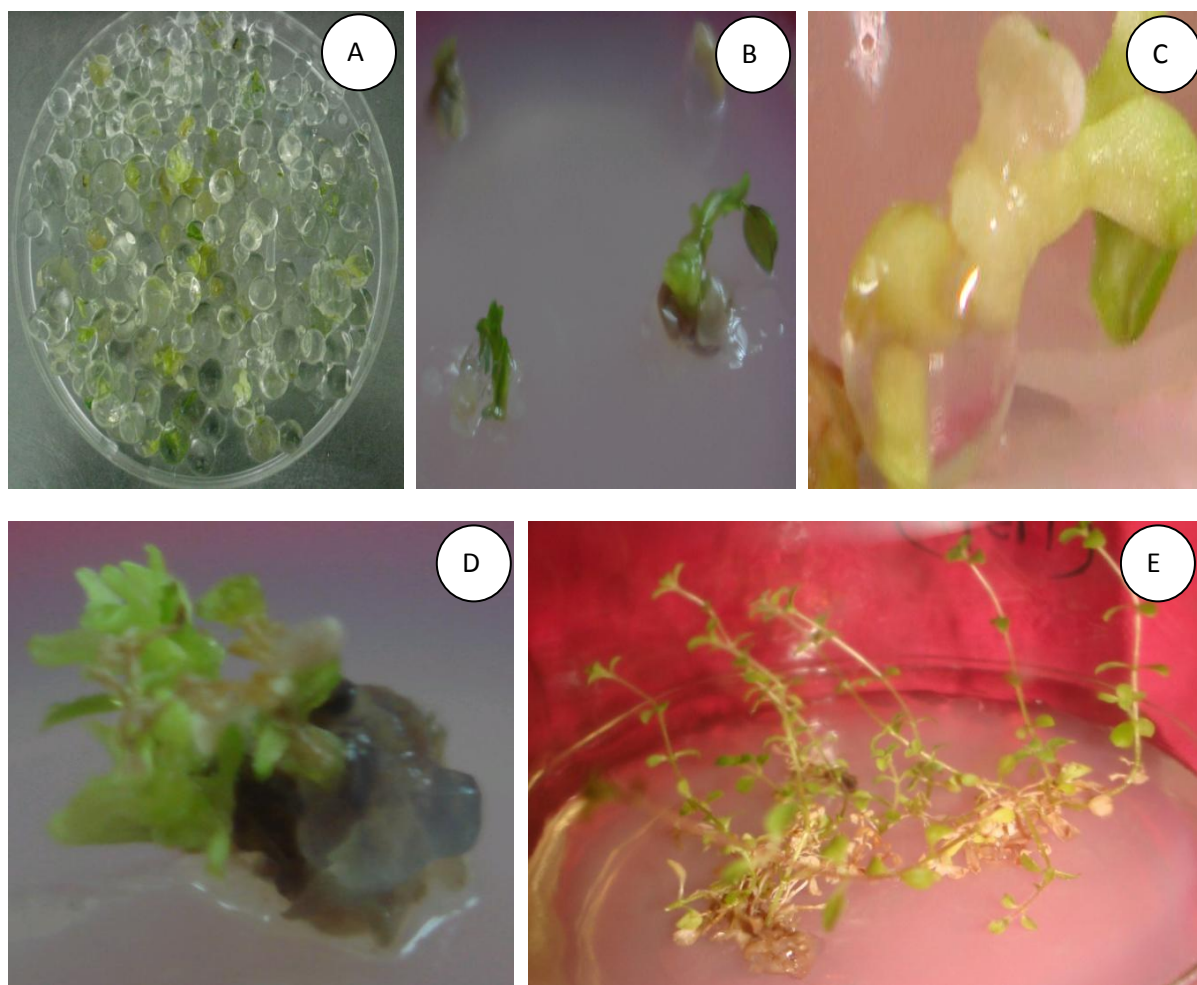


Fig. 12.

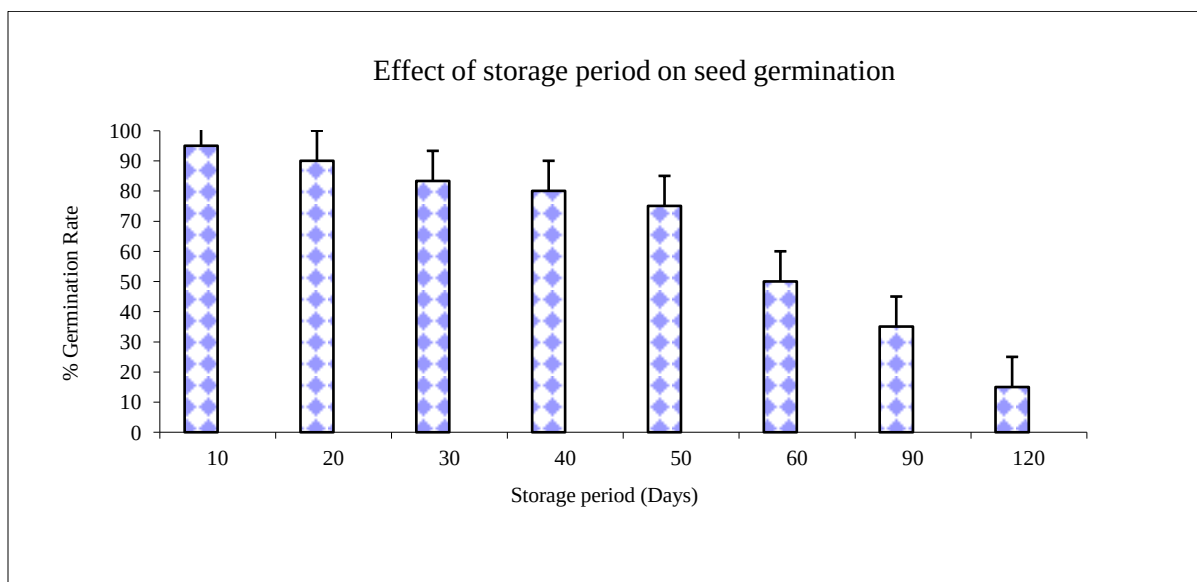


Fig. 13. Effect of storage period on seed viability

Fig. 14. Rooting of microshoots on MS medium supplemented and devoid of auxins.

- A. Formation of thin long roots on IBA (9.84 μ M) supplemented medium after 10 days of culturing.
- B. Formation of 4-6 light green colored healthy roots on half strength MS medium.
- C. Formation of thick-branched roots on half strength liquid MS medium after 12 days of the culturing.
- D. Further growth and elongation of shoots and roots on the liquid medium.

Fig. 15. Acclimatization of plantlets.

- A. Transfer of plantlet to culture bottles containing moist cotton under growth room conditions.
- B. Plantlet in plastic cups containing potting mixture of soil: vermicompost (1:1).
- C & D. Hardened plantlets under green house conditions.
- E Well acclimatized plants in open field conditions.



Fig.14.



Fig.15. A, B



Fig. 15. C, D & E

4.2 Micropropagation of *Tylophora indica*:

For establishing sterile cultures of *Tylophora indica*, different explants like leaf, stem, root and nodal explants were excised from 3 yr old mature healthy field grown plant. After initial disinfection with bavistin (0.1% w/v) for 10-12 minutes, explants were subjected to surface sterilization with 0.1% (w/v) mercuric chloride for 2 minutes for leaves and 4 minutes for root, internodal and nodal explants in laminar airflow bench. Explants were trimmed after sterilization to remove unwanted or dead portions. These were then planted on MS medium supplemented with various auxins and cytokinins either alone or in various combinations.

4.2.1 Leaf Culture

4.2.1.1 Direct *de novo* adventitious shoot regeneration

Leaf segments (4-5 mm) in size were cultured on variously supplemented MS medium for *de novo* adventitious shoot formation directly from the explant. MS medium supplemented with 6-benzyladenine (8.8 μ M) either alone or in combination with adenine sulphate (1.35 μ M) produced greatest number of shoots/explant. Nodular meristemoids differentiated from the cut ends and from the abaxial and adaxial surface of leaf lamina after 8 days (Fig 16 A) and within 3 weeks the entire surface was covered with these nodular meristemoids that eventually grew into green leafy shoots. Figure 16 B shows differentiation of a few shoots formed from nodular meristemoids after 4 weeks, which elongated, developed many leaves and grew into green leafy shoots after 5 weeks of culturing. Initially fewer shoots were formed from these meristemoids (Fig. 16 C) but in due course, these multiplied further forming many shoots (Fig. 16 D). The highest regeneration frequency (85%) and maximum number of shoots (55 ± 2.88 per culture) was achieved on BA (8.8 μ M) and adenine sulphate (1.35 μ M). Repeated subculturing accelerated the formation of shoots without any decline in their proliferation (Fig. 16 E) upto 3 subculture passages. Effect of different concentrations of BA and adenine sulphate on adventitious shoot formation from leaf explant is depicted in Table 16.

Table 16: Effect of BA and adenine sulphate on *de novo* adventitious shoot formation from leaf explant in *T.indica* (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level).

S.No	Plant growth regulator	Concentration (μM)	Explant forming shoots (% age)	Shoots per explant (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.40	62	27.66 ± 1.45^c	3.2 ± 0.05^a
		8.80	70	42.60 ± 1.45^b	3.7 ± 0.05^a
		17.6	58	21.00 ± 0.57^e	2.8 ± 0.05^a
2.	BA + Adenine Sulphate	$4.40 + 1.35$	59	25.00 ± 1.73^d	3.9 ± 0.05^a
		$8.80 + 1.35$	85	55.00 ± 2.88^a	4.4 ± 0.05^a
		$17.6 + 1.35$	70	23.33 ± 0.88^e	3.3 ± 0.05^a

BA: 6-benzyl adenine

Histological studies: Histological investigations revealed the formation of globular meristemoids from the leaf explants after 8-10 days (Fig. 17 A) which developed into shoot bud initials (Fig. 17 B). Figure 17 C shows the formation of shoot buds having dome shaped apical meristems and young leaf primordia. Figure 17 D depicts a developed shoot bud showing shoot apical meristem with subtending leaf primordia.

Direct Root induction: Direct rooting from the leaf explant was observed on MS medium supplemented with different concentrations of NAA with K or BA. A few roots regenerated on NAA (9.0- 29.4 μM) + K (4.65 μM) medium after 10 days in 60 % of the cultures. Rooting was more or less simultaneous with callus induction. Profuse rooting occurred at the cut ends as well as the entire surface of the explant after 12-15 days of planting on NAA (19.4 μM) and BA (4.4 μM) supplemented medium (Fig. 18 A). Initially fewer roots regenerated and within 4 weeks, the entire explant was covered with thick growth of roots. The roots were white, branched and bore profuse root hairs.

4.2.1.2 Callus Induction and Growth

Among the auxins (NAA, 2, 4-D, IBA and IAA) tested for establishing callus cultures, NAA and 2, 4-D in combination with K proved most effective. Good callus growth from the explant occurred on NAA (9.0- 29.4 μM) supplemented medium, however, the addition of K enhanced the callus production many folds. Optimal callusing occurred on NAA (29.4 μM) and K (4.65

μM) hereby designated as NK medium where 98 % of the explants callused either at the cut ends or along the entire surface (Fig. 18 B). The callus grew further and within 30 days, the entire explant turned into a mass of green and compact callus (Fig. 18 C). Callus when subcultured on the same medium proliferated further and showed sustained growth.

Callus induction from the leaf explants also occurred on 2, 4-D (19.48 μM) and K (4.65 μM) supplemented medium but the growth of callus was slow and took nearly 6 weeks for the explant to turn into a mass of callus. The callus was deep green in colour and compact in texture. Effect of different concentrations of NAA or 2, 4- D with or without K on induction and growth of callus from leaf explant is shown in Table 17.

Table 17: Effect of different concentrations of NAA, 2, 4-D and K on callus induction from leaf explants of *T.indica*

S. No	Plant growth regulator	Concentration (μM)	Explant forming callus (Mean ± SE)	Nature of Callus	Colour of callus
1.	NAA	7.35	75	Solid	Green
		14.7	80	Solid	Green
		22.0	75	Solid	Green
		29.4	72	Solid	Green
2.	NAA + K	7.35 + 4.65	98	Solid	Green
		14.7 + 4.65	70	Solid	Green
		22.0 + 4.65	78	Solid	Green
		29.4 + 4.65	98	Solid	Green
3.	2,4-D + K	4.87 + 4.65	62	Solid	Deep green
		9.74 + 4.65	70	Non-Friable	Deep green
		19.12 + 4.65	88	Non-Friable	Deep green

NAA: α-naphthalene acetic acid, K; kinetin, 2, 4-D: 2,4-dichloroacetic acid

Callus was also induced from the abaxial, adaxial and cut surfaces of leaf lamina on MS medium supplemented with different concentrations of NAA (9.0 – 29.4 μM) with K (4.65-18.6 μM) under dark conditions. The callus formed was light green in color, friable and capable of sustained growth.

Study of callus

The leaf callus obtained on NK medium was extremely hard and compact and could not be easily broken into single cells or groups when placed in water. The callus was teased forcibly with the help of thin strong needles. Microscopic examination of callus indicated its heterogeneous nature showing cells of various sizes and shapes. The cells were round, oval and elongated (Fig. 19 A) each having numerous starch granules scattered throughout the cytoplasm (Fig. 19 B). Elongated filaments of callus cells were also observed which were formed by mitotic divisions and failure of cells to separate (Fig. 19 C).

Three week old leaf callus revealed xylem differentiation in the form of tracheids, which occurred either singly or in groups and possessed reticulate thickenings on their walls (Fig. 19 D). Callus also revealed the presence of organized tissue formed prior to organogenesis (Fig. 19 E).

4.2.1.3 Organogenetic differentiation from the callus

Rhizogenesis

Root differentiation occurred in 95% of callus cultures on NK medium after 25 days of culturing. Initially a few roots were formed (Fig. 20 A) but with further proliferation of the callus more and more roots were organized (Fig. 20 B). The roots were thick, white and bore profuse root hairs. Likewise, root differentiation occurred when leaf callus raised on NAA and K medium was transferred to different concentrations of IBA (4.92 – 19.68 μ M).

Caulogenesis

Solid culture

Shoot differentiation occurred in 20-30 % of callus cultures on NK medium after 7-8 weeks where 4-5 shoots differentiated from the callus. To obtain high frequency shoot differentiation, calli were transferred to different concentrations of BA or K used alone and in combination with each other. Best results were obtained on 8.8- 9.84 μ M BA where 86 % of the cultures resulted in excellent shoot induction (40.0 ± 1.45 per culture) upto 3-4 subcultures. Initially 6-7 shoot initials differentiated from the callus after 2 weeks of culturing (Fig. 20 C) which grew further

and developed many leaves. Figures 20 D show the formation of numerous healthy green leafy shoots after 4 weeks. There was a steady increase in the number of shoots per culture on subsequent subculturing. Equally good results were obtained on 9.3 μM K supplemented medium forming an average of 36.0 ± 1.45 shoots/ culture. Effect of different concentrations of cytokinins on caulogenesis from leaf callus is depicted in Table 18.

Table 18: Effect of BA and K on shoot regeneration from leaf callus of *T.indica* (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level).

S. No.	Plant growth regulator	Concentration (μM)	Explant forming shoots (%)	Shoots per culture (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.40	76	32.0 ± 1.15^a	9.4 ± 0.24^a
		8.80	87	42.0 ± 1.45^b	9.0 ± 0.05^b
		9.84	86	40.0 ± 1.45^b	8.9 ± 0.05^b
		13.2	69	22.0 ± 1.15^c	6.4 ± 0.05^c
2.	K	4.65	72	25.0 ± 1.45	7.4 ± 0.05^c
		9.30	84	36.0 ± 1.45	7.8 ± 0.04^d
		13.95	68	24.0 ± 1.15^c	6.4 ± 0.05^c
		18.60	65	28.0 ± 1.15^c	6.8 ± 0.05^c

BA: 6-benzyl adenine, K: kinetin

Liquid culture

Callus raised on solid NK medium when transferred to liquid MS medium containing different concentrations of BA (4.4 -17.6 μM) or K (4.65 – 23.25 μM) exhibited prolific shoot organogenesis. Differentiation of shoots from callus occurred on 8.8 μM BA supplemented medium after 15 days of culturing which proliferated further (Fig. 20 E) leading to an average number of 48 ± 1.15 shoots/culture after 5 weeks of culturing in 80 % of the cultures (Fig. 20 F).

Fig. 16. *De novo* adventitious shoot formation from leaf explants of *T.indica* on BAP (8.8 μ M) + adenine sulphate (1.35 μ M) supplemented medium.

- A. Formation of nodular meristemoids (arrowheads) from leaf surface after 8 days of culturing.
- B. Adventitious shoot formation (arrowheads) from nodular meristemoids after 4 weeks.
- C. 6 weeks old culture showing elongation of regenerated shoots (arrowhead).
- D & E. Prolific shoot differentiation from nodular meristemoids on subsequent subculturing.



Fig. 16.

Fig. 17. Histological section of leaf depicting various stages of shoot regeneration in *Tylophora indica*.

- A. Formation of globular meristemoid from leaf explant.
- B. Multiple shoot bud primordia developing from these meristemoids.
- C. Shoot bud primordia with shoot apical meristems and young leaf primordia.
- D. Fully developed shoot apex with two-leaf primordia.

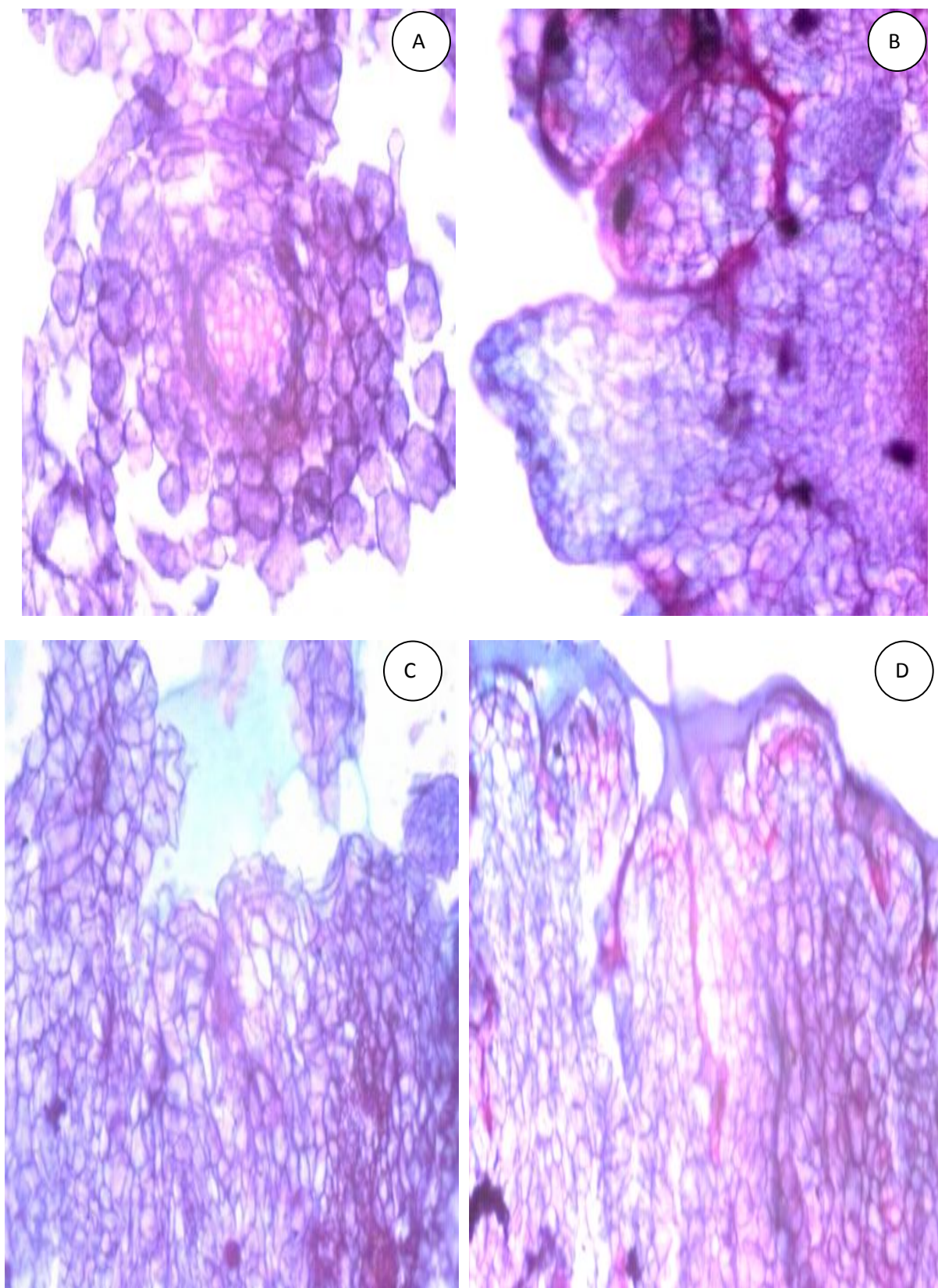


Fig.17.

Fig. 18. Direct root induction and callus growth from leaf explants on different media combinations.

- A. Direct root formation from leaf explants on NAA (19.4 μ M) and BA (4.4 μ M) after 15 days of culturing (arrowheads).
- B. Actively growing callus on NAA (29.4 μ M) + K (4.65).
- C. 4 weeks old leaf callus showing sustained growth.

Fig. 19. Study of leaf callus of *Tylophora indica*

- A. Heterogeneous group of callus cells with scattered starch granules (100 X).
- B. Magnified callus cell with numerous starch grains scattered throughout the cytoplasm (400 X).
- C. Elongated filaments of callus cells (400 X).
- D. Group of tracheids isolated from leaf callus showing reticulate thickenings (400 X).
- E. Callus showing organized tissue prior to organogenesis (400 X).

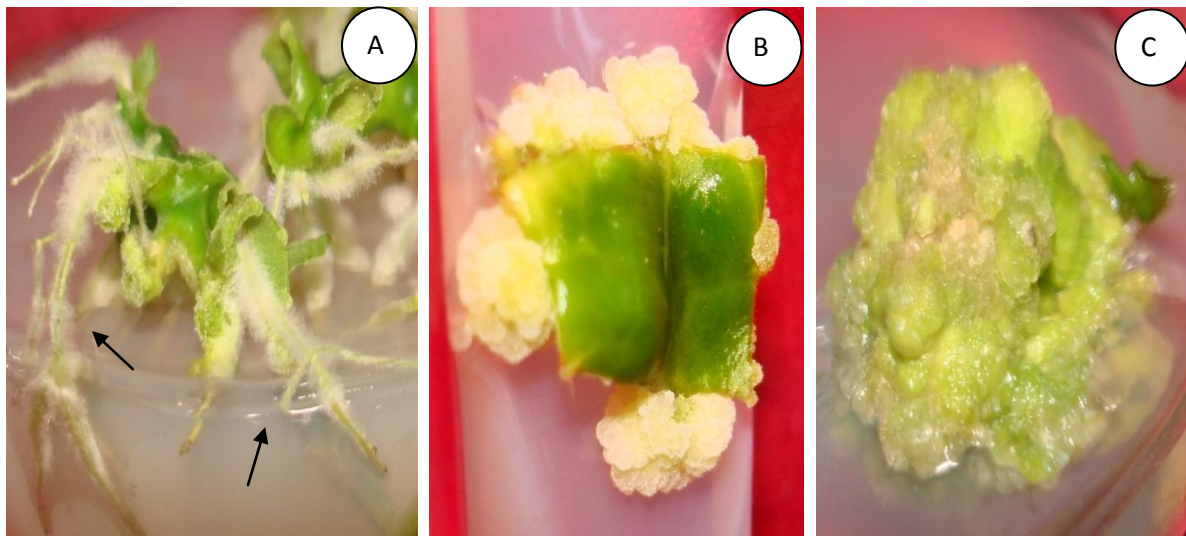


Fig.18.

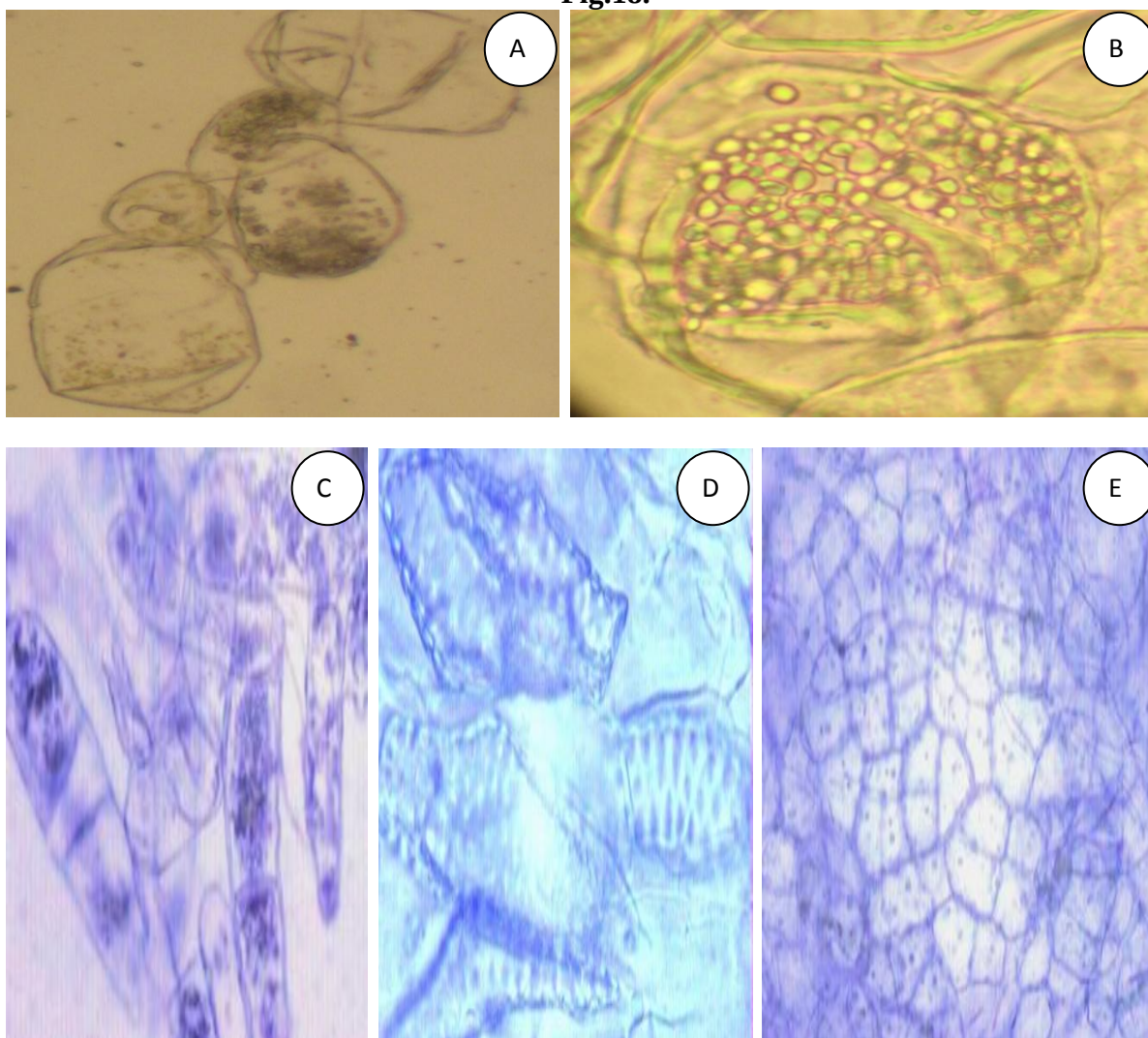


Fig.19.

Fig. 20. Organogenesis from leaf callus on different media combinations.

- A. 4 weeks old leaf callus showing root differentiation on NAA (29.4 μ M) + K (4.65 μ M) medium (arrowheads).
- B. Differentiation of roots with profuse root hairs after 6 weeks of culturing (arrowheads).
- C. Differentiation of a few shoots from leaf callus on 9.84 μ M BA after 2 weeks.
- D. Formation of numerous healthy green leafy shoots after 4 weeks.
- E & F. Formation of numerous green leafy shoots on liquid MS + BA (8.8 μ M) medium after 3 and 5 weeks respectively.

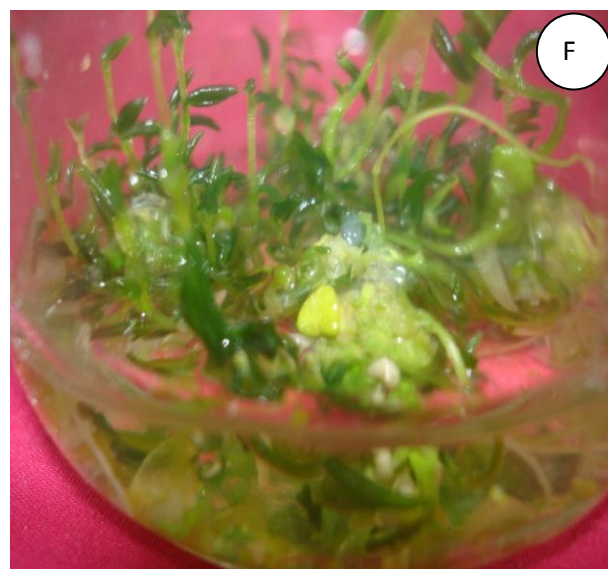
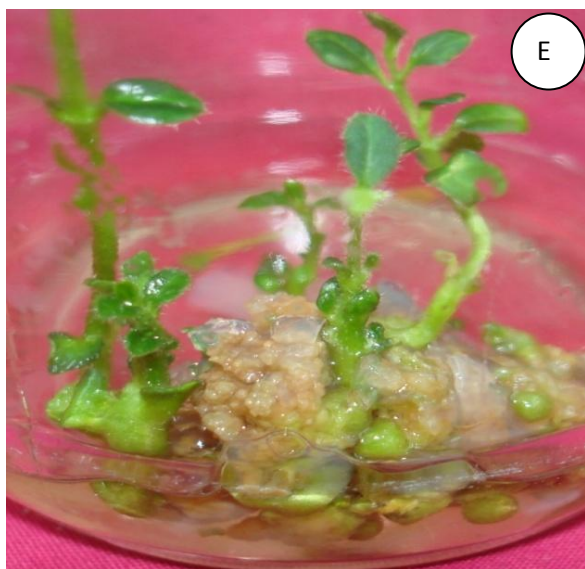
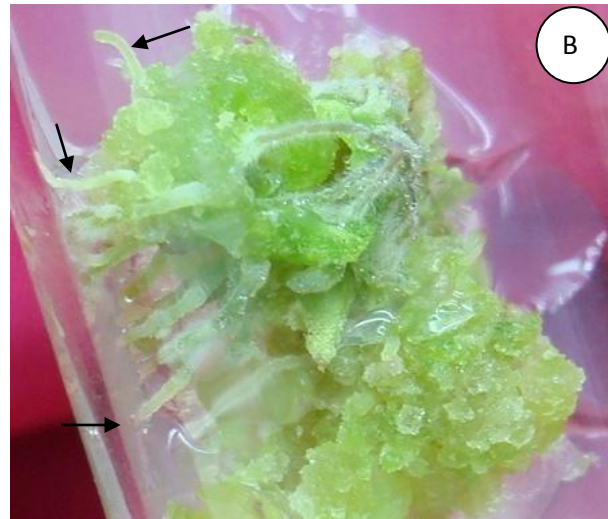
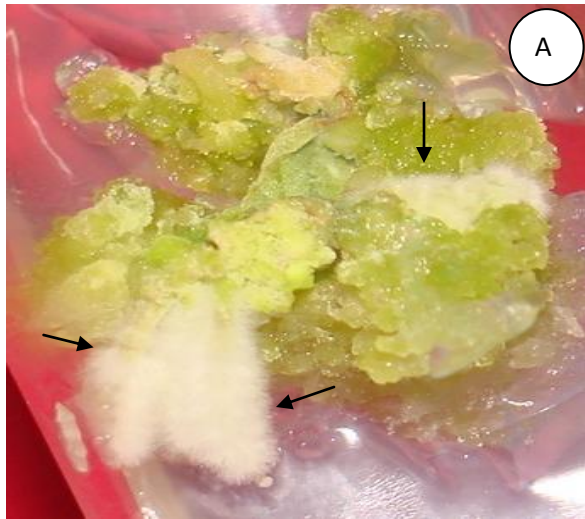


Fig. 20.

4.2.1.4 Somatic embryogenesis from leaf explants

Fast growing, green and compact callus obtained from the leaf explants on NK medium remained non-embryogenic even after repeated subculturing. For the induction of embryogenesis, the callus was transferred to different concentrations of 2, 4-D (4.87 – 38.96 μM) and sucrose (1-5%) either singly or in combination with TDZ. 2, 4- D at the concentration of 19.48 μM and 3 % sucrose proved very effective in inducing somatic embryos in 95 % of the cultures. Embryogenic but slow growing callus could be induced directly by culturing leaf explants on MS medium supplemented with 2, 4-D (9.74 - 19.48 μM) and 3% sucrose. Initiation of embryogenesis occurred within 15 days of culturing showing the formation of green globular structures appearing as protuberances from the surface of embryogenic callus (Fig. 21 A), which formed numerous (average of 45 embryos/culture) prominent globular shaped embryos after 4 weeks (Fig. 21 B). These globular shaped embryos passed through heart, torpedo and cotyledonary developmental stages. Only 70% of the globular structures developed into cotyledonary embryos after 6-7 weeks of culturing (Fig. 21 C) which developed further into tiny plantlets (Fig. 21 D). An increase in 2, 4- D concentration beyond 29.22 μM caused an inhibitory effect on embryo induction and decrease in sucrose concentration also resulted in the reduction of embryogenesis. Somatic embryogenesis also occurred on medium supplemented with 2, 4- D (7.3 μM) and TDZ (1.35 μM) and 3 % sucrose, where 80 % of the cultures formed clusters of somatic embryos.

Stereozoom microscopy was carried out to ascertain various stages of embryo development in the callus cultures. Figure 21 E shows numerous globular shaped embryos after 4 weeks, which further developed into heart, torpedo and cotyledonary shaped embryos (Figures 21 F-H). Cotyledonary embryos formed developed into tiny plantlets with distinct root and shoot axis (Fig. 21 I). An isolated complete plantlet showing the emergence of root axis into well-developed radical is depicted in Figure 21 J.

The different stages of embryogenesis were also studied histologically to confirm the formation of somatic embryos in the cultures. The early stages showed differentiation of embryogenic cells with prominent nuclei and dense cytoplasm (Fig. 21 K). These embryogenic cells divided further and gave rise to various stages of embryo formation. All the developmental stages like globular,

heart, torpedo and cotyledonary were seen histologically in the cultures (Fig. 21 L- O). Figure 21 P shows an embryogenic mass depicting developing embryos at different stages of development.

Transfer of embryos from 2, 4-D/ TDZ supplemented medium to BMS medium

On 2, 4-D (9.74- 19.48 μ M) and 3% sucrose supplemented medium, 70% of globular embryos and on 2, 4- D (1.5 μ M) and TDZ (0.3 μ M) and 3% sucrose supplemented medium, 60% of globular embryos reached up to cotyledonary stage. In order to increase the efficacy of embryo development, the embryogenic callus at globular stage was transferred to BMS medium with 3% sucrose only which resulted in the development of embryos into plantlets in all the cultures (Fig. 21 Q & R).

4.2.1.5 Synthesis of artificial seeds

In the present investigation, somatic embryos and shoot buds formed directly from the leaf explants were used for the construction of synthetic seeds by encapsulating them either in sodium alginate or in chitosan. Seeds were constructed by encapsulating somatic embryos mostly at early cotyledonary stage and shoot buds (3-4 mm in size) in different concentrations of sodium alginate (1-4 %) and calcium chloride (2%) as the gel matrix. Sodium alginate at 2% concentration was found to be the most appropriate for encapsulation resulting in the formation of clear, transparent isodiametric beads approximate 5 mm in diameter (Fig. 22 A). Figures 22 B and C show the magnified view of somatic embryos and shoot bud-encapsulated beads respectively. Lower concentrations (1 %, 1.5 %) of sodium alginate resulted in the formation of fragile beads and higher concentrations (3 %, 3.5 %, 4%), favored the formation of hard beads, which had a marked effect on the germination or conversion process later on. Somatic embryos and shoot buds were also encapsulated in chitosan, where best complex was formed using 0.1% chitosan with 0.1% TPP solution (Fig. 22 D). The encapsulated beads were collected on a sterile filter paper in a Petridish, which was sealed with a parafilm. The seeds were then stored in refrigerator at 4°C.

For retrieving plantlets, the encapsulated shoot buds were cultured on basal MS medium with 2% sucrose. The alginate coat started dissolving after 3-4 days followed by sprouting of shoot bud

(Fig. 22 E), which elongated further (Fig. 22 F) and formation of complete plantlets with well developed root and shoot system was observed within 18 days (Fig. 22 G). Numerous well-developed plantlets showing normal phenotype were formed from shoot bud encapsulated beads (Fig. 22 H). Similarly, germination of somatic embryo encapsulated seeds occurred after 7-8 days of culturing on basal MS medium, where emergence of radical occurred first (Fig. 22 I) and complete plantlet was formed after 15 days (Fig. 22 J). A number of plantlets formed from encapsulated somatic embryos are shown in Figure 22 K.

The frequency of conversion of these synthetic seeds into plantlets was evaluated after different storage periods of 0, 20, 30, 40, 60 and 90 days respectively. Synthetic seeds could be stored at 4°C for more than 90 days but there was a gradual reduction in the conversion rates and plantlet regeneration thereafter. The highest conversion rates for encapsulated somatic embryos and shoot buds was 95 and 98% after 0 day of storage which decreased to 80 and 85% respectively after 20 days and it was only 10 to 15 % after 90 days. The percentage response for conversion of these synthetic seeds stored for different periods is shown in Figure 23. Somatic embryos and shoot buds encapsulated in chitosan complex were transferred to basal MS medium to evaluate the viability of these synthetic seeds, which however failed to show germination.

Fig. 21. Somatic embryogenesis from leaf explants of *Tylophora indica*.

- A. Initiation of somatic embryogenesis in the callus cultures showing differentiation of numerous embryos.
- B. 4-week old culture showing prominent globular embryos.
- C. 5 weeks old culture showing cotyledonary development stage (arrowheads).
- D. Development of embryo into tiny plantlets showing root and shoot axis (arrowheads).

Stereozoom pictures of different developmental stages (80 X)

- E. 4-weeks old culture showing globular shaped embryos.
- F, G, H. Depicts different stages of embryogenesis like heart, torpedo and cotyledonary
- I. Cotyledonary embryo developing into tiny plantlet (arrowheads).
- J. A plantlet showing well developed shoot and root axis isolated from the culture (arrowheads).

Histological evidences of somatic embryogenesis.

- K. Differentiation of embryogenic cells with prominent nuclei and dense cytoplasm (100 X).
- L-O. Different developmental stages like globular, heart, torpedo and cotyledonary during somatic embryogenesis (400 X)
- P. Embryogenic mass showing embryos at different stages of development (100 X).

Development of plantlets on Basal MS medium

- Q & R. Germination of embryos into plantlets on BMS after 2 weeks and their further growth into green leafy shoots after 4 weeks.

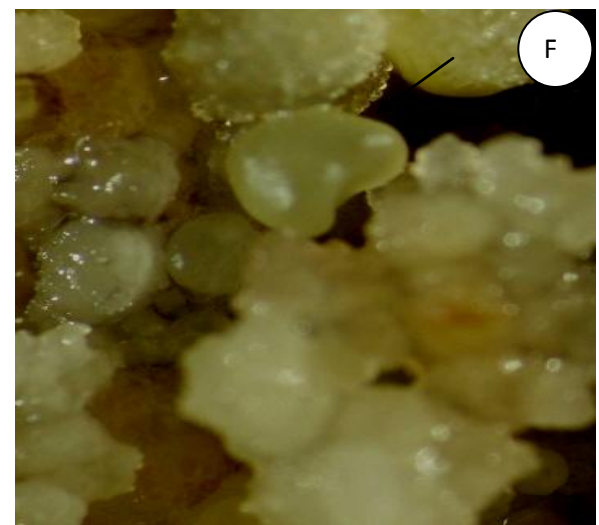
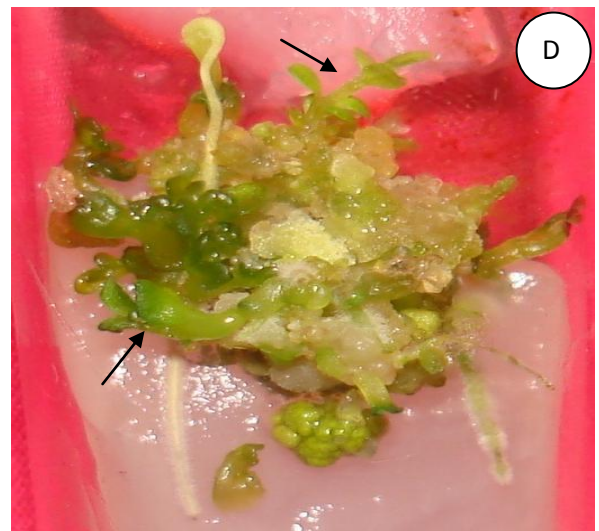
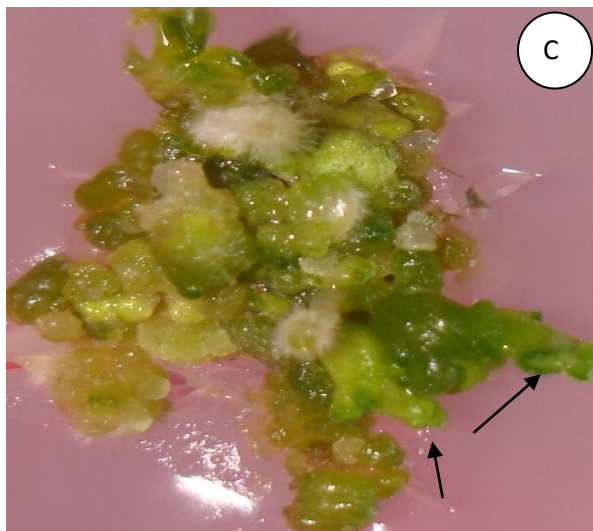
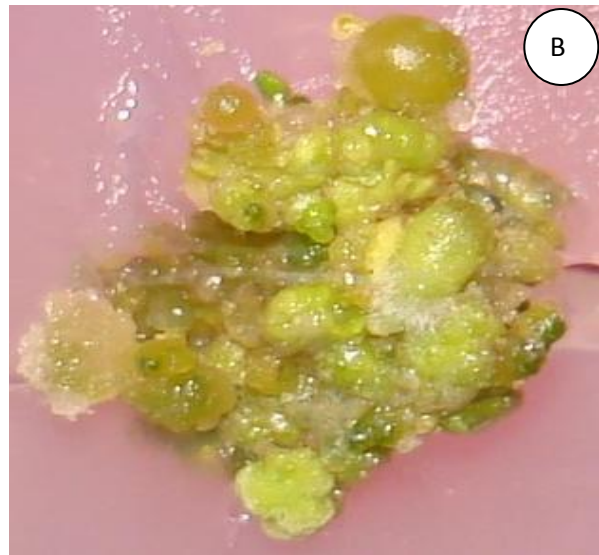
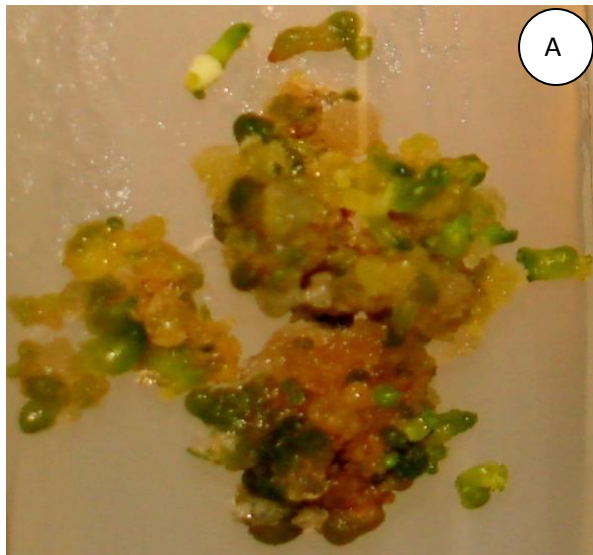


Fig. 21. A-F

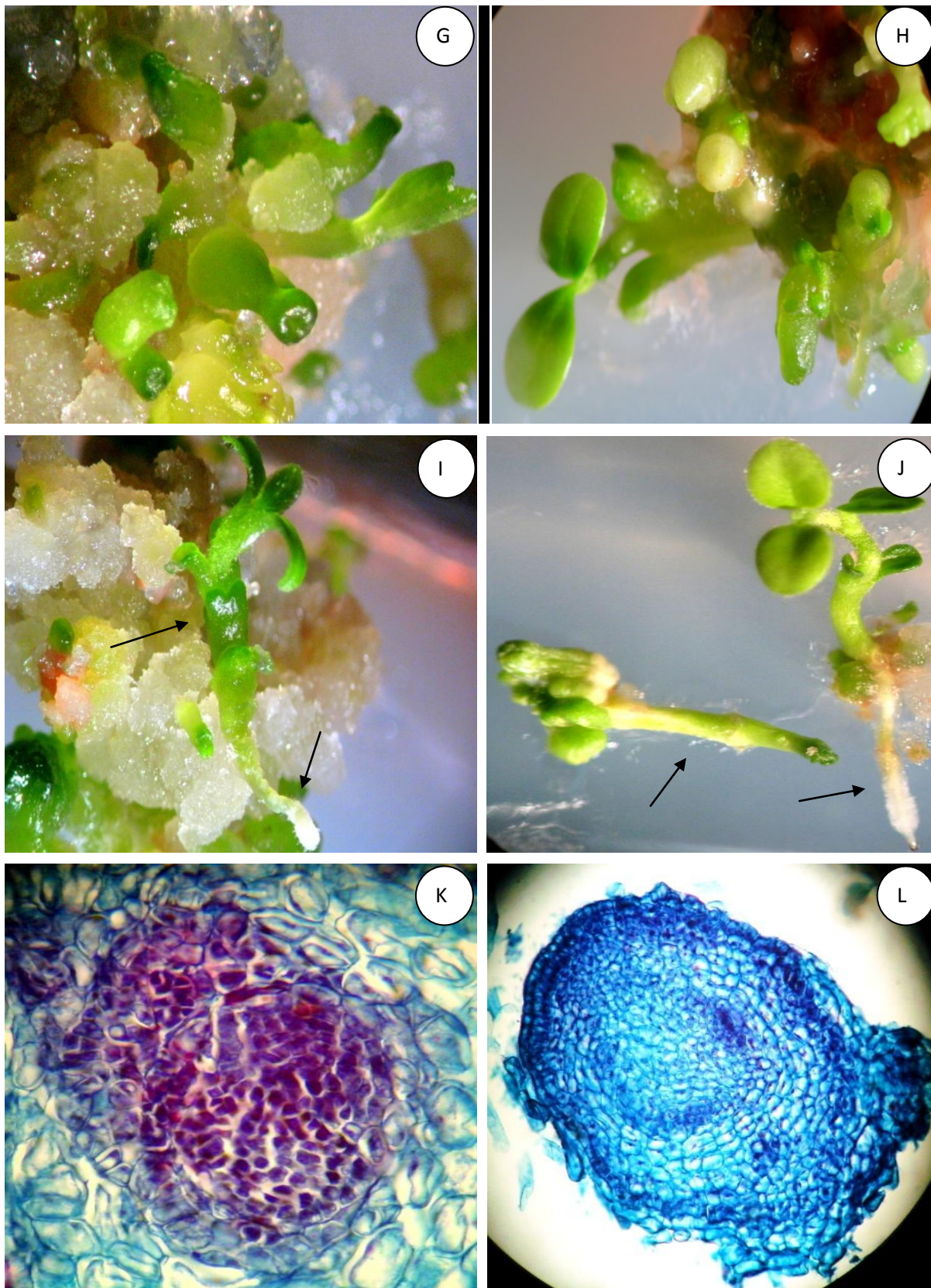


Fig. 21. G-L

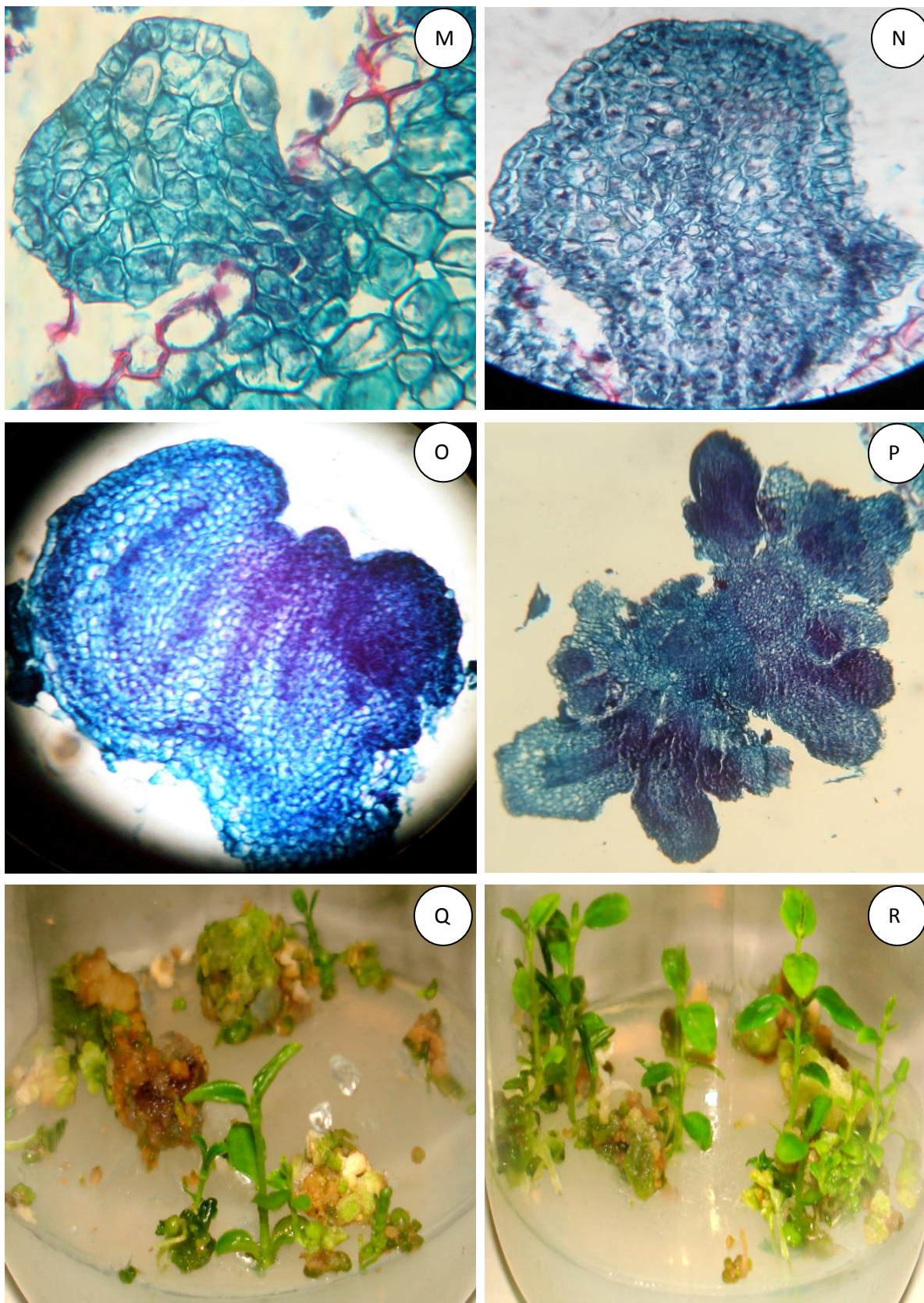


Fig. 21. M-R

Fig. 22. Artificial seed production from somatic embryos and shoot buds of *Tylophora indica*

- A. Artificial seeds formed by encapsulation with sodium alginate (2%) and pre chilled CaCl_2 (2%).
- B & C. Encapsulated somatic embryos and shoot buds shown on a magnified scale.
- D. Chitosan encapsulated embryos.
- E. Sprouting of shoot bud encapsulated seed on basal MS after 7 days of culturing.
- F. Further elongation and growth of shoot.
- G. A plantlet formed after 15 days.
- H. Numerous well-developed plantlets formed from encapsulated shoot buds
- I. Germination of somatic embryo encapsulated bead after 8 days of culturing.
- J. A plantlet with distinct root and shoot axis formed after 15 days.
- K. Formation of large number of well developed plantlets

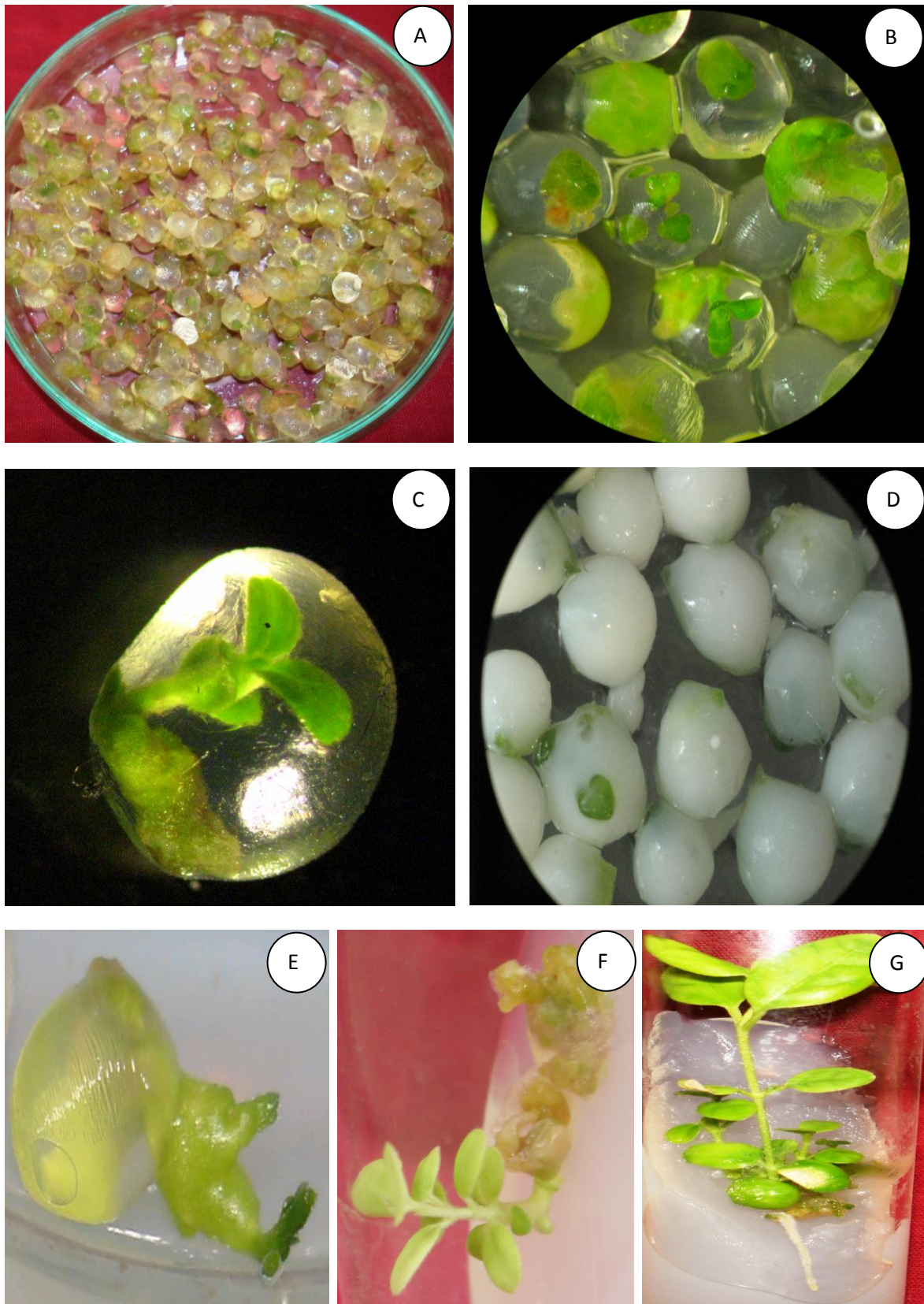


Fig. 22. A-G

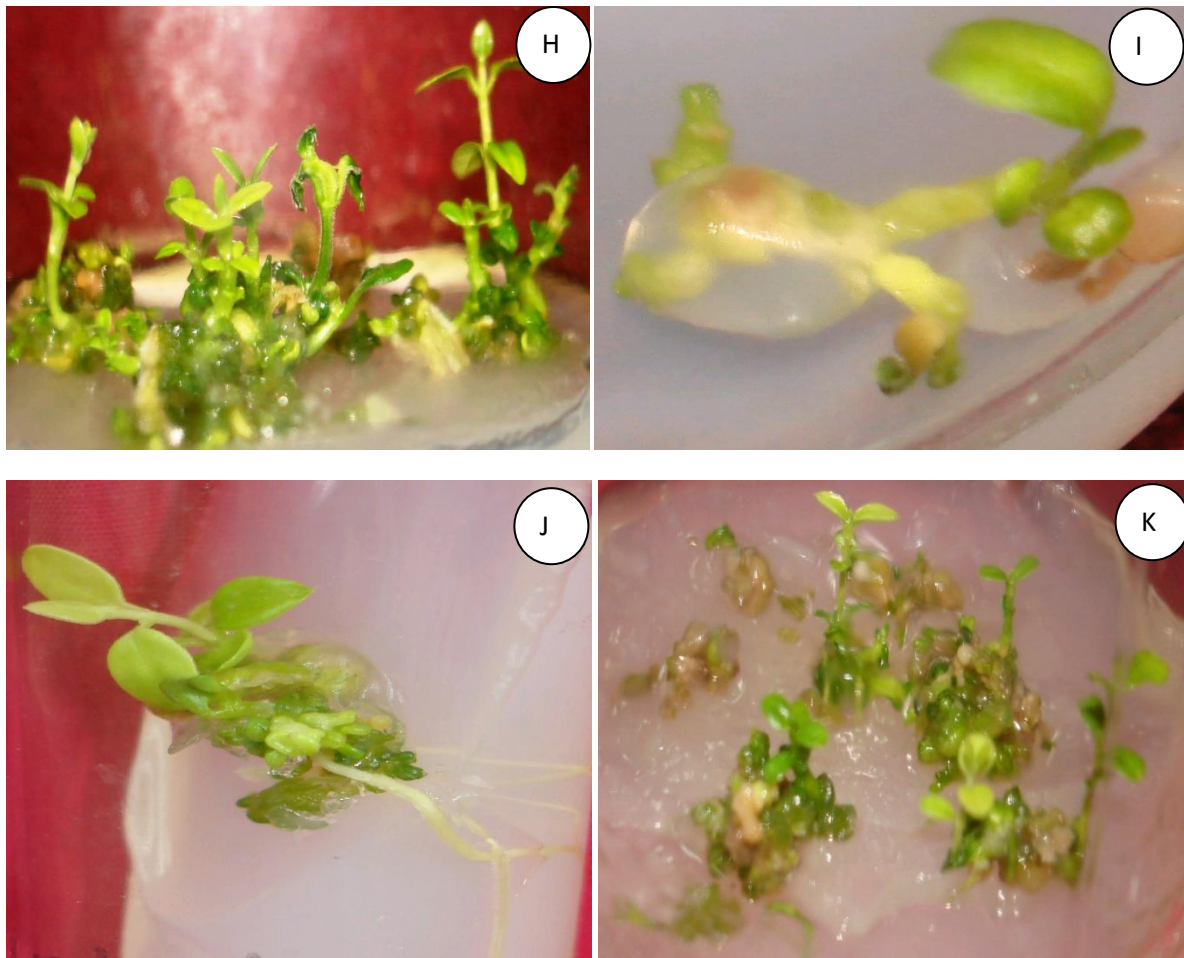


Fig. 22. H-K

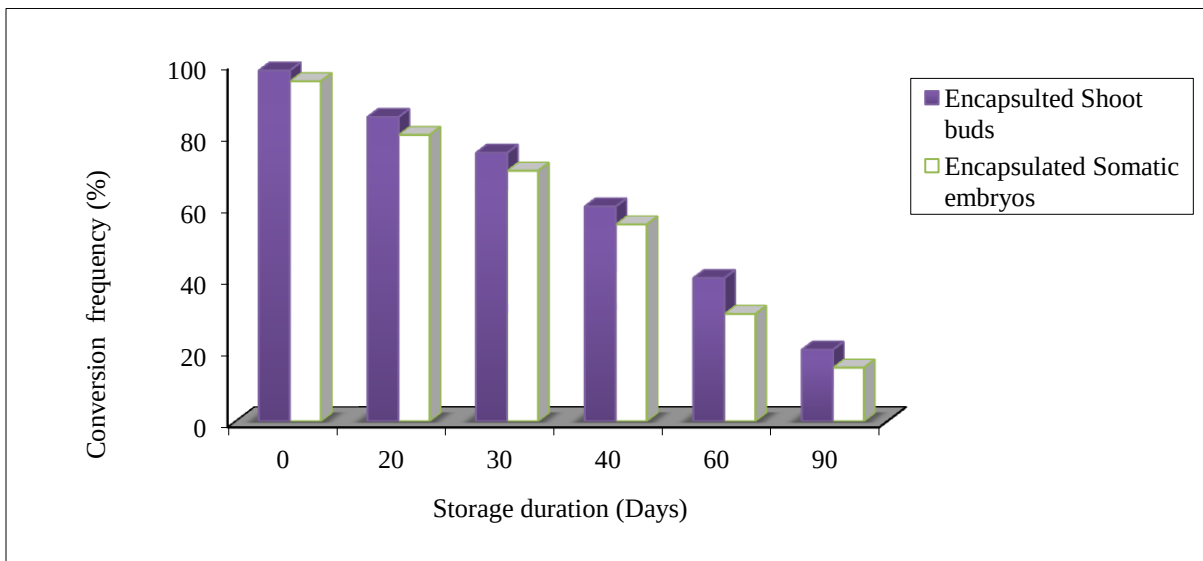


Fig. 23. Percentage conversion of encapsulated seeds after different storage periods

4.2.2 Stem Culture

4.2.2.1 Direct Adventitious Shoot induction

Direct shoot induction from the stem segments occurred when cultured on MS medium supplemented with different concentrations of BA either alone or in conjunction with adenine sulphate. 8.8 μ M of BA produced greatest number of shoots per explant where initially 5-8 shoots regenerated from the meristemoids formed at the cut ends of stem segments after 3-4 weeks (Figures 24 A and B). The number increased to an average of 48.0 ± 0.57 shoots/explant with average shoot length of 6.60 ± 0.12 cm after 7-8 weeks (Fig. 24 C). The shoots so produced were excised into small groups and were subcultured for further multiplication. MS medium supplemented with BA (8.8 μ M) in conjunction with adenine sulphate (2.7 μ M) also generated good response in terms of shoot bud induction forming an average number of 38 ± 1.45 shoots per culture after 7 weeks. Response of stem explants for direct shoot induction on different concentrations of BA with or without adenine sulphate is demonstrated in Table 19.

Table 19: *De novo* adventitious shoot formation on different plant growth combinations (Mean followed by same letter are not significantly different by the Duncan's multiple range test at 0.05% probability level).

S. No	Plant growth regulator	Concentration (μ M)	Explant forming shoots (% age)	Shoots per explant (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.40	64	19.0 ± 0.57^f	1.53 ± 0.08^b
		8.80	90	48.0 ± 0.57^b	6.60 ± 0.12^b
		17.6	70	23.0 ± 0.57^e	4.56 ± 0.14^b
		26.4	63	21.0 ± 0.57^h	4.03 ± 0.08^b
		35.2	58	18.0 ± 0.57^g	4.21 ± 0.08^b
2.	BA + Adenine sulphate	$4.40 + 2.71$	74	28.0 ± 0.57^e	5.62 ± 0.14^b
		$8.80 + 2.71$	80	38.0 ± 1.45^a	3.53 ± 0.08^b
		$17.6 + 2.71$	66	27.0 ± 0.57^d	4.93 ± 0.08^b
		$26.4 + 2.71$	62	26.3 ± 0.80^c	6.40 ± 0.05^b
		$35.2 + 2.71$	57	11.0 ± 0.57^i	3.26 ± 0.08^b

BA: 6-benzyl adenine

4.2.2.2 Callus Induction and differentiation

For callus induction, stem segments (4-5 mm) were transferred to MS medium supplemented with different growth regulators. Best callus growth took place on MS medium supplemented with 29.4 μ M NAA and 4.65 μ M K or NK medium where callusing initiated after 8 days (Fig.

24 D) and within 4 weeks, the entire segment turned into green, compact and non-friable callus mass in about 85% of the cultures (Fig. 24 E). Likewise, synergistic action of 2, 4-D with BA or K was found to be effective in inducing callus from the stem explants (Table 20). About 74% of the cultures callused at the cut ends on 2, 4-D (14.6 μ M) + K (9.3 μ M) supplemented medium after 12 days and turned into mass of compact non friable callus within 8 weeks.

Table 20: Effects of different plant growth regulators on callus induction from stem explants

S.No	Plant growth regulator	Concentration (μ M)	Explant forming callus (% age)	Nature of Callus	Colour of callus
1.	NAA + K	7.35 + 4.65	70	Non- Friable	Green
		14.7 + 4.65	78	Non- Friable	Green
		29.4 + 4.65	85	Non- Friable	Green
2.	2, 4-D + K	4.87 + 9.3	54	Non- Friable	Light green
		9.74 + 9.3	60	Non- Friable	Light green
		14.6 + 9.3	74	Non- Friable	Light green
		19.4 + 9.3	66	Non- Friable	Light green
3.	2, 4 -D + BA	4.87 + 8.8	66	Non- Friable	Light green
		9.74 + 8.8	72	Non- Friable	Light green
		14.6 + 8.8	62	Non- Friable	Light green
		19.4 + 8.8	54	Non- Friable	Light green

NAA: α -naphthalene acetic acid, K: kinetin, 2, 4-D: 2, 4- dichlorophenoxy acetic acid, BA: 6-benzyl adenine

Xylogenesis

Three weeks old stem callus revealed differentiation of xylem in the form of tracheids, which occurred singly, or in groups forming nodules. Initially only a few tracheids could be located but as the callus proliferated, more and more of them appeared among the callus cells. The tracheids possessed sclariform thickenings on their walls (Fig. 25 A).

Rhizogenesis

On NK medium, formation of roots occurred from the proliferating callus with roots piercing deep into the medium (Fig. 25 B). These roots were white and bore thick crop of root hairs. Root differentiation from stem callus was also observed when transferred onto IBA (9.84 μ M) after 2 weeks of culturing. Initially fewer roots were formed but with further proliferation of callus more and more roots appear randomly in about 80% of the cultures. The roots were long, thin, white or green in colour.

Caulogenesis

Solid culture

Shoot differentiation from callus was observed on NK supplemented medium but the number of shoots formed was less and took longer time to differentiate (10-12 weeks). Prolific shoot differentiation occurred when callus was transferred to BA (8.8 μ M) supplemented medium where initially 5-6 shoots differentiated from the callus after 3 weeks (Fig. 25 C) which increased to an average of 35 ± 1.15 shoots/explant after 7 weeks (Fig. 25 D). Repeated subculturing enhanced shoot multiplication rates without any decline in their growth thereafter. The shoots elongated and grew further and developed many leaves. Differentiation of many shoots occurred on 9.3 μ M K supplemented medium after 8 weeks of culturing where initially few shoots were formed and with the passage of time, numerous shoots differentiated from the callus. The shoots elongated further and developed many leaves.

Liquid culture

The maximum number of shoots were formed on liquid MS supplemented with 8.8 μ M BA. Initiation of shoots occurred after 12 days of transfer to liquid medium in about 80% of the cultures (Fig. 25 E), which latter on resulted in the formation of large number of green healthy shoots (Fig. 25 F).

Fig. 24. Direct adventitious shoot formation and callus induction from stem explants

- A. 3 week old culture showing formation of adventitious shoots from nodular meristemoids on BA (8.8 μ M) supplemented medium.
- B. 4 week old culture showing further proliferation of shoots.
- C. 8 week old culture showing healthy green leafy shoots.
- D. Induction of callus from the cut ends of stem explants on NAA (29.4 μ M) and K (4.65 μ M) after 8 days.
- E. Formation of non-friable callus mass after 4 weeks.



Fig. 24.

Fig. 25. Differentiation from stem callus

- A. 3 weeks old callus showing differentiation of tracheids with sclariform thickenings.
- B. Emergence of roots from proliferating callus mass (arrowheads).
- C. Differentiation of shoots on 8.8 μ M BA after 3 weeks of culturing.
- D. Large number of shoots differentiated after 7 weeks of culturing.
- E. Differentiation of green leafy shoots on liquid MS supplemented with 8.8 μ M BA.
- F. Further growth and proliferation of shoots in large number.

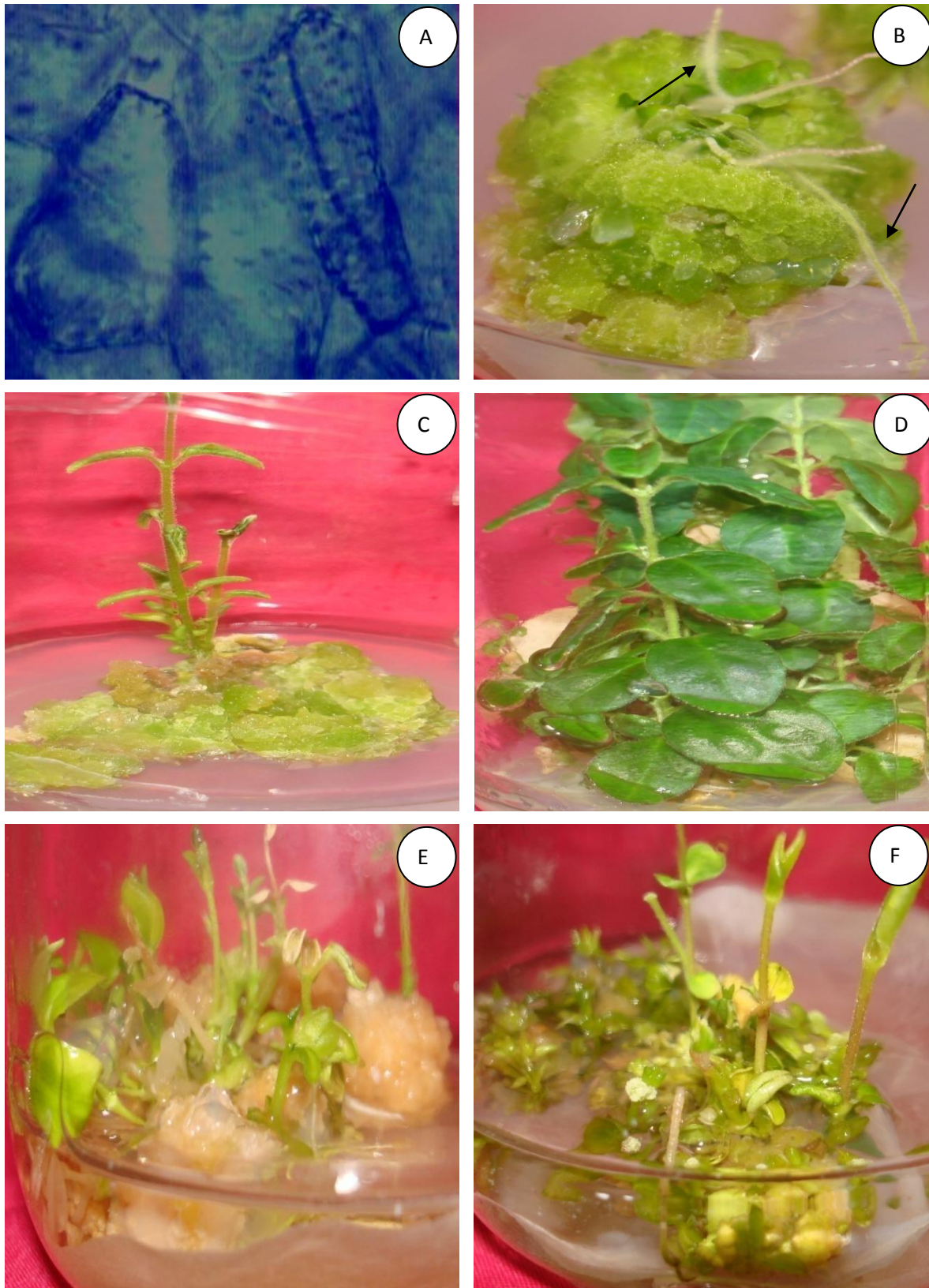


Fig. 25.

4.2.3 Root culture

4.2.3.1 Direct Adventitious Shoot induction from root explant

Root explants (5-6 mm in length) were excised from field grown and *in vitro* raised plants and were cultured on MS medium supplemented with NAA (7.35-29.4 μM), TDZ (2.25 – 9.0 μM), BA (4.4- 17.6 μM) either singly or in combinations with L-Ascorbic acid (5.6- 22.4 μM). Nodular meristemoids differentiated from the cut ends as well as on the surface of the root segments when cultured on BA (8.8 μM) supplemented medium and covered the entire explant within 2-weeks of culture. In 80-90% of the cultures, these meristemoids differentiated into green, leafy shoots after 25-30 days of culturing (Fig. 26 A). Initially fewer shoots were formed and the number increased to 20-25 shoots per culture after 7 weeks (Fig. 26 B). Repeated subculturing accelerated the formation and proliferation of shoots in large numbers forming 40 - 45 shoots/culture. High frequency shoot regeneration also occurred on 13.2 μM BA but the percentage response was comparatively less. Likewise, root segments cultured on MS medium supplemented with α -naphthalene acetic acid (7.35 μM) also induced nodular meristemoids but their initiation and subsequent proliferation into leafy shoots was comparatively slow.

Direct regeneration of adventitious shoots was also observed without meristemoid formation on MS medium supplemented with TDZ (2.25 μM) and L-Ascorbic acid (8.4 μM). Initiation of multiple shoots occurred directly from the explant after 6-7 days of planting in 88% of the cultures (Fig. 26 C). The number increased further and clusters of shoots (average shoot length 3.9 ± 0.57 cm) were formed after 4 weeks of culturing (Fig. 26 D).

4.2.3.2 Induction of callus

Solid culture

Callusing of the root segments occurred on MS medium supplemented with plant growth regulators such as 2, 4-D (4.87- 19.48 μM), IBA (4.92 -24.6 μM), TDZ (4.5- 9.0 μM) either alone or in combination with BA (4.4-13.2 μM). Synergistic action of IBA (24.6 μM) with BA (13.2 μM) was most effective in the initiation and sustained growth of callus. The callusing occurred along the entire surface of the explant after 10 days of planting (Fig. 27 A) and within 3

weeks the entire segment turned into a mass of greenish white callus (Fig. 27 B). The callus was fast growing, compact and proliferated further on subculturing.

Good callus growth also occurred on 9.74 μM of 2, 4- D supplemented medium where initiation of callus occurred after 12 days leading to the formation of greenish white callus. Deep green callus masses were formed on MS medium supplemented with TDZ (9.0 μM) and BA (8.8 μM). An increase in the concentration of TDZ, however, slowed down the pace of callus formation. Table 21 shows effect of different PGRs on callus induction.

Table 21: Impact of auxin and cytokinin on callus induction from root explants of *T.indica*

S.No	Plant growth regulator	Concentration (μM)	Explant forming callus (% age)	Nature of Callus	Colour of callus
1.	IBA + BA	12.8 + 8.8	67	Friable	Greenish white
		24.6 +13.2	88	Friable	Greenish white
2.	2, 4-D	4.87	76	Friable	Greenish white
		9.74	80	Friable	Greenish white
		14.6	72	Friable	Greenish white
3.	TDZ + BA	3.0 + 8.8	70	Friable	Deep green
		9.0 + 8.8	76	Friable	Deep green

IBA: indole 3- butyric acid, BA: 6- benzyl adenine, 2, 4- D: 2, 4- dichlorophenoxy acetic acid, TDA: thidiazuron

Liquid Culture Medium

Callus was induced on liquid MS augmented with different concentrations of IBA (4.92 -19.68 μM) and BA (4.4- 17.6 μM), best results being obtained on 14.76 μM IBA and 13.2 μM BA. On this medium 80 % of the explants callused along the entire surface after 15 days of culturing (Fig. 27 C) and masses of whitish green compact calli were formed after 4 weeks (Fig. 27 D). On 2, 4-D (9.74 μM) supplemented medium, callus formation also occurred but the growth was comparatively slow.

4.2.3.3 Shoot regeneration from root callus

Solid medium

Shoot bud differentiation occurred from the callus on IBA (24.6 μM) and BA (13.2 μM) supplemented medium but better results were obtained when latter was transferred to MS

medium supplemented with BA (8.8 μ M) alone (Fig. 28 A). Emergence of 4-5 shoots was observed initially after 10 days of culturing which further increased in number (Fig. 28 B) and developed into 5-6 cm long shoots after 25 days of culturing (Fig. 28 C). Differentiation of many shoots (average no. 69 ± 1.5 shoots/culture) was observed after subsequent subculturing of calli on fresh media (Fig. 28 D). Good frequency of shoot differentiation was also observed on K supplemented MS medium where addition of TDZ further enhanced the rate of shoot differentiation from the root callus. Table 22 shows effects of various plant growth regulators on shoot regeneration from root callus.

Table 22: Effects of various concentrations of cytokinin alone or in combination with auxins on shoot regeneration from root callus (Means followed by same letters are not significantly different by the Duncan's multiple range test at 0.05% probability level).

S. No	Plant growth regulator	Concentration (μ M)	Explant forming shoots (% age)	Shoots per explant (Mean $\pm$ SE)	Shoot length (cm) (Mean $\pm$ SE)
1.	BA	4.4	76	51.3 ± 1.76^b	6.2 ± 0.05^a
		8.8	90	69.0 ± 1.50^b	7.2 ± 0.05^a
		13.2	88	60.6 ± 1.76^c	6.9 ± 0.05^a
		17.6	80	56.0 ± 1.15^e	6.7 ± 0.12^a
2.	K	4.65	76	56.6 ± 1.45^e	6.0 ± 0.08^a
		9.30	86	64.0 ± 1.15^a	6.6 ± 0.14^a
		13.9	82	62.3 ± 0.88^a	6.4 ± 0.08^a
		18.6	74	59.0 ± 0.57^b	5.9 ± 0.15^a
3.	TDZ + K	$4.5 + 9.3$	89	62.4 ± 1.50^b	6.9 ± 0.12^a
		$9.0 + 9.3$	76	52.3 ± 1.76^b	5.3 ± 0.05^a
		$13.5 + 9.3$	72	52.3 ± 1.85^d	5.0 ± 0.12^a

BA: 6 –benzyladenine, K: kinetin, TDZ: thidiazuron

Caulogenesis on liquid medium

Differentiation of shoot initials from root callus occurred on liquid MS supplemented with BA (8.8 μ M) after 20 days of culturing (Fig. 28 E). Within 6 weeks of inoculation an average number of 65 ± 1.15 shoots/flask were formed in nearly 87% of the cultures (Fig. 28 F). The shoots so produced were excised into small groups and were subcultured on the same medium for further multiplication.

Fig. 26. *De novo* adventitious shoot formation from root explants on variously supplemented MS medium.

- A. Differentiation of nodular meristemoids into green leafy shoots after 3 weeks of culturing on 8.8 μM BA.
- B. 7 week old culture showing formation of large number of shoots from the meristemoids.
- C. Formation of few shoots on TDZ (2.25 μM) and L-Ascorbic acid (8.4 μM) after 6 days of culturing (arrowhead).
- D. Cluster of shoots formed after 4 weeks on the same medium.

Fig. 27. Induction and growth of callus from root explants on MS supplemented with different concentrations of IBA and BA.

- A. Induction of callus on 24.6 μM IBA and 13.2 μM BA after 10 days of culturing.
- B. Greenish white callus formed within 3 weeks.
- C. Formation of whitish green compact callus on liquid MS supplemented with 14.76 μM IBA and 13.2 μM BA.
- D. 4 weeks old root callus on liquid medium.

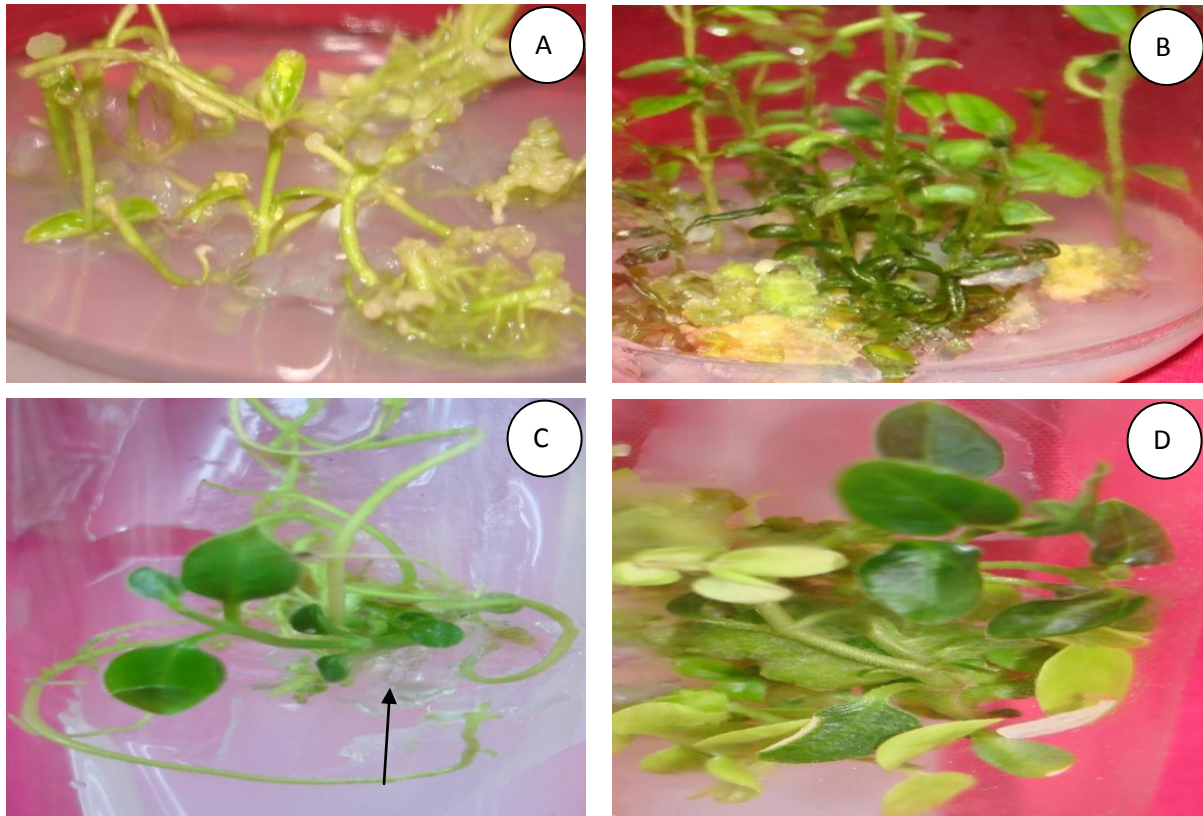


Fig. 26.

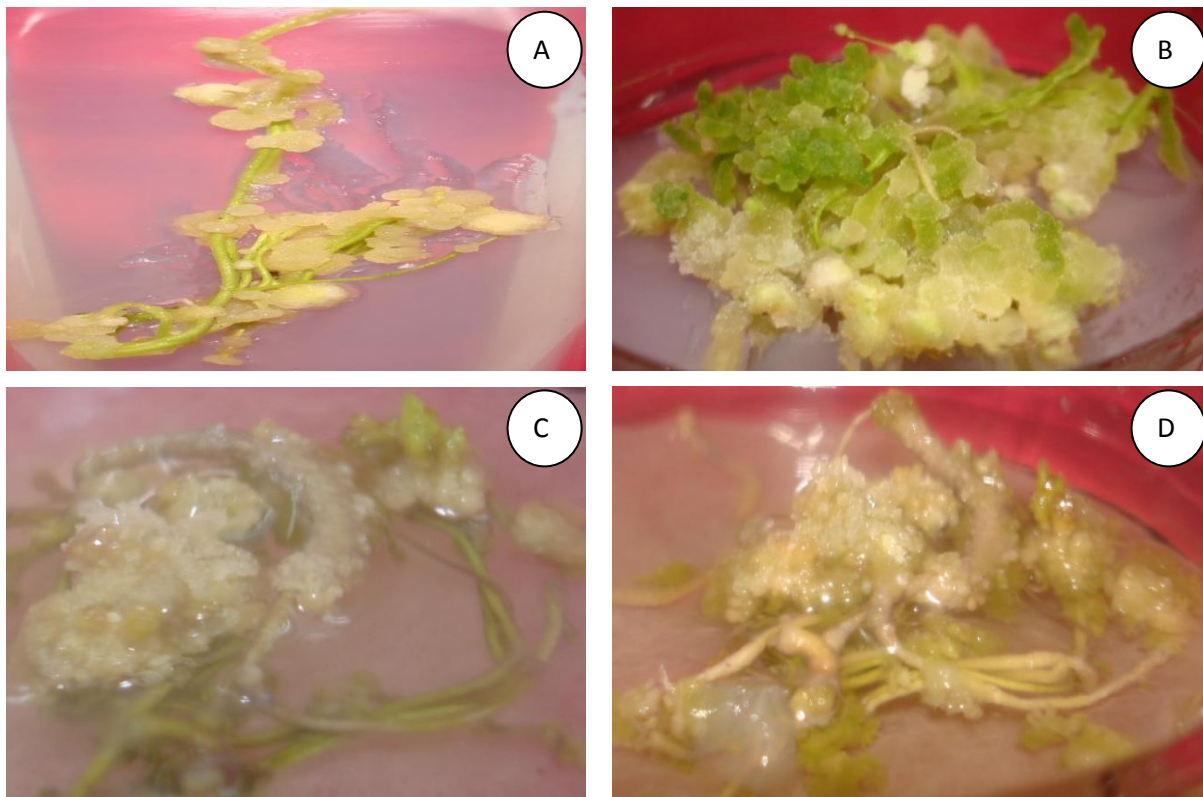


Fig. 27.

Fig.28. Shoot differentiation from root callus on BA 8.8 μ M supplemented medium.

- A. Differentiation of shoot initials on 8.8 μ M BA alone.

- B & C. 3 and 4 week old cultures showing green leafy shoots and their further elongation.

- D. Large number of shoots formed after 7 weeks of culturing.

- E. Formation of shoots on liquid MS with 8.8 μ M BA after 20 days of culturing.

- F. Further growth and proliferation of shoots.

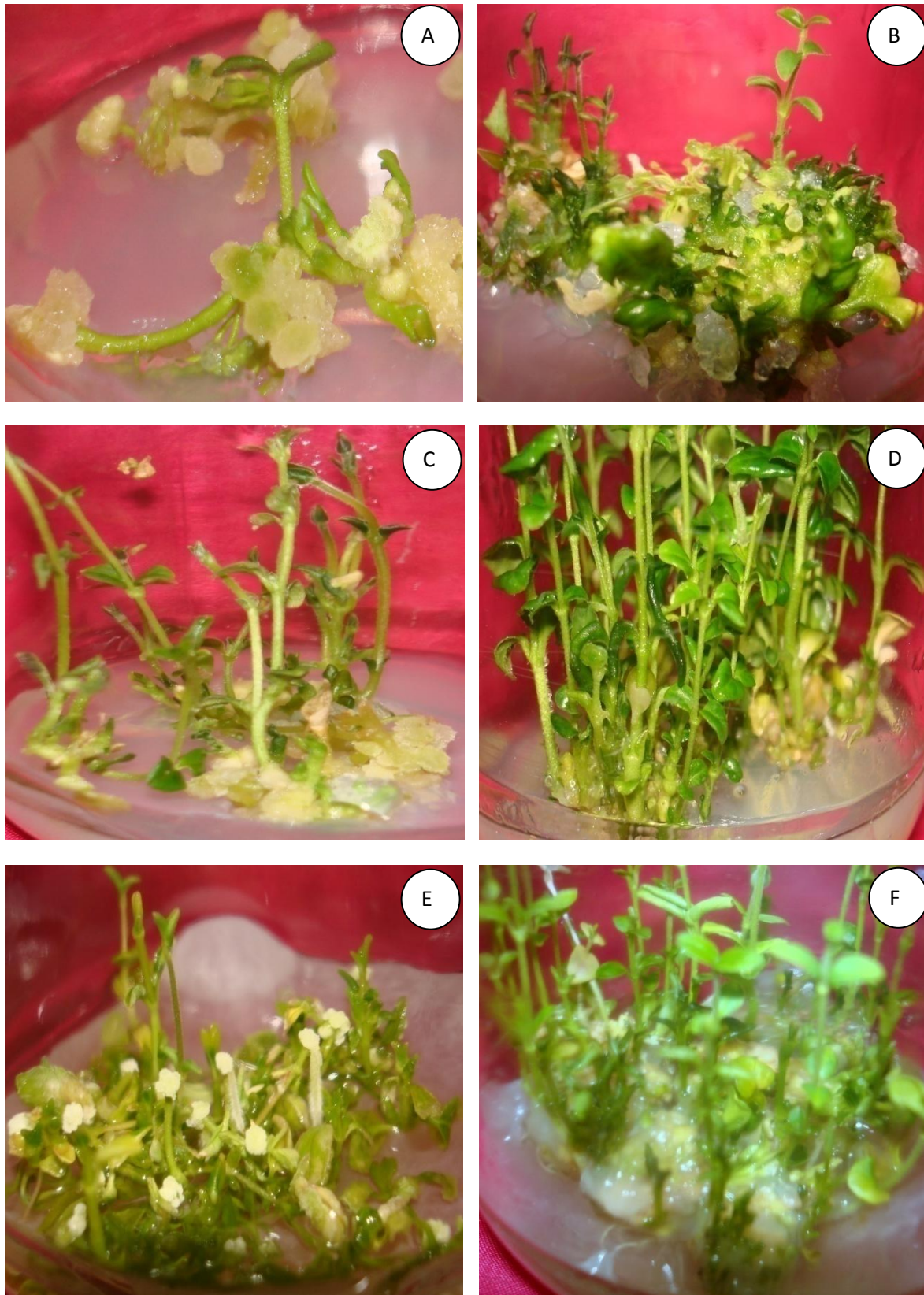


Fig. 28.

4.2.4 Nodal Explant Culture

4.2.4.1 Multiple shoot proliferation

Fresh nodal explants each holding one dormant lateral bud were collected from mature field grown healthy *Tylophora* plant. After sterilization, the damaged internodal tissues on both sides were cut off and the nodal segments 3-4 mm in size were cultured on MS medium supplemented with various growth regulators. The axillary shoot proliferation was remarkably influenced by the type and concentration of growth regulator used. Multiple shoot induction occurred on MS medium supplemented with 8.8 μM of BA, where initial bud break occurred after 10-15 days of inoculation (Fig. 29 A) leading to the formation of 8-10 shoots from the axillary position after 3 weeks. At the same time, lower cut end of the explant formed a cluster of globular meristemoids (Fig. 29 B) which developed into shoots latter on. Nearly 20-25 shoots originated from a single nodal segment after 6 weeks. When nodal segments bearing theses meristemoids were planted on the fresh BA supplemented medium, the globular meristemoids proliferated further and grew into many shoots (Fig. 29 C).

MS medium supplemented with BA (13.2 μM) in conjunction with NAA (3.67 μM) and L-Ascorbic acid (8.4 μM) gave the best proliferation rate. About 75 % of the cultures showed globular meristemoid formation at the cut ends and 40-45 vegetative regenerants were produced after 5-6 weeks of inoculation. On frequent subculturing there was a marked increase in the number of meristemoids and the shoots formed. Figure 30 depicts effects of different concentrations of BA alone and in combination with NAA or Ascorbic acid on shoot proliferation from nodal explant.

Fig. 29. Multiple shoot proliferation from nodal explants on MS medium containing BA (8.8 μ M).

- A. Bud break from nodal segment after 10 days of culturing.
- B. Formation of shoots from nodular meristemoids after 3 weeks of culture (arrowheads).
- C. Further elongation and proliferation of shoots.



Fig. 29.

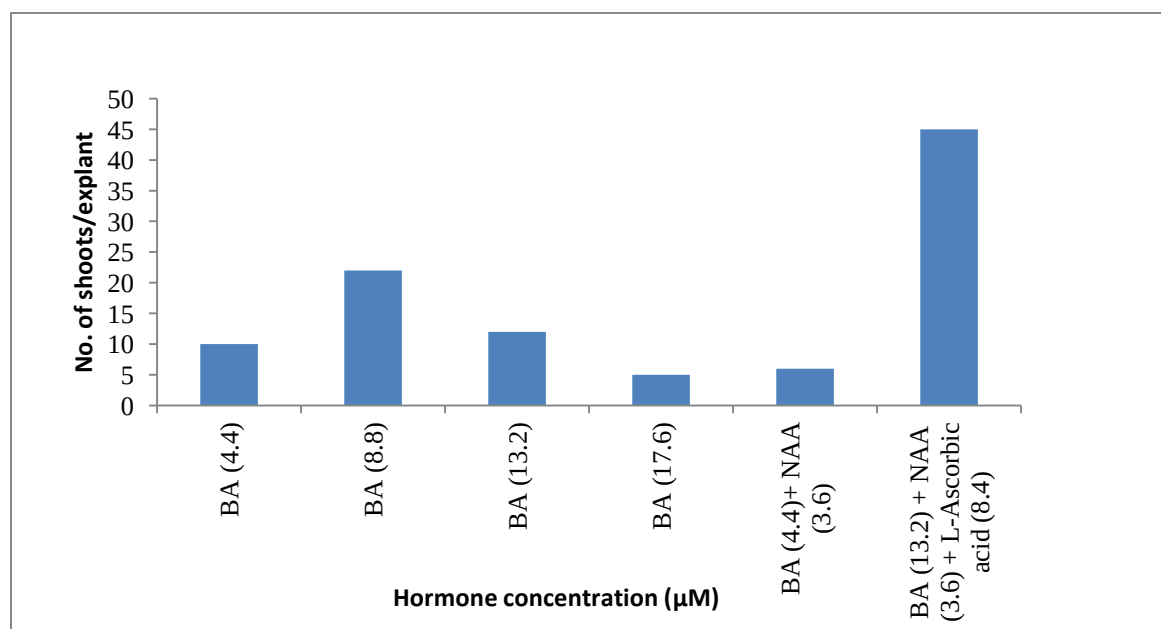


Fig. 30. Effect of different concentrations of benzyl adenine alone and in combination with naphthalene acetic acid or ascorbic acid on shoot proliferation from nodal explants.

4.2.5 Rooting of *in vitro* regenerated shoots

For root induction, individual regenerated healthy shoots (5-6 cm) were carefully rescued from culture vessels and transferred onto to half strength MS medium, full strength MS medium and MS medium supplemented with different concentrations of IAA, NAA & IBA. Among the various growth regulators tested, IBA (9.84 μM) showed good results, where roots initiated after 20 days of culture. Best root initiation, however, occurred on half strength MS medium, where 4-6 healthy roots emerged in 90% of the cultures after 10 days (Fig. 31 A). The roots were long, white and branched. Roots were also formed on half strength liquid basal MS medium, where 3-4 healthy long roots emerged in 80% of the cultures (Fig. 31 B).

4.2.6 Acclimatization of plantlets and optimization of potting mixture

Rooted plantlets were transferred successfully to the field conditions through successive hardening stages. Firstly, the plantlets were acclimatized on moist cotton for 12-15 days kept under growth room conditions (Fig. 31 C). Plantlets kept on moist cotton registered increase in shoot and root length, increase in the number of leaves and also exhibited better survival percentage in the subsequent hardening stages. Physiochemical properties of soil sample used as potting mixtures in different combinations and analyzed using standard protocols are shown in Table 23. Plantlets were transferred to plastic cups containing different potting mixtures in equal ratios i.e. soil (T0), soil: vermicompost (T1), soil: vermicompost: *Azotobacter* (N_2 fixers): *Pseudomonas* (phosphate solubilisers) (T2), soil: *Azotobacter*: *Pseudomonas* (T3), soil: *Azotobacter* (T4), soil: *Pseudomonas* (T5). The cups were covered with perforated plastic bags to maintain internal humidity and aeration and were kept inside the growth room for 15 days (Fig. 31 D). Thereafter, the plantlets were shifted to poly bags containing the same potting mixture and were kept in growth room for another 2 weeks (Fig. 31 E) and were watered periodically.

The hardened plantlets in plastic bags were then transferred to green house for 2 weeks before their final transfer to full sunlight outdoor (Fig. 31 F). By this time, plants became sturdy, developed an efficient root system, formed new leaves and became photosynthetically active. Different parameters like plant height, number of photosynthetically active leaves and percentage

survival of the plants were evaluated (Table 24). Out of various potting mixtures used for hardening, the highest survival percentage was found in T2 (92%), followed by T1 (88%), T3 (65%), T4 (61%) and T5 (59%) respectively. Poor growth of plants occurred on control (soil without any organic matter) as compared to other treatments. Use of biofertilizers along with basic potting mixture exhibited better shoot growth and number of leaves formed per plant and the mortality rate was reduced by 4-5 % on transplantation to the field. Although the inclusion of biofertilizers in the soil: vermicompost potting mixture gave the best results in term of plant growth and percentage survival (92%), soil: vermicompost potting mixture was also closer to it giving 88% survival of regenerated plants. Hence, after acclimatization, the plants were transplanted to earthen pots containing only soil: vermicompost and were successfully established under field conditions (Fig. 31 G). The acclimatized plants showed well-developed shoot and root systems in field conditions with no phenotypic variations (Fig. 31 H).

Table 23: Physiochemical properties of soil used in potting mixture.

Parameters	Mean $\pm$ SE
pH	8.16 $\pm$ 0.03
EC(μ S/cm)	208 $\pm$ 0.26
Organic carbon (%)	0.40 $\pm$ 0.01
Water holding capacity (%)	40.31
Sand %	64.97
Silt %	8.53
Clay %	22.04

Table 24: Effect of different potting mixtures on hardening of *in vitro* raised plantlets of *T.indica*

Potting mixtures	Number of plantlets transferred	Survival (%)	Shoot height (cm) (Mean $\pm$ SE)	Number of leaves/plant (Mean $\pm$ SE)
Soil: vermicompost (T1)	40	88 $\pm$ 0.57	15 $\pm$ 0.03	25 $\pm$ 0.50
Soil: vermicompost: <i>Azotobacter</i> : <i>Pseudomonas</i> (T2)	40	92 $\pm$ 0.57	19 $\pm$ 0.03	31 $\pm$ 0.33
Soil: <i>Azotobacter</i> : <i>Pseudomonas</i> (T3)	40	65 $\pm$ 0.88	14 $\pm$ 0.03	18 $\pm$ 0.7
Soil: <i>Azotobacter</i> (T4)	40	61 $\pm$ 0.88	13 $\pm$ 1.73	15 $\pm$ 0.7
Soil: <i>Pseudomonas</i> (T5)	40	59 $\pm$ 0.50	10 $\pm$ 1.13	10 $\pm$ 0.5

Data in terms of mean $\pm$ SE values

Fig. 31. Rooting of microshoots and acclimatization of plantlets.

- A. Healthy thick long roots formed on half strength basal MS medium.
- B. Rooting of microshoots after 20 days of culture on half strength liquid MS medium.
- C. Plantlet transferred to moist cotton for initial acclimatization.
- D. Plantlets in plastic cups containing different potting mixtures.
- E. Plantlet in poly bags under growth room conditions.
- F. Hardened plantlets in green house.
- G. Plantlets in earthen pots after 60 days of acclimatization.
- H. 6 months old well acclimatized plants in soil.

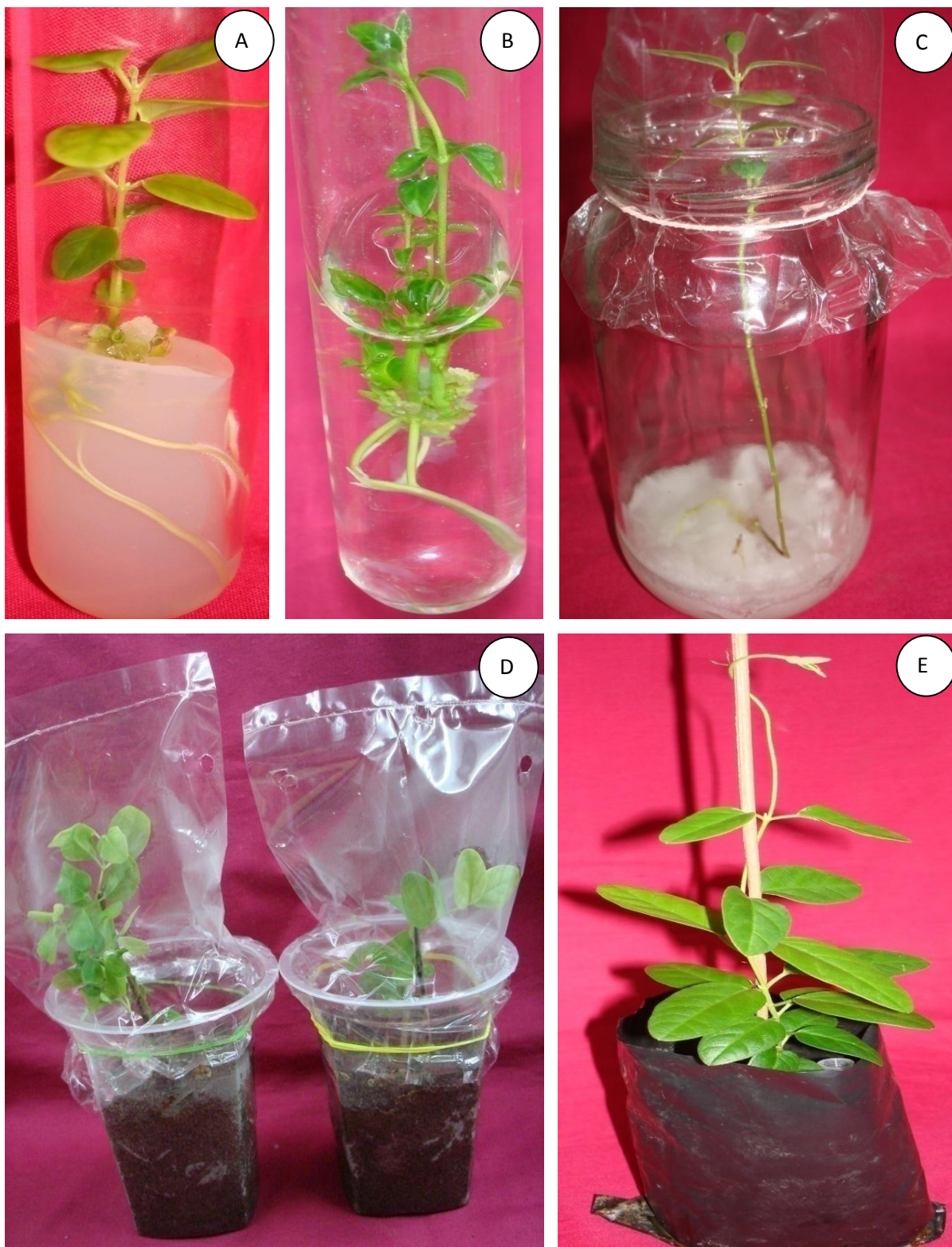


Fig. 31. A-E



Fig. 31. F, G & H

Objective 2: Extraction and characterization of major secondary metabolites from *in vitro* and *in vivo* cultures

4.3 *Stevia rebaudiana*

4.3.1 Estimation of carbohydrates, proteins and lipids (primary metabolites) from vegetative plant parts

Among different vegetative parts of *in vitro* raised plants of *Stevia rebaudiana*, the highest amount of total sugars was found in leaf explants (3.0 %) followed by stem and root which had 0.41 and 0.36 % of total sugars respectively. Likewise, the leaf contained the highest amount of reducing sugars (1.90 %) followed by stem (1.40 %) and root (1.24 %). Sucrose also followed a similar trend with highest content (2.16 %) present in the leaf followed by stem and root, which had 1.80 and 1.67 % sucrose respectively. The amount of starch was 1.26 % in leaves and 0.56 % in stem and root parts. There was no significant difference found in the level of starch in root and stem.

Protein and lipid contents showed almost similar trend in different vegetative parts of *Stevia rebaudiana* with maximum content found in the leaves, followed by almost comparable concentrations both in stem and root (Table 25). Proteins were 0.96 % in leaves and 0.64 % in stem and roots, while lipids were found to be 0.94 % in leaves, 0.68 and 0.69 % in stem and root respectively.

Table 25: Carbohydrate, protein and lipid contents (g/100g DW) in different vegetative parts of *in vitro* raised plants of *Stevia rebaudiana* (values are average of three replicates).

Experimental material		Total sugars	Reducing Sugars	Sucrose	Starch	Proteins	Lipids
<i>Stevia rebaudiana</i>	Leaf	3.00 ± 0.12	1.90 ± 0.17	2.16 ± 0.14	1.26 ± 0.01	0.96 ± 0.01	0.94 ± 0.08s
	Stem	0.41 ± 0.01	1.40 ± 0.06	1.80 ± 0.11	0.56 ± 0.01	0.64 ± 0.08	0.68 ± 0.01
	Root	0.36 ± 0.01	1.24 ± 0.01	1.67 ± 0.01	0.56 ± 0.01	0.64 ± 0.05	0.69 ± 0.08

4.3.2 Estimation of sterols, phenols and flavonoids (secondary metabolites) from vegetative plant parts

In *Stevia rebaudiana*, sterols, phenols and flavonoids were found to be highest in the leaves as compared to other plant parts (Table 26). The level of sterols and flavonoids were maximum in leaves (1.72 and 1.74 %) followed by stem (1.54 and 0.97%) and root (1.24 and 0.55%) respectively. The amount of phenols however, was maximum in leaves (3.90%) followed by root (2.20%) and stem (1.90 %).

Table 26: Secondary metabolites (g/100g DW) in different explants of *in vitro* raised plants of *Stevia rebaudiana* (values are average of three replicates).

Experimental material		Sterols	Phenols	Flavonoids
<i>Stevia rebaudiana</i>	Leaf	1.72 ± 0.09	3.90 ± 0.04	1.74 ± 0.09
	Stem	1.54 ± 0.09	1.90 ± 0.04	0.97 ± 0.06
	Root	1.24 ± 0.06	2.20 ± 0.08	0.55 ± 0.04

4.3.3 Extraction and purification of major secondary metabolite- stevioside from *Stevia rebaudiana*

The procedures for the extraction of sweet tasting stevioside from the leaves of *Stevia rebaudiana* is described in details in the material and methods section 3.5. The final extracts of different extraction protocols were evaporated in rotary flash evaporator at 60°C and the concentrates thus obtained, were resuspended in methanol for further purification to obtain pure stevioside fractions using various chromatographic techniques (discussed in detail in M & M section 3.7). For carrying out analysis of test samples, firstly the optimization of different parameters such as sample volume, developing solvent system and wavelength for standard (stevioside) was done using HPTLC.

4.3.3.1 Parameter optimization for HPTLC analysis of standard (stevioside)

For the optimization of developing solvent system, loading volume and wavelength of standard (stevioside), different volumes of stevioside standard (1, 2, 3, 4, 5 and 6 µl) were loaded on

HPTLC pre coated fluorescent (10 x 10 cm) plate. The plate was developed in mobile phase comprising of ethyl acetate: methanol: n-hexane, chloroform: methanol: water and ethyl acetate: methanol: water in different ratios and was scanned over wavelength range of 200 - 210 nm. Among all the trials, the best results in terms of percentage area were observed with 6 µl of standard in developing solvent system comprising of ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm. The plate developed in the above solvent system showed different peaks with fourth peak corresponding to stevioside when scanned at 210 nm (Fig. 32 A). UV absorbance spectra of the plate captured at 254 and 366 nm also showed stevioside bands (Fig. 32 B and C). The end position of stevioside peak was detected at R_f 0.50 covering 47.62 % peak area (Table 27). Thus, 6µl of stevioside was used as the optimum volume of standard in future experiments.

Fig. 32. HPTLC chromatograph of standard (stevioside) developed in solvent system comprising of ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm.

- A. Track showing the standard peak corresponding to stevioside (arrowhead).
- B. Photodocumentation pictures of plate at UV 254 nm showing stevioside spot.
- C. Photodocumentation pictures of plate at UV 366 nm showing stevioside spot.

Fig. 32. HPTLC chromatogram of standard (stevioside)

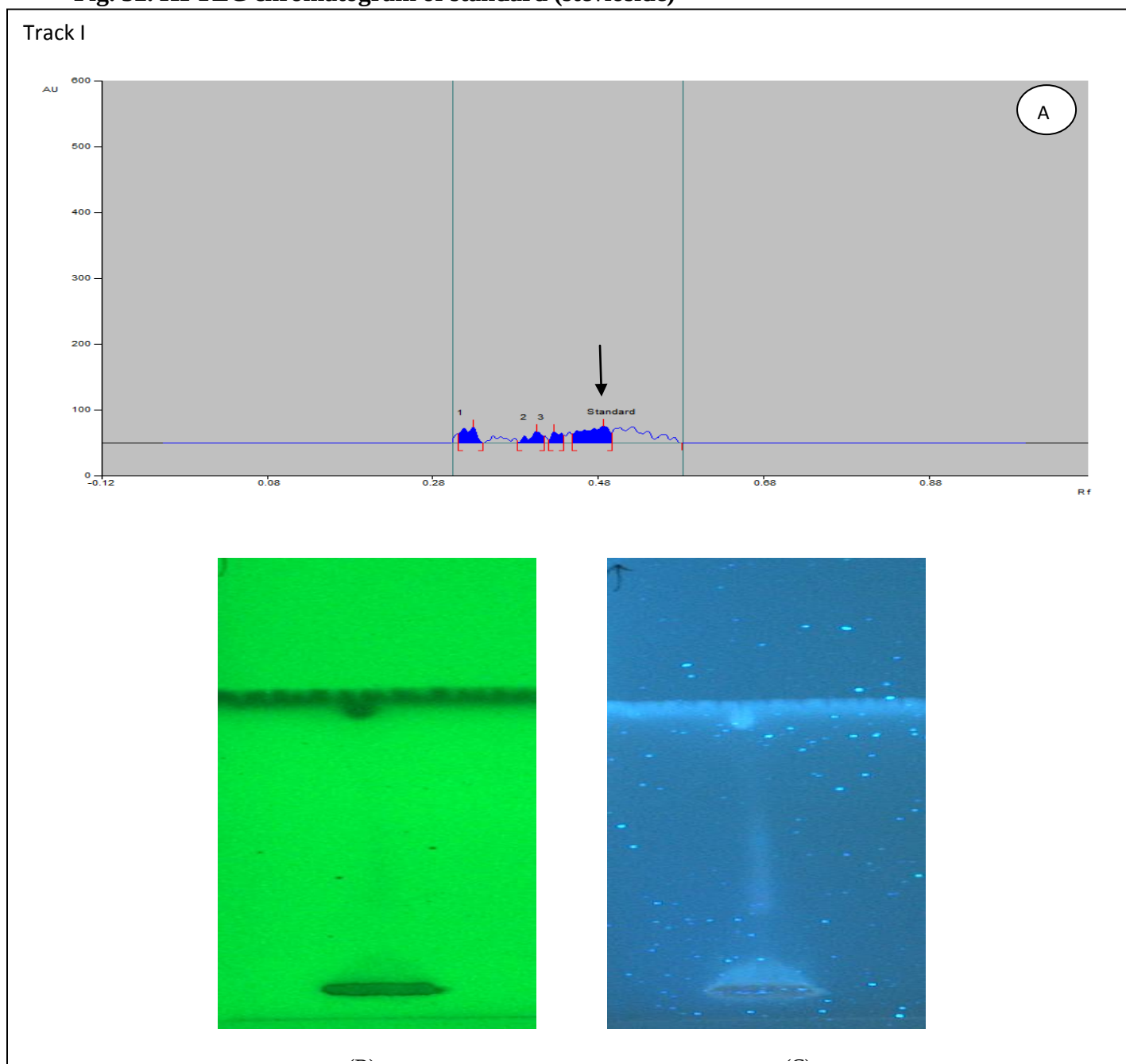


Table 27. Stevioside in standard track (in terms of Rf and peak area) when developed in ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm.

Track ID	Peak	End Position (Rf)	Area (AU)	Area %
Fig. 32 Track I (A)	4	0.50	908.9	47.62

4.3.3.2 Optimization of extraction protocol using HPTLC

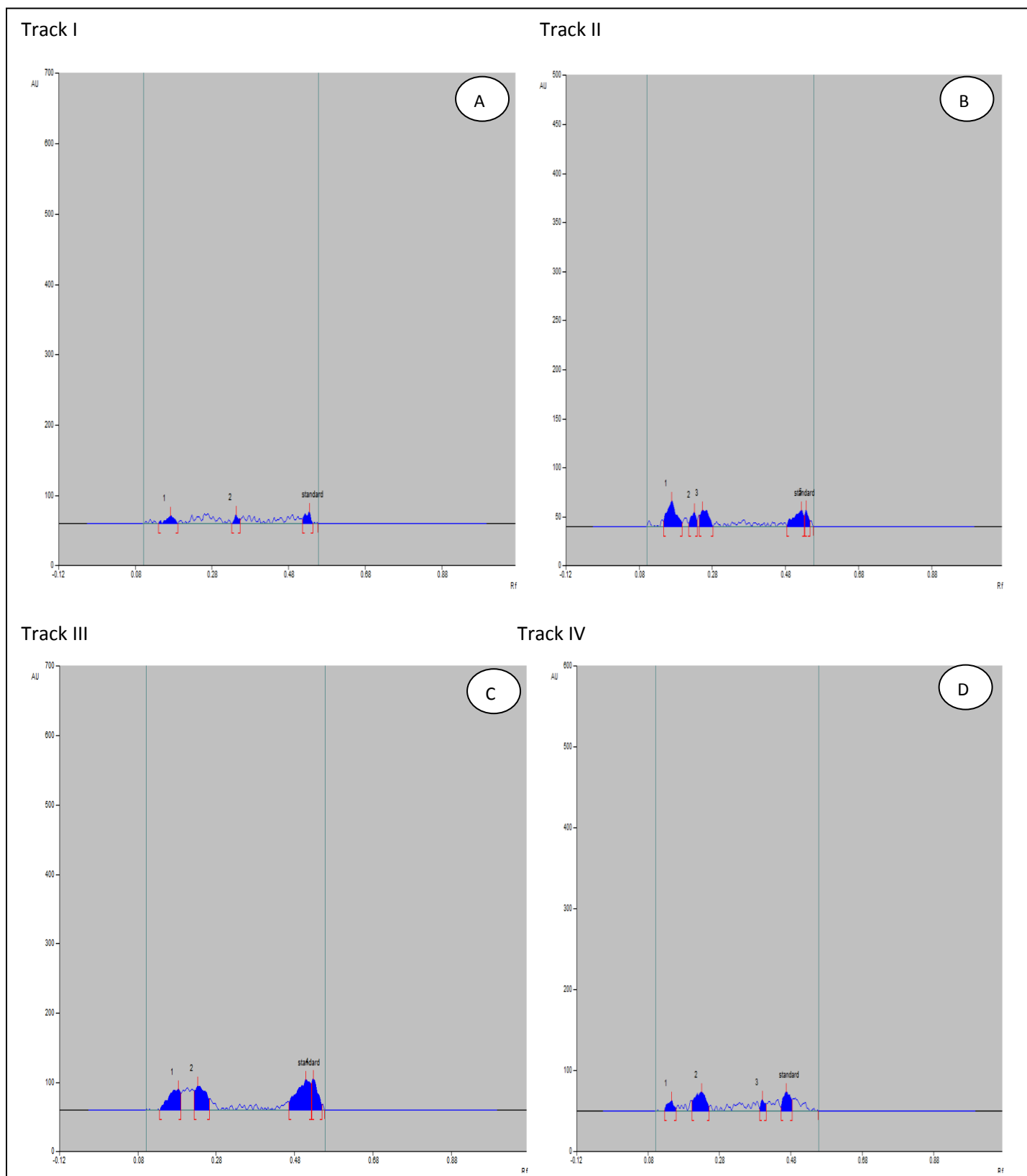
Methanolic fractions (6 µl each) of leaf extracts obtained using protocols I to IX and stevioside (standard) were loaded on pre coated silica gel 60 F 254 plate (20 X 20 cm) in separate tracks (Fig. 33 A- J). The plate was developed in various solvent systems comprising of different combinations and ratios of ethyl acetate: methanol: n-hexane, chloroform: methanol: water, ethyl acetate: methanol: ethanol: water and toluene: chloroform: methanol: water based upon the polarities of the solvents. Multi wavelength scanning of the plate was done from 200-700 nm to identify the most suitable wavelength of scan. Based on these trials, optimum conditions identified includes solvent system comprising of chloroform: methanol: water (7: 3:1) and wavelength 210 nm. Plate under these sets of conditions yielded following results:

On comparison of different tracks with the standard, it was found that track II, III, IV, VI, VII, VIII, X showed the presence of stevioside peaks. Three-dimensional plot of the plate at 210 nm was used to compare the stevioside peak in all the samples (Fig. 33 K). Photodocumentation pictures of the plate captured at two different UV ranges (254 and 366 nm) identified the stevioside bands in different tracks (Fig. 33 L and M). Quantitative analysis showed that maximum amount of stevioside (89.9 µg/ml) was detected in samples obtained through extraction protocol V whereas, least amount of stevioside (8 µg/ml) was obtained from leaf samples processed using protocol IX (Table 28). Extraction protocol V gave best results in terms of stevioside concentration at 210 nm with mobile phase consisting of chloroform: methanol: water (7:3:1).

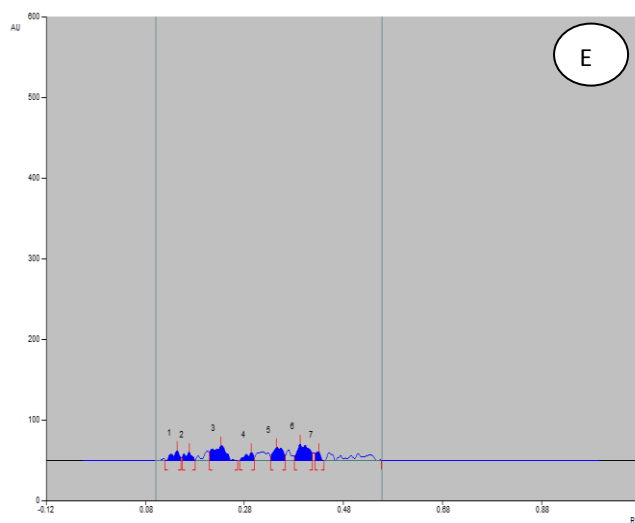
Fig. 33. Chromatograph of leaf extract of various extraction protocols, when the plate was developed in solvent system comprising of chloroform: methanol: water (7: 3:1) and scanned at 210 nm.

- A. Track I loaded with stevioside standard.
- B. Track II contains leaf sample processed using protocol I.
- C. Track III contains sample extracted as per protocol II.
- D. Track IV contains sample extracted as per protocol III.
- E. Track V contains sample processed using protocol IV.
- F. Track VI contains extract obtained using protocol V.
- G. Track VII contains extract obtained using protocol VI.
- H. Track VIII contains extract obtained using protocol VII.
- I. Track IX contains extract obtained using protocol VIII.
- J. Track X contains extract obtained using protocol IX.
- K. 3-Dimensional scan of all tracks at 210 nm.
- L. Photodocumentation pictures at 254 nm showing bands of stevioside in various tracks.
- M. Photodocumentation pictures at 366 nm showing bands of stevioside in various tracks.

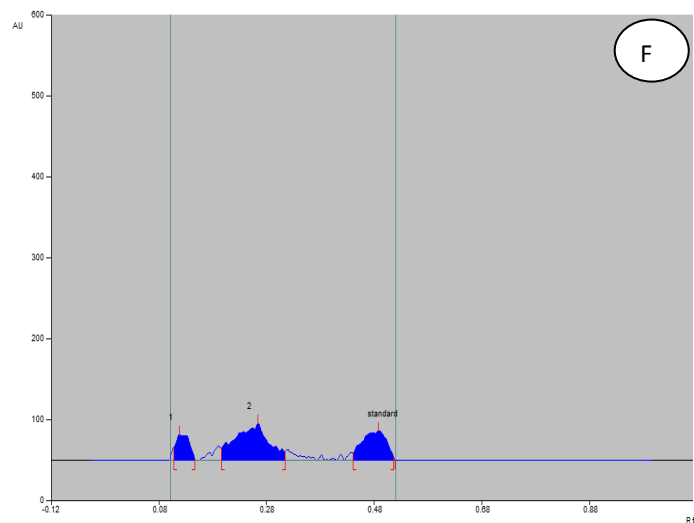
Fig. 33. HPTLC of leaf extract processed using different extraction protocols



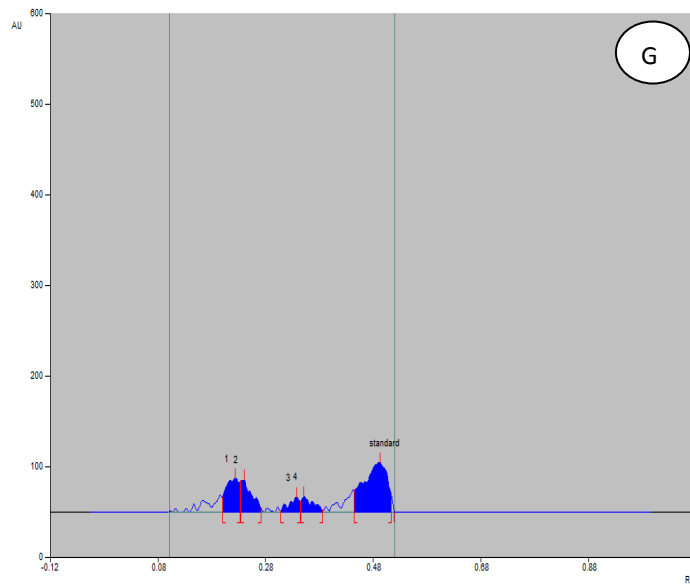
Track V



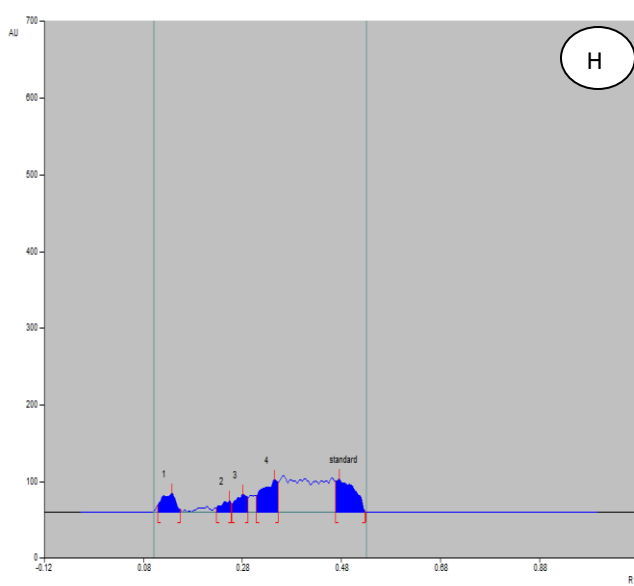
Track VI



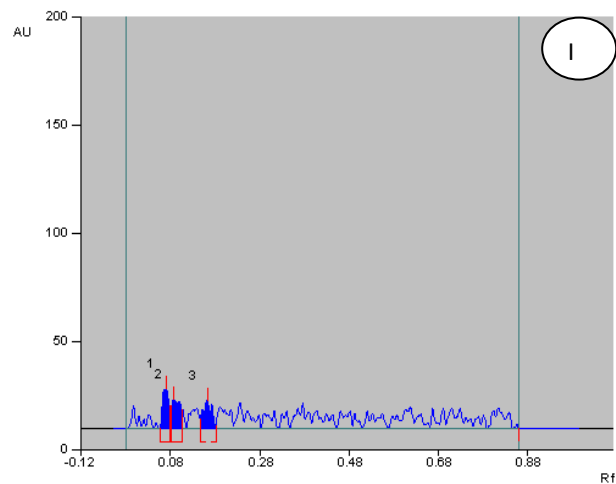
Track VII



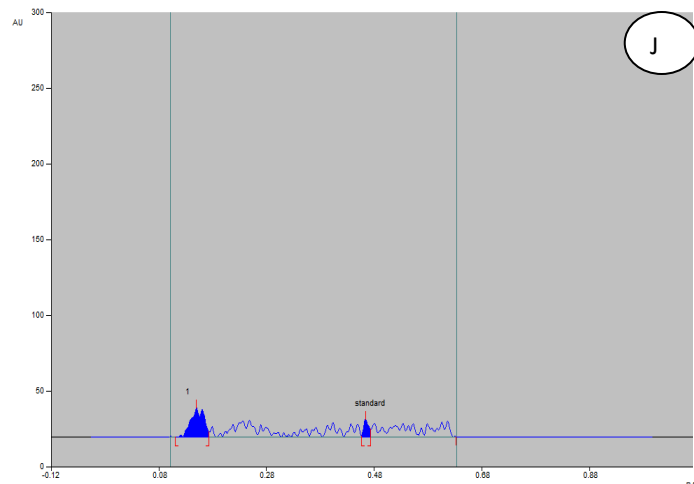
Track VIII

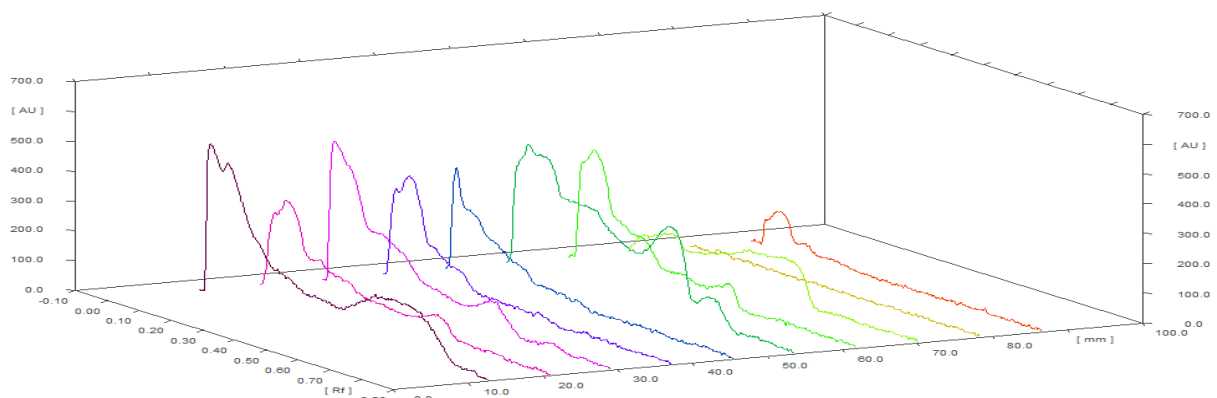


Track IX

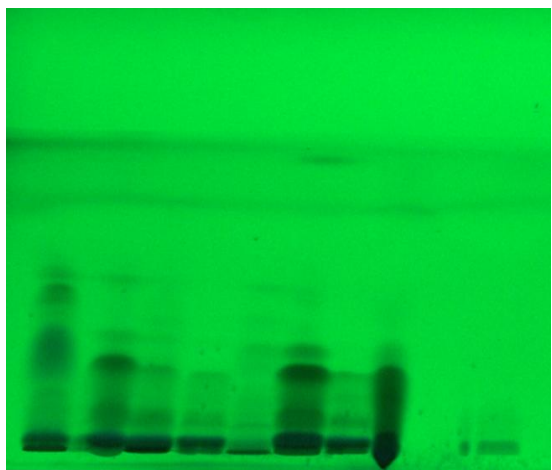


Track X

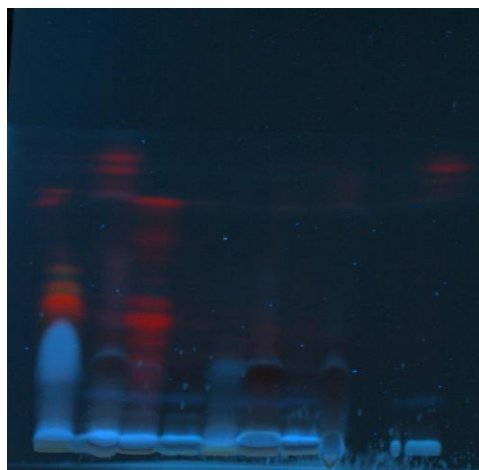




(K)



(L)



(M)

Table 28. Stevioside ($\mu\text{g/ml}$) in various tracks when developed in chloroform: methanol: water (7: 3:1) at 210 nm. Standard stevioside applied at a working concentration of 20.0 $\mu\text{g/ml}$.

Track ID	Peak	End Position (Rf)	Area (AU)	Area %	Stevioside ($\mu\text{g/ml}$)
Fig. 33 Track I (A)	3	0.52	191.8	41.80	-----
Fig. 33Track II (B)	4	0.53	332.3	23.28	34.61
Fig. 33Track III (C)	3	0.52	746.0	37.17	77.70
Fig. 33Track IV (D)	4	0.52	384.6	30.76	40.10
Fig. 33Track VI (F)	3	0.52	863.0	77.75	89.90
Fig. 33Track VII (G)	5	0.52	512.0	55.37	53.38
Fig. 33Track VIII (H)	5	0.52	391.0	30.00	40.77
Fig. 33Track X (J)	2	0.49	76.12	17.20	8.000

4.3.3.3 Stevioside in leaves of *in vitro* raised and *in vivo* plants after different intervals of time

4.3.3.3.1 Stevioside in leaf samples of three, four and five months old *in vitro* raised plants

Stevioside was extracted from the leaves of *in vitro* raised mother plants of *Stevia* harvested after different time intervals. Leaf samples of *in vitro* raised three, four and five months old plants processed using optimized extraction protocol V were primarily chromatographed on thin layer glass plate developed in solvent system comprising of chloroform: methanol: water (7:3:1). Track I was loaded with three months old leaf sample, track II with four months old and track III with five months old sample (Fig. 34). On comparison of all the spots in various tracks with the standard, it was observed that stevioside spot corresponding to Rf 0.50 was found in all the tracks (Table 29). The spots were visualized in iodine chamber.

For quantitative analysis, samples were analyzed using HPTLC. Methanolic extract (6 µl) of all the test samples were loaded on pre coated silica gel (5 X 10 cm) plate in three separate tracks. Track I contained leaf extract of three month old plant, track II contained extract of four month old and track III contained extract from five month old plants whereas, track IV was loaded with stevioside (standard) (Fig. 35 A-D). The plate was developed in same solvent system as used for TLC i.e. chloroform methanol: water (7:3:1) and was scanned at 210 nm. On comparison of all the tracks with the standard (Rf 0.50), it was found that all the samples contained the peaks corresponding to stevioside with similar Rf values and different area percentage. Auto spectra scan at 210 nm also revealed the presence of stevioside in different tracks (Fig. 35 E). UV absorbance spectra of plate captured at 254 and 366 nm showed distinct stevioside bands in all the tracks (Fig. 35 F and G). Highest amount of stevioside (68 µg/ml) was detected in three month old plant extract, followed by four month old and five month old plant samples, which contained 52.7 and 44.1 µg/ml of stevioside respectively (Table 30).

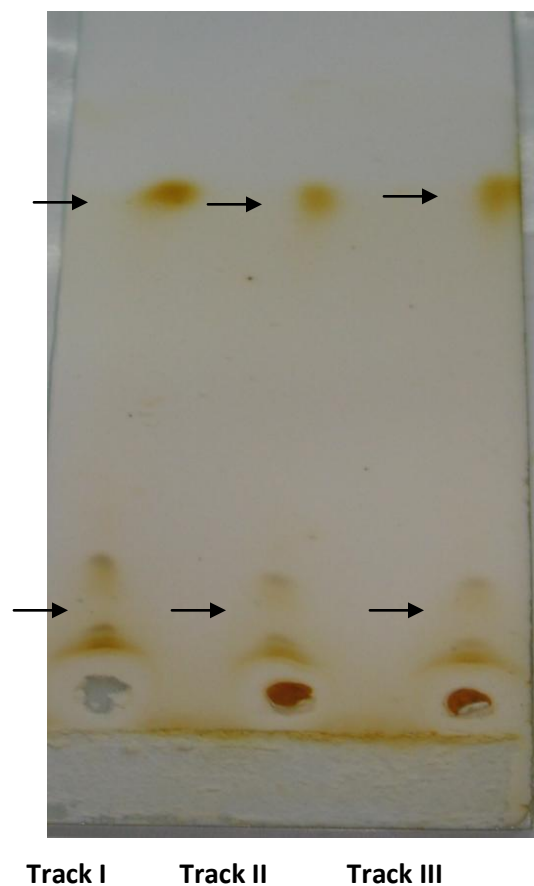


Fig. 34. TLC profile of leaf samples from three, four and five month's old plants of *Stevia rebaudiana*

Track I: Leaf sample from three months old plant

Track II: Leaf sample from four months old plant

Track III: Leaf sample from five months old plant

Table 29. Stevioside spots in different tracks when developed in iodine chamber.

Table 5

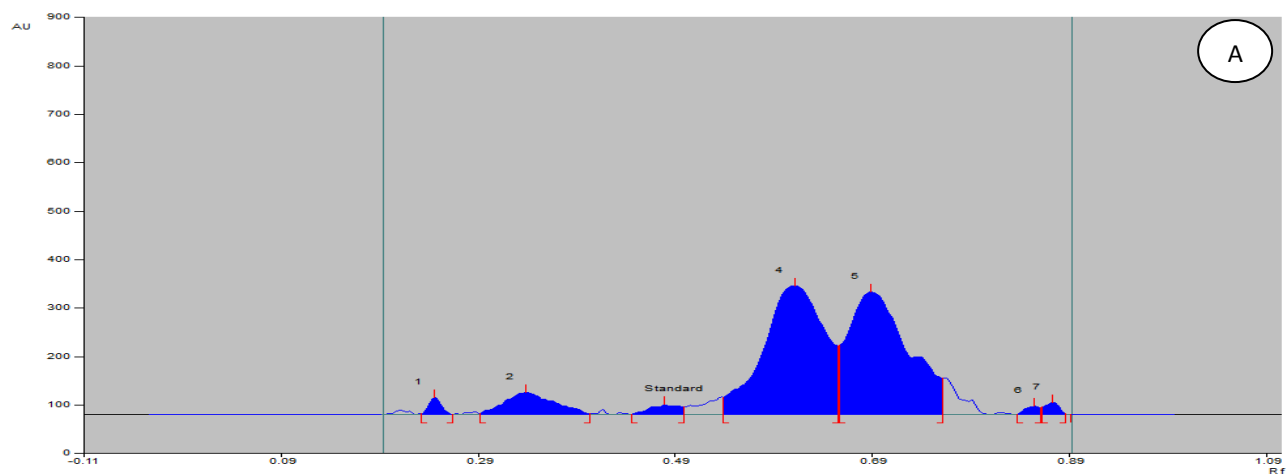
Track ID	Number of spots	Rf values	Colour with iodine vapors
I	1	0.17	Light Brown
	2	0.50	Dark Brown
II	1	0.14	Light Brown
	2	0.50	Dark Brown
III	1	0.15	Light Brown
	2	0.50	Dark Brown

Fig. 35. Chromatogram of leaf samples of three, four and five months old plants on pre coated HPTLC plate developed in chloroform: methanol: water (7:3:1) and scanned at 210 nm.

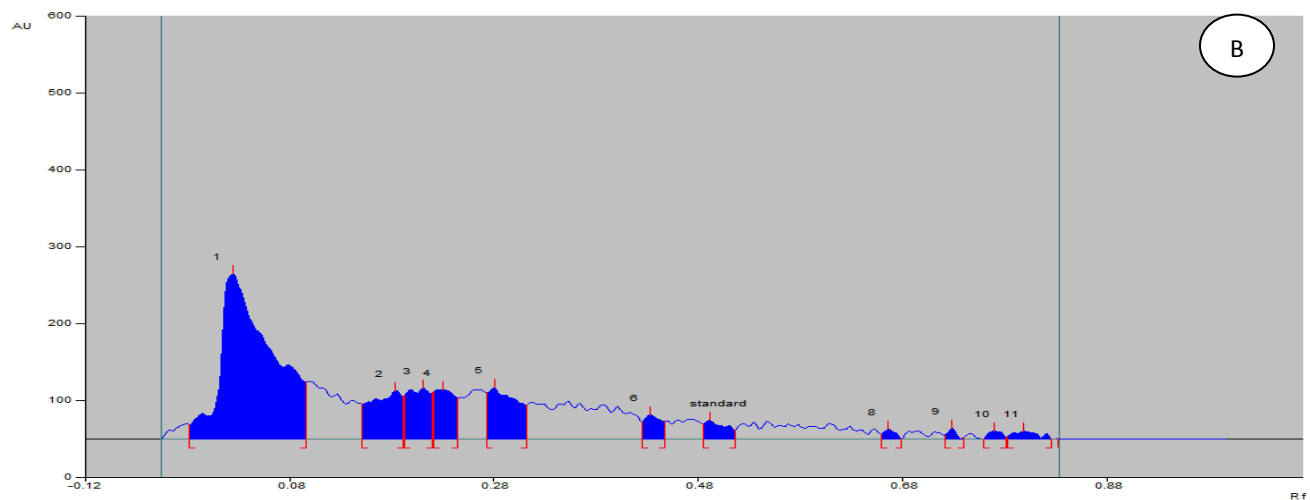
- A. Track I loaded with leaf sample from three month old plant.
- B. Track II loaded with leaf sample from four month old plant.
- C. Track III loaded with leaf sample from five month old plant.
- D. Track IV loaded with stevioside (standard).
- E. 3-D spectra showing stevioside peaks in all the tracks at 210 nm.
- F. UV absorbance spectra at 254 nm showing distinct bands of stevioside in different tracks.
- G. UV absorbance spectra at 366 nm showing bands of stevioside in various tracks.

Fig. 35. HPTLC chromatograms of three, four and five months old leaf samples

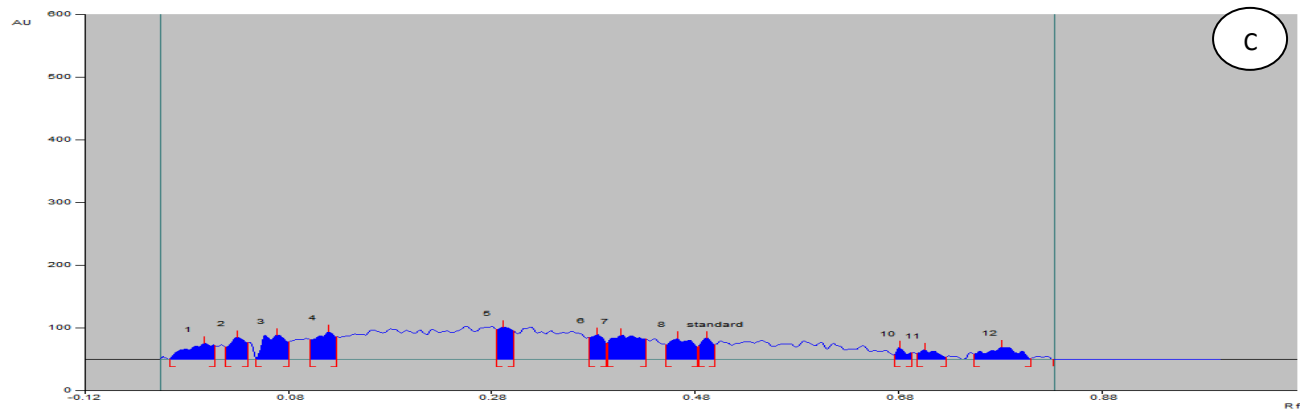
Track I



Track II



Track III



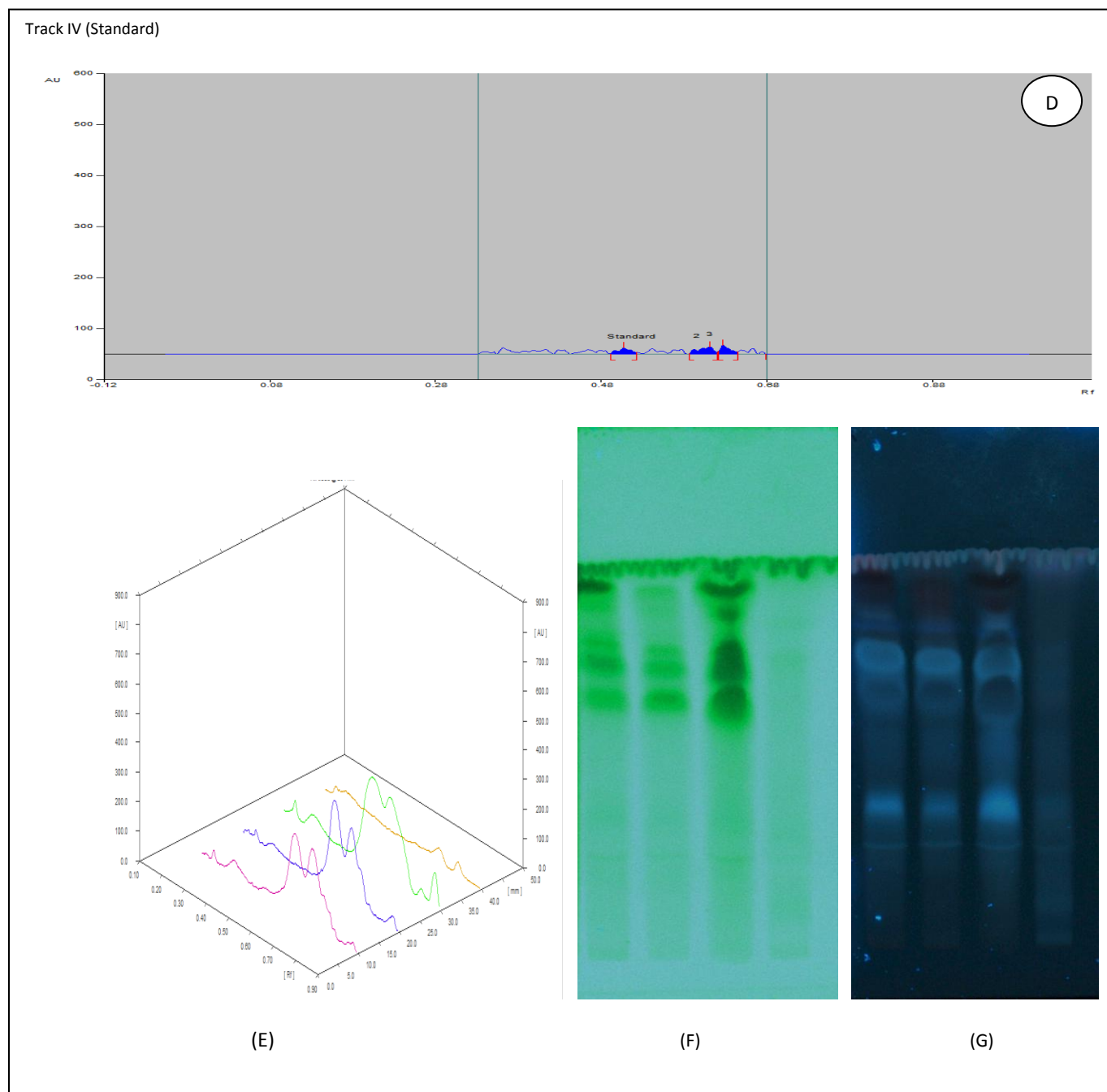


Table 30. Stevioside ($\mu\text{g/ml}$) in different tracks developed using chloroform: methanol: water (7:3:1). Standard stevioside applied at a working concentration of $20.0 \mu\text{g/ml}$.

Track ID	Peak	End Position (Rf)	Area (AU)	Area %	Stevioside ($\mu\text{g/ml}$)
Fig. 35 Track I (A)	3	0.49	518.0	1.69	68.0
Fig. 35 Track II (B)	7	0.50	401.0	2.70	52.7
Fig. 35 Track III (C)	9	0.50	335.2	5.61	44.1
Fig. 35 Track IV (D)	1	0.50	151.9	29.4	-----

4.3.3.3.2 Stevioside in leaves of eighteen and thirty months old *in vitro* raised field established and *in vivo* plants

Stevioside was also analyzed from leaf samples excised from eighteen and thirty month's old *in vitro* raised and *in vivo* plants. Processing of leaves was done using extraction protocol V and purification was done primarily with TLC followed by HPTLC and HPLC. For TLC analysis, leaf samples of eighteen and thirty months old *in vitro* raised plants were loaded in track I and II whereas, track III and IV contained samples of eighteen and thirty months old *in vivo* plants (Fig. 36). The plate was developed in solvent system comprising of chloroform: methanol: water (7:3:1). On comparison of all the tracks with standard, it was observed that stevioside spot was found in all the tracks (Table 31). Further analysis was done using HPLC and HPTLC.

Crude leaf extract (6 µl each) from eighteen and thirty months old *in vitro* raised plants were loaded on pre-coated (10 X10 cm) HPTLC plate in track I and track II respectively. Track III and IV contained 4 µl and 6 µl of standard whereas track V and VI were loaded with leaf extract (6 µl each) obtained from eighteen and thirty months old *in vivo* mother plants. The plate was developed in two different solvent systems comprising of chloroform: methanol: water (7:3:1) and ethyl acetate: methanol: n-hexane (2:2:5) and was scanned at 210 nm. Plate in first solvent system showed the presence of five peaks in track I, twenty different peaks in track II, eighteen and eight different peaks in track V and VI respectively (Fig 37 A-F). On comparing the R_f with standard showing stevioside peak at R_f 0.50, it was concluded that all the tracks contained the stevioside peaks. However, standard in track III at lower concentration (4 µl) did not show any peak corresponding to stevioside.

Another plate was loaded with same samples from eighteen and thirty months old *in vitro* plants and *in vivo* plants in separate tracks and was developed in solvent system comprising of ethyl acetate: methanol: n-hexane (2:2:5) (Fig. 37 G-L). Stevioside standard in track IV showed the presence of three different peaks with second peak corresponding to stevioside at R_f value of 0.47 whereas standard in track III failed to show the stevioside peak. On comparison of all the sample peaks with the standard, presence of stevioside peak was found in all the tracks. 3-D comparison of peaks in all the tracks was done at 210 nm, which clearly showed the presence of

stevioside peaks in different tracks (Fig. 37 M). To study the banding pattern for stevioside the plate was photographed at two different UV ranges 254 nm and 366 nm (Fig. 37 N and O).

Quantitative analysis of all the peaks showed the presence of stevioside in both the samples obtained from eighteen and thirty month's old *in vitro* raised and *in vivo* plant samples. Crude samples in solvent system of ethyl acetate: methanol: n-hexane (2:2:5) and scanned at 210 nm showed 92.7, 60 µg/ml of stevioside in thirty and eighteen months old *in vitro* raised plants respectively whereas thirty and eighteen months old *in vivo* plant samples contained 23.7 and 14.2 µg/ml of stevioside (Table 32). Further it was concluded that crude sample in solvent system of chloroform: methanol: water (7:3:1) and scanned at 210 nm offers optimum set of conditions for quantification of stevioside with highest amount (94.9 µg/ml) being found in thirty months old *in vitro* raised plants and 86.8 µg/ml in eighteen month old plants. Thirty months old *in vivo* plants contained 52.6 µg/ml while eighteen months old contained 48.0 µg/ml of stevioside when chromatographed using same solvent system.

HPLC analysis of isolated stevioside samples (10 µl) from thirty months old *in vitro* raised and *in vivo* plants was carried using methanol: water (8:2) as mobile phase and C₁₈ column. The HPLC column was maintained at thermostat temperature of 50° C. On comparison of sample with standard (retention time of 16 min and 79.90 % peak area), peaks 11 and 12 corresponded to stevioside peaks both *in vitro* and *in vivo* plants with retention time of 15.947 minutes with 46.24 % peak area and 15.861 minutes with 28.97 % peak area respectively (Fig. 38). Table 33 shows the HPLC chromatogram analysis of different fractions for isolated stevioside. Thus, both *in vitro* and *in vivo* plant samples showed the presence of stevioside. Thus, leaf samples obtained from thirty months old *in vitro* raised plants of *Stevia rebaudiana* yielding maximum amount of stevioside were selected for further purification using column chromatography to obtain pure stevioside fraction.

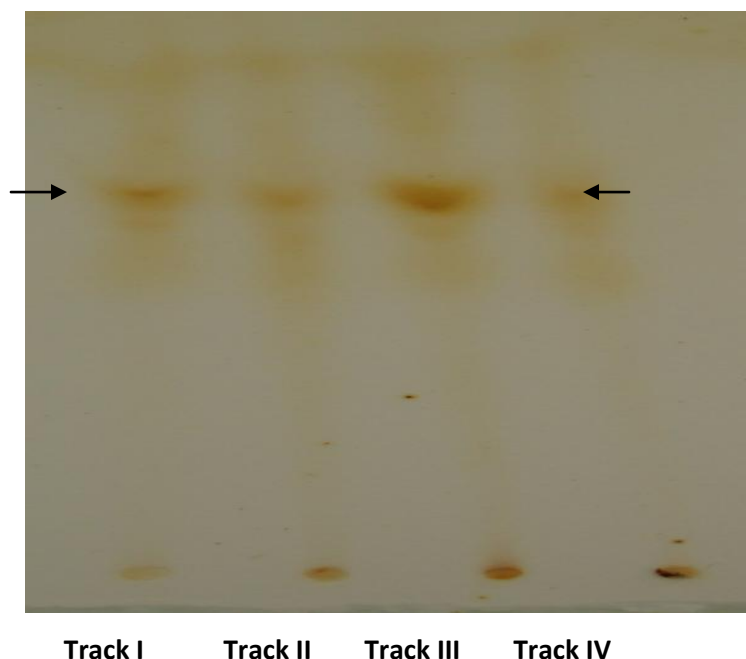


Fig. 36. TLC profile of leaf samples of *in vitro* and *in vivo* plant samples

Track I: Leaf samples from eighteen months old *in vitro* plants

Track II: Leaf samples from thirty months old *in vitro* plants

Track III: Leaf samples from eighteen months old *in vivo* plants

Track IV: Leaf samples from thirty months old *in vivo* plants

Table 31. Stevioside spots when visualized in iodine chamber

Track ID	Number of spots	Rf values	Colour with iodine vapors
Fig. 36. Track I	1	0.51	Light Brown
Fig. 36. Track II	1	0.50	Light Brown
Fig. 36. Track III	1	0.52	Dark Brown
Fig. 36. Track IV	1	0.50	Light Brown

Fig. 37. HPTLC chromatogram of leaf extracts of *in vitro* and *in vivo* plants.

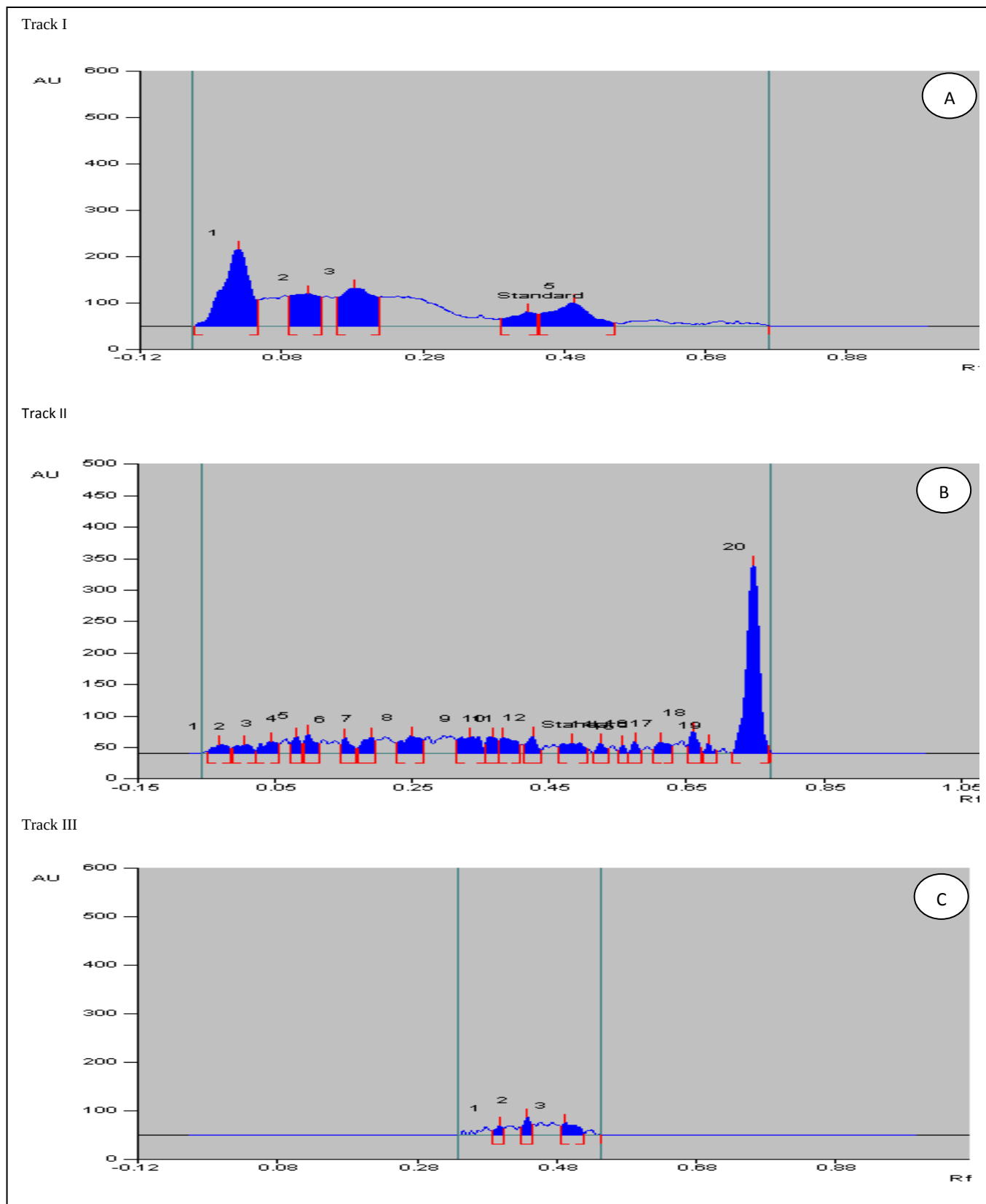
Leaf extract in solvent system comprising of chloroform: methanol: water (7:3:1).

- A. Track I loaded with leaf sample from eighteen months old *in vitro* raised plant.
- B. Track II loaded with leaf sample from thirty months old *in vitro* raised plant.
- C. Track III contained 4µl of stevioside standard.
- D. Track IV contained 6µl of stevioside standard.
- E. Track V contains leaf sample from eighteen months old *in vivo* plant.
- F. Track VI contains leaf sample from thirty months old *in vivo* plant.

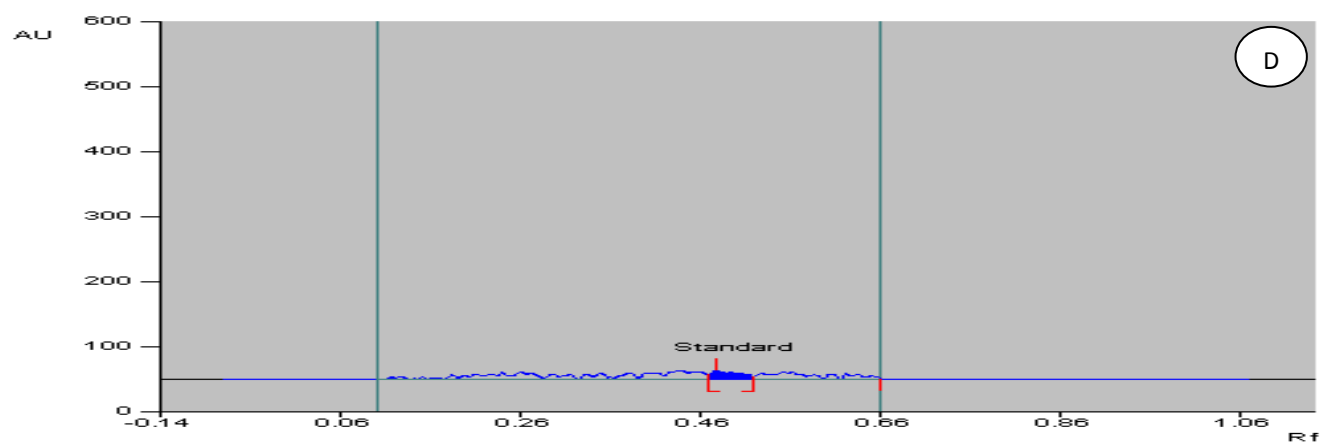
Leaf extract in solvent system comprising of ethyl acetate: methanol: n-hexane (2:2:5)

- G. Track I loaded with leaf extract of eighteen months old *in vitro* raised plant.
- H. Track II loaded with leaf extract of thirty months old *in vitro* raised plant.
- I. Track III loaded with 4µl of stevioside standard.
- J. Track IV loaded with 6µl of stevioside standard.
- K. Track V loaded with leaf extract of eighteen months old *in vivo* mother plant.
- L. Track VI loaded with sample from thirty months old *in vivo* mother plant.
- M. 3-D Display of all tracks at 210 nm.
- N. Photodocumentation pictures showing stevioside band at 254 nm.
- O. Photodocumentation pictures of plate at 366 nm showing stevioside bands in various tracks.

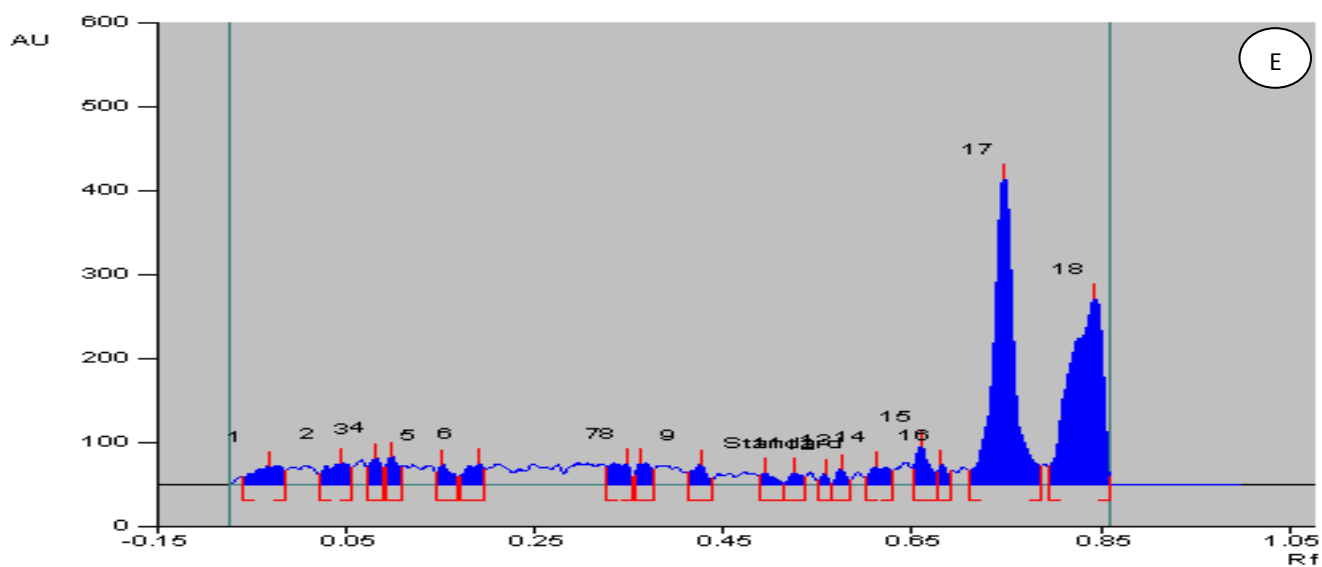
Fig. 37. Crude leaf extract of eighteen and thirty months old *in vitro* raised and *in vivo* plants



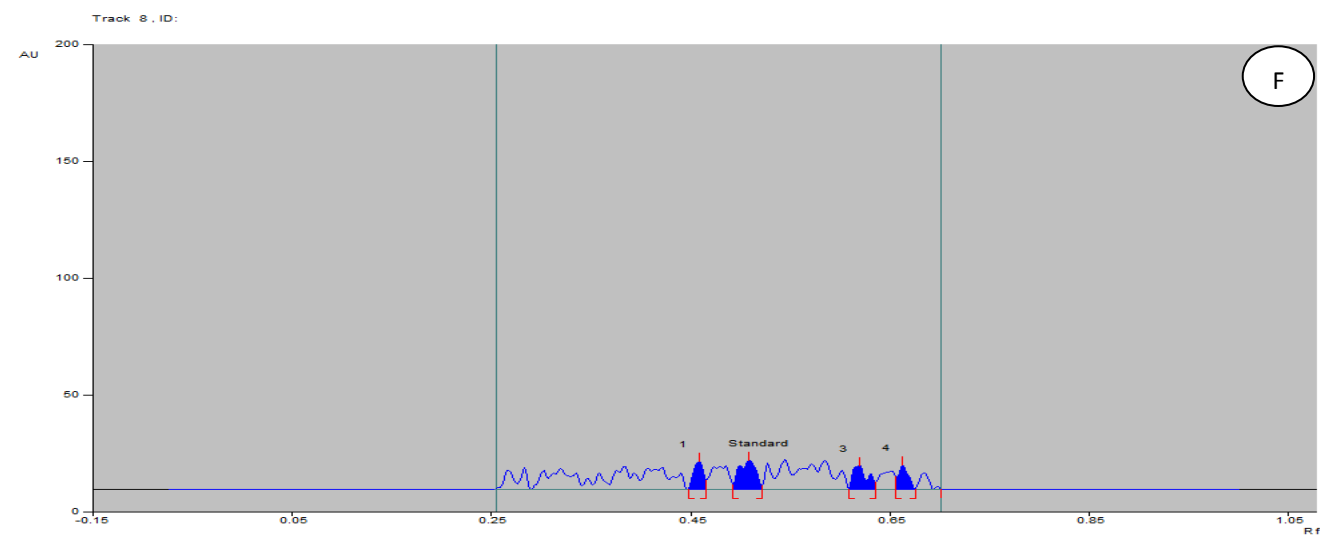
Track IV



Track V

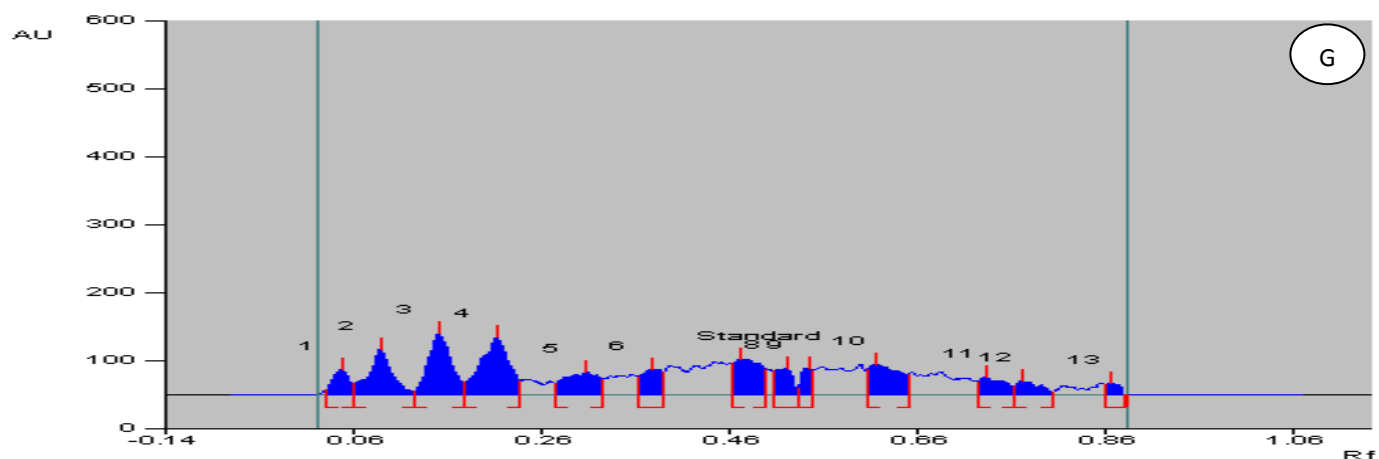


Track VI

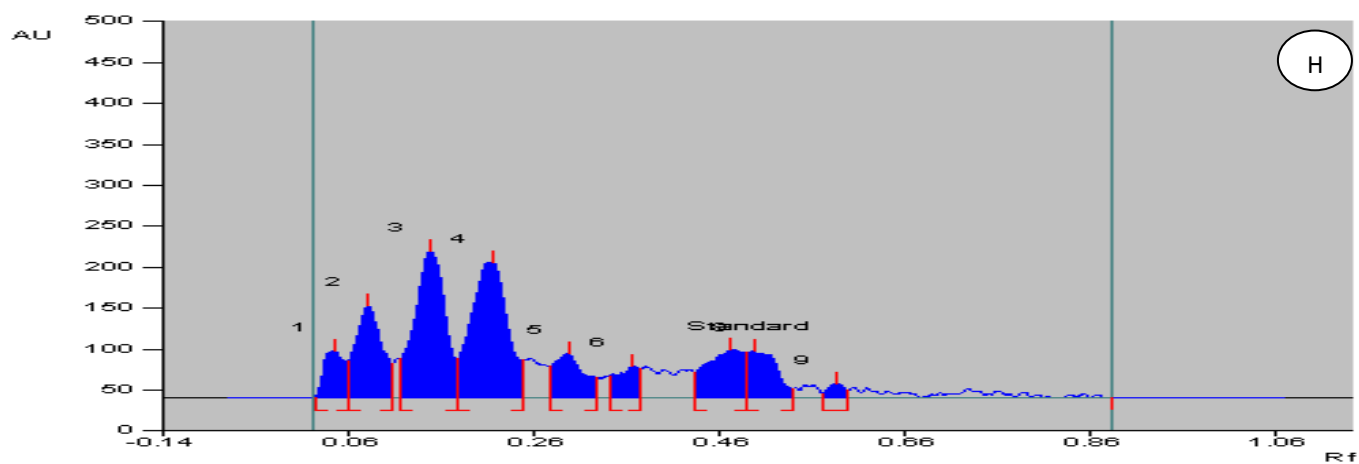


HPTLC chromatogram in ethyl acetate: methanol: n-hexane (2:2:5)

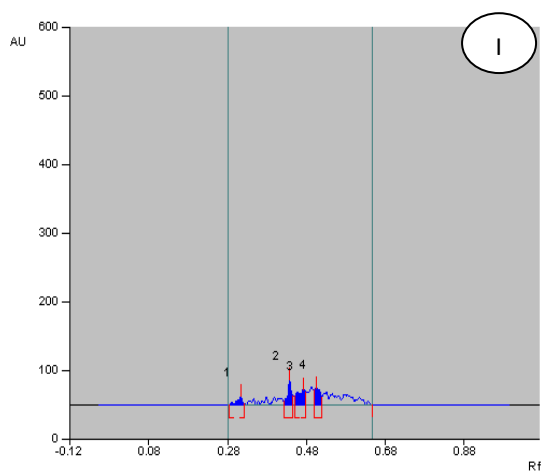
Track I



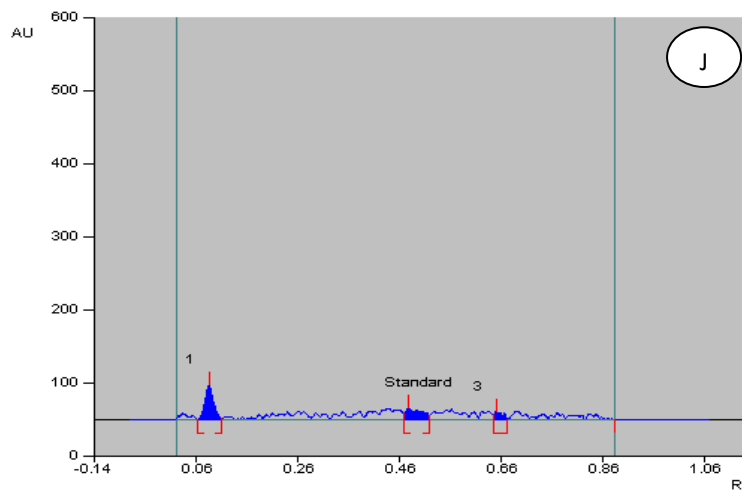
Track II



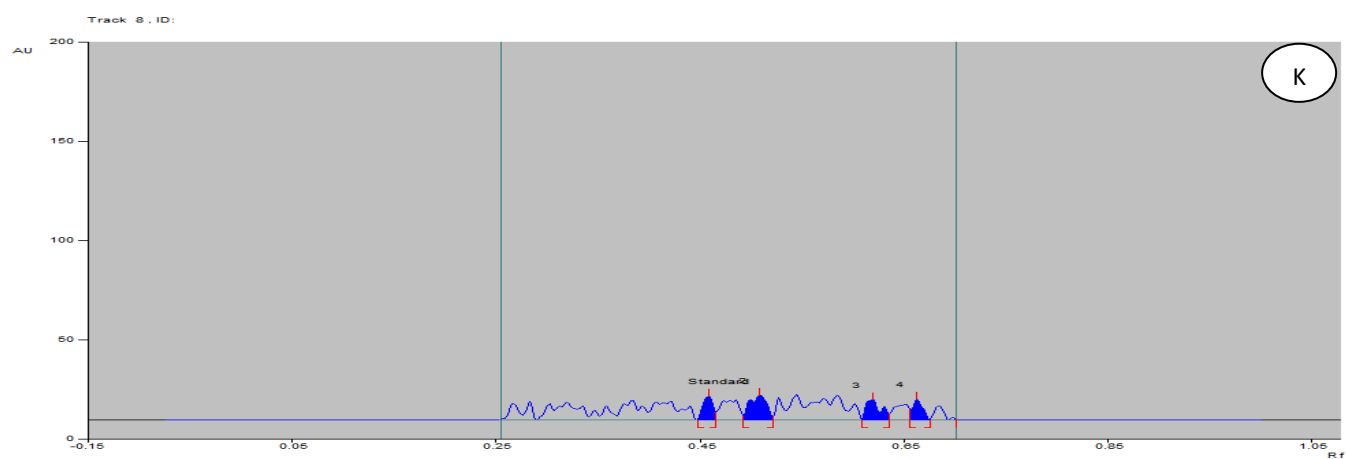
Track III



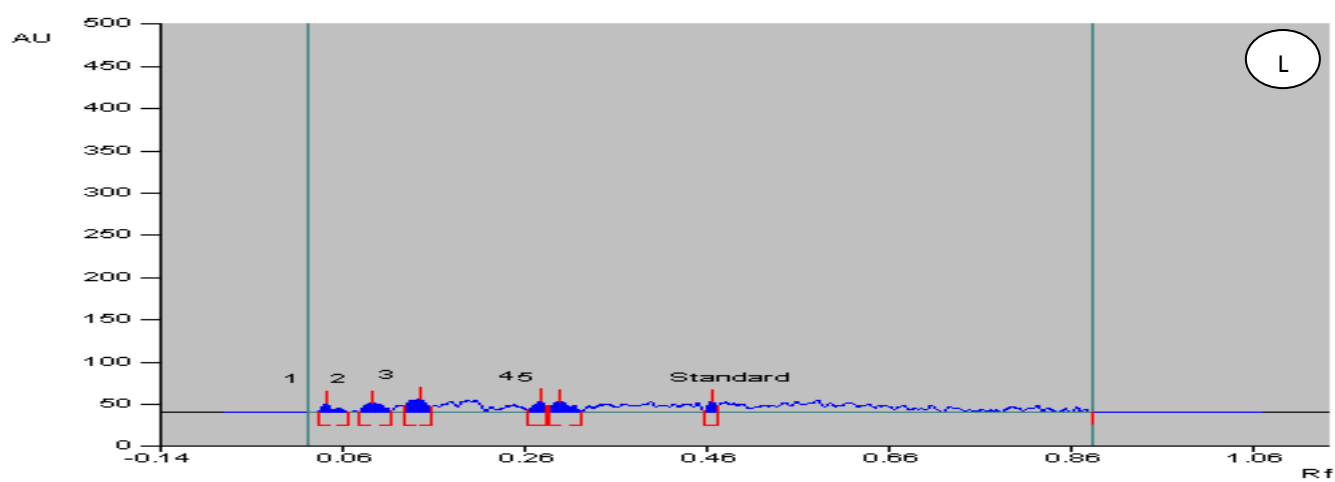
Track IV



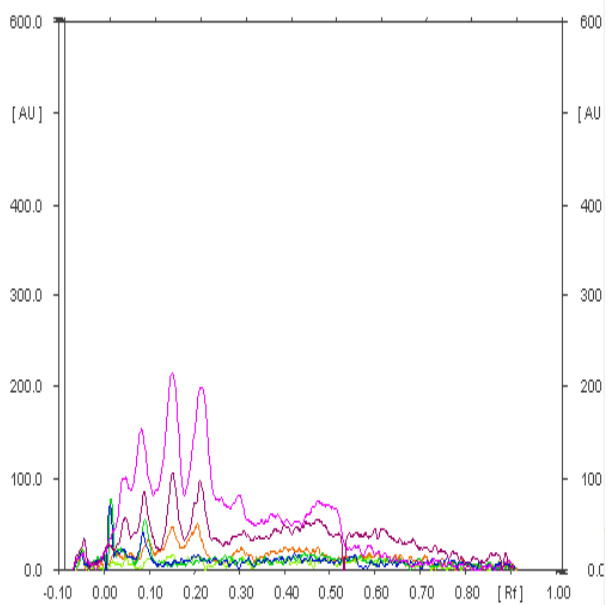
Track V



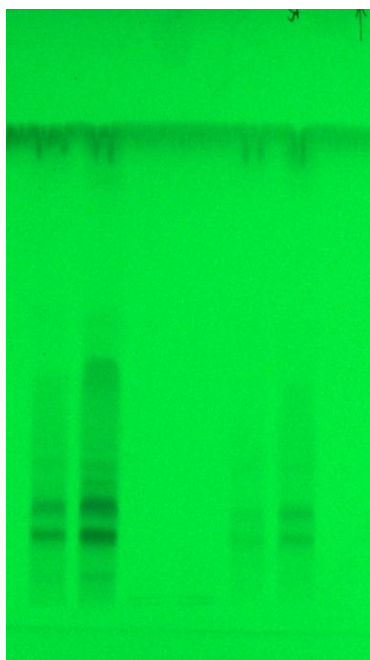
Track VI



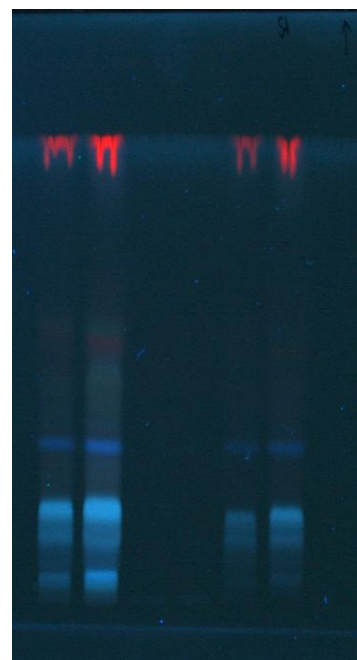
All tracks @ 210 nm



(M)



(N)



(O)

Table 32. Stevioside ($\mu\text{g/ml}$) in various tracks developed using different solvent systems. Standard stevioside applied at a working concentration of 20.0 $\mu\text{g/ml}$.

Track ID	Solvent system	Peak	End Position (Rf)	Area (AU)	Area %	Stevioside ($\mu\text{g/ml}$)
Fig. 37. Track 1 (A)	Chloroform: methanol: water (7:3:1)	4	0.48	262.5	6.68	86.8
Fig. 37. Track II (B)	Chloroform: methanol: water (7:3:1)	13	0.51	286.2	4.02	94.9
Fig. 37. Track IV (D)	Chloroform: methanol: water (7:3:1)	1	0.51	60.30	100	---
Fig. 37. Track V (E)	Chloroform: methanol: water (7:3:1)	10	0.51	147.2	0.92	48.0
Fig. 37. Track VI (F)	Chloroform: methanol: water (7:3:1)	2	0.51	158.8	35.87	52.6
Fig. 37. Track I (G)	Ethyl acetate: methanol: n-hexane (2:2:5)	7	0.47	1225	9.79	60.0
Fig. 37. Track II (H)	Ethyl acetate: methanol: n-hexane (2:2:5)	8	0.47	1892	10.9	92.7
Fig. 37. Track IV (J)	Ethyl acetate: methanol: n-hexane (2:2:5)	2	0.47	408.0	32.6	-----
Fig. 37. Track V (K)	Ethyl acetate: methanol: n-hexane (2:2:5)	1	0.47	291	20.6	14.2
Fig. 37. Track VI (L)	Ethyl acetate: methanol: n-hexane (2:2:5)	6	0.46	485.0	8.90	23.7

Fig. 38. HPLC Chromatogram of leaf extract from *in vitro* and *in vivo* plants

- A. Chromatogram of extract of thirty months old *in vitro* raised plant.
- B. Chromatogram of extract of thirty months old *in vivo* plant.
- C. Chromatogram of standard stevioside.

Fig. 38. HPLC chromatogram of leaf samples from thirty months old *in vitro* and *in vivo* plants.

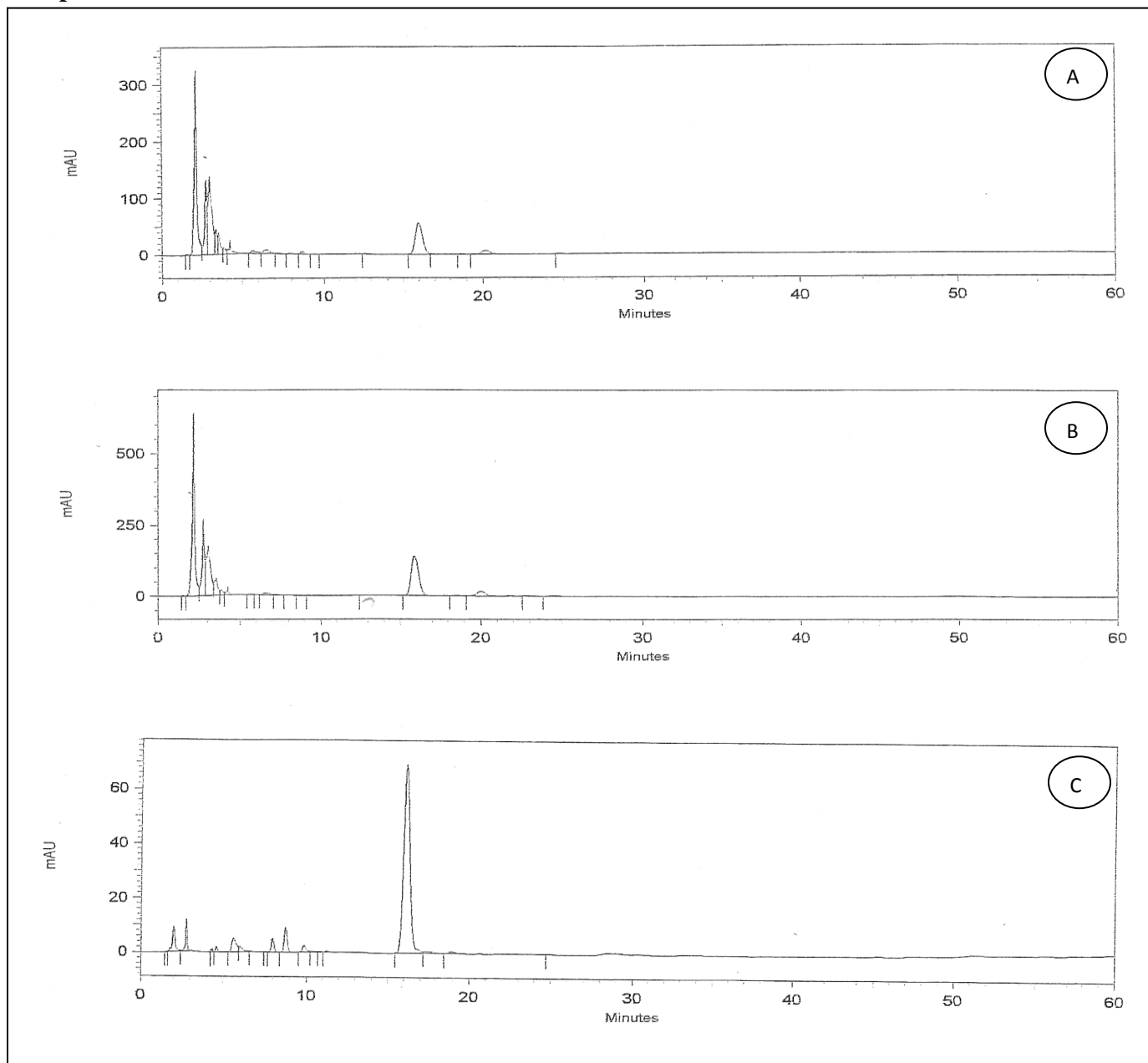


Table 33. HPLC chromatogram analysis of different fractions

Track ID	Peak number	Retention Time (minutes)	Area (mAU)	Area Percent
Fig. 38 (A)	12	15.947	2102806	46.24
Fig. 38 (B)	11	15.861	2994173	28.97
Fig. 38 (C)	10	16.000	2125769	79.90

4.3.3.4 Purification using column chromatography

The crude leaf mother extract of thirty months old *in vitro* raised plant of *Stevia rebaudiana* obtained through extraction protocol V was fractionated by Silica gel (60-120 mesh size) column chromatography. The column was eluted with increasing polarities of n-hexane and ethyl acetate (10, 20, 30, 40 and 50%). Elutes collected regularly as different fractions contained a number of compounds. The mixture was subjected to HPTLC analysis on pre coated silica gel 60 F 254 (20 X 20 cm) plate.

The collected fractions were loaded in separate tracks and were scanned at 210 nm using chloroform: methanol: water (7:3:1) and ethyl acetate: methanol: n-hexane (2:2:5) as developing solvents. In first set, 20 different fractions (6 µl each) were developed using chloroform: methanol: water (7:3:1). On comparison of peaks with the standard, it was observed that stevioside peak is present in track IX, X, XI, XII and XIII (Fig. 39 A - F). Highest area percentage of stevioside (43.71%) was observed in second peak in track X whereas lowest area percentage of stevioside (4.18 %) was observed in ninth peak of XIII track. Same set of twenty fractions were loaded on another pre-coated silica gel plate and were developed in solvent system comprising of ethyl acetate: methanol: n-hexane (2:2:5). On comparison of different fractions peaks with the standard, presence of stevioside was observed in track IV, VI, IX, XIV, XVI, XVIII and XIX (Fig. 39 G-N). Highest area percentage of stevioside (14.72 %) was observed in track IV whereas lowest percentage of stevioside (4.96 %) was in track XVI. Table 34 showed the percentage area of stevioside peak in different tracks. 3-D spectra of all the peaks at 210 nm were used to compare the stevioside peaks in different tracks (Fig. 39 O and P). Fingerprint pictures were captured to study the banding pattern of stevioside in different tracks. Distinct stevioside bands were seen when the plate was scanned at UV 366 nm (Fig. 39 Q). Among all the different column fractions showing stevioside peaks, fraction tenth showed the highest percentage of peak area of stevioside when developed in chloroform: methanol: water (7:3:1). Thus, tenth fraction was further loaded on preparative glass plates for next stage of purification using preparative TLC.

Fig. 39. HPTLC chromatogram of different column fractions of thirty months old *in vitro* raised plants.

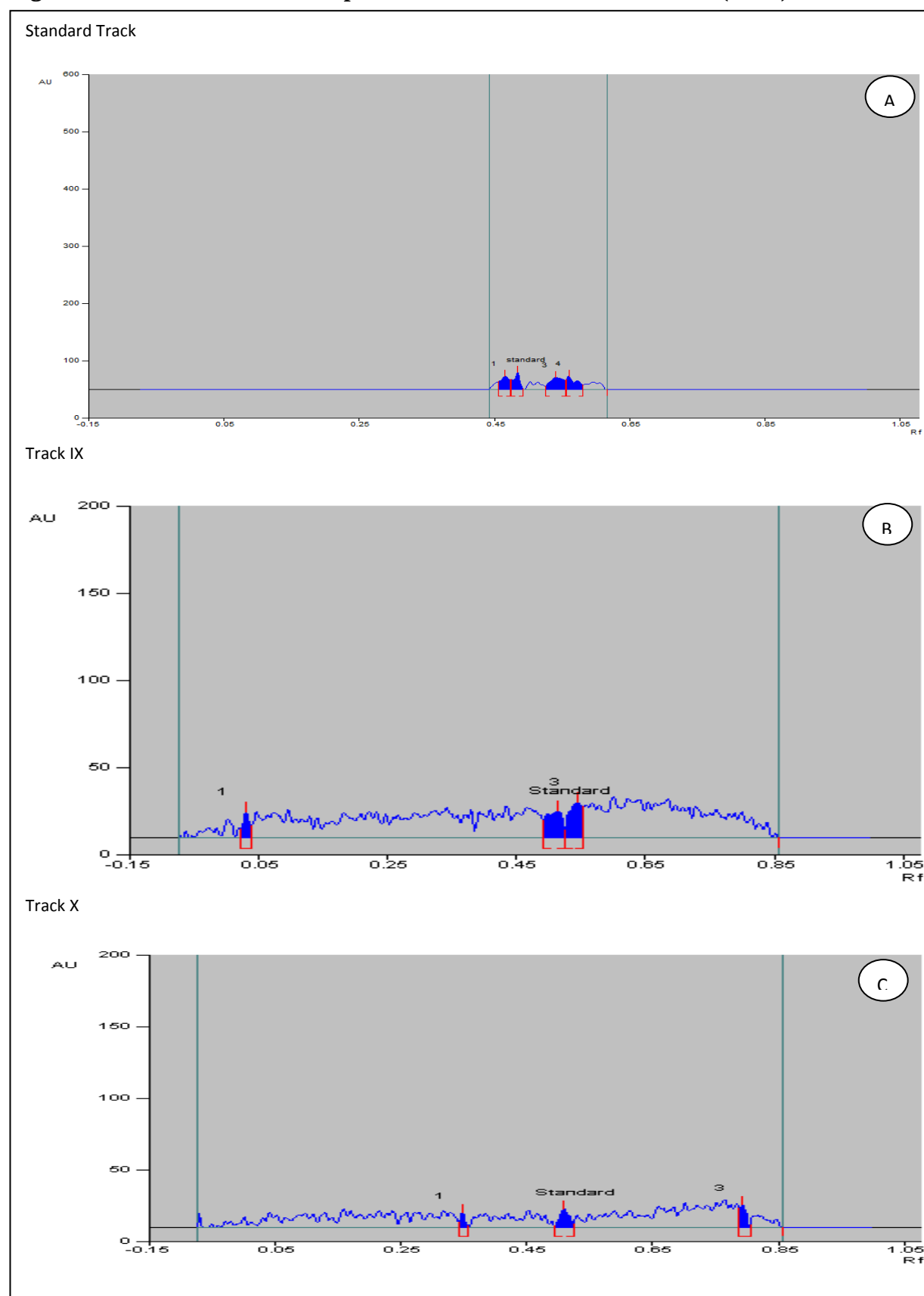
Column fractions developed in chloroform: methanol: water (7:3:1)

- A. Standard track loaded with stevioside (6µl).
- B. Track IX showing stevioside peak with 39.14% peak area.
- C. Track X showing stevioside peak with 43.71% peak area.
- D. Track XI showing stevioside peak with 5.61% peak area.
- E. Track XII showing stevioside peak with 6.98% peak area.
- F. Track XIII showing stevioside peak with 4.18% peak area.

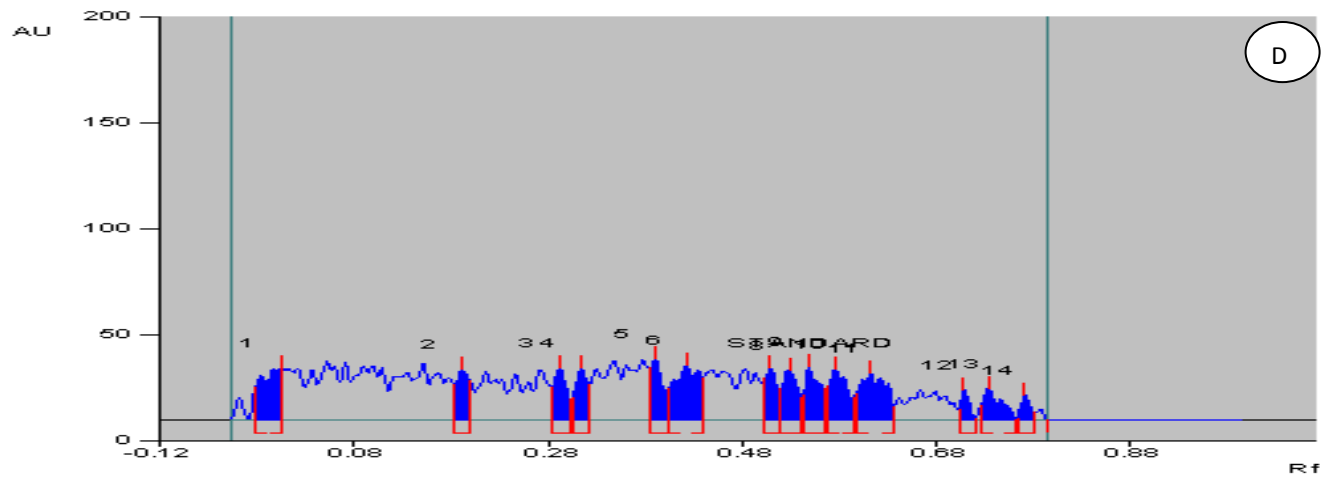
Column fractions in ethyl acetate: methanol: n-hexane (2:2:5)

- G. Track IV showing stevioside peak with 14.72% peak area.
- H. Track VI showing stevioside peak with 6.91% peak area.
- I. Track IX showing stevioside peak with 13.79% peak area.
- J. Track XIV showing stevioside peak with 13.29% peak area.
- K. Track XVI showing stevioside peak with 4.96% peak area.
- L. Track XVIII showing stevioside peak with 6.81% peak area.
- M. Track XIX showing stevioside peak with 9.26% peak area.
- N. Standard track loaded with stevioside.
- O. 3-D spectra of fractions (1-10) at 210 nm.
- P. 3-D spectra of fractions (11-20) at 210 nm.
- Q. Photodocumentation pictures of plate at 366 nm showing stevioside bands in different tracks.

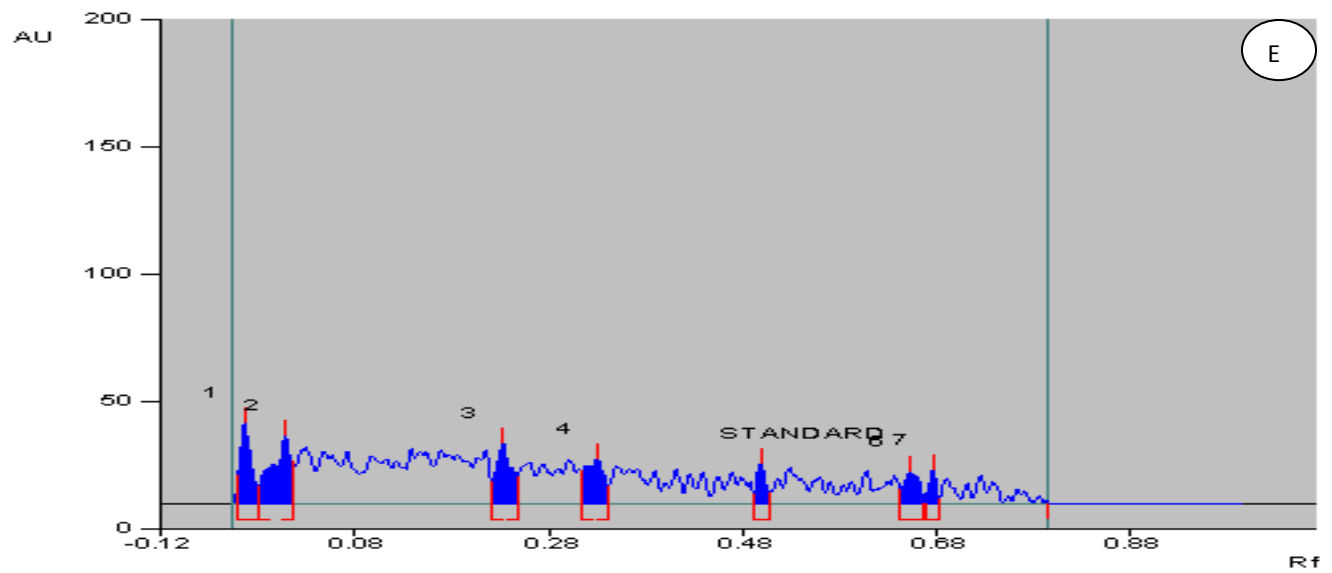
Fig. 39. Column fractions developed in chloroform: methanol: water (7:3:1)



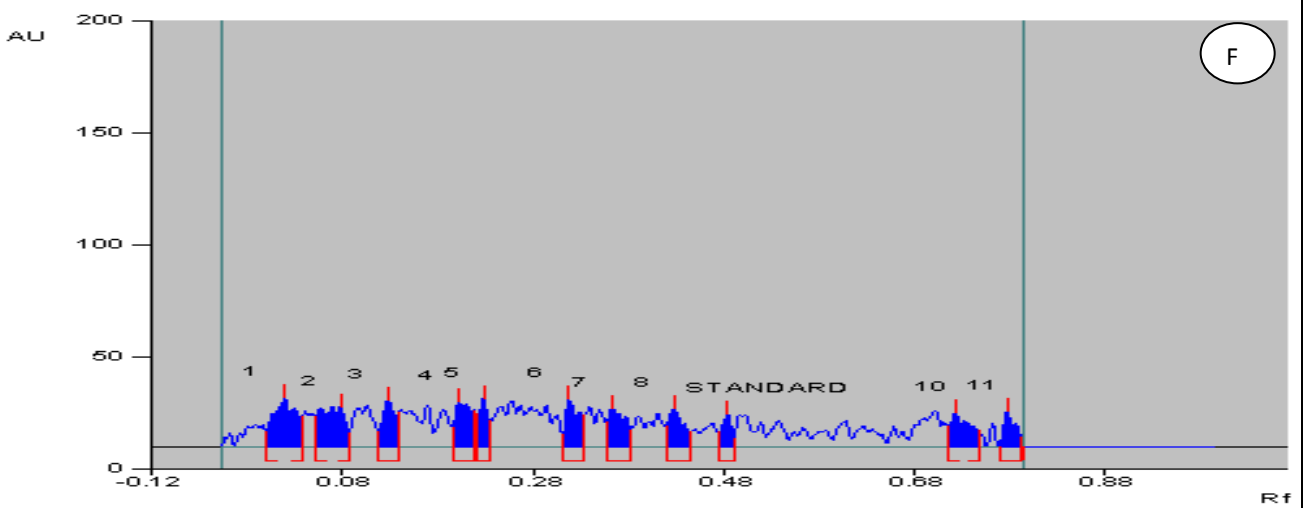
Track XI



Track XII

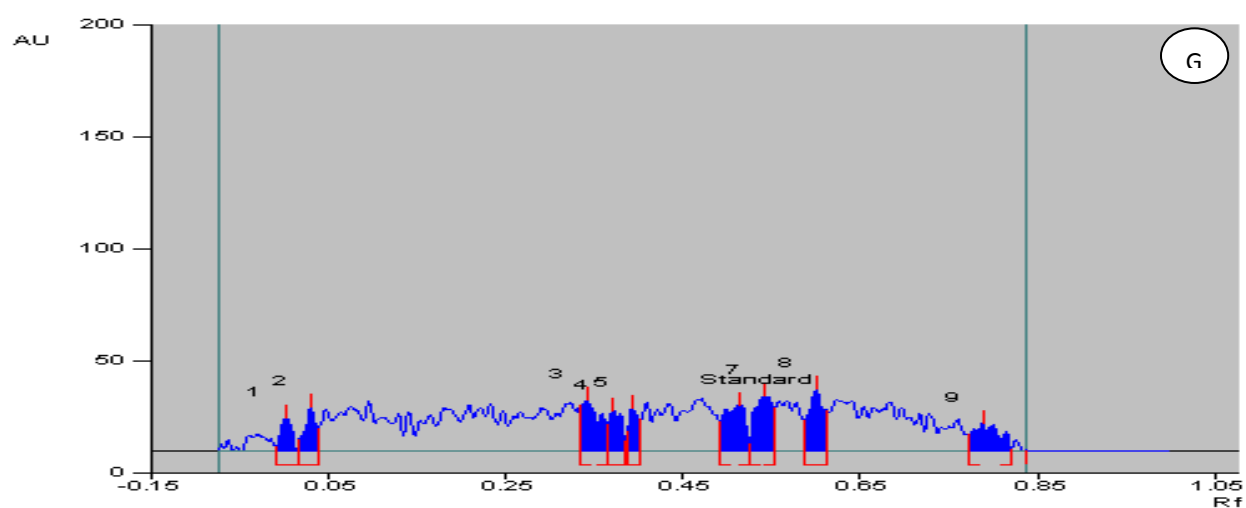


Track XIII

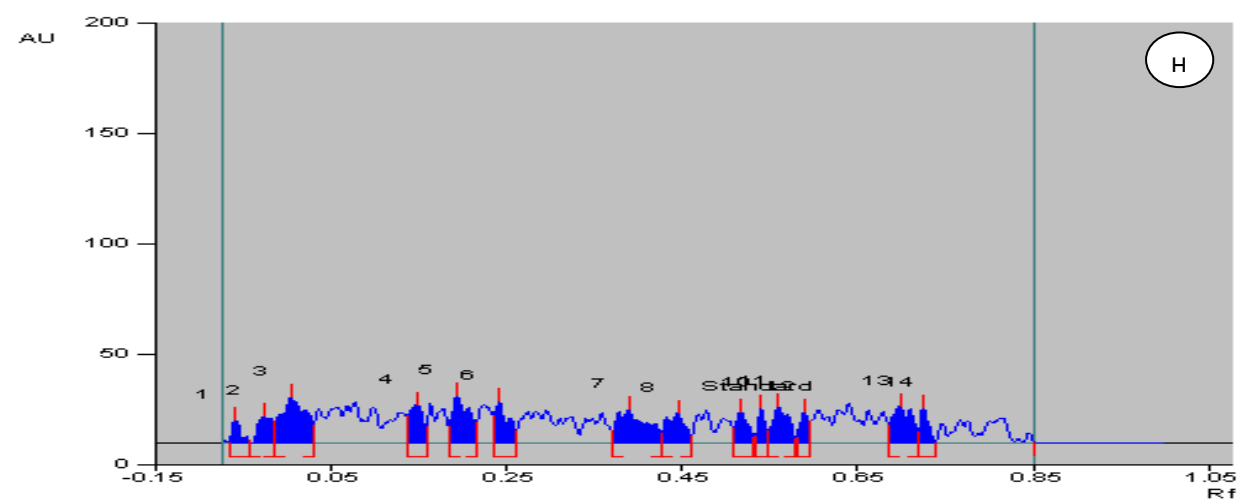


Column fractions in ethyl acetate: methanol: n-hexane (2:2:5)

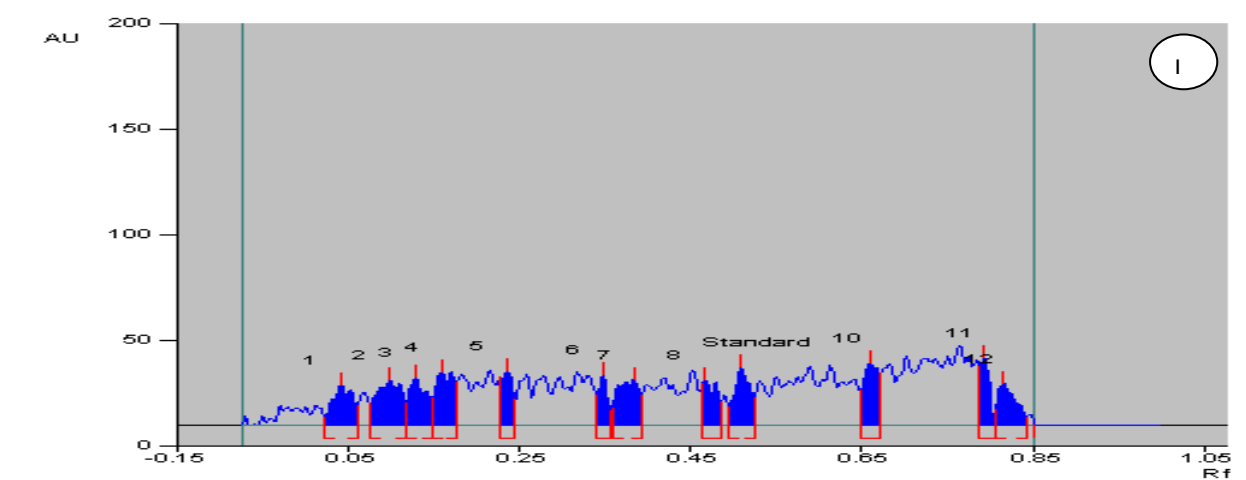
Track IV



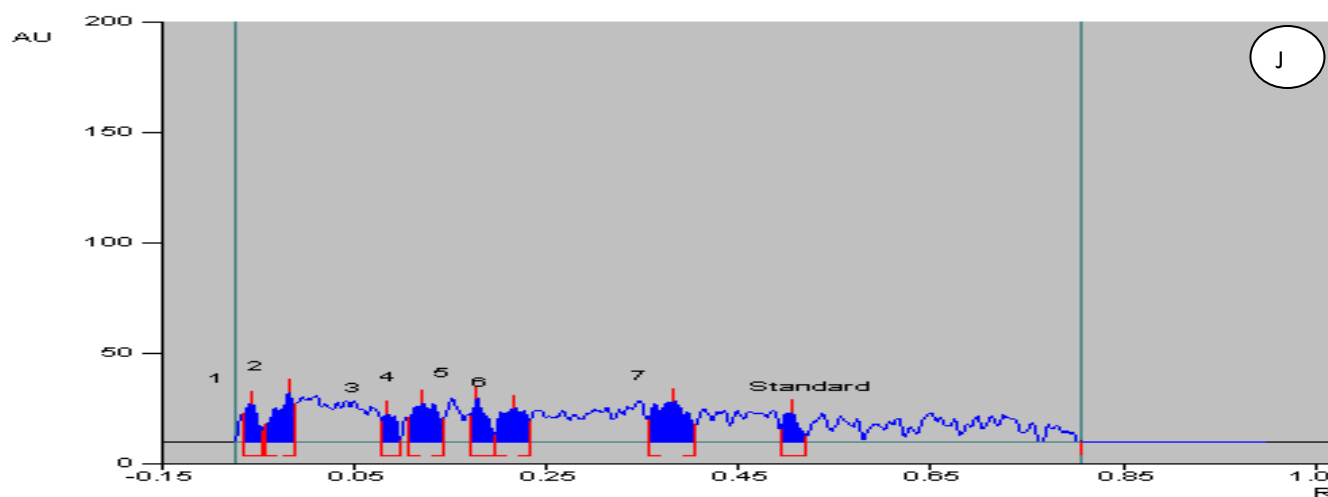
Track VI



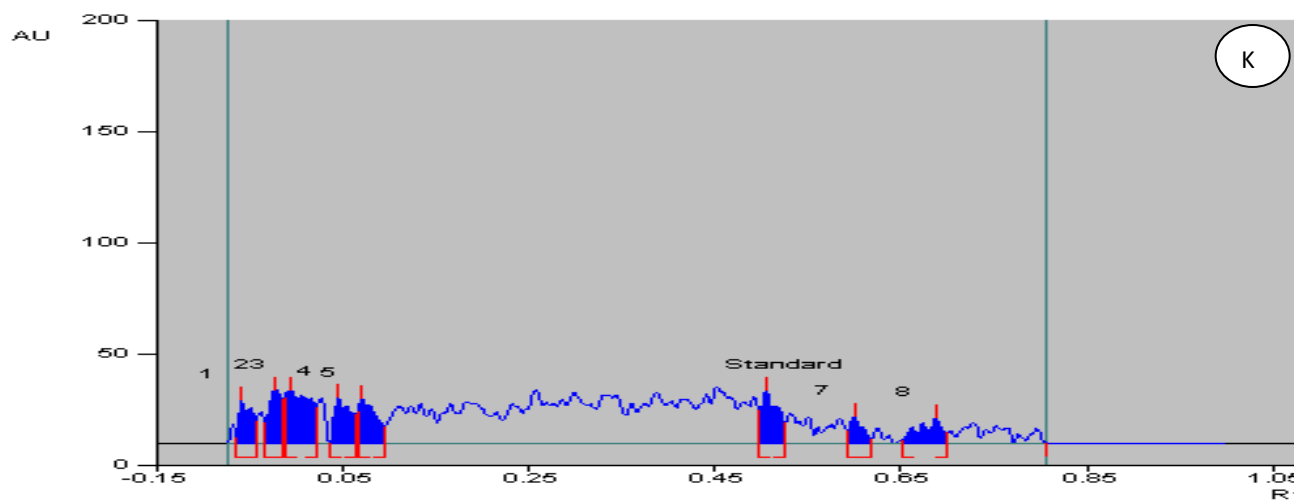
Track IX



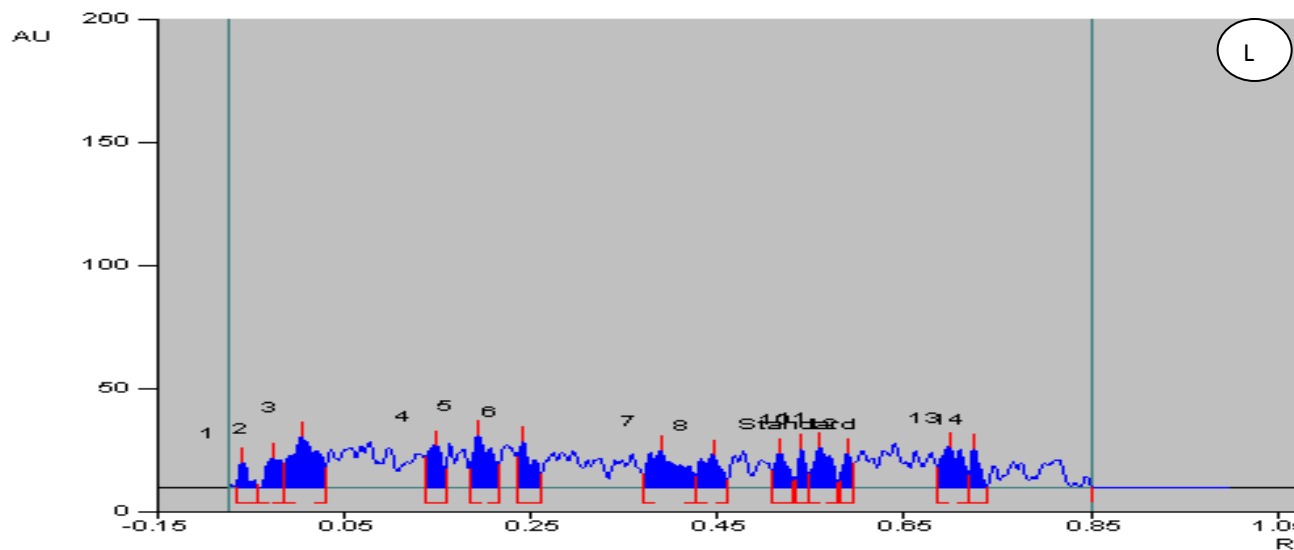
Track XIV



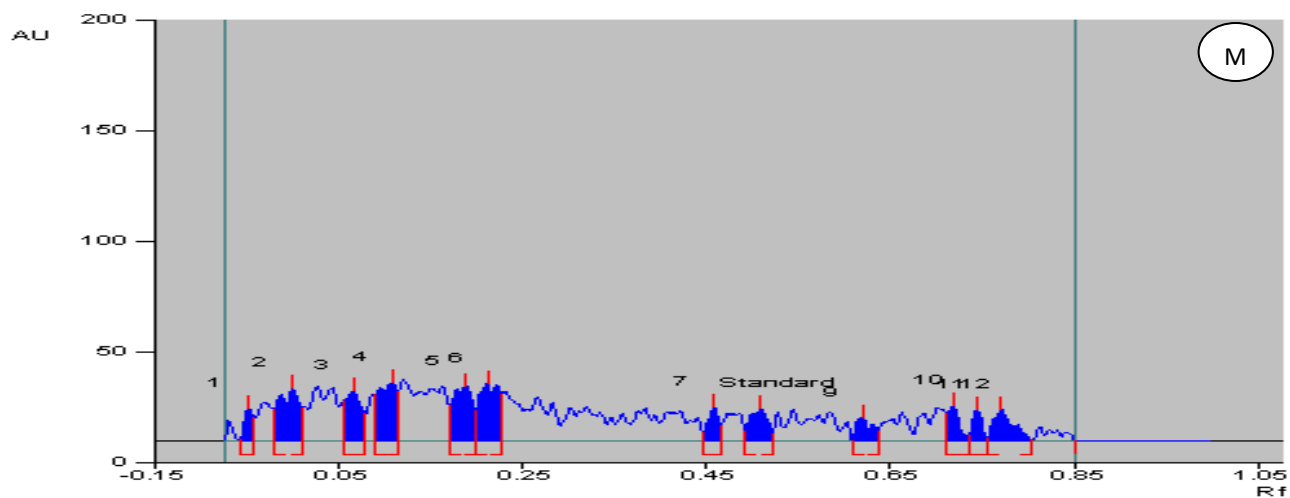
Track XVI



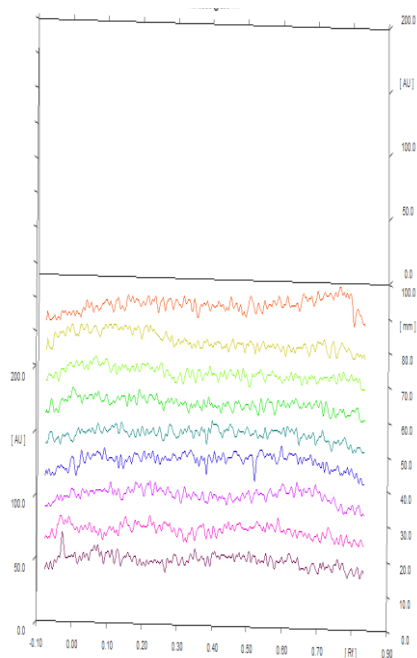
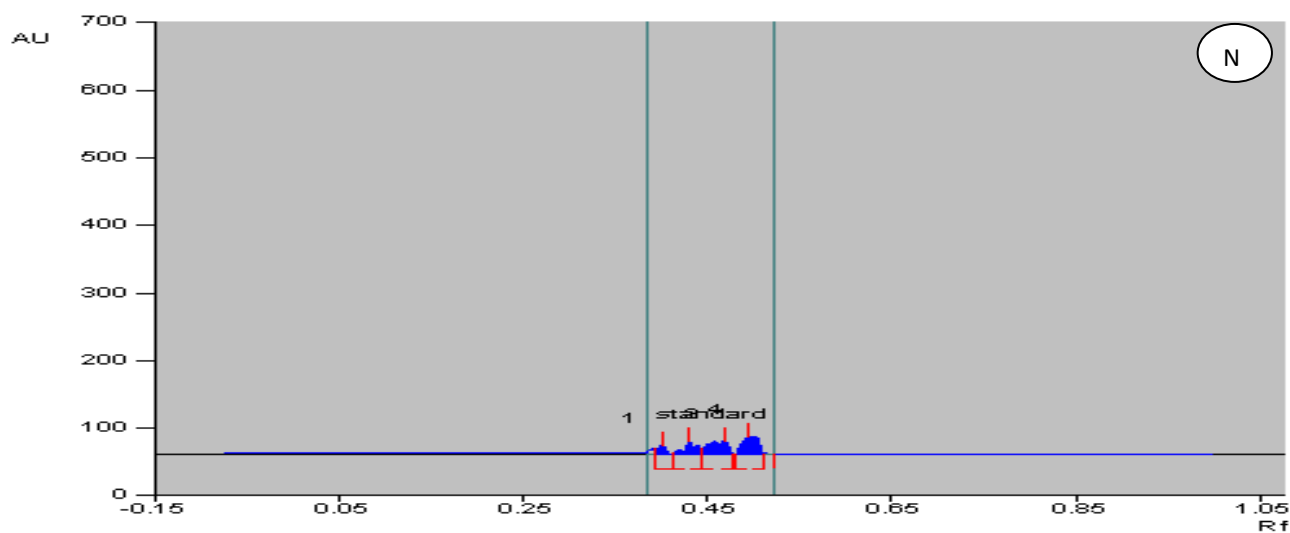
Track XVIII



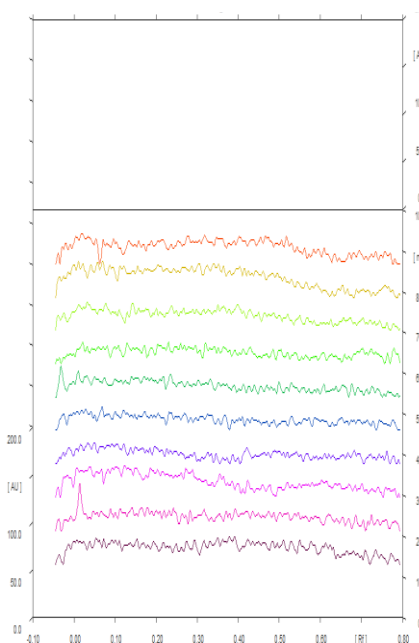
Track XIX



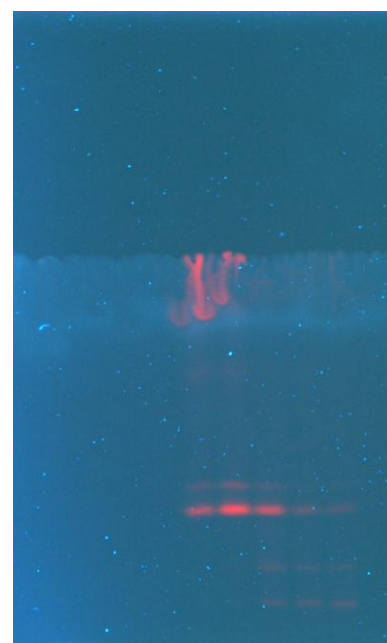
Standard Track



(O)



(P)



(Q)

Table 34. Stevioside peaks in various tracks when developed using different solvent systems.

Track ID	Solvent system	Peak	End Position (Rf)	Area (AU)	Area %
Fig. 39. Standard (A)	Chloroform: methanol: water (7:3:1)	2	0.52	139.0	44.8
Fig. 39. Track IX (B)	Chloroform: methanol: water (7:3:1)	2	0.52	274.3	39.1
Fig. 39. Track X (C)	Chloroform: methanol: water (7:3:1)	2	0.51	148.2	43.7
Fig. 39. Track XI (D)	Chloroform: methanol: water (7:3:1)	7	0.52	219.0	5.61
Fig. 39. Track XII (E)	Chloroform: methanol: water (7:3:1)	5	0.51	108.4	6.98
Fig. 39. Track XIII (F)	Chloroform: methanol: water (7:3:1)	9	0.50	112.0	4.18
Fig. 39. Track IV (G)	Ethyl acetate: methanol: n-hexane (2:2:5)	6	0.52	349.3	14.7
Fig. 39. Track VI (H)	Ethyl acetate: methanol: n-hexane (2:2:5)	6	0.52	133.7	6.91
Fig. 39. Track IX (I)	Ethyl acetate: methanol: n-hexane (2:2:5)	5	0.52	291.0	13.7
Fig. 39. Track XIV (J)	Ethyl acetate: methanol: n-hexane (2:2:5)	7	0.52	369.6	13.2
Fig. 39. Track XVI (K)	Ethyl acetate: methanol: n-hexane (2:2:5)	9	0.52	141.7	4.96
Fig. 39. Track XVIII (L)	Ethyl acetate: methanol: n-hexane (2:2:5)	8	0.52	202.4	6.81
Fig. 39. Track XIX (M)	Ethyl acetate: methanol: n-hexane (2:2:5)	9	0.52	369.0	9.26
Fig. 39. Standard (N)	Ethyl acetate: methanol: n-hexane (2:2:5)	2	0.52	228.0	25.6

4.3.3.5 Preparative Chromatography

Preparative TLC of tenth column fraction was done initially on glass plates and then on pre coated silica gel plate. Optimization of solvent system was done using glass TLC where plates were developed in solvent systems comprising of hexane: ethyl acetate: methanol in ratios of 9:0.5:0.5, 8:1:1, 7:1:2, 6:2:2 and chloroform: methanol: water in ratios of 7:3:1, 6:3:1, 4:6:2 and 5:3:2 (Fig. 40 A-I). However, the optimum solvent system comprised of ethyl acetate: methanol: hexane (6:2:2) where spot showed R_f comparable to that of the stevioside (Table 35). Spot thus, obtained was scraped using fine end of spatula and was dissolved in methanol followed by its filtration using syringe filter to remove silica carried with the spot. The solvent was evaporated in rotary and the remaining extract was chromatographed using HPTLC to confirm the banding pattern.

Preparative HPTLC was performed with the extract obtained from glass preparative plate. The extract thus, obtained was loaded at different concentrations and developed using different solvent systems to develop optimum banding pattern at a particular wavelength range. Extract (6 µl each) was loaded in three different tracks I, II and III and was developed in ethyl acetate: methanol: n-hexane (2:2:5) (Fig. 41 A- C). Each track was scanned at different wavelengths i.e. 210, 215 and 205 nm (Fig. 41 D-F). From the different peaks obtained, it was very clear that track I scanned at 210 nm showed the peak corresponding to the stevioside whereas, none other track showed the stevioside peak. Photodocumentation at 366 nm showed the presence of single distinct stevioside band (Fig. 41 G). The stevioside spot thus, obtained was scraped using fine end of spatula and was again dissolved in methanol followed by its filtration using syringe filter to remove silica carried with the spot. The solvent was evaporated in rotary yielding pure stevioside fraction. Structural studies of stevioside fraction were further carried through NMR analysis.

Fig. 40. Preparative chromatography of column fraction in different solvent systems

Prep-TLC fractions on glass plate in different solvent systems

- A. Column fraction in solvent system of hexane: ethyl acetate: methanol (9:0.5:0.5).
- B. Fraction in solvent system of hexane: ethyl acetate: methanol (8:1:1).
- C. Fraction in solvent system consisting of hexane: ethyl acetate: methanol (7:2:1).
- D. Fraction in solvent system consisting of hexane: ethyl acetate: methanol (6:2:2).
- E. Fraction developed in solvent system consisting of 100% hexane.
- F. Fraction in solvent system consisting of chloroform: methanol: water (7:3:1).
- G. Fraction in solvent system consisting of chloroform: methanol: water (6:3:1).
- H. Fraction in solvent system consisting of chloroform: methanol: water (4:6:2).
- I. Fraction in solvent system consisting of chloroform: methanol: water (5:3:2).

Fig.40 Preparative glass TLC in different solvent systems

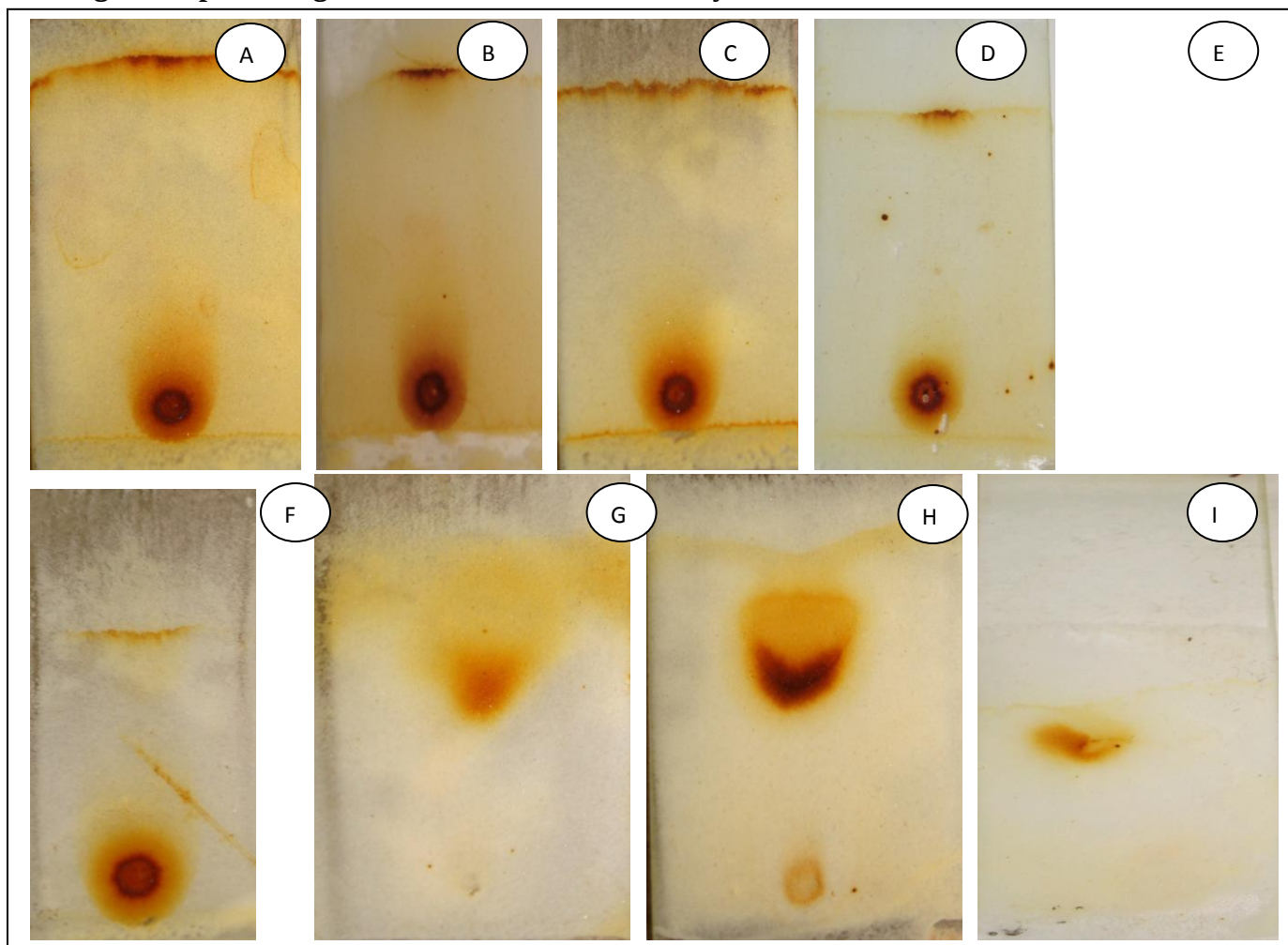


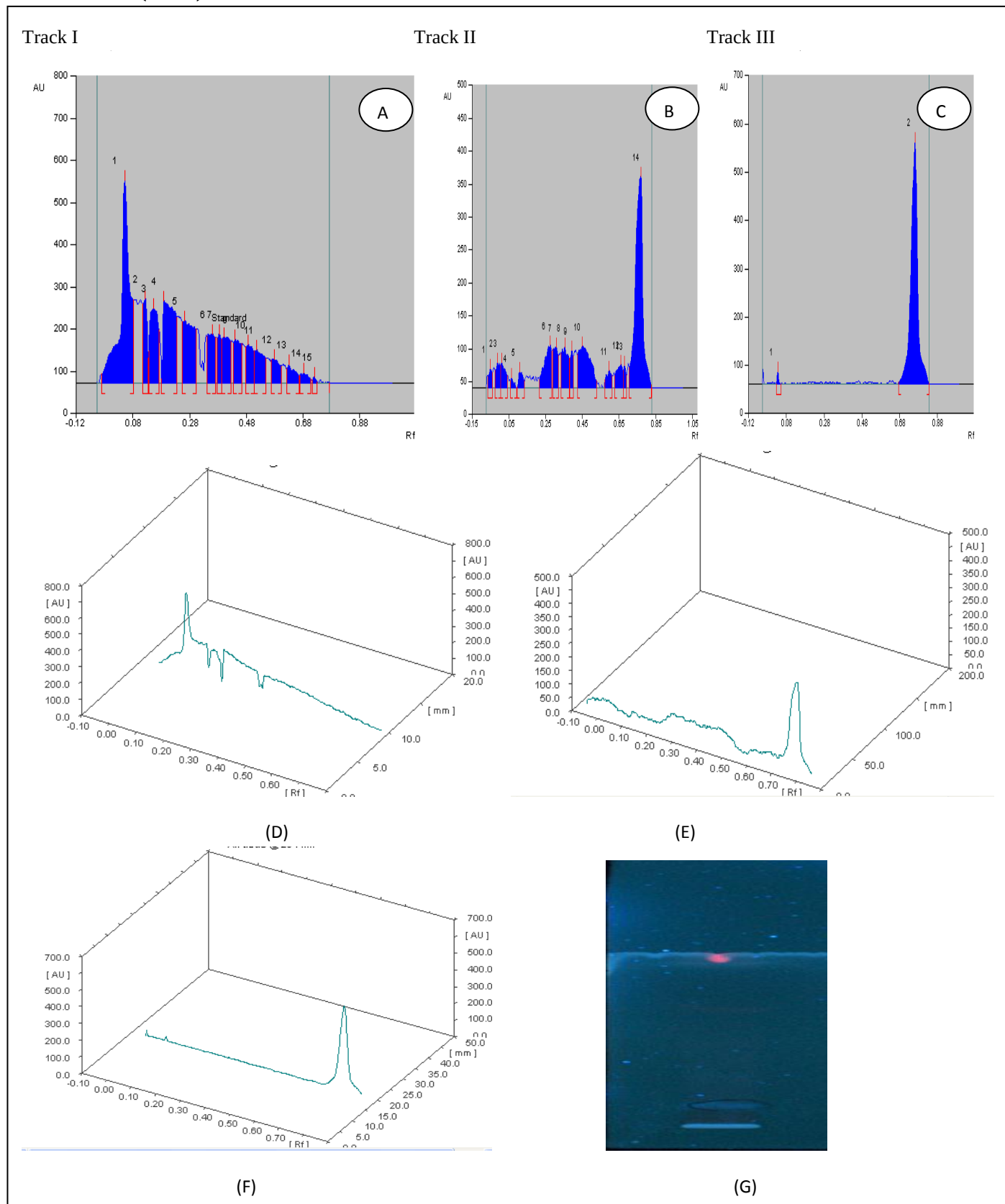
Table 35 Preparative TLC of column fraction in different solvent systems

Fig. No.	Solvent system used	Solvent Ratio	Rf
40 (A)	Hexane :ethyl acetate: methanol	9:0.5:0.5	0.8
40 (B)	Hexane :ethyl acetate: methanol	8:1:1	0.8
40 (C)	Hexane :ethyl acetate: methanol	7:2:1	0.7
40 (D)	Hexane :ethyl acetate: methanol	6:2:2	0.5
40 (E)	Hexane	10	0.4
40 (F)	Chloroform: Methanol : Water	7:3:1	0.6
40 (G)	Chloroform :Methanol : Water	6:3:1	0.6
40 (H)	Chloroform :Methanol : Water	4:6:2	0.6
40 (I)	Chloroform :Methanol : Water	5:3:2	0.4

Fig. 41. HPTLC chromatograms of preparative sample in ethyl acetate: methanol: n-hexane

- A. Track I loaded with column fraction showing stevioside peak at 210nm.
- B. Track II loaded with column fraction scanned at 215 nm failed to show stevioside peak.
- C. Track III loaded with fraction scanned at 205 nm also failed to show stevioside peak.
- D. 3-D spectra of track I at 210 nm.
- E. 3-D spectra of track II at 215 nm.
- F. 3-D spectra of track III at 205 nm.
- G. Pictured captured under UV 366 nm, showing distinct single stevioside band.

Fig. 41. HPTLC chromatograms of preparative sample in ethyl acetate: methanol: n-hexane (2:2:5)



4.3.3.6 NMR Study

The ^1H NMR spectra of stevioside isolated from the leaves of thirty months old *in vitro* raised plants of *Stevia rebaudiana* was measured in a 5mm ampule on a Bruker spectrophotometer (Model AVANCE DPX-400 MHz) (Fig. 42). On comparison of NMR spectra of stevioside with that of standard stevioside following observations were made. The ^1H NMR signals showed three anomeric protons as doublets at δ 5.24 ($J = 8.12$, 1H), δ 4.44 ($J=7.6$, 1H), δ 4.34 ($J=7.6$, 1H), suggesting the presence of three sugar residues in the structure. Signal at δ 1.37-1.47 showed the presence of CH_2 group, δ 3.5-3.9 ppm showed -CH groups, δ 4.3-4.7 ppm showing the presence of hydroxyl groups. The signal at δ 5.25 and δ 5.23 appeared as doublet ($J = 8.12$, tetra hydropyran group). Detailed structural elucidation was however not conducted but the basic interpretation of data was done to confirm the presence of stevioside (Table 36).

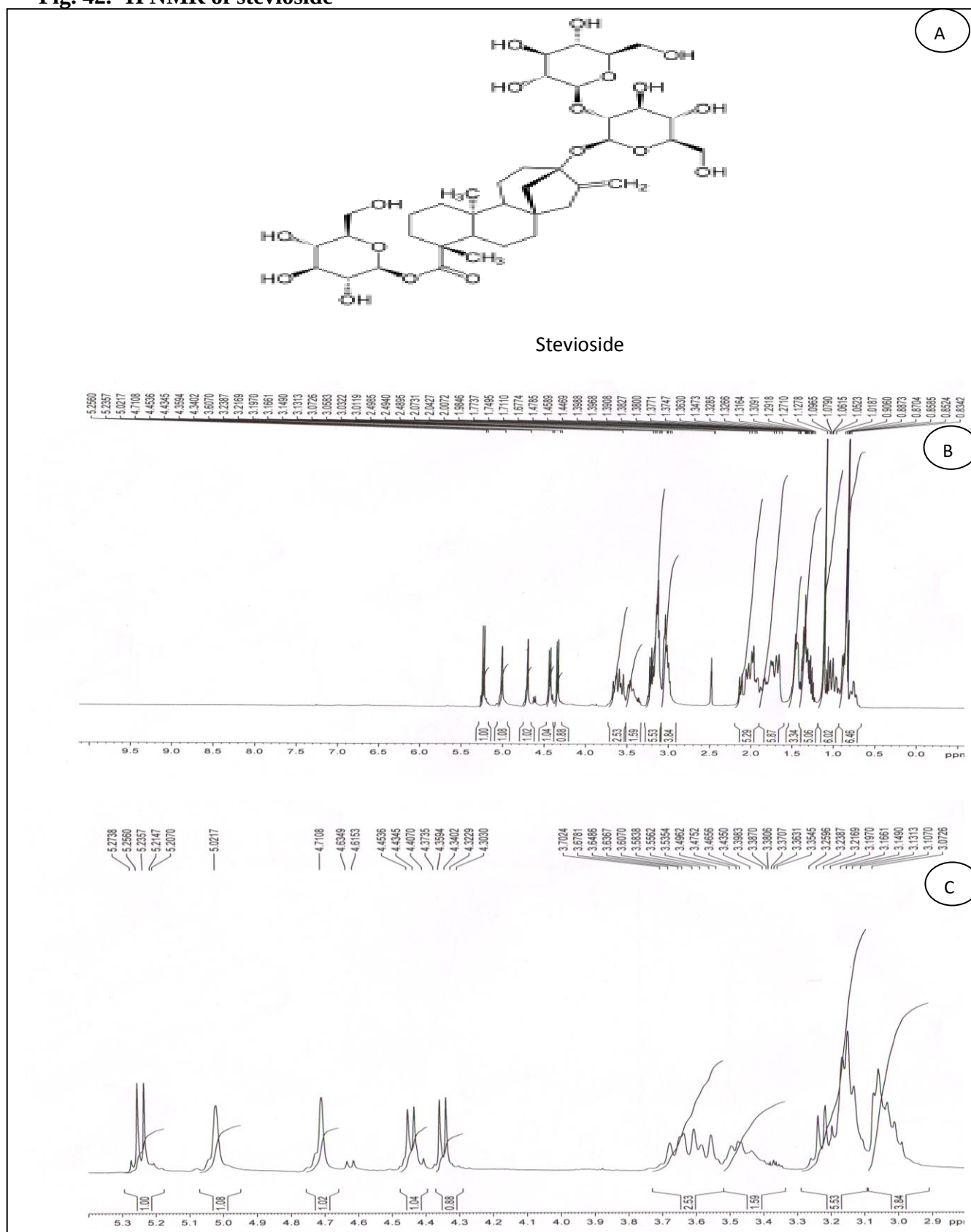
Table 36 Interpretation of basic groups of stevioside through ^1H NMR

S. No.	Signals (ppm)	Types of Proton
1.	1.37-1.47	R- CH_2
2.	3.5-3.9	CH
3.	0.7-0.98	R- CH_3
4.	5.2-5.27	H
5.	4.3-4.7	C-H-OH

Fig. 42. ^1H NMR scan of stevioside

- A. Stevioside structure.
- B. NMR scan of stevioside at 400 MHz.
- C. Enlarged view of stevioside scan.

Fig. 42. ^1H NMR of stevioside



4.4 *Tylophora indica*:-

4.4.1 Estimation of carbohydrates, proteins and lipids (primary metabolites) from vegetative plant parts

Among different plant parts of *in vitro* raised plants of *Tylophora indica*, the highest concentration of reducing sugars was found in leaf explants (1.39%) whereas almost equal amounts (1.30%) were present in stem and root parts. Similarly, no variation for total sugars was observed among different vegetative parts of the plant. Sucrose showed distinct variation in the concentration among different plant parts with highest being in leaves (1.70%) followed by stem (1.21%) and root parts (1.01 %). Protein concentration was highest in leaf explants (0.61%) and lowest in stem parts (0.34%). Both starch and lipids contents were maximum in root (3.20 and 2.40 %) and minimum (1.43 and 1.46 %) in leaves (Table. 37).

Table 37: Carbohydrate, protein and lipid contents (g/100g DW) in different explants of *in vitro* raised plants of *Tylophora indica* (values are average of three replicates)

Experimental material		Total sugars	Reducing Sugars	Sucrose	Starch	Proteins	Lipids
<i>Tylophora indica</i>	Leaf	0.23 ± 0.01	1.39 ± 0.01	1.70 ± 0.01	1.43 ± 0.01	0.61 ± 0.01	1.46 ± 0.02
	Stem	0.22 ± 0.02	1.30 ± 0.01	1.21 ± 0.01	2.60 ± 0.05	0.34 ± 0.02	1.56 ± 0.02
	Root	0.21 ± 0.01	1.31 ± 0.02	1.01 ± 0.02	3.20 ± 0.11	0.43 ± 0.02	2.40 ± 0.14

4.4.2 Estimation of sterols, phenols and flavonoids (secondary metabolites) from vegetative plant parts

In *Tylophora indica*, sterols were found to be maximum (2.2 %) in leaf explants and minimum (0.7 %) in stem parts. Equal concentration (0.98 %) of phenols was found in leaf and root whereas flavonoids were found to be maximum (4%) in roots and minimum (1.70%) in stem (Table 38).

Table 38: Sterols, phenols and flavonoids (g/100g DW) in different explants of *in vitro* raised plants of *Tylophora indica* (values are average of three replicates)

Experimental material		Sterols	Phenols	Flavonoids
<i>Tylophora indica</i>	Leaf	2.2 ± 0.09	0.98 ± 0.04	2.30 ± 0.04
	Stem	0.7 ± 0.06	0.77 ± 0.04	1.70 ± 0.02
	Root	1.8 ± 0.04	0.98 ± 0.02	4.00 ± 0.02

4.4.3 Extraction and purification of major secondary metabolite- tylophorine from *T. indica*

The procedure for the extraction of major alkaloid tylophorine from leaves and root of *T.indica* is described in detail in the material and method section 3.6. The final extract obtained after solvent extraction using extraction protocol I to III was evaporated in rotary flash evaporator at 60°C and the concentrate thus obtained, was resuspended in methanol for further purification to obtain pure tylophorine fractions using various chromatographic techniques (discussed in detail in M & M section 3.7). For carrying out analysis of test samples, firstly optimization of standard tylophorine using different parameters such as sample volume, developing solvent system and wavelength was done using HPTLC.

4.4.3.1 Optimization of parameters for standard tylophorine using HPTLC analysis

For the optimization of developing solvent system, loading volume and wavelength of standard, different volumes (1, 2, 3, 4, 6 and 8 µl) were loaded on pre coated silica gel HPTLC 60 F 254 plate on different tracks (Fig. 43 A-F). The plate was developed in different developing solvent systems comprising of different ratios of toluene: ethyl acetate: diethyl amine, toluene: chloroform: ethanol: ammonia, chloroform: methanol: ammonium hydroxide and was scanned at a wavelength range of 200-700 nm. Among all the solvent combinations, toluene: ethyl acetate: diethyl amine (7:2:1) showed tylophorine peaks in track V and VI when scanned at 258 nm whereas, none of the other tracks showed the presence of tylophorine. UV spectra of tylophorine recorded at 200-400 nm revealed that tylophorine peak in all the tracks showed quite similar UV absorbance (Fig. 43 G). Figure 43 H showed 3-D spectra scan of tylophorine peaks in all the tracks at 258 nm, which resembled the visual impression of the plate under UV 254 and 366 nm (Figures 43 I and J). Thus, tylophorine peak was observed at two different concentration levels

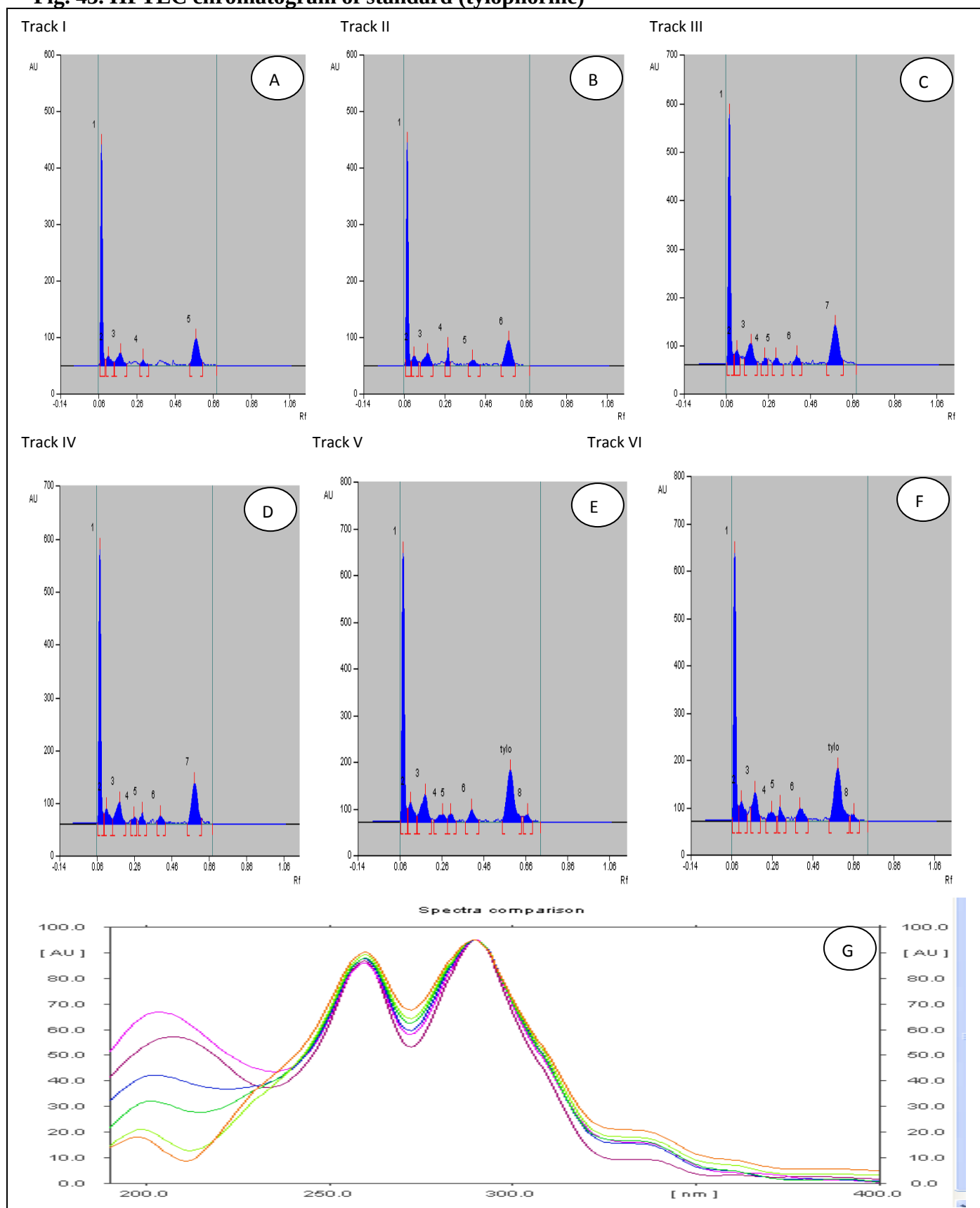
i.e. 6 and 8 μ l, when developed in toluene: ethyl acetate: diethyl amine (7:2:1) and scanned at 258 nm (Table 39).

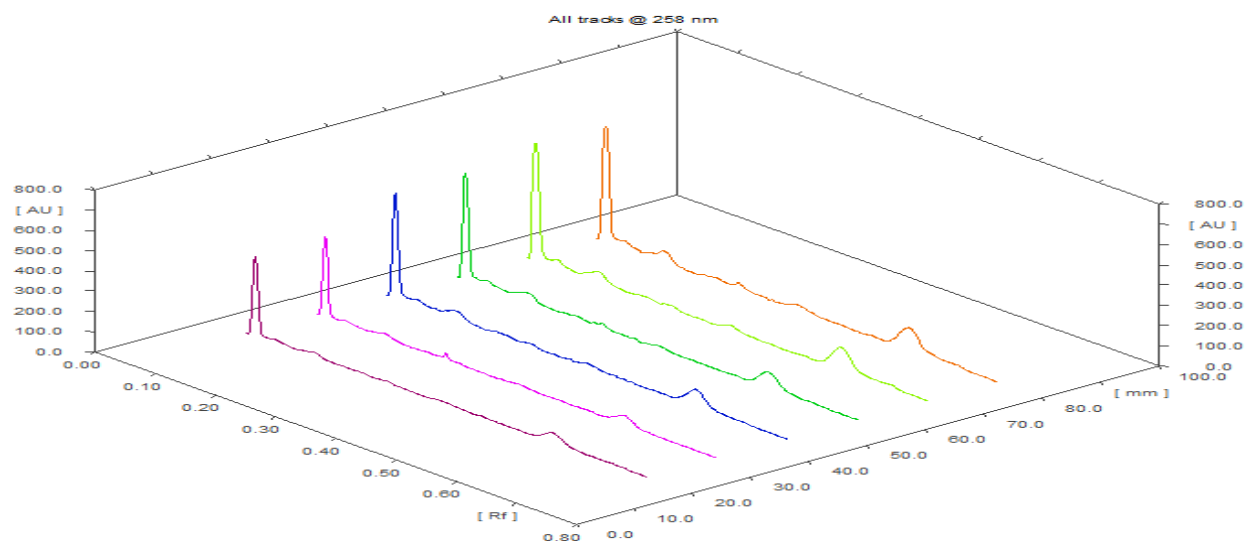
Further leaves and root were excised from 2 yrs old healthy *in vitro* raised plants of *T indica* and were processed using extraction protocols I to III to precondition the parameters as well as the plant part yielding maximum amount of tylophorine. For the extraction of tylophorine, fine grounded powder of different vegetative plant parts were solvent extracted and the final extract thus, obtained was analyzed using high performance thin layer chromatography. The optimum extraction protocol was selected for all future extraction studies.

Fig. 43. HPTLC chromatogram of standard (tylophorine)

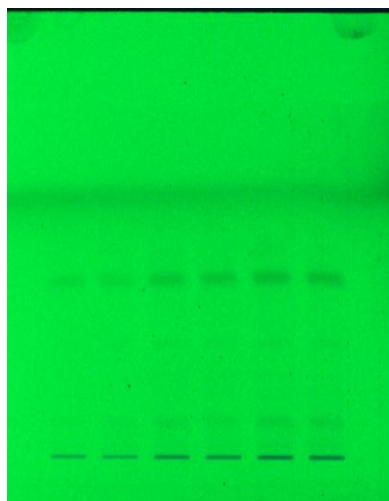
- A. Track I loaded with 1 μ l of standard (tylophorine).
- B. Track II loaded with 2 μ l of standard.
- C. Track III loaded with 3 μ l of standard.
- D. Track IV loaded with 4 μ l of standard.
- E. Track V loaded with 6 μ l of standard.
- F. Track VI loaded with 8 μ l of standard.
- G. Spectra comparison of tylophorine peaks in all the tracks.
- H. 3-D spectra of all the peaks at 258 nm.
- I. UV absorbance spectra at 254 nm showing distinct bands of tylophorine in different tracks.
- J. UV absorbance spectra at 366 nm showing distinct bands of tylophorine in different tracks.

Fig. 43. HPTLC chromatogram of standard (tylophorine)





(H)



(I)



(J)

Table 39. Tylophorine in standard track (in terms of Rf and peak area) when developed in toluene: ethyl acetate: diethyl amine (7:2:1).

Track ID	Peak	End Position (Rf)	Area (AU)	Area (%)
Fig. 43. Track V(E)	7	0.64	3166	24.87
Fig. 43. Track VI (F)	7	0.64	3330	26.01

4.4.3.2 Extraction and estimation of tylophorine from leaf explants of *in vitro* raised field established plants of *T.indica*

Plants of *T.indica* were obtained directly from the leaf explants as well as from the calli obtained from them on variously supplemented Murashige and Skoog's medium and were successfully established in the field. Thereafter, the leaves were collected from twelve months old plants for the quantification of tylophorine. The excised leaves were powdered and processed using optimized extraction protocol I and the final extract obtained was analyzed using HPTLC. Samples along with the standard tylophorine were loaded in separate tracks on pre coated silica gel fluorescent (5X5cm) plate (Fig. 44 A-C). The plate was developed in solvent system comprising of toluene: chloroform: ethanol: ammonia (4: 3.5: 1.5: drop) and scanned in scanner III at 258 nm. On comparison of R_f values of standard tylophorine (R_f 0.68) with sample peaks it was observed that tylophorine peak (R_f 0.68) was present in both the samples. Banding pattern of the plate was observed under UV scan at 254 and 366 nm, which clearly showed tylophorine bands in both the test samples (Fig. 44 D and E). Lane I shows tylophorine band in directly obtained plants whereas lane II shows tylophorine in callus regenerated plants.

Similarly, plants of *T.indica* were raised from the calli obtained from root explants and were successfully transferred and established under field conditions. The leaves were excised from these healthy twelve months old plants and processed using extraction protocol I. The extract obtained was analyzed for the presence of tylophorine using HPTLC. Leaf samples were applied on pre coated silica gel plate (5 X 5cm) and developed using solvent system comprising of toluene: ethyl acetate: diethyl amine (7: 2: 1) and scanned at 254 nm (Fig. 44 F). On comparison of sample with standard, presence of tylophorine was observed at R_f 0.63 with 22.52% peak area. Both 3-D scan and UV visuals clearly showed the presence of tylophorine in the samples. A comparable peak spectrum was observed in both samples and standard peaks (Fig. 44 G). UV visuals also showed tylophorine band in the sample corresponding to standard tylophorine band at R_f 0.63. Lane I showed tylophorine band in the sample whereas lane II showed tylophorine band in the standard (Fig. 44 H).

Quantitative analysis of all the samples showed that maximum amount of tylophorine (80 µg/ml) was detected in leaf callus-regenerated plants of *T.indica* whereas, directly obtained plants contained 71 µg/ml of tylophorine. Root regenerated plants contained 35 µg/ml of tylophorine under optimal set of conditions (Table 40).

Fig. 44. HPTLC chromatogram of tylophorine extracted from leaf explants of *in vitro* raised plants of *T.indica*

A.Track I loaded with standard tylophorine.

B.Track II loaded with leaf extract of leaf explant regenerated plants.

C.Track III loaded with leaf extract of leaf callus-regenerated plants.

D.UV visuals at 254 nm showing bands of tylophorine (arrowhead) in both the samples.

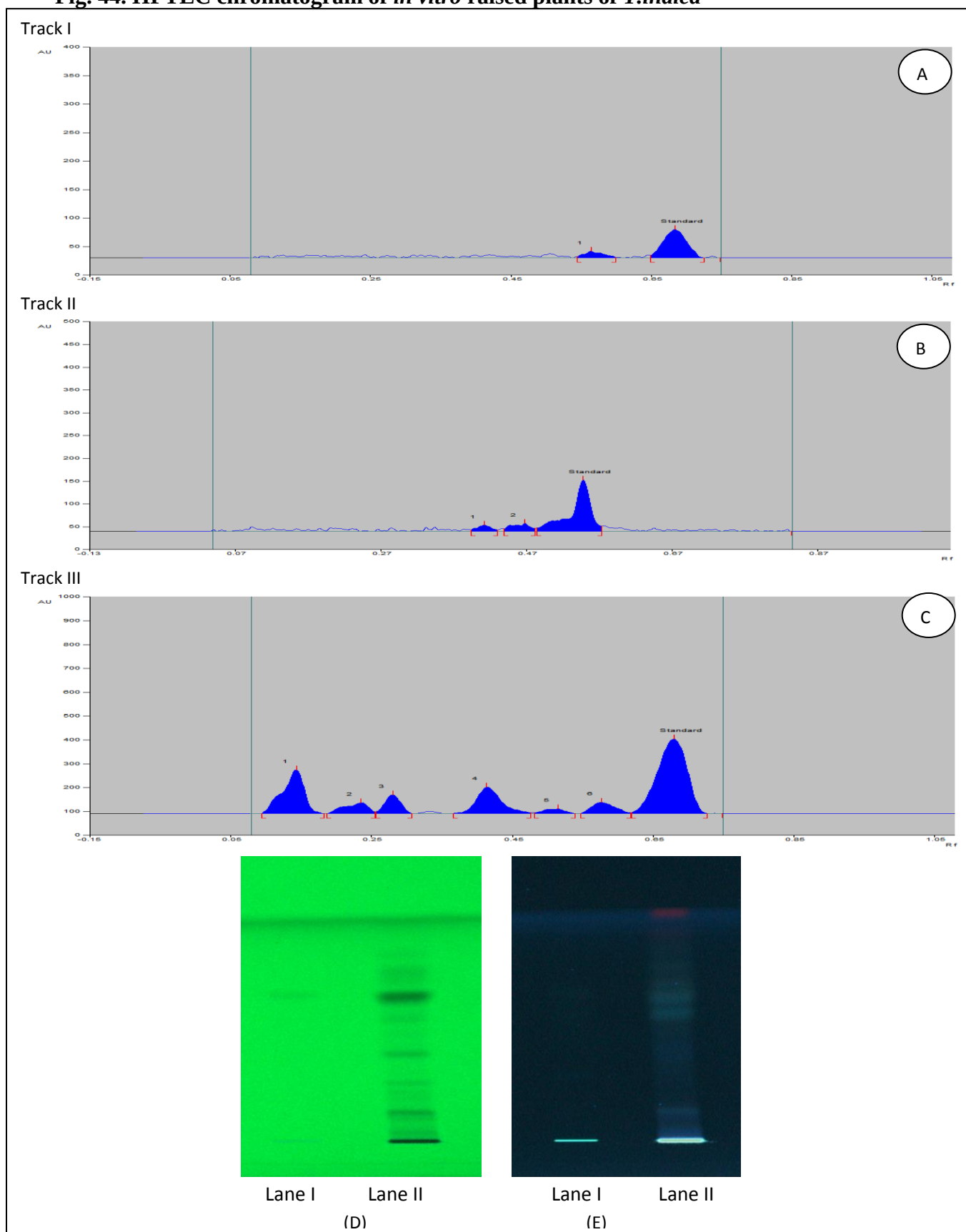
E.UV visuals at 366 nm showing bands of tylophorine in both the samples.

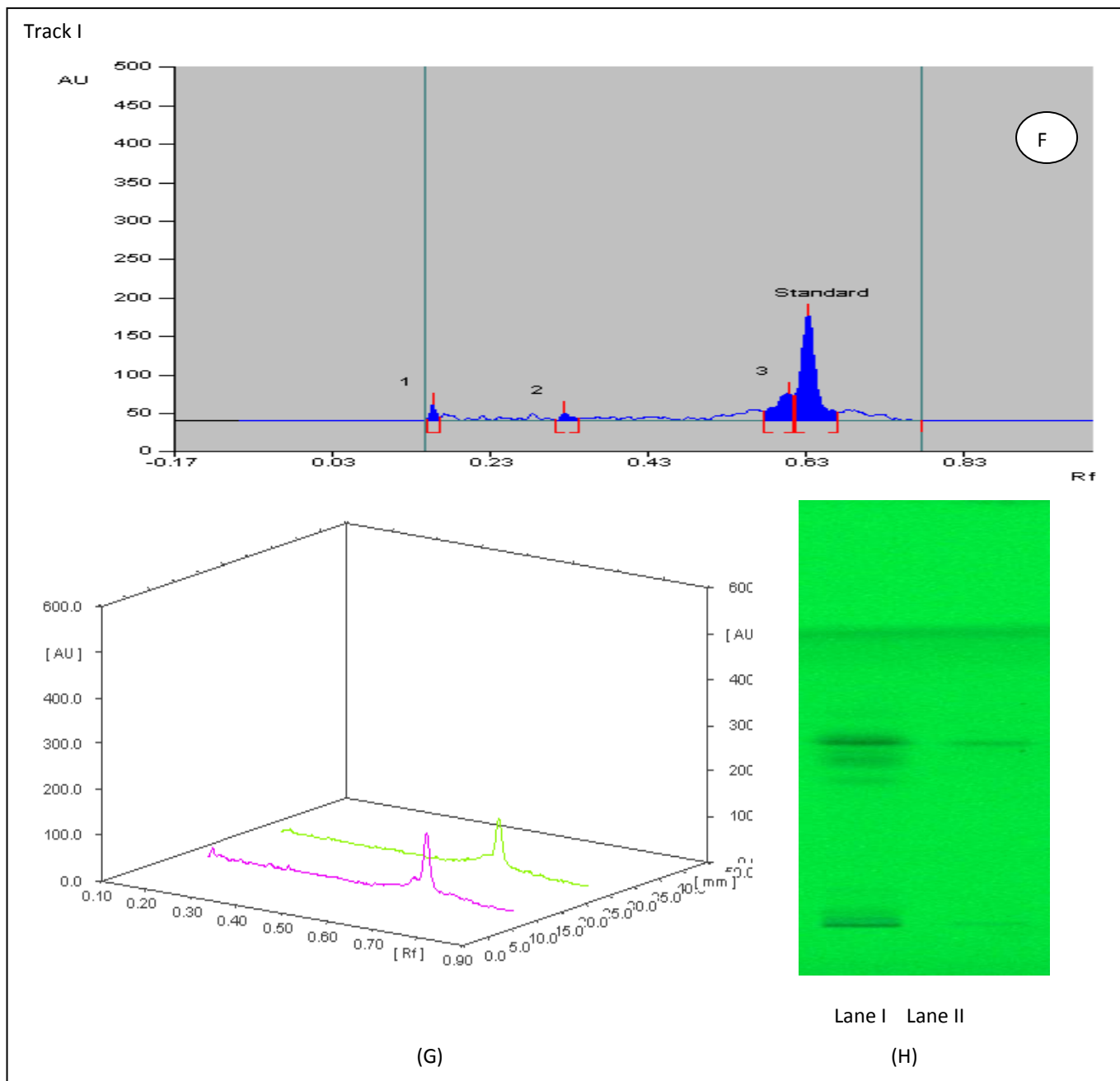
F.Track I loaded with leaf extract obtained from root callus regenerated plants.

G.3-D spectra of leaf extract of root-regenerated plant along with the standard at 254 nm showing similar tylophorine spectra for sample and standard.

H.UV visuals at 254 nm showing tylophorine band (arrowhead) in both sample and standard at similar R_f.

Fig. 44. HPTLC chromatogram of *in vitro* raised plants of *T.indica*





Track ID	Solvent System	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorine (µg/ml)
Fig 44 Track I (A)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	2	0.68	1246.6	49.00	-----
Fig 44 Track II (B)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	3	0.68	1099.6	69.00	71
Fig 44 Track III (C)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	7	0.68	9888.1	80.00	80
Fig. 44 Track I (F)	Toluene: ethyl acetate:	4	0.63	573.50	22.52	35

	diethyl amine (7:2:1)					

Table 40 Tylophorine ($\mu\text{g/ml}$) in different tracks developed using different solvent systems. Standard tylophorine applied at a working concentration of $20.0\mu\text{g/ml}$.

4.4.3.3 Extraction and estimation of tylophorine from roots of *in vitro* raised and *in vivo* plants of *T.indica*

Since highest amount of tylophorine is reported to be present in the roots of the plant, a comparative study of tylophorine present in the roots of *in vitro* raised field established and *in vivo* plants was made.

4.4.3.3.1 Tylophorine in twelve months old plants of *T.indica*

The root explants were excised from twelve months old *in vitro* raised and *in vivo* plants of *T.indica* and were processed using extraction protocol I. All the fractions were analyzed for the presence of tylophorine initially on glass TLC followed by HPTLC. For TLC analysis, both the samples of *in vitro* and *in vivo* extracts were loaded in track I and III respectively whereas, standard was loaded in track II on silica gel glass plate (Fig. 45). The plate was developed using various solvent systems comprising of different combinations and ratios of toluene: chloroform: ethanol: ammonia, chloroform: methanol: ammonium hydroxide and toluene: ethyl acetate: diethyl amine based upon the polarities of the solvents. The optimum combination identified was toluene: ethyl acetate: diethyl amine (7:2:1) which showed the presence of tylophorine in all the tracks. On comparison of all the tracks with the standard, presence of tylophorine band was observed at R_f 0.64 and 0.65 in track I and III respectively (Table 41).

All these samples were then loaded on pre coated silica gel plate for HPTLC analysis and were developed using the same solvent system i.e. as toluene: ethyl acetate: diethyl amine (7:2:1). The plate when scanned at 258 nm showed the presence of tylophorine peaks in both the test samples (Fig. 46 A-C). Track I loaded with standard showed the presence of tylophorine band at R_f value of 0.64 with 28.05% peak area. Track II loaded with extract from *in vitro* raised plant showed tylophorine peak covering 70.39% peak area whereas tylophorine peak in track III loaded with extract of *in vivo* plant sample covered 55.34 % peak area. Auto spectra scans of all the tracks at 258 nm resembled the UV visuals at 254 and 366 nm, clearly indicating the presence of tylophorine in the samples (Fig. 46 D and E).

4.4.3.3.2 Tylophorine in twenty four months old plants of *T.indica*

Root from *in vitro* raised and *in vivo* mother plants of twenty-four months old *T.indica* were selected for tylophorine analysis using HPTLC. Crude extracts obtained through the optimized extraction protocol I were spotted on silica gel glass plate using fine capillary tubes. Track I was loaded with root samples from *in vitro* raised plant whereas, track II was loaded with root samples of *in vivo* mother plant. The plate was developed in solvent system comprising of toluene: ethyl acetate: diethyl amine (7:2:1). Track I showed two different spots corresponding to Rf 0.64 and 0.89 whereas, track II showed two spots with Rf value 0.60 and 0.87 (Fig. 47). On comparison of different spots with the standard, it was concluded that tylophorine spot is visualized in track I with Rf corresponding to 0.64 whereas no spot in track II corresponded to tylophorine spot (Table 42). The samples were analyzed further through HPTLC. For HPTLC analysis, *in vitro* and *in vivo* samples were loaded along with the standard tylophorine on pre coated silica gel fluorescent (5X5cm) plate (Fig. 48 A-C). The plate was developed in same solvent system as employed for glass TLC i.e. toluene: ethyl acetate: diethyl amine (7:2:1) and was scanned at 254 nm. On comparison of different tracks with the standard tylophorine, presence of tylophorine peak was detected in both the samples. Track I containing root samples from *in vitro* raised plant showed tylophorine peak at Rf 0.63 with 35.81% peak area whereas track II containing root samples of *in vivo* mother plant showed tylophorine peak at Rf 0.64 with 35.43% peak area. UV spectra of tylophorine recorded at 200-400 nm revealed that tylophorine peak in all the tracks showed nearly similar UV absorbance (Fig. 48 D). Figure 48 E showed 3-D spectra scan of tylophorine peaks in all the tracks at 254 nm, which resembled the visual impression of the plate under UV 254 and 366nm (Fig. 48 F and G).

Quantitative analysis of all the test samples showed that highest amount of tylophorine i.e. 90.75 µg/ml was detected in root samples of twenty four months old *in vitro* raised plant whereas *in vivo* plants of same age yielded 59.40 µg/ml of tylophorine (Table 43). Extracts from twelve months old *in vitro* and *in vivo* plants contained 88.34 and 57.47 µg/ml of tylophorine. Thus, root extract of twenty four months old *in vitro* raised plant yielding maximum amount of tylophorine

was selected for further purification using column chromatography to obtain pure fractions of tylophorine.

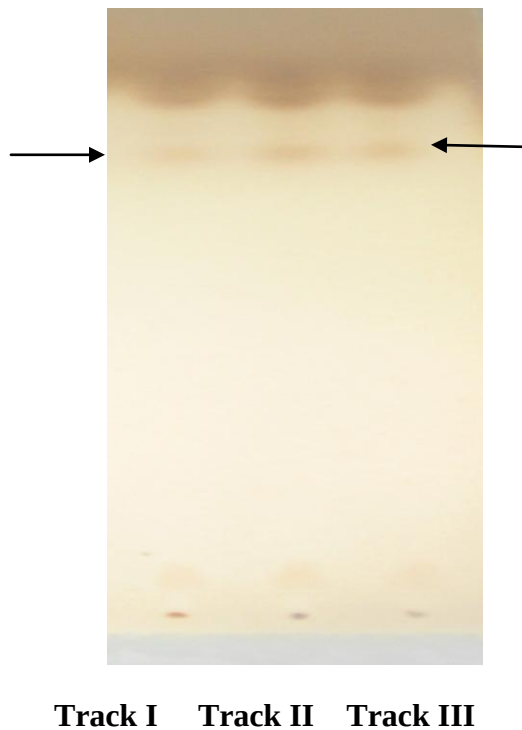


Fig. 45. TLC profile of root extract of twelve months old *in vitro* raised and *in vivo* plants of *T.indica*.

Track I: Root samples from twelve months old *in vitro* raised plants

Track II: Standard Tylophorine

Track III: Root samples from twelve months old *in vivo* plants

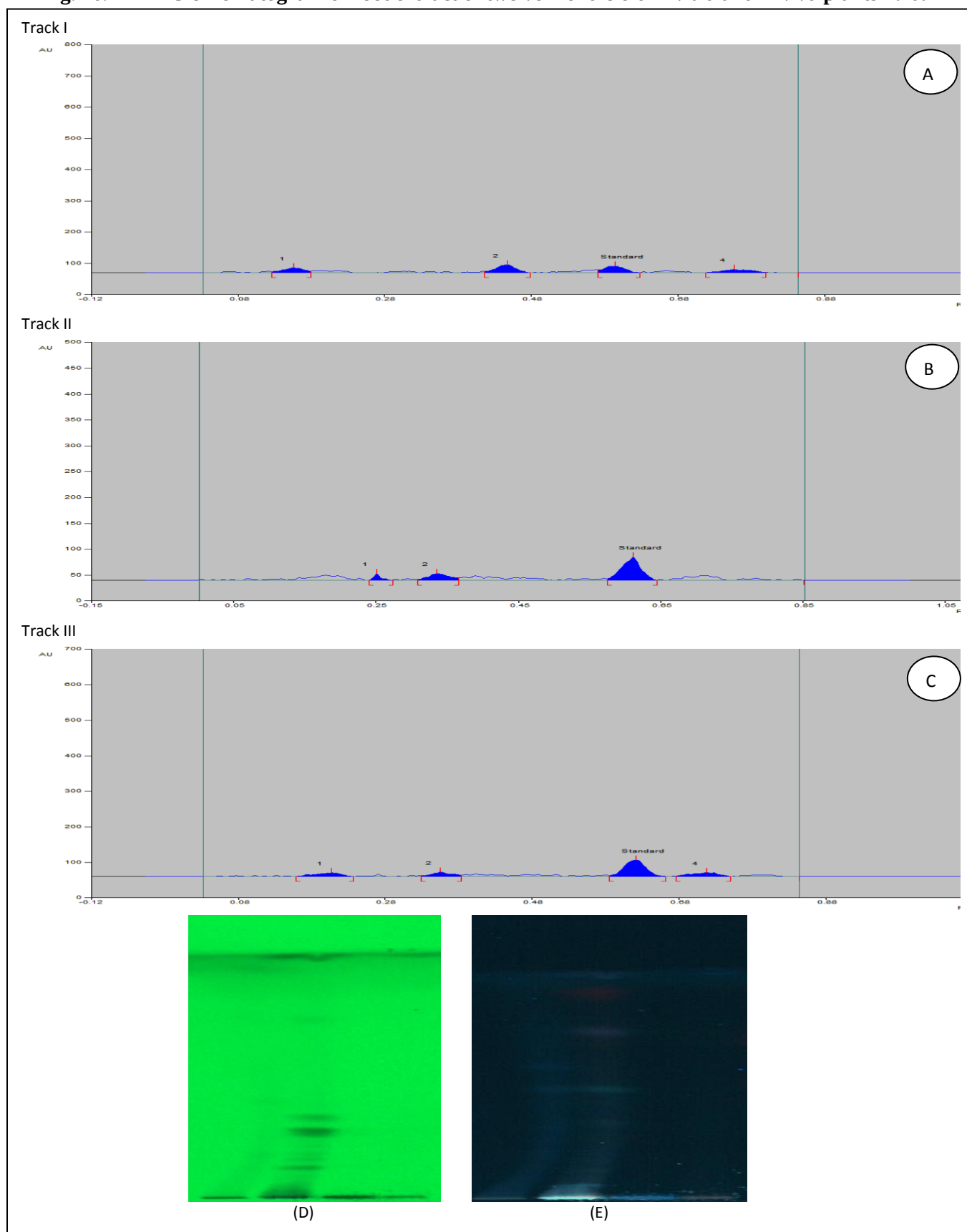
Table 41. Tylophorine spots in different tracks when developed in iodine chamber

Track ID	Number of spots	Rf values	Color with iodine vapours
I	1	0.17	Light Brown
	2	0.64	Dark Brown
II	1	0.14	Light Brown
	2	0.64	Dark Brown
III	1	0.15	Light Brown
	2	0.65	Dark Brown

Fig. 46. HPTLC chromatograms of root extracts of twelve months old *in vitro* raised and *in vivo* plants of *T.indica*.

- A. Track I loaded with standard tylophorine.
- B. Track II containing root extract of *in vitro* raised plant.
- C. Track III contained extract of *in vivo* plant.
- D. UV visual at 254 nm showing tylophorine bands in different tracks.
- E. UV visual at 366 nm showing bands of tylophorine in different tracks.

Fig. 46. HPTLC chromatogram of root extract of twelve months old *in vitro* and *in vivo* plants *ndica*



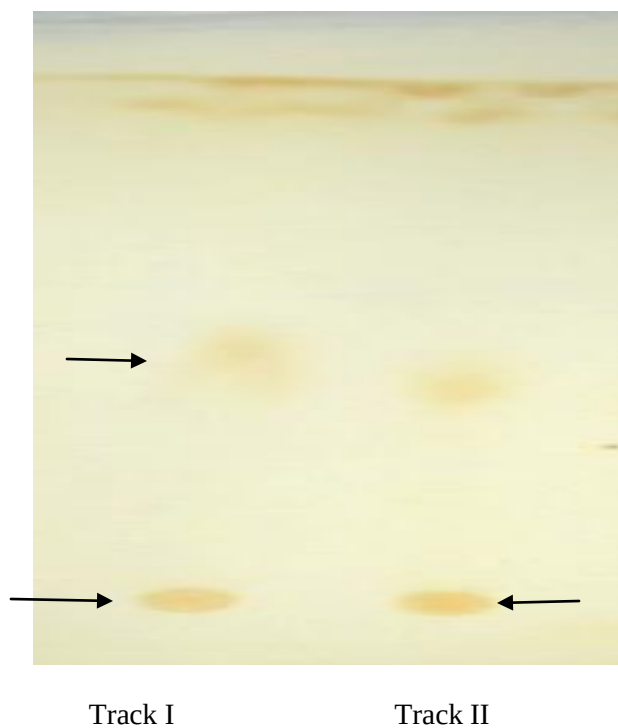


Fig. 47. TLC profile of root samples of twenty four months old *in vitro* and *in vivo* plants

Track I: Root sample from twenty four months old *in vitro* plants.

Track II: Root sample from twenty four months old *in vivo* plants.

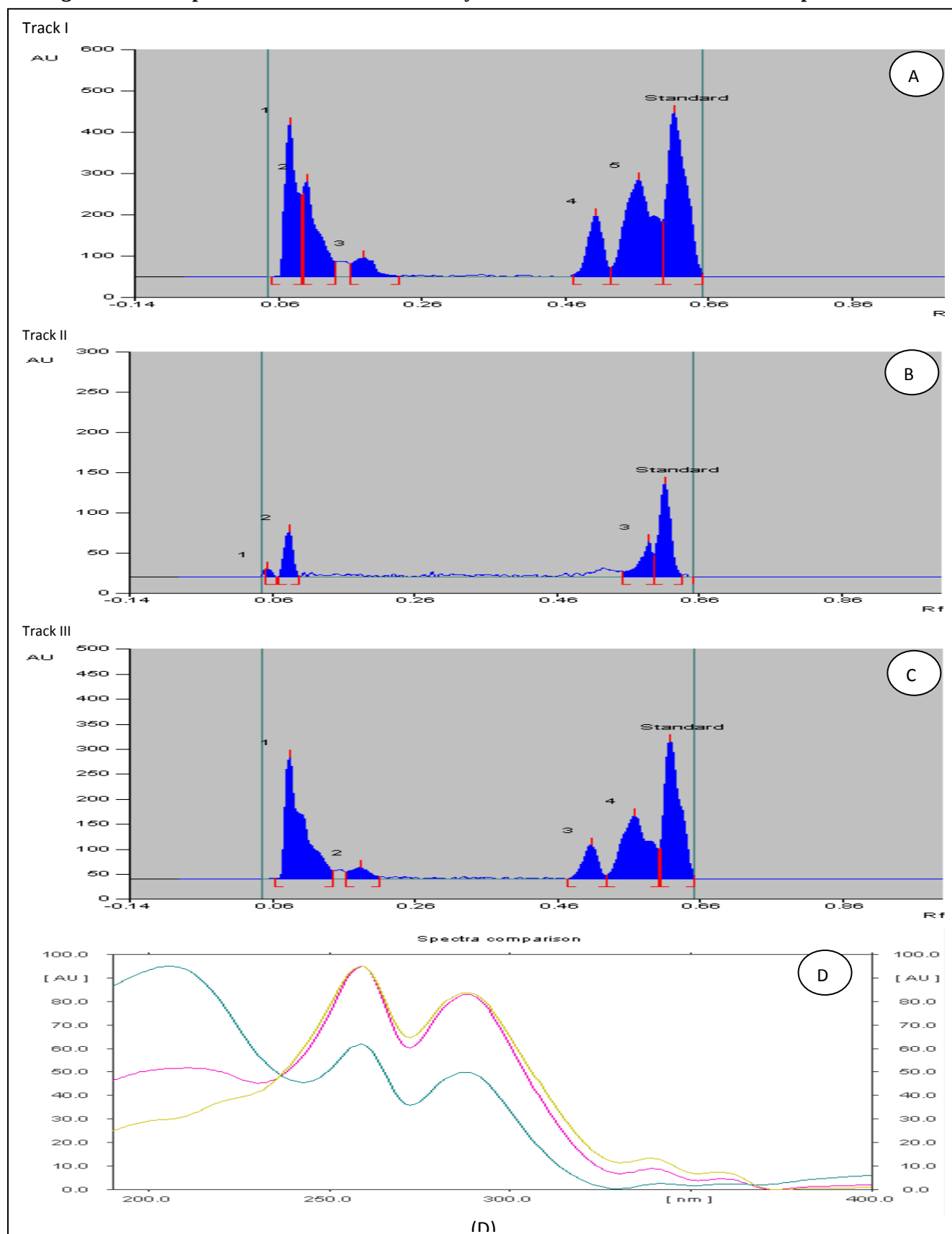
Table 42. Tylophorine spots in different tracks when developed in iodine chamber.

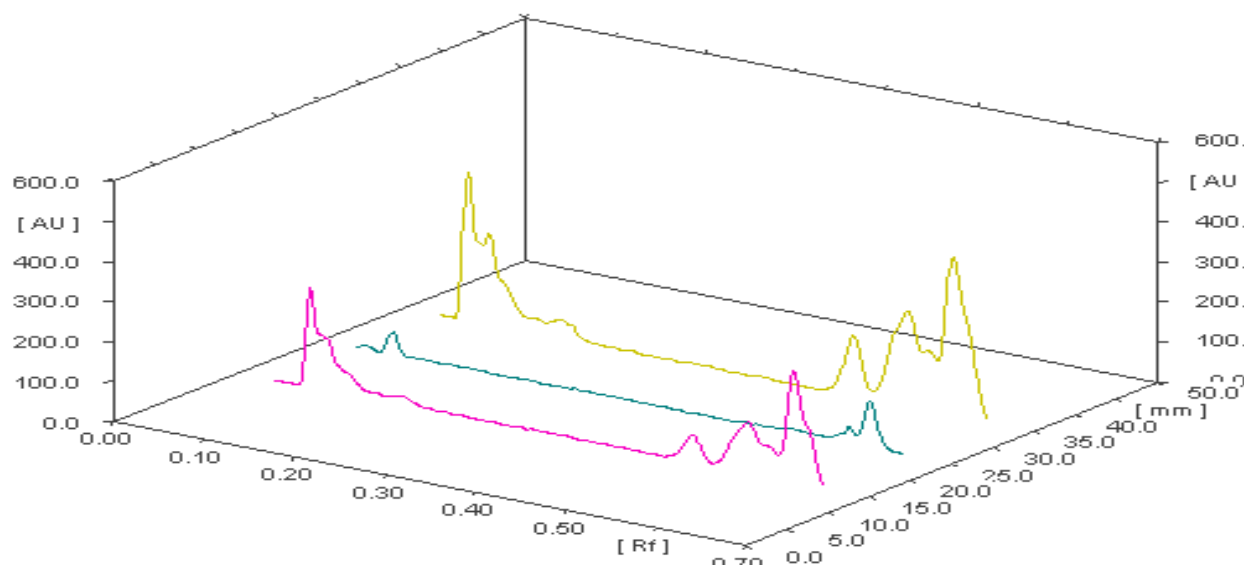
Track ID	No. of spots	Rf value	Colour in iodine atmosphere
I	1	0.64	Light Brown
	2	0.89	Dark Brown
II	1	0.60	Light Brown
	2	0.87	Dark Brown

Fig. 48. Chromatogram of root extracts of twenty four months old *in vitro* raised and *in vivo* mother plants of *T.indica*

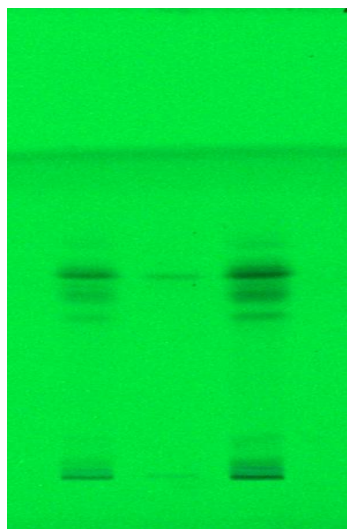
- A. Track I loaded with root extract obtained from *in vitro* raised plant.
- B. Track II loaded with standard tylophorine.
- C. Track III contained root extract from *in vivo* mother plant.
- D. UV spectra of tylophorine peaks recorded from 200-400 nm.
- E. 3- D spectra comparison of all the peak at 258 nm.
- F. UV visual at 254 nm showing distinct tylophorine (arrowheads) bands in all the tracks.
- G. UV visual at 366 nm showing tylophorine (arrowhead) bands in different tracks.

Fig. 48. HPTLC profile of root extract of twenty four months old *in vitro* and *in vivo* plants

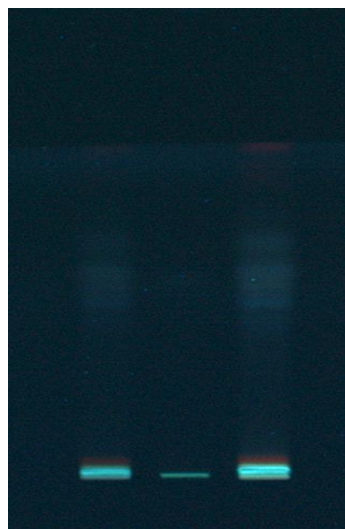




(E)



(F)



(G)

Table 43. Tylophorine ($\mu\text{g/ml}$) in different tracks developed using toluene: ethyl acetate: diethyl amine (7:2:1). Standard tylophorine applied at a working concentration of $20.0\mu\text{g/ml}$.

Track ID	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorine ($\mu\text{g/ml}$)
Fig. 46. Track I (A)	3	0.64	198.40	28.05	-----
Fig. 46. Track II (B)	3	0.64	876.40	70.39	88.34
Fig. 46. Track III (C)	3	0.64	569.00	55.34	57.47
Fig. 48. Track I (A)	6	0.63	7537.0	35.81	90.75
Fig. 48. Track II (B)	4	0.63	1661.6	53.07	-----

Fig. 48. Track III (C)	5	0.64	4932.2	35.43	59.40

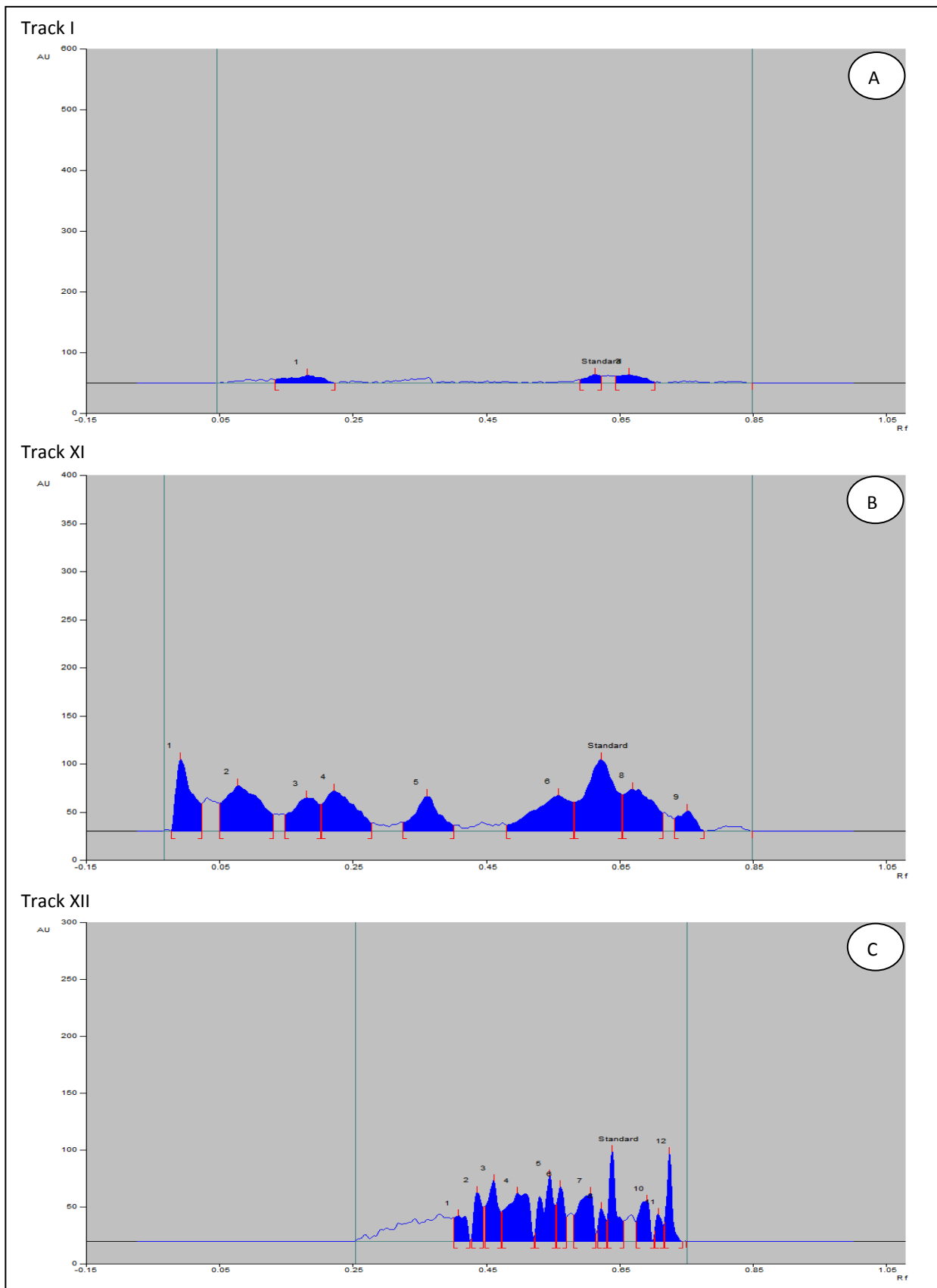
4.4.3.4 Purification using column chromatography

The crude extract from roots of twenty four months old *in vitro* raised plant was further purified by column chromatography. The fraction obtained was loaded on silica gel column, which was eluted with increasing polarities of mixture of chloroform and methanol (5, 15 and 25%). The elutes obtained by using chloroform (100%) followed by increasing concentrations of methanol contained number of alkaloids. The mixture was subjected to high performance thin layer chromatography on pre coated silica gel fluorescent plate for the detection of tylophorine peak in various tracks. All the different fractions (6 µl each) along with the standard tylophorine were loaded as separate tracks and developed using toluene: ethyl acetate: diethyl amine (7:2:1). The optimum wavelength of scan was 258 nm. On comparison of different tracks with the standard, presence of tylophorine was observed only in track XI and XII (Fig. 49 A-C). Standard track showed tylophorine peak at Rf 0.63 with 21.25% peak area. A comparison of standard peak with samples clearly showed that sixth peak (with 28.49 % peak area) in track XI and ninth peak (10.05% peak area) in track XII corresponded to tylophorine peaks (Table 44). 3-D spectra scan at 258 nm also showed distinct tylophorine peaks in both the tracks (Fig. 49 D). UV visuals at 254 and 366 nm showed clear bands in both the tracks corresponding to Rf 0.63 confirming the presence of tylophorine in both the samples (Fig. 49 E and F). Hence, track XI showing maximum area percentage of tylophorine peak was analyzed further using preparative TLC and HPTLC to obtain pure tylophorine fractions.

Fig. 49. Chromatogram of column fractions of twenty four months old *in vitro* raised plant of *T.indica*

- A. Standard track loaded with tylophorine (6µl).
- B. Track XI showing tylophorine peak with 28.49 % peak area.
- C. Track XII showing tylophorine peak with 10.05% peak area
- D. 3-D spectra comparison tylophorine peaks at 258 nm
- E. Photodocumentation pictures of plate at 254 nm showing bands of tylophorine (arrowhead) in different tracks.
- F. Photodocumentation pictures of plate at 366 nm showing bands of tylophorine (arrowhead) in different tracks.

Fig. 49. HPTLC chromatogram of column chromatography fractions



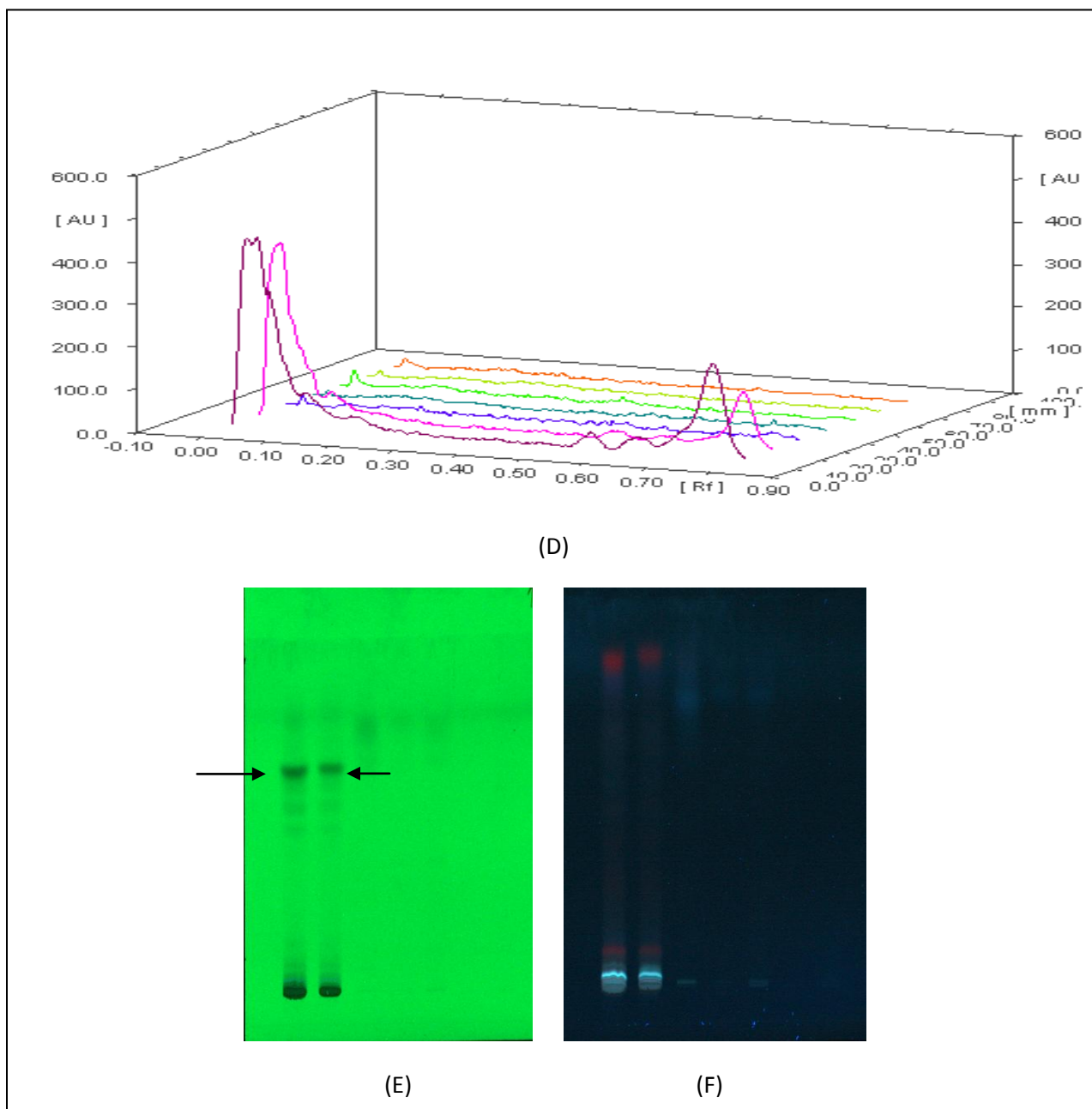


Table 44. Tylophorine ($\mu\text{g/ml}$) in different tracks developed using toluene: ethyl acetate: diethyl amine (7:2:1).

Track ID	Peak	End Position (Rf)	Area (AU)	Area %
Fig. 49. Track I (A)	2	0.63	229.6	21.25
Fig. 49. Track XI (B)	7	0.63	2347	28.49
Fig. 49. Track XII (C)	9	0.63	621.0	10.05

4.4.3.5 Purification using preparative TLC

Preparative TLC of eleventh column fraction was done initially on glass plates and then on pre coated silica gel plates. For carrying preparative TLC, glass plates were prepared with silica gel using the same method as done in TLC. Optimization of solvent system was done using glass TLC where plates were developed in different sets of solvents systems comprising of toluene: chloroform: ethanol: ammonia (7: 4: 3: drop and 6: 2:3: drop) and toluene: ethyl acetate: diethyl amine (6:3:1 and 7:2:1) (Fig. 50 A-D). However, the optimum solvent system comprised of toluene: ethyl acetate: diethyl amine (7:2:1) where tylophorine spot with R_f 0.63 comparable to that of the standard tylophorine was seen (Table 45). Spot thus, obtained was scraped using fine end of spatula and was dissolved in methanol followed by its filtration using syringe filter to remove silica carried with the spot. The solvent was evaporated in rotary and the remaining extract was chromatographed using HPTLC to confirm the banding pattern.

Plate developed in toluene: ethyl acetate: diethyl amine (7:2:1) with optimum wavelength of scan at 258 nm, showed tylophorine peak at R_f 0.63 when compared with the standard (Fig. 51 A). Also, 3-D spectra scan at 258 nm showed comparable peaks with the UV visual tylophorine band at 366 nm, hereby confirming the presence of tylophorine in the sample (Fig. 51 B and C). Spot thus, obtained was again scraped using fine end of spatula and was dissolved in methanol followed by its filtration using syringe filter to remove silica carried with the spot. The solvent was evaporated in rotary and the pure fraction obtained was finally studied through NMR analysis.

Fig. 50. Preparative chromatography of column fraction in different solvent systems

- A. Column fraction in solvent system of toluene: ethyl acetate: diethyl amine (6:4:1).
- B. Column fraction in solvent system of toluene: ethyl acetate: diethyl amine (7:2:1).
- C. Column fraction in solvent system of toluene: chloroform: ethanol: ammonia (6:2:3: drop).
- D. Column fraction in solvent system of toluene: chloroform: ethanol: ammonia (7:4:3: drop).

Fig. 50. Preparative TLC and HPTLC analysis of pure tylophorine

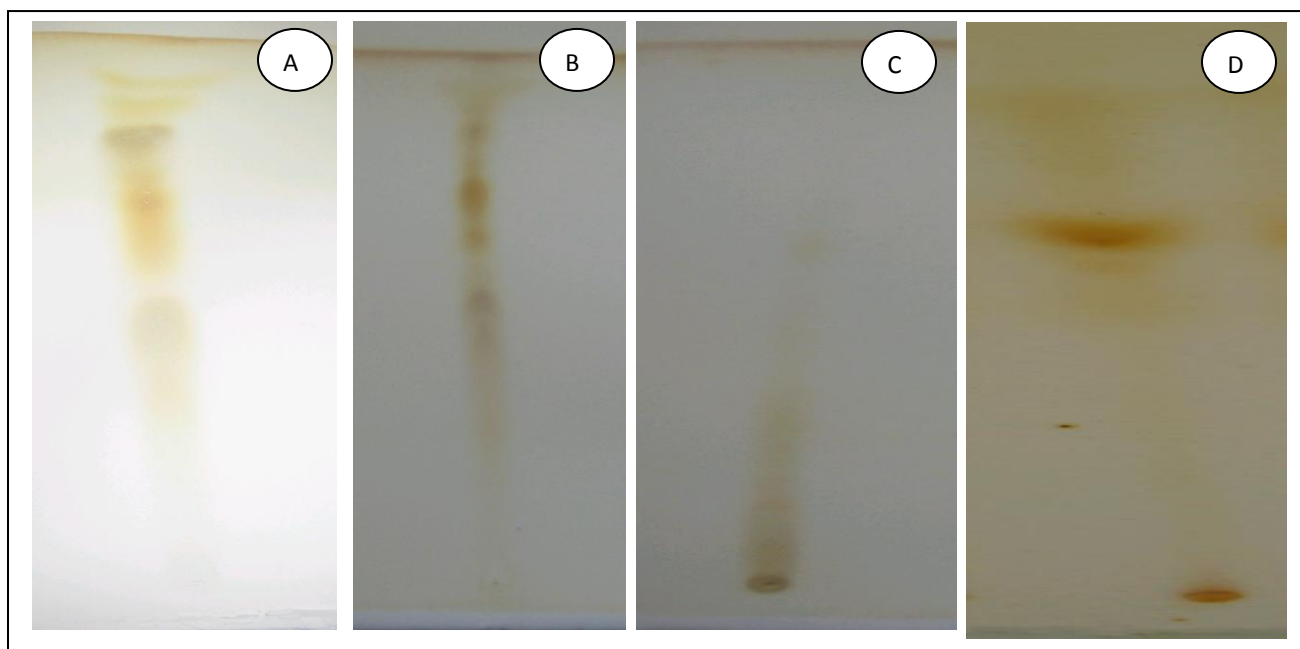


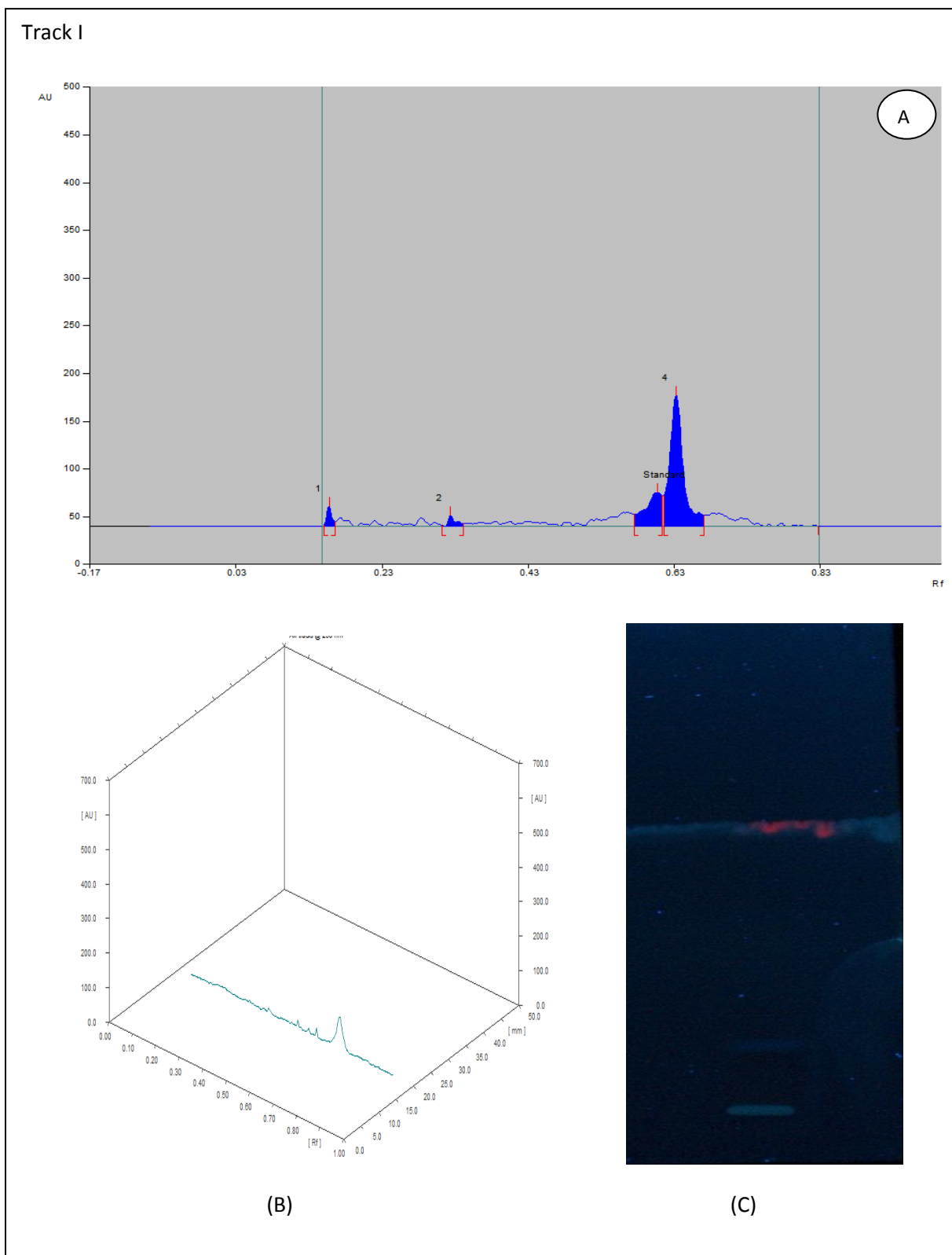
Table 45. Preparative TLC of column fraction in different solvent systems

Fig. No	Solvent system	Solvent Ratio
50 (A)	Toluene: ethyl acetate: diethyl amine	6:4:1
50 (B)	Toluene: ethyl acetate: diethyl amine	7:2:1
50 (C)	Toluene: chloroform: ethanol: ammonia	6: 2: 3: drop
50 (D)	Toluene: chloroform: ethanol: ammonia	7: 4: 3: drop

Fig. 51. HPTLC chromatograms of preparative sample in toluene: ethyl acetate: diethyl amine (7:2:1)

- A. Track I loaded with column fraction showing tylophorine peak at 258 nm.
- B. 3-D spectra scan of track showing tylophorine peak.
- C. Photodocumentation picture of plate at 366 nm showing distinct tylophorine band.

Fig. 51. Chromatogram of preparative sample in toluene: ethyl acetate: diethyl amine (7:2:1)



4.4.3.6 NMR Study

The ^1H NMR spectra of tylophorine isolated from the roots of 2 yr old *in vitro* raised plant of *T.indica* was measured on a Bruker spectrophotometer (Model AVANCE DPX-400 MHz) using methanol as the solvent (Fig. 52). On comparison of NMR spectra of tylophorine with that of standard tylophorine following observations were made. Spectra signal at δ 1.68-2.30 showed the presence of CH_2 group, δ 7.20-7.44 showed CH group, δ 3.30-3.87 CH_3 group. Detailed structural elucidation was however not conducted but the basic interpretation of data was done to confirm the presence of tylophorine (Table 46).

Table 46: ^1H NMR interpretation of tylophorine

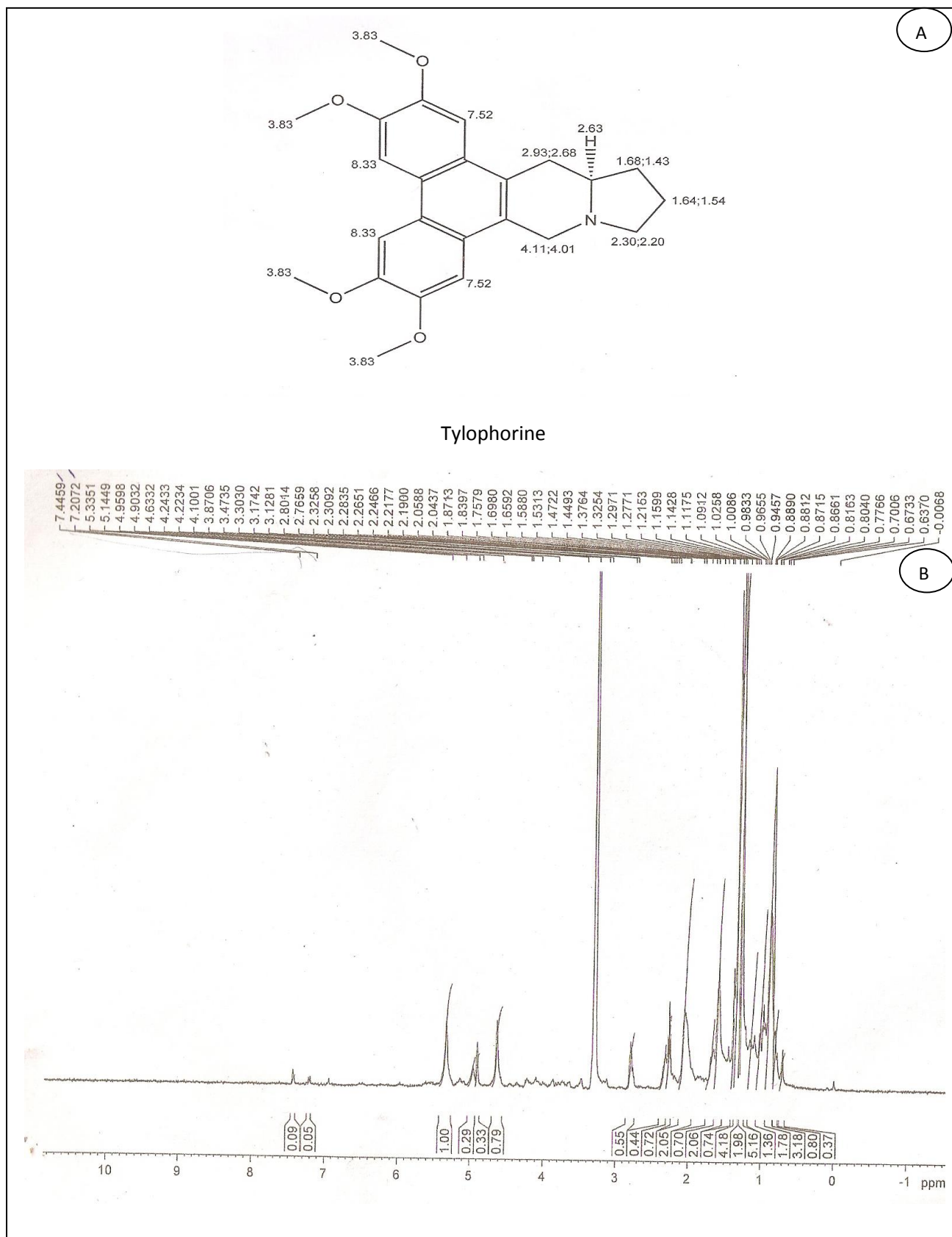
S. No.	Signals (ppm)	Types of Proton
1.	1.68-2.30	R- CH_2
2.	7.20-7.44	CH
3.	3.30--3.87	R- CH_3
4.	2.32-2.04	H

Fig. 52. ^1H NMR scan of tylophorine

A. Structure of tylophorine

B. NMR scan at 400 MHz

Fig. 52. ^1H NMR scan of tylophorine



Objective 3: To develop protocol for mass extraction of secondary metabolites

4.5 Mass extraction of Stevioside from callus and suspension cultures of *Stevia rebaudiana*

4.5.1 Callus cultures

Based on the results obtained on different combinations and concentrations of plant growth regulators used for callus induction, it was found that the maximum growth of leaf callus occurred on MS medium supplemented with 2, 4-D (19.48 μ M) and K (4.65 μ M) where callusing occurred in 98% of the cultures. Callusing initiated after 6-7 day of culturing and within 3 weeks, the entire explant turned into greenish white, soft and friable callus mass capable of sustained growth. Likewise, good callus growth occurred on NAA (29.4 μ M) and K (4.65 μ M) supplemented MS medium, where light brown soft callus was formed in 95% of the cultures.

Callusing from stem explants also occurred on 29.4 μ M NAA and 4.65 μ M K supplemented MS medium, where callusing initiated at the cut ends of stem segments after 8-10 days of culturing and within 6 weeks, large friable callus masses were formed. The calli remained light green and showed sustained growth on repeated subculturing. The proliferation rate of calli of leaf explants was significantly higher than that of stem explants (Table 47).

Table 47: Effects of different combinations of auxins and cytokinins on callus culture

Type of Explant	Media Composition	Fresh Weight (g/l)	Dry weight (g/l)
Leaf	2, 4-D (19.48 μ M) + K (4.65 μ M)	340	21.8
	NAA (29.4 μ M) + K (4.65 μ M)	298	18.9
Stem	NAA (29.4 μ M) + K (4.65 μ M)	214	16.7
	2, 4-D (19.48 μ M) + K (4.65 μ M)	166	9.6

Leaf callus (40g) growing on 2, 4-D + K medium and stem callus (40g) on NAA + K supplemented medium was dried, extracted using protocol V as discussed in material and method section and purified using HPTLC. Leaf and stem calli extracts (6.0 μ l each) were loaded on pre-coated silica gel 60 F 254 (5 x10cm) HPTLC plate in different tracks (Fig. 53 A-C). Optimization of solvent system was done using various combinations of polar and non-polar

solvents (Table 48). However, the best identified solvent system comprises of ethyl acetate: methanol: n-hexane (2:2:5) wherein both the samples showed the presence of stevioside when compared with standard. UV spectra of stevioside recorded at 200-400 nm revealed that stevioside peak in all the tracks showed quite similar UV absorbance (Fig. 53 D). Figure 53 E showed 3-D spectra scan of stevioside peaks in all the tracks at 210 nm, which resembled the visual impression of the plate under UV 254 and 366 nm (Fig. 53 F and G).

Similarly, leaf callus raised on NAA + K and stem callus raised on 2, 4-D + K were processed using same extraction protocol and were applied onto another plate developed in same solvent system of ethyl acetate: methanol: n-hexane (2:2:5). Here also, stevioside peaks were observed in both the test samples when compared with the standard (Fig. 53 H-J). Spectra comparison of stevioside peaks recorded at 200-400 nm showed quite similar UV absorbance (Fig. 53 K). 3-D spectra scan of all the tracks at 210 nm, showed the presence of stevioside peaks in both the samples (Fig. 53 L). Visual impression of the plate captured under UV 254 and 366 nm showed stevioside bands in different tracks (Fig. 53 M and N). Quantitative analysis of the samples showed that the highest amount of stevioside i.e. 44.37µg/ml was found in leaf callus raised on 2, 4-D + K combination whereas stem callus cultured on NAA + K yielded 26.9 µg/ml of stevioside (Table 49).

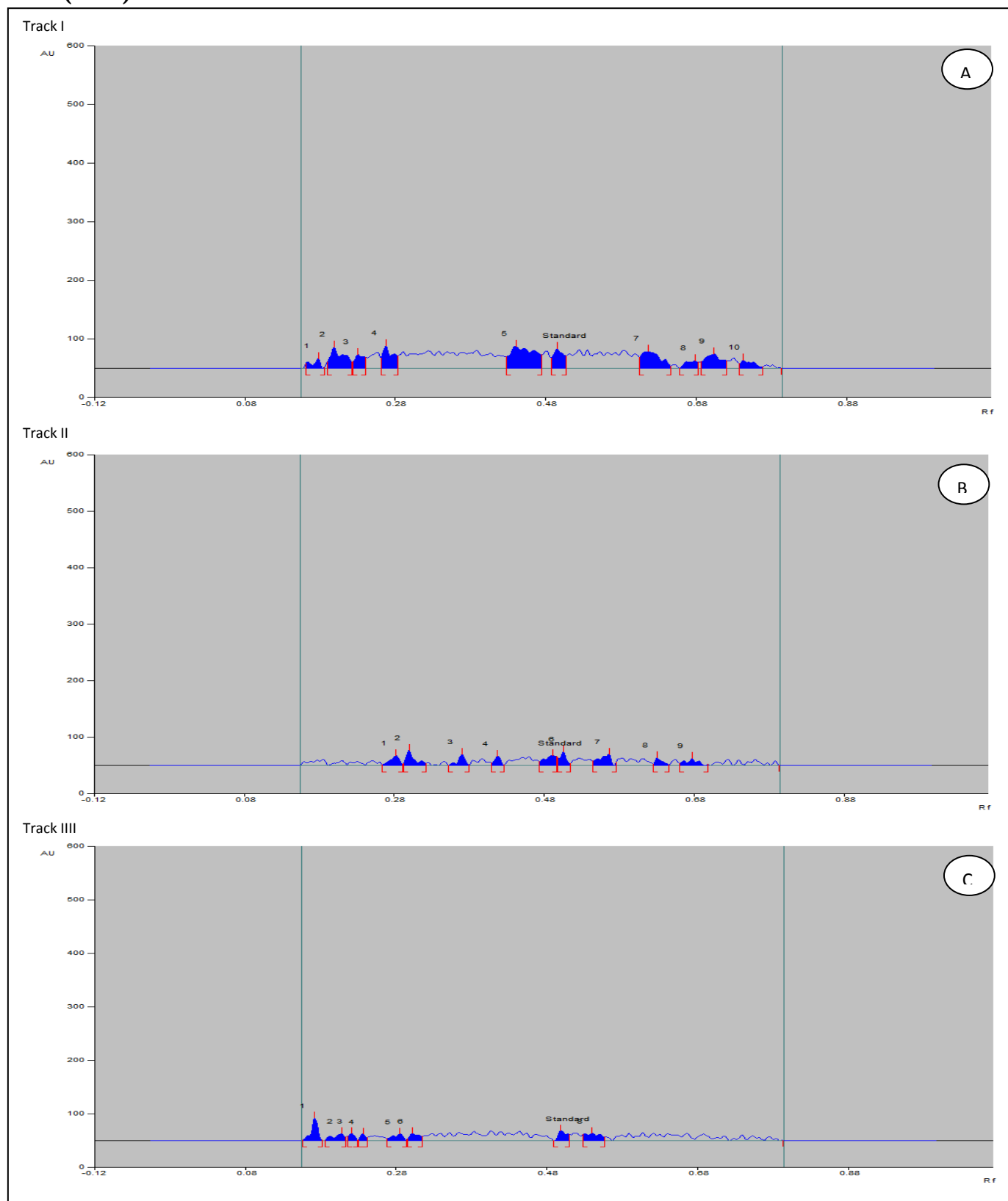
Table 48 Different ratios of solvents for optimizing solvent system

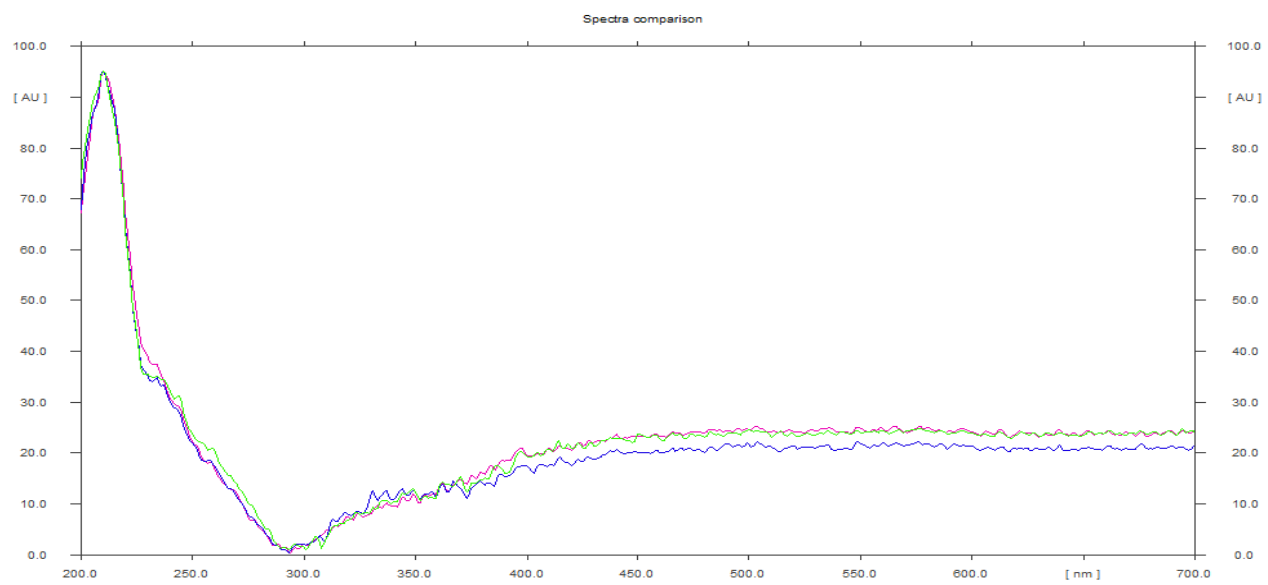
S. No.	Developing solvent	Ratio
1	Ethyl acetate: methanol: n-hexane	2:1:7
2	Ethyl acetate: methanol: n-hexane	1:2:7
3	Ethyl acetate: methanol: n-hexane	1:4:5
4	Ethyl acetate: methanol: n-hexane	2:3:6
5	Ethyl acetate: methanol: n-hexane	2:3:7
6	Ethyl acetate: methanol: n-hexane	1:1:6
7	Ethyl acetate: methanol: n-hexane	1:1:8

Fig. 53. HPTLC chromatogram of leaf and stem callus in ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm.

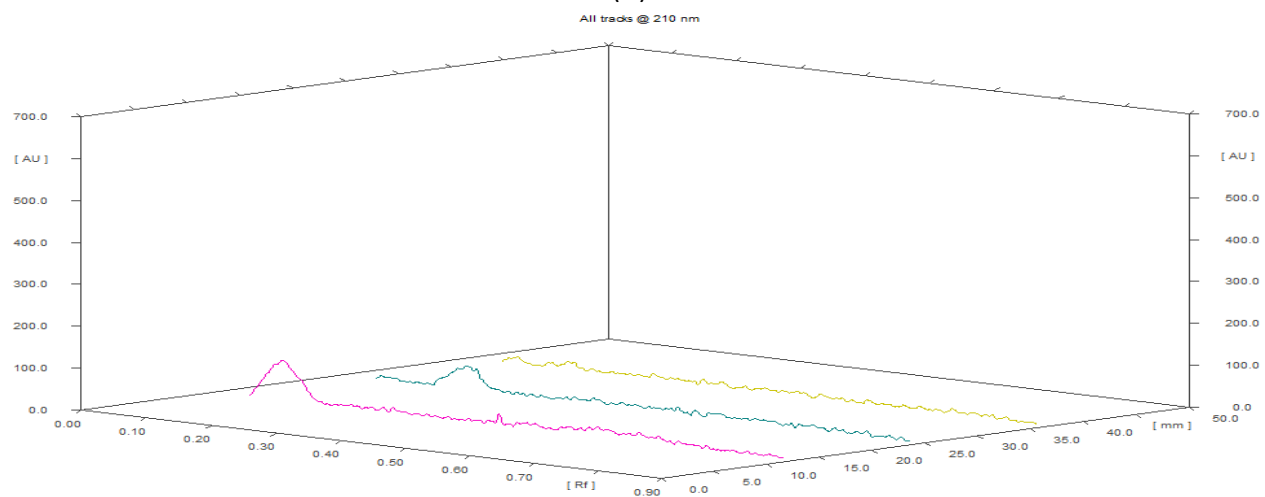
- A. Track I loaded with extract of leaf callus raised on 2, 4-D + K combination.
- B. Track II loaded with extract of stem callus raised on NAA + K combination
- C. Track III loaded with stevioside standard.
- D. Spectra comparison of stevioside peak in different samples.
- E. 3-D spectra scan of all peaks at 210 nm showing stevioside peaks in different samples.
- F. UV visuals at 254 nm showing stevioside band in both the samples and standard at similar R_f.
- G. UV visuals at 366 nm showing stevioside band in both the samples and standard at similar R_f.
- H. Track I loaded with extract of leaf callus raised on NAA + K.
- I. Track II loaded with extract of stem callus raised on 2,4-D + K.
- J. Track III loaded with standard (stevioside).
- K. Spectra comparison of stevioside peak in different samples.
- L. 3-D spectra scan of all peaks at 210 nm showing stevioside peaks in different samples.
- M. UV visuals at 254 nm showing stevioside band in both the samples and standard at similar R_f.
- N. UV visuals at 366 nm showing stevioside band in both the samples and standard at similar R_f.

Fig. 53. HPTLC chromatogram of leaf and stem callus in ethyl acetate: methanol: n-hexane (2:2:5)

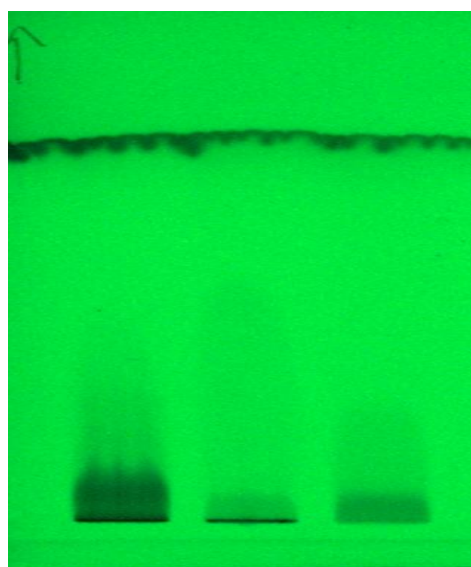




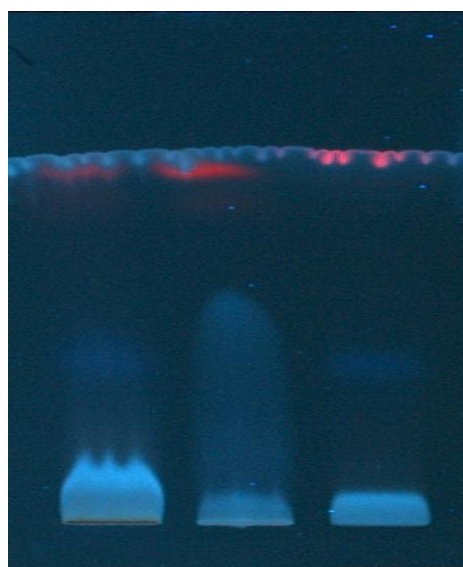
(D)



(E)

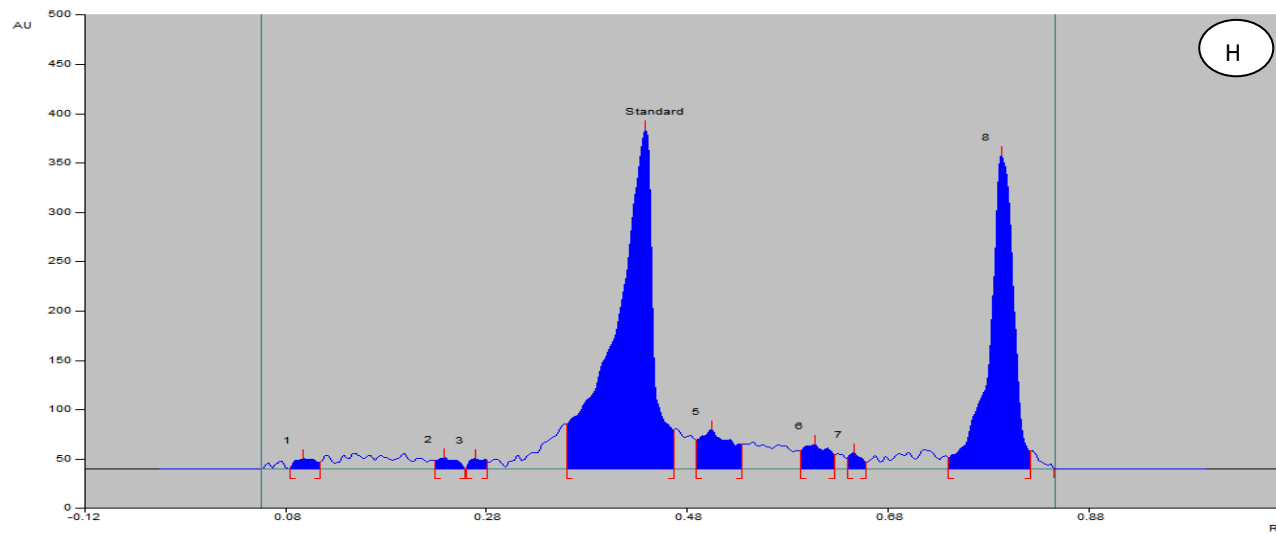


(F)

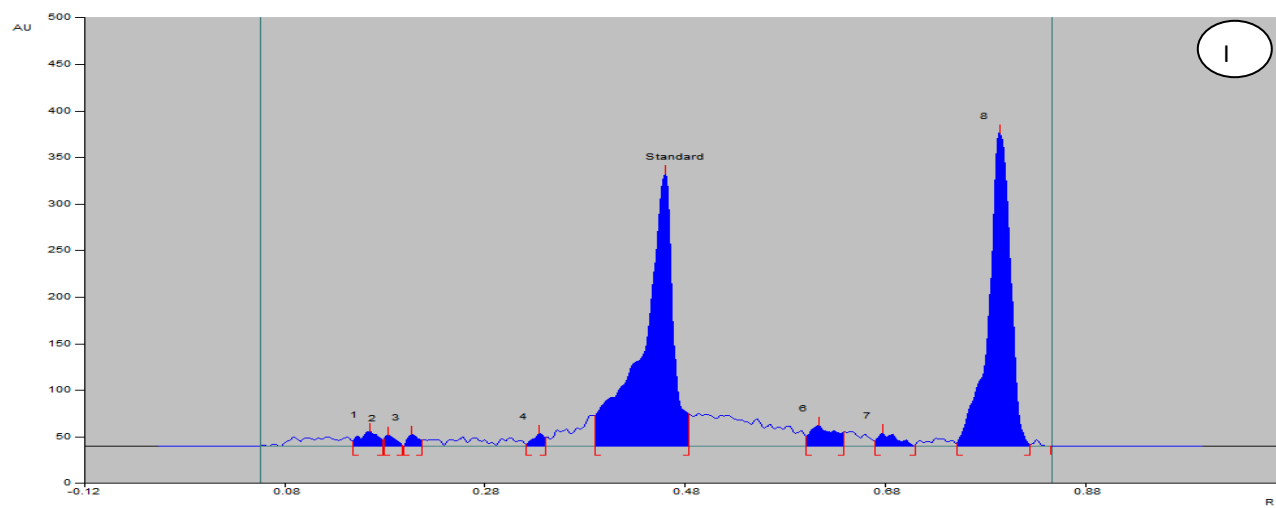


(G)

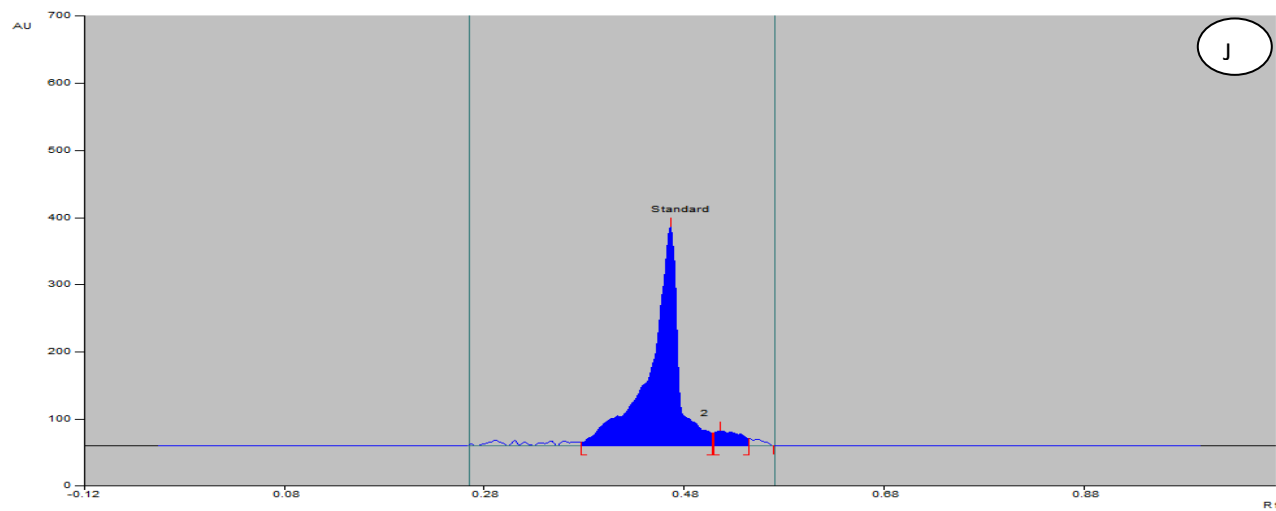
Track I



Track II



Track III



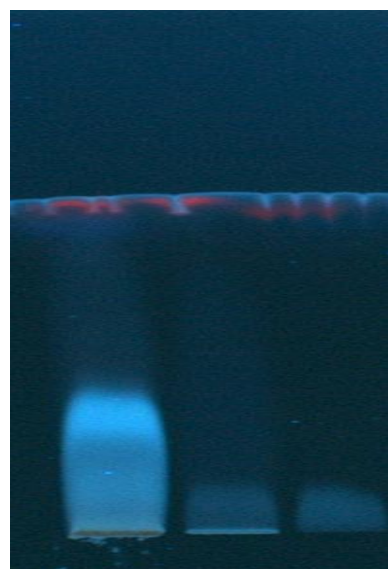
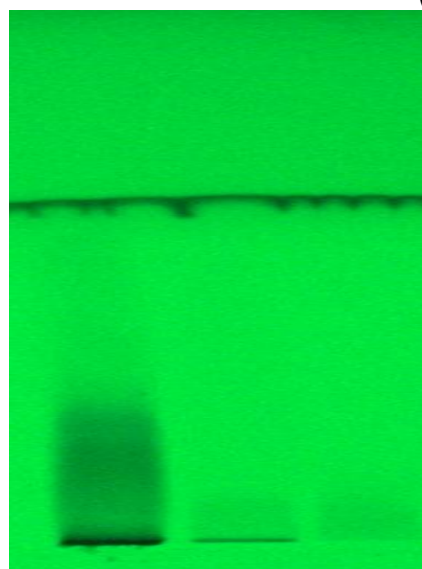
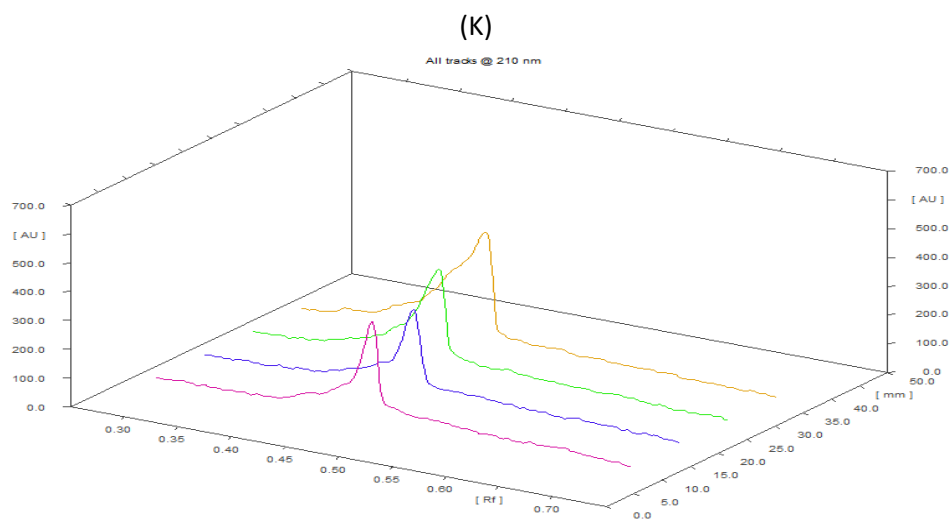
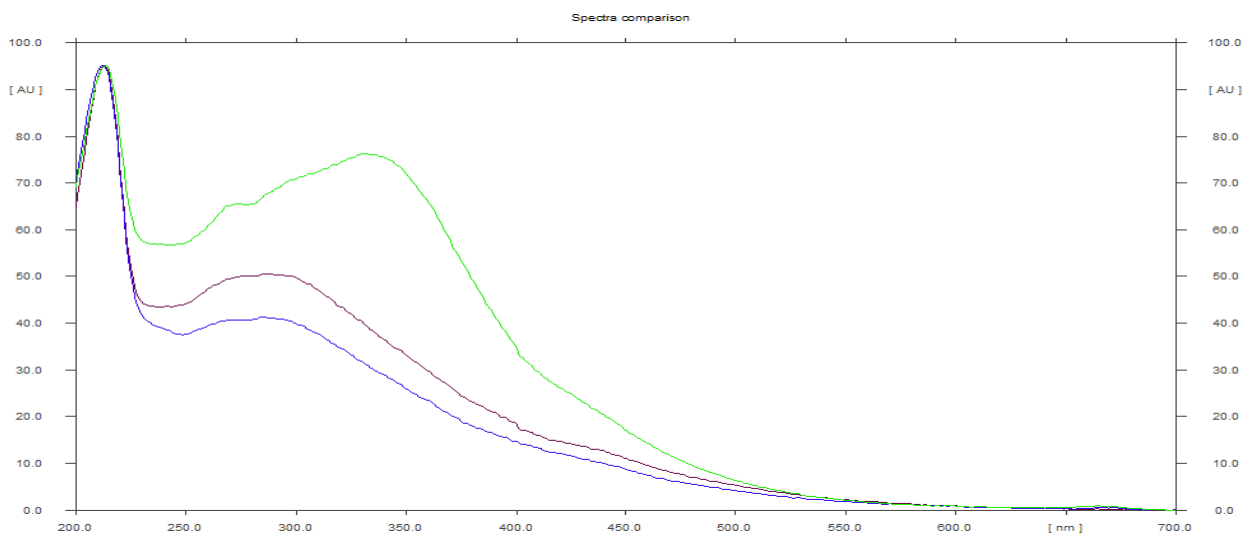


Table 49. Stevioside (µg/ml) in various tracks when developed in ethyl acetate: methanol: n-hexane (2:2:5) at 210 nm. Standard stevioside applied at a working concentration of 20.0 µg/ml

Track	Peak	End Position (Rf)	Area (AU)	Area %	Stevioside (µg/ml)
Fig. 53. Track I (A)	6	0.47	359.4	9.19	44.37
Fig. 53. Track II (B)	5	0.47	218.5	14.4	26.90
Fig. 53. Track III (C) Standard	7	0.47	162.0	13.1	-----
Fig. 53. Track I (H)	5	0.47	9389	88.2	24.60
Fig. 53. Track II (I)	4	0.47	6612	92.6	17.30
Fig. 53. Track III (J) Standard	2	0.47	7623	86.9	-----

4.5.2 Suspension cultures

Well-grown leaf callus (1 g) was subjected to 100 ml liquid MS medium containing 2, 4-D (19.48 μM) + K (4.65 μM) for the generation of suspension cultures. The cultures were maintained on rotator shaker at 120 rpm and regular biomass examination was done after an interval of 3 days (Table 50 and Fig. 54). There was no apparent visible change observed initially, but later the cells grew in volume and mass. Just after lag phase of 9 days, apparent increase in growth in terms of increased biomass occurred till 21st day which corresponded to log phase. Between 22nd and 24th day hardly any change was observed in the biomass and this phase represented the stationary phase. Similarly, actively growing stem callus (1000 mg) was inoculated in 250 ml conical flask containing 100 ml of liquid MS supplemented with NAA (29.4 μM) and K (4.65 μM) for the growth of suspension cultures. Observations were recorded at 3 day interval from the ninth day after incubation up to the 36th day (Table 51 and Fig. 54). Lag phase was much extended in stem cultures as compared to leaf cultures, which lasted for 15 days. Maximum biomass yield was observed between 15th to 27th day and thereafter there was no significant increase. During stationary or decline phase which started after 27th day, accumulation of secondary metabolites occurred. To scale up the production of secondary metabolite, the inoculum was transferred to 5 lt conical flask containing liquid MS medium supplemented with similar combination of growth regulators and monitored regularly till the stationary phase was observed.

Table 50: Growth of leaf cell suspension culture from *S.rebaudiana* in MS liquid medium

S. No	Age of culture (Days)	Callus biomass Fresh weight (g/l)	Callus biomass Dry weight (g/l)
1	6	22	1.8
2	9	28	1.9
3	12	36	2.0
4	15	58	3.0
5	18	74	3.8
6	21	86	4.8
7	24	88	4.7
8	27	80	4.3
9	30	74	3.9

Fig. 54. Growth of leaf cell suspension culture from *S.rebaudiana* in MS liquid medium

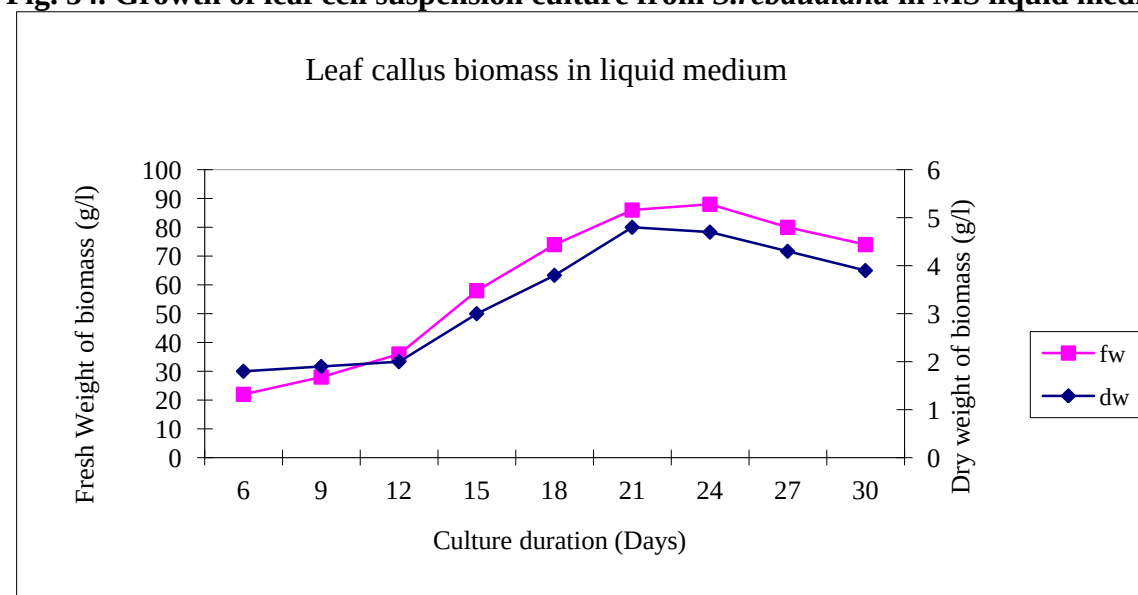
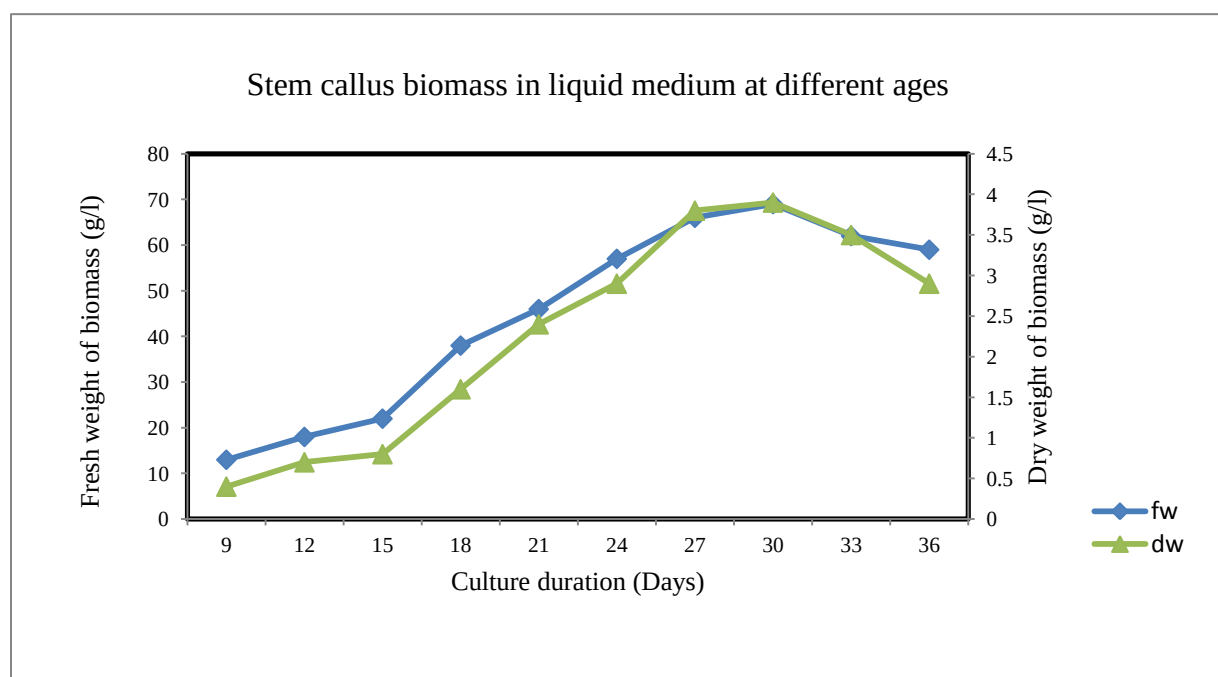


Table 51: Growth of stem cell suspension culture from *S.rebaudiana* in MS liquid medium

S. No	Age of culture (Days)	Callus biomass Fresh weight (g/l)	Callus biomass Dry weight (g/l)
1	9	13	0.4
2	12	18	0.7
3	15	22	0.8
4	18	38	1.6
5	21	46	2.4
6	24	57	2.9
7	27	66	3.8
8	30	69	3.9
9	33	62	3.5
10	36	59	2.9

Fig. 55. Growth of stem cell suspension culture from *S.rebaudiana* in MS liquid medium



Suspension cultures were harvested at the stationary phase using the method described in section 3.8.2. The dried biomass of suspension cultures were extracted using extraction protocol V (as discussed in material and method section 3.5) and was purified by HPTLC. Crude suspension extracts of leaf and stem (6 μ l) were loaded on pre-coated silica gel (5 X10 cm) plate and developed in ethyl acetate: methanol: n-hexane in ratios of 2:2:5 and 2:4:6. Plate developed in solvent system consisting of ethyl acetate: methanol: n-hexane (2:2:5) was loaded with extract of leaf suspension culture in track I, whereas track II contained extract of stem suspension culture. On comparison of both the samples with the standard in track III, it was found that stevioside peak is present in both the samples at Rf 0.53 (Fig. 56 A-C). Figure 56 D showing 3-D spectra scan of stevioside peaks in all the tracks at 210 nm, resembling the visual impression of the plate under UV 254 and 366 nm (Fig. 56 E and F). Similarly, another plate was loaded with same test samples and was developed in solvent system of ethyl acetate: methanol: n-hexane (2:4:6) and stevioside peaks were seen in all the tracks (Fig. 56 G-I). Spectra scan of all the peaks at 210 nm showed the presence of stevioside with similar Rf value of 0.51 (Fig 56 J). Quantitative analysis of all the samples showed that solvent system used in chromatogram development consisting of ethyl acetate: methanol: n-hexane (2:4:6) yielded 55.13 and 43.80 μ g/ml of stevioside in leaf and stem suspension extracts respectively. However, highest amount of stevioside 69.40 and 57.69 μ g/ml was found in leaf and stem suspension extracts respectively, when developed in ethyl acetate: methanol: n-hexane in ratio of 2:2:5 (Table 52).

Fig. 56. HPTLC chromatogram of stevioside from suspension culture of *Stevia rebaudiana*

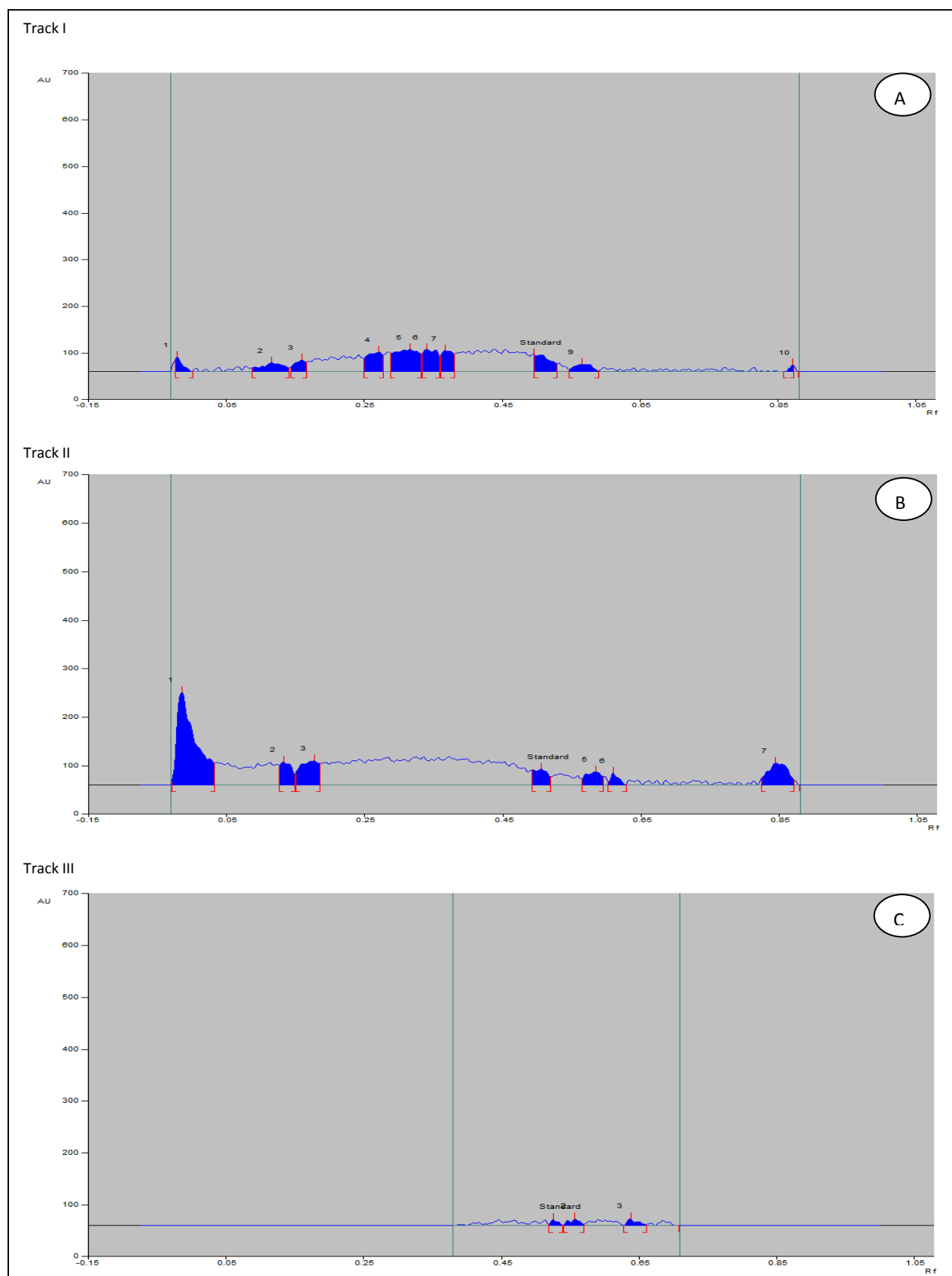
Plate was developed in ethyl acetate: methanol: n-hexane in ratios of 2:2:5

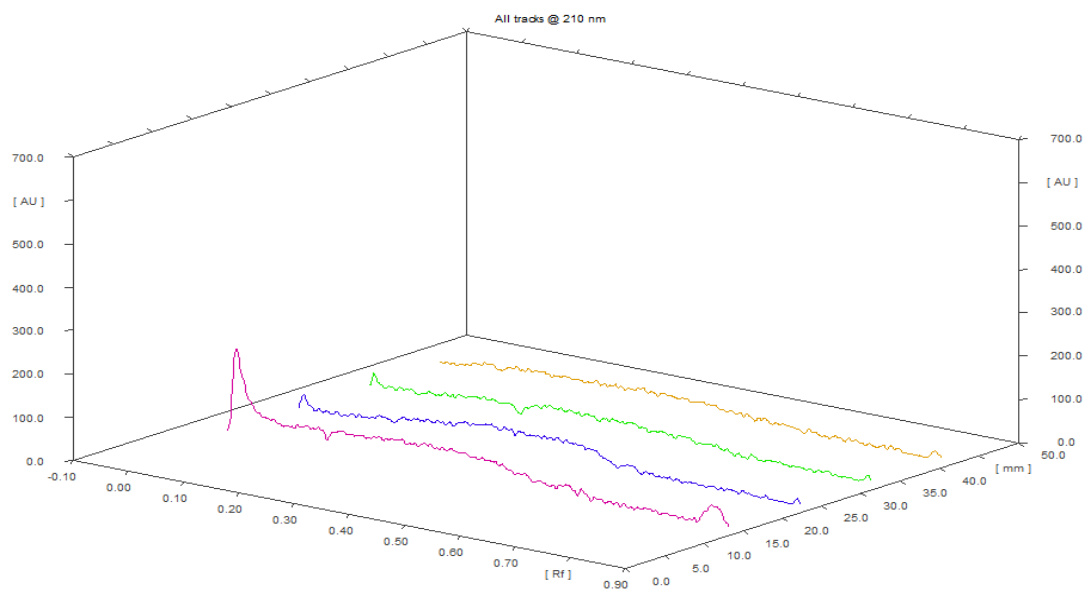
- A. Track I loaded with suspension extract of leaf explants.
- B. Track II loaded with suspension extract of stem explants
- C. Track III loaded with stevioside standard.
- D. 3-D spectra of all the tracks at 210 nm.
- E. Photodocumentation pictures at 254 nm showing bands of stevioside in various tracks.
- F. Photodocumentation pictures at 366 nm showing bands of stevioside in various tracks.

Plate was developed in ethyl acetate: methanol: n-hexane in ratios of 2:4:6.

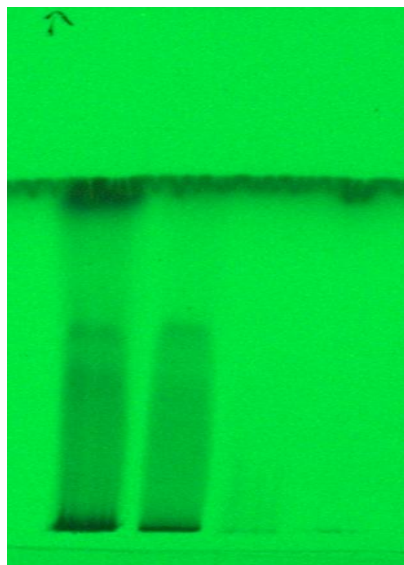
- G. Track I loaded with suspension extract of leaf explants.
- H. Track II loaded with suspension extract of stem explants
- I. Track III loaded with stevioside standard.
- J. 3-D spectra of all the tracks at 210 nm.

Fig. 56. HPTLC chromatogram of suspension culture of *Stevia rebaudiana*

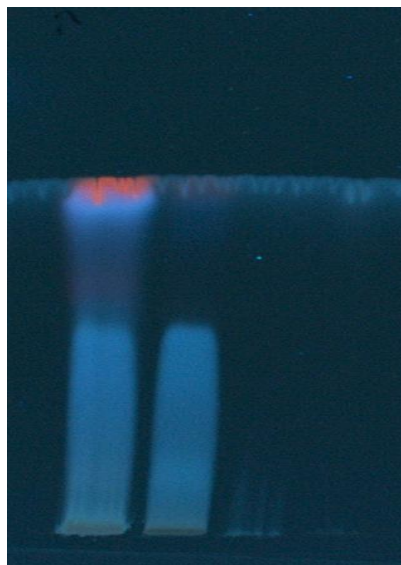




(D)



(E)



(F)

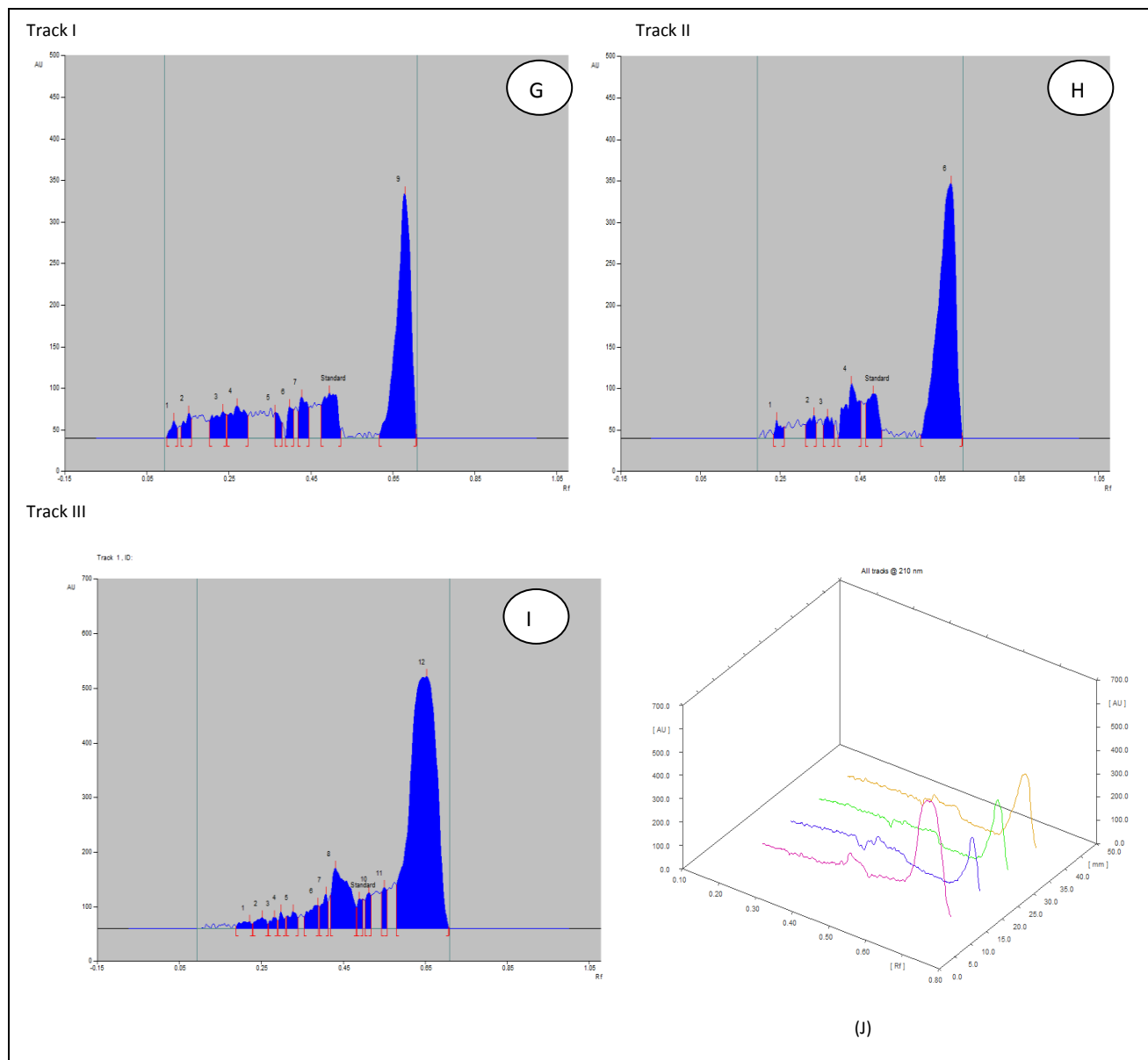


Table 52. Stevioside ($\mu\text{g/ml}$) in different tracks. Standard stevioside applied at a working concentration of $20.0 \mu\text{g/ml}$.

Track ID	Solvent System	Peak	End Position (Rf)	Area (AU)	Area %	Stevioside ($\mu\text{g/ml}$)
Fig. 56. Track I (A)	Ethyl acetate: methanol: n-hexane (2:2:5)	4	0.53	644.3	12.02	69.40
Fig. 56. Track II (B)	Ethyl acetate: methanol: n-hexane (2:2:5)	8	0.53	535.1	6.92	57.69
Fig. 56. Track III (C)	Ethyl acetate: methanol: n-hexane (2:2:5)	4	0.53	185.5	6.67	-----
Fig. 56. Track I (G)	Ethyl acetate: methanol: n-hexane (2:4:6)	6	0.51	1428	12.93	55.13
Fig. 56. Track II (H)	Ethyl acetate: methanol: n-hexane (2:4:6)	5	0.51	1135	8.22	43.80
Fig. 56. Track III (I)	Ethyl acetate: methanol: n-hexane (2:4:6)	8	0.51	518.1	1.73	-----

4.6 Mass extraction of tylophorine from callus and suspension cultures of *Tylophora indica*

4.6.1 Callus culture

Based on the results obtained on different combinations and concentrations of plant growth regulators used for callus induction, it was found that the maximum growth of leaf callus occurred on 29.0 μM NAA + 4.65 μM K supplemented MS medium. For root callus synergistic action of IBA (24.6 μM) with BA (13.2 μM) was most effective in the initiation and sustained growth of callus. The proliferation rate of calli from both the explants after 30 days is shown in Table 53.

Table 53: Effects of different combinations of auxins and cytokinins on callus culture of *T.indica*.

Type of Explant	Media Composition	Fresh Weight (g/l)	Dry weight (g/l)
Leaf	NAA (9.0 μM) + K (4.65 μM)	548	36.90
Root	IBA (24.6 μM) + BA (13.2 μM)	448	30.09

Leaf callus (40g) growing on NAA + K medium and root callus (40g) on IBA + BA supplemented medium was dried, extracted using protocol I as discussed in material and method section and purified using HPTLC. Leaf and root calli extracts (6.0 μl each) were loaded on pre-coated silica gel 60 F 254 (5 x10cm) HPTLC plate along with the standard tylophorine (Fig. 57 A-C). Plate was developed using different solvent systems (Table 54), and the optimum developing solvent system identified consisted of toluene: ethyl acetate: diethyl amine (7:2:1) at 258 nm. Tylophorine was detected in both the sample, when compared with the standard. Spectra comparison of tylophorine peaks recorded at 200-400 nm showed quite similar UV absorbance (Fig. 57 D). Visual impression of the plate captured under UV 254 and 366 nm showed tylophorine bands in different tracks (Fig. 57 E and F). Quantitative analysis revealed highest amount of tylophorine i.e. 26.42 $\mu\text{g/ml}$ in root callus whereas leaf callus yielded 24.46 $\mu\text{g/ml}$ of tylophorine (Table 55).

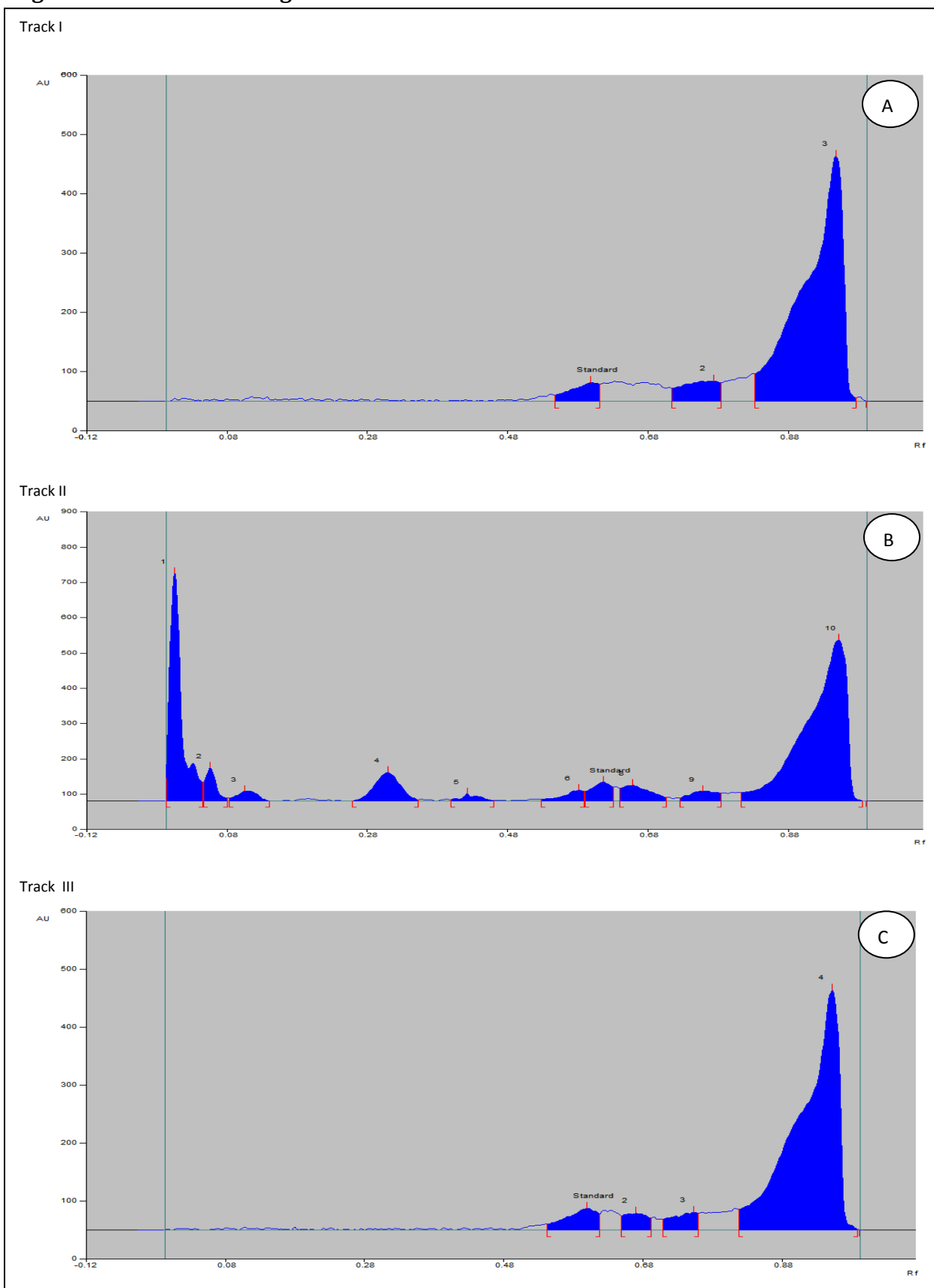
Table 54. Different ratios of solvents for optimizing solvent system

S. No.	Developing solvent	Ratios
1	Toluene: ethyl acetate: diethyl amine	6:4:3
2	Toluene: ethyl acetate: diethyl amine	5:3:2
3	Toluene: ethyl acetate: diethyl amine	7:2:1
4	Toluene: ethyl acetate: diethyl amine	7:3:2
5	Toluene: chloroform: ethanol: ammonia	4:3.5:1.5:drop
6	Toluene: chloroform: ethanol: ammonia	6:3:3:drop
7	Toluene: chloroform: ethanol: ammonia	6:2:2:drop
8	Toluene: chloroform: ethanol: ammonia	7:3:2:drop

Fig. 57. HPTLC chromatogram of leaf and root callus of *T.indica* using toluene: ethyl acetate: diethyl amine (7:2:1).

- A. Track I loaded with standard (tylophorine).
- B. Track II loaded with extract of leaf callus raised on NAA + K.
- C. Track III loaded with extract of root callus raised on IBA + BA.
- D. Spectra comparison of tylophorine peaks in all the tracks.
- E. UV visuals at 254 nm showing tylophorine band in all the samples at similar R_f.
- F. UV visuals at 366 nm showing tylophorine band in all the samples at similar R_f.

Fig. 57. HPTLC chromatograms of leaf and root callus of *T.indica*



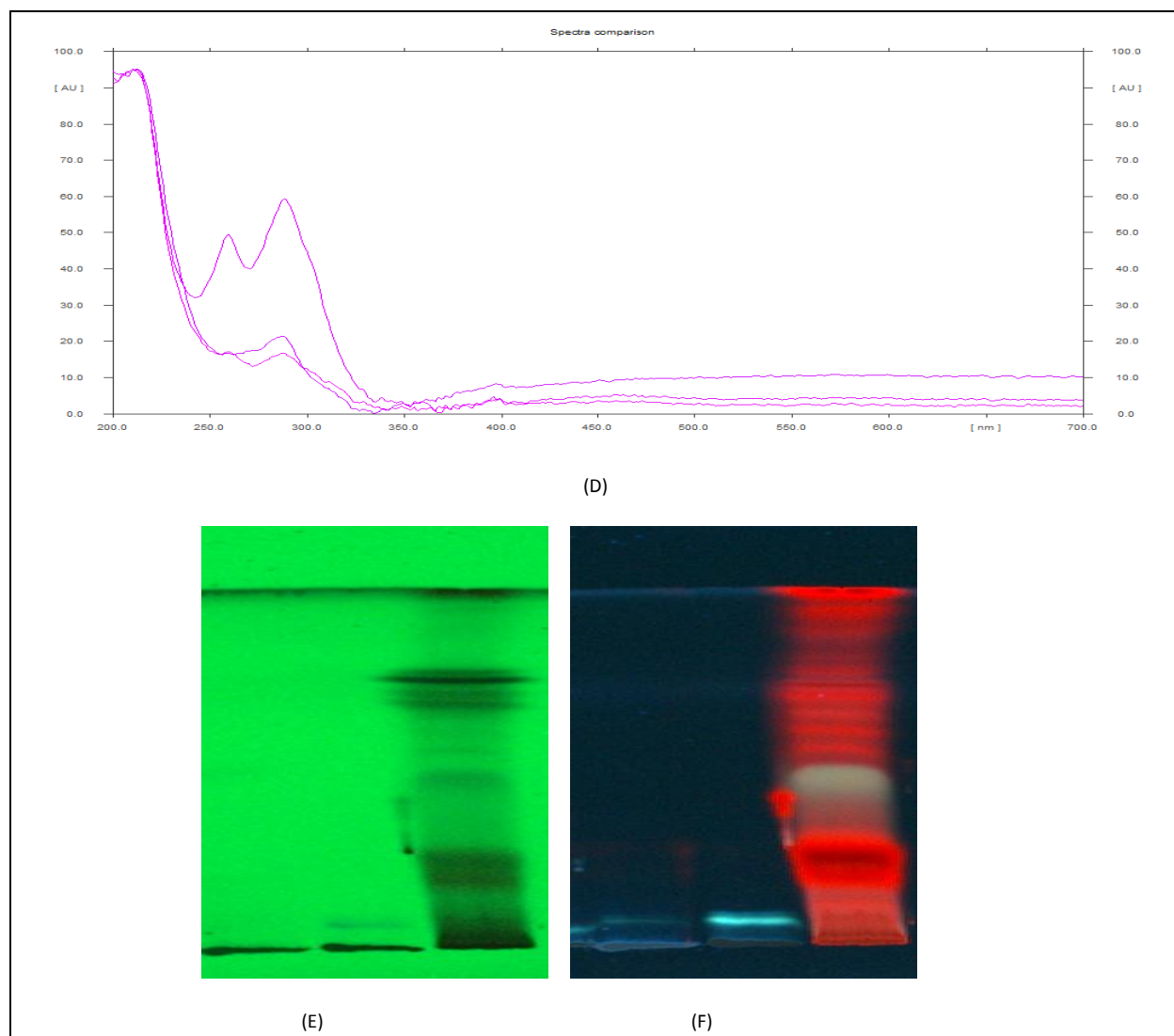


Table 55. Tylophorine ($\mu\text{g/ml}$) in different samples. Standard tylophorine applied at a working concentration of $20.0 \mu\text{g/ml}$.

Track ID	Solvent system	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorine ($\mu\text{g/ml}$)
Fig. 57. Track I (A)	Toluene: ethyl acetate: diethyl amine (7:2:1)	1	0.62	981	5.07	-----
Fig. 57. Track II (B)	Toluene: ethyl acetate: diethyl amine (7:2:1)	7	0.62	1200	3.26	24.46
Fig. 57. Track III (C)	Toluene: ethyl acetate: diethyl amine (7:2:1)	1	0.62	1296	6.25	26.42

4.6.2 Suspension cultures

Leaf callus (1 g) fresh weight was inoculated in 250 ml conical flask containing 100 ml of liquid MS supplemented with NAA (29.0 μM) and K (4.65 μM) for the growth of suspension cultures. The cultures were maintained on shaker at 120 rpm and observations were made from the sixth day after incubation until 33rd day with 3 days interval (Table 56 and Fig. 58). The period of maximum biomass yield was during 12th to 24th day after that the biomass yield did not show any significant increase and remained almost stationary until 27th day. Similarly, root callus (1 g) was subjected to 100 ml liquid MS medium containing IBA (24.6 μM) with BA (13.2 μM) for the growth of suspension culture. Root cultures showed an extended lag phase of 15 days, which was otherwise 12 days in leaf cultures. Following this phase, period of maximum biomass yield occurred which lasted until 27th day. After that death phase started in which decrease in biomass was observed (Table 57 and Fig. 59).

To scale up the production of secondary metabolite the inoculum was transferred to 5lt conical flask containing liquid MS supplemented with similar combination of hormone and were harvested at stationary phase using the method described in section 3.8.2. The dried biomass suspension cultures were extracted using extraction protocol I (as discussed in material and method section 3.6). The final mother extract thus obtained, was purified using HPTLC. Crude suspension extracts of root and leaf (6 μl each) were loaded on pre-coated silica gel (5 X10 cm) plate and developed in toluene: ethyl acetate: diethyl amine (7:2:1) (Fig. 60 A-C). All the tracks showed the presence of tylophorine peak at R_f 0.63 when compared with the standard. Spectra comparison of tylophorine peaks recorded at 200-400 nm showed quite similar UV absorbance (Fig. 60 D). 3-D spectra scan of all the tracks at 258 nm showed the presence of tylophorine peaks in both the samples (Fig. 60 E). Visual impression of the plate captured under UV 254 and 366 nm showed tylophorine bands in different tracks (Fig. 60 F and G). Quantitative analysis of the samples showed that the highest amount of tylophorine (34.71 $\mu\text{g/ml}$) was found in root suspension extract whereas leaf suspension extract contained 28.30 $\mu\text{g/ml}$ of tylophorine (Table 58).

Table 56: Growth of leaf cell suspension culture from *T. indica* in MS liquid medium

S. No	Age of culture (Days)	Callus biomass Fresh weight (g/l)	Callus biomass Dry weight (g/l)
1	6	28	2.0
2	9	34	2.2
3	12	39	2.5
4	15	56	2.9
5	18	62	3.4
6	21	73	4.1
7	24	89	4.8
8	27	91	4.9
9	30	84	4.2
10	33	79	3.6

Fig. 58. Growth of leaf cell suspension culture from leaf callus of *T.indica* in MS liquid medium

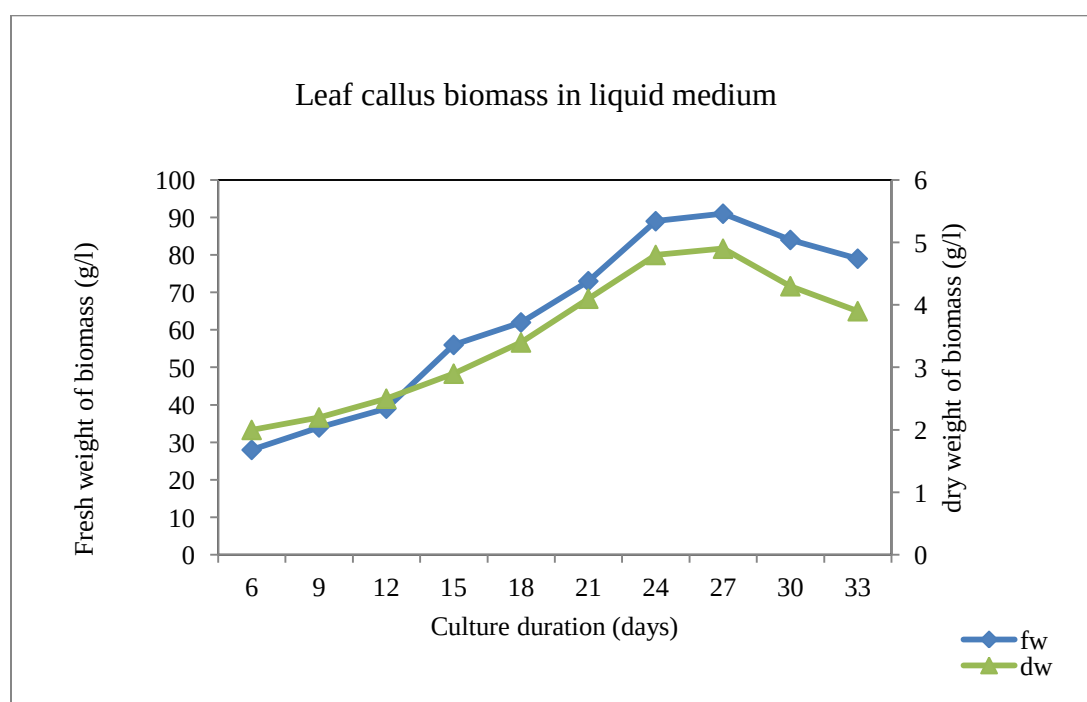


Table 57: Growth of root cell suspension culture from *T. indica* in MS liquid medium

S. No	Age of culture (Days)	Callus biomass Fresh Weight (g/l)	Callus biomass Dry weight (g/l)
1	6	10	0.2
2	9	13	0.4
3	12	18	0.7
4	15	22	0.8
5	18	38	1.6
6	21	46	2.4
7	24	57	2.9
8	27	66	3.8
9	30	69	3.9
10	33	62	3.5
11	36	59	2.9

Fig. 59. Growth of root cell suspension culture from *T. indica* in MS liquid medium

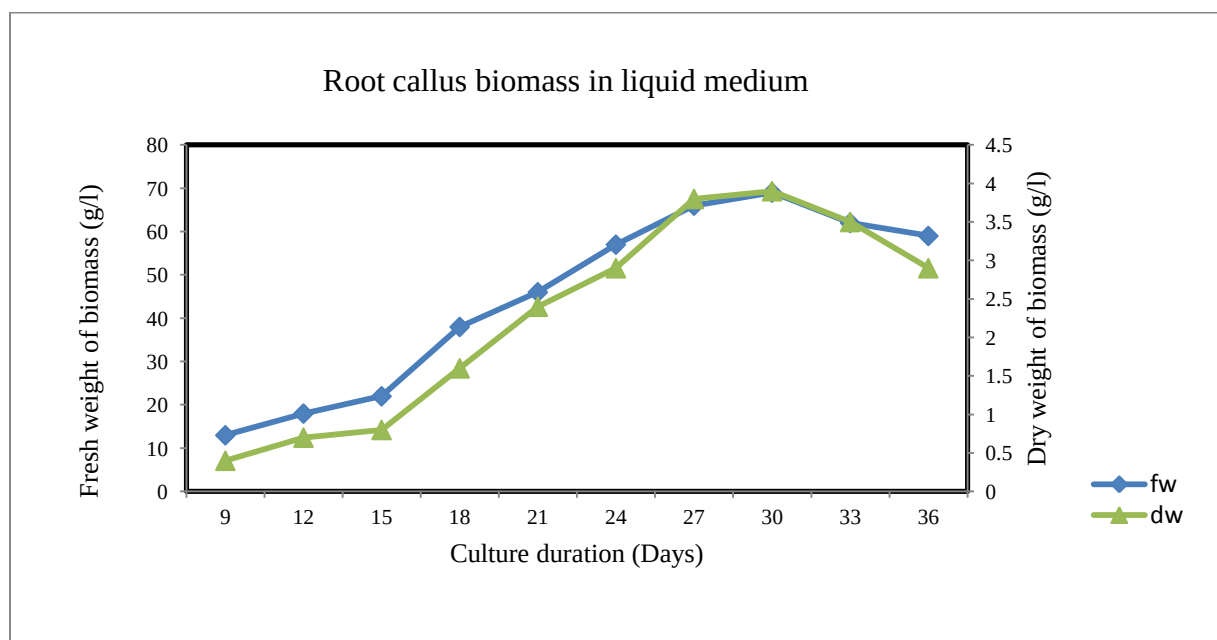
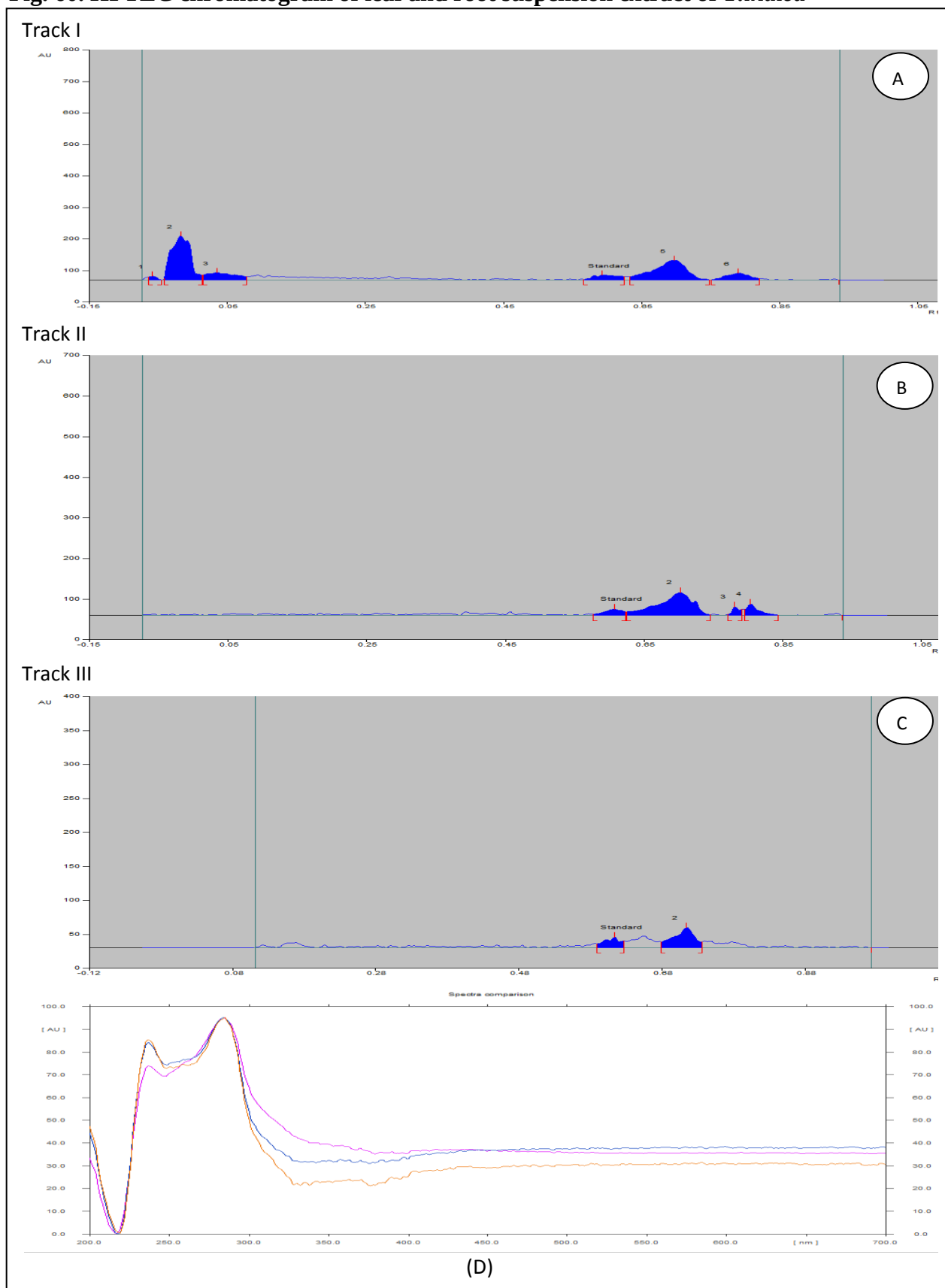


Fig. 60. HPTLC chromatogram of leaf and root suspension extracts

- A. Track I loaded with extract of root suspension culture.
- B. Track II loaded with extract of leaf suspension culture.
- C. Track III loaded with standard (tylophorine).
- D. Spectra comparison of tylophorine peaks in all the tracks.
- E. 3-D spectra comparison of all peaks at 258nm.
- F. UV visuals at 254nm showing tylophorine band in all the samples at similar Rf.
- G. UV visuals at 366 nm showing tylophorine band in all the samples at similar Rf.

Fig. 60. HPTLC chromatogram of leaf and root suspension extract of *T.indica*



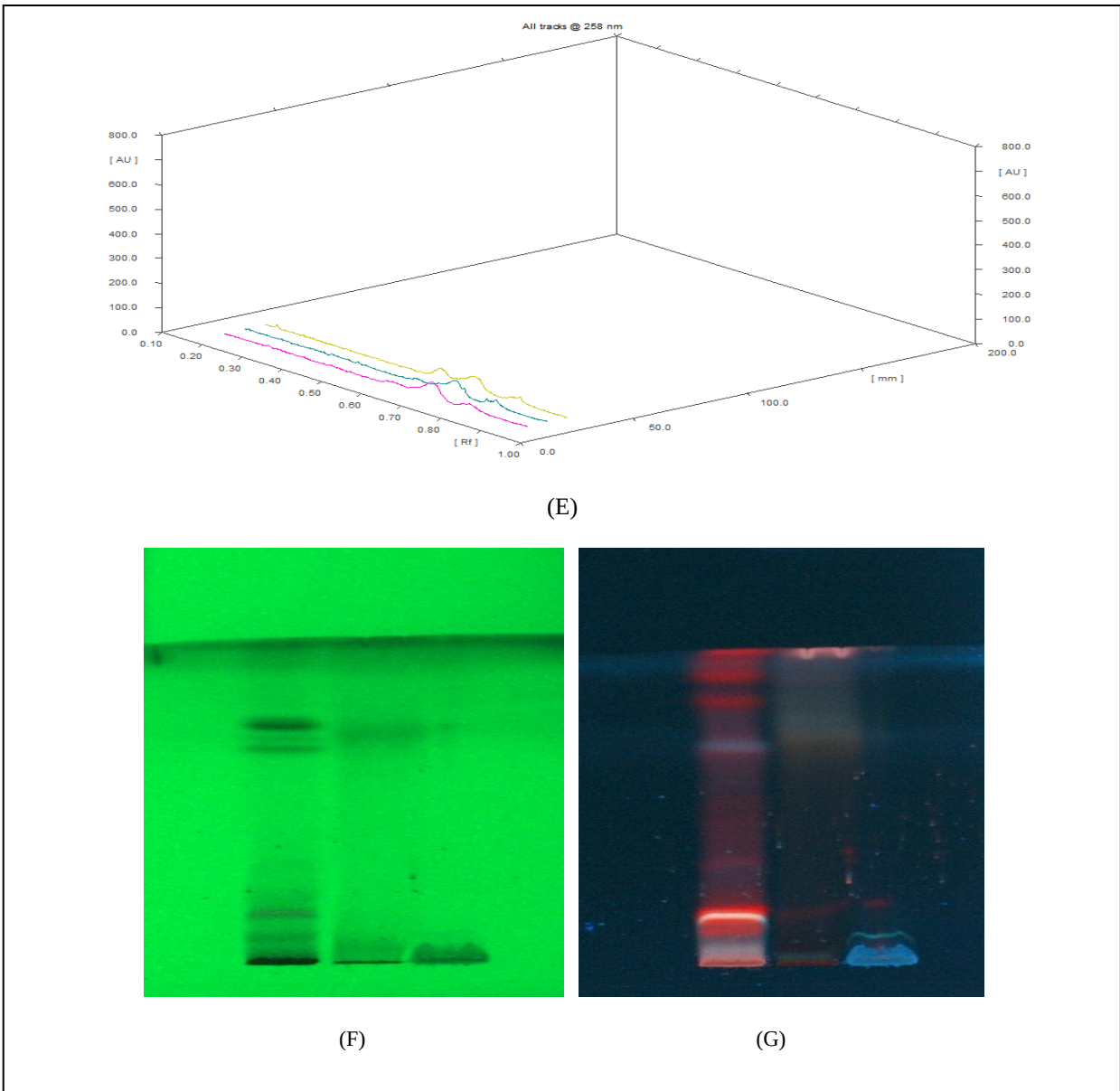


Table 58. Tylophorine ($\mu\text{g/ml}$) in various tracks when developed in toluene: ethyl acetate: diethyl amine (7:2:1) at 258 nm. Standard tylophorine applied at a working concentration of 20.0 $\mu\text{g/ml}$.

Track ID	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorine ($\mu\text{g/ml}$)
Fig. 60. Track I (A)	1	0.63	454.8	13.96	34.71
Fig. 60. Track II (B)	1	0.63	376.4	10.37	28.30
Fig. 60. Track III (C)	1	0.63	265.3	28.66	-----

DISCUSSION

The present investigation was carried out on two medicinal plants namely- *Stevia rebaudiana* and *Tylophora indica* with a view to develop a reproducible protocol for their mass production under *in vitro* conditions and extract bioactive compounds.

Stevia rebaudiana “The wonder herb of Paraguay” has become a potential and renewable raw material as health conscious individuals have helped in boosting up the international market for this high quality non-caloric natural sweetener. However, there appears to be no large-scale mechanized method for the production of *Stevia* due to difficulties in producing the crop through seeds, as seeds are not viable enough for germination and establishment of successful plants. Also, the conventional method by “stem cuttings” limits the number of propagules for large scale propagation. Likewise, the commercial plantation of *Tylophora indica* has not been attempted and only wild populations are exploited for secondary metabolite extraction. The plant has low seed viability and germination rate and the destruction of plant caused by harvesting the roots as a source of drug has threatened the very survival of this plant (Faisal *et al.*, 2007).

To meet the growing demands of these important medicinal plant species, it is necessary to develop an efficient regeneration system for their effective conservation and large-scale multiplication through *in vitro* culture technology.

5.1 MICROPROPAGATION

Under the scope of present investigation, micropropagation protocols for both the plant species were standardized using different techniques of micropropagation.

5.1.1 *De novo* adventitious shoot formation directly from the explants

Direct *de novo* adventitious shoot formation is regarded as the most reliable method for clonal propagation as it upholds genetic uniformity among the progenies unlike those regenerated from callus tissue. In our study on *Stevia rebaudiana*, an exhaustive range of combinations of auxins and cytokinins were employed for *de novo* adventitious shoot formation from different organs.

Out of all the combinations tried, synergistic action of BA (8.8 μ M) with 2, 4- D (9.74 μ M) was most pronounced in forming innumerable healthy, green shoots in nearly 88% of leaf cultures. MS containing 4.4 μ M BA with 7.35 μ M NAA also yielded significant number of shoots per culture with manifold increase in the number of shoots after subsequent subculturings. Similar results showing the importance of BA with NAA in forming maximum number of adventitious shoots have been studied in *Crepis novoana*, another member of family Asteraceae (Corral *et al.*, 2011).

Sivaram and Mukundan, 2003 and Anbazhagan *et al.*, 2010 reported combined effect of BA with IAA on shoot regeneration directly from the leaf explants of *Stevia rebaudiana* whereas Preethi *et al.*, 2011 formed maximum numbers of 28.7 ± 0.84 shoots/culture when leaf explants were inoculated on MS containing BA + IAA along with K. Effects of benzyladenine on *in vitro* growth (that is estimated in term of shoot number, shoot length and leaf number) in combination with auxins are necessary for the commitment of cultured cells towards organogenesis (Ahmed *et al.*, 2007). The duo combination complements each other as cytokinins enhance cell division, stimulate adventitious shoot proliferation whereas auxins regulate cell elongation and shoot expansion. Effects of both the growth regulators on shoot regeneration has been reported in several plant species such as *Catalpa ovata* (Lisowska and Wysokinska, 2000), *Echinacea purpurea* (Koroch *et al.*, 2002), *Daphne georum* (Mala and Bylinsky, 2004), *Murraya koenigii* (Deepu and Prasad, 2007) and *Trichodesma indicum* (Verma *et al.*, 2010).

In the present study on *Tylophora indica*, cytokinin BA with or without adenine sulphate was found to be most effective in inducing *de novo* adventitious shoots from almost all the vegetative parts including leaf, stem and root explants (Kaur *et al.*, 2011 a, b & c). Leaf explants induced maximum number of 55 ± 2.88 shoots/ culture on MS supplemented with 8.8 μ M BA and 1.35 μ M adenine sulphate. Addition of adenine sulphate to the culture medium can stimulate cell growth, greatly enhance shoot proliferation as it has a base structure similar to that of the cytokinin and hence show cytokinin like activity. *De novo* adventitious shoot formation from stem explants is a new observation of the current study, which has not been reported previously. High propensity of *de novo* adventitious shoot formation occurred from the stem segment of *T. indica* through meristemoids on BA (8.8 μ M) supplemented medium where 48 ± 0.57

shoots/culture were formed after 7-8 weeks of culturing (Kaur *et al.*, 2011 b). Interestingly, only lower concentrations were effective in inducing large number of shoots and higher concentrations were found to either reduce or inhibit shoot growth. Subsequent subculturing further accelerated the formation of shoots in large number without any decline in their proliferation. Periodic subculturing was essential as otherwise the growth potential was affected which may be due to the accumulation of waste metabolic products in the medium and/or exhaustion of chemicals. Likewise, prolific shoot bud formation and plant regeneration via meristemoids from the root segments of *T. indica* was observed on MS medium supplemented with BA (8.8 μ M). Direct regeneration of adventitious shoots was also observed without meristemoid formation on MS supplemented with TDZ (2.25 μ M) and L-ascorbic acid (8.4 μ M) where clusters of shoots (average shoot length 3.9 ± 0.57 cm) were formed after 4 weeks of culturing. So far a single report on the micropropagation of *T. indica* from root explant has been reported by Chaudhari *et al.*, 2004. They reported the formation of organogenic nodular meristemoids from the root segments cultured on MS medium supplemented with BA (10.72 – 26.80 μ M) which developed into shoot buds in 42% of cultures.

A wide range of cytokinins have been employed for shoot formation and a wider survey suggest that BA is the most reliable and effective cytokinin. The caulogenic effect of BA as described in the present study is in consonance with other reports as well. Bera and Roy, 1993 reported multiple shoot formation directly from leaf explants of *Tylophora indica* on MS medium supplemented with 22 μ M BA with 0.65 μ M adenine sulphate. Effect of BA either alone or in combination with auxins has been demonstrated in many medicinal plants of family Asclepiadaceae viz. *Ceropegia jainii* and *Ceropegia bulbosa* (Patil, 1998), *Holostemma annulare* (Sudha *et al.*, 1998), *Hemidesmus indicus* (Sreekumar *et al.*, 2000) *Holostemma adakodien* (Martin, 2002), *Ceropegia pusilla* (Kondamudi *et al.*, 2010) and *Hylotelephium tatarinowii* (Wang *et al.*, 2010).

There are variable reports regarding the potentiality of differentiation of plant tissues in culture but none of the observations in the literature reported direct differentiation of roots from the leaf explants of *Tylophora indica*. The current work reports for the first time direct root regeneration from leaf explants when cultured on different combinations of NAA with K or BA. Profuse

rooting, however, occurred on NAA (19.4 μ M) and BA (4.4 μ M) supplemented medium. The roots formed were white in color and bore profuse root hairs.

5.1.2 Callus induction and Differentiation

Majority of the plant tissues growing *in vitro* require exogenous hormones in the nutrient medium for dedifferentiation. The reaction of an isolated tissue to auxin depends upon its endogenous auxin level at the time of excision and its genetic capacity for its synthesis. In the present work, the MS medium was supplemented with various concentrations of different auxins. It was observed that the level and type of auxin required for dedifferentiation and optimal callusing varied among the two species. In *Stevia rebaudiana*, both NAA and 2, 4- D alone could initiate callusing from different vegetative parts but the callus was slow growing and did not show sustained growth. Incorporation of K considerably enhanced the callus growth when used in conjunction with auxins. Out of all the auxins tried, synergistic action of 2, 4- D or NAA with K was most pronounced for leaf and shoot apex calli. Leaf explants also exhibited callusing on IBA + K supplemented medium, whereas shoot apices and stem callus cultures grew well on NAA + K supplemented medium. Stem explants callused best on MS + NAA + K supplemented medium but did not form sustainable callus on 2, 4- D or IBA medium supplemented with K. The role of auxin 2, 4- D alone in inducing callus growth from leaf, nodal and internodal segments of *Stevia rebaudiana* was demonstrated by Uddin *et al.*, 2006; Naz, 2009; Sadeak *et al.*, 2009 and Tiwari, 2010. However, Gupta *et al.*, 2010 induced whitish green callus from leaf explants of *S.rebaudiana* on MS medium containing 4.87 μ M 2, 4 –D with 5.5 μ M NAA while no callus growth was observed when same hormones were used separately. Das *et al.*, 2006 reported that NAA with K gave satisfactory results for callus initiation and growth while NAA with BA was superior for callus maintenance in *Stevia rebaudiana*. The experiments clearly demonstrate that the nature and concentration of auxins have marked influence on callusing of the explants. The growth of callus in all the cases varied according to the composition of the medium as well as the type of explant. In the present investigation, leaf explants callused more often than internodal and shoot apices, which are contrary to the observations made by Din *et al.*, 2006 who reported internodal segments to be the most frequently callused explant as compared to leaves.

In *Tylophora indica*, different organs exhibited different sensitivity to exogenously supplied chemical stimuli. While leaf and stem segments responded best on NAA + K supplemented medium, roots showed best callus growth on IBA + BA medium (Kaur *et al.*, 2011 a, b & c). The results can be explained on the basis that different plants and even different organs of the same plant are characterized not only by their unique intrinsic biochemical make up but also by the sensitivity to the exogenously supplied chemical stimuli. The calli formed were fast growing, compact and green which did not turn brown even after many passages in the culture. The leaf and stem calli were more green as compared to the light green callus of root segments. The poor rate of chlorophyll synthesis in the root tissue may be the result of inherent bio-chemical deficiencies of the cells. Another new finding of the present investigation was induction of callus from root explants on liquid MS medium in combination with different growth regulators. Root explants formed light green, compact callus masses in nearly 80% of the cultures on liquid MS containing 14.76 μM IBA and 13.2 μM BA. Good callus induction was also observed from different plant parts on 2, 4- D supplemented medium either alone or in combination with K or BA but the growth rate was slow. Faisal and Anis, 2003, 2005 reported the production of highly proliferative light yellow callus from the leaf and stem explants of *T. indica* on the medium containing 2, 4, 5-T (10 μM) whereas optimum callus induction from petiole segments occurred on MS supplemented with 2, 4- D (10 μM) + TDZ (2.5 μM). Thomas and Philip, 2005 reported callus induction from the immature leaf explants on 7.5 μM 2, 4- D and 1.5 μM BA where 92% explants produced callus. In contrast to the above results, Verma *et al.*, 2010 reported callus induction from leaf, stem and petiole explants of *T. indica* with highest response of 95% from leaf explants on MS medium supplemented with BA (8.8 μM) + IBA (2.4 μM).

Nature of Callus

All the calli of *Tylophora indica* and *Stevia rebaudiana* comprised of heterogeneous population of cells exhibiting a wide variations in cell sizes and geometric shapes. It is opined that with the onset of callusing, the capacity for orderly growth is lost causing divisions in irregular planes, which lead to the formation of cells assuming different shapes and sizes. Also the biophysical factors obtaining in intact tissues is lacking in callus cultures.

Differentiation

In the present study, histogenetic differentiation was observed in the form of tracheids from all the calli of *Tylophora indica* and *Stevia rebaudiana* and in no case phloem differentiation was recorded. The calli from all the vegetative parts showed this propensity on a sucrose- auxin rich medium. Tracheids were of variable shapes and sizes showing reticulate and sclariform thickenings on their walls. They were either loosely scattered or grouped together in the form of nodules or nests.

A wide spectrum of differentiation from the calli of different organ origin of *T.indica* was observed in the form of rhizogenesis, caulogenesis and differentiation of embryoids. It seems not improbable that different explants have certain biochemical environment within their cells in the intact plant, which became accentuated during callus growth resulting in preferential differentiation of roots, leaves and shoots from their respective calli. Control of differentiation has been based on the hypothesis of Skoog and Miller, 1957 who showed that differentiation of roots or shoots is a function of interaction between the two plant growth regulators, auxin and cytokinin. A relatively high auxin to cytokinin ratio favors root formation whereas the reverse is true for shoot formation. NAA at a concentration of 29.4 μM with 4.65 μM of K was most effective growth regulator combination for inducing rhizogenesis from leaf and stem calli. Rhizogenesis also occurred when callus was transferred from NK medium to IBA supplemented medium. To the author's knowledge, no report is available on the rhizogenesis in *Tylophora* so far. Similar report on rhizogenesis from leaf callus was observed in *Huernia hystrix* where NAA in combination with BA was found to be effectual for root differentiation (Amoo *et al.*, 2009). The report of Shasthree *et al.*, 2009 also showed good rooting response from callus of *Erythrina variegata* when planted on different concentrations of IBA.

The present investigation demonstrated the great organogenic potential of *Tylophora* and *Stevia*, as they exhibited high efficiency shoot formation and plant regeneration from various calli under *in vitro* conditions. Hundreds of plants were differentiated from calli under present cultural conditions employed. In *T. indica*, high frequency shoot differentiation from the leaf, stem and root calli occurred on 8.8 μM BA or 9.3 μM K.

A perusal of literature reveals similar observations by Faisal and Anis, 2003, 2005 who reported high shoot regeneration from leaf and stem calli in *T. indica* when subcultured onto MS medium containing either K or BA, but better results in terms of number and growth of regenerants was reported on 5 μ M K. Sahai *et al.*, 2010, reported high frequency shoot induction (26.8 ± 0.97 shoots/culture) when calli were transferred to MS medium containing BA (5 μ M) alone. The stimulatory role of cytokinins BA or K for satisfactory shoot bud organogenesis and plantlet production was also advocated in a number of other medicinal plants like *Psoralea cordifolia* (Anis and Faisal, 2005), *Mucuna pruriens* (Faisal *et al.*, 2006), *Ophiorrhiza prostrata* (Beegum *et al.*, 2007) and *Sinapis alba* (Abbasi *et al.*, 2011). In the present study, caulogenic role of TDZ in combination with K was also demonstrated for root callus of *T.indica*. Good frequency shoot differentiation with an average number of 62.4 ± 1.50 shoots/culture was observed on 4.5 μ M TDZ and 9.3 μ M K supplemented medium. Earlier, Thomas and Philip, 2005 reported 8.0 μ M TDZ optimum for shoot regeneration from leaf callus of *Tylophora* with an average 66 ± 0.7 shoots/culture. Faisal *et al.*, 2007 reported shoot induction from the surface of petiole callus on MS containing 2.5 μ M TDZ. A number of reports have highlighted the role of TDZ in modulating endogenous level of plant growth regulators either directly or as a result of induced stress and exposure of plant tissue to TDZ for a short period being sufficient to stimulate shoot regeneration (Hutchinson and Saxena, 1996, Murthy *et al.*, 1998 and Jones *et al.*, 2007). The feature not documented previously includes differentiation of shoots in liquid MS with different cytokinins. Current study reports for the first time differentiation of shoots from leaf, stem and root calli on variously supplemented liquid MS medium. Large numbers of healthy shoots from calli were formed when cultured on 8.8 μ M BA supplemented medium. The importance of liquid medium in enhancing the micropropagation efficiency in terms of higher multiplication rates, improved productivity, reduced time of multiplication and cost effectiveness has also been documented in many research articles (Sood *et al.*, 2000; Mehrota *et al.*, 2007 and Pati *et al.*, 2011).

In *Stevia rebaudiana*, different growth hormones used for shoot multiplication displayed significant variations among the calli of different organ origin with regards to earliness in shoot induction, percentage of cultures initiating shoot buds and number and length of shoots. Although hormonal combination of NAA and 2, 4-D with different cytokinins i.e. K or BA

showed differentiation of green leafy shoots from the callus masses but BA or K either alone or together ranked superior in initiating maximum number of shoots in the cultures. In leaf explants, 18.6 μM K was most effective in inducing 90% of the cultures to form uncountable numbers of shoots per culture. Excellent shoot differentiation from leaf and stem calli also occurred on 8.8 μM BA whereas shoot apex callus favored optimum caulogenesis on 17.6 μM BA with 4.65 μM K supplemented medium. Higher doses of BA or K were found deleterious to the regenerative capacity of *in vitro* cultures when applied singly or in combination. Utilization of BA either alone or in combination with other plant growth regulators for inducing caulogenesis in *S. rebaudiana* is well supported by different observations from literature. Swanson *et al.*, 1992 observed differentiation of shoots from friable leaf callus on 8.8 μM BA either alone or in combination with reduced concentration of NAA. Similarly, Naz, 2009 reported shoot differentiation from leaf callus on MS containing 8.8 μM BA with 4.84 μM IAA supplemented medium. Smitha *et al.*, 2005 and Ahmad *et al.*, 2011 obtained green leafy shoots when leaf callus was cultured on MS containing BA as a lone growth regulator.

The significant observation of current study, which is the first report of its kind, is the induction of shoot buds from leaf-derived calli when cultured on liquid MS having BA in combination with K, where the entire callus mass differentiated into large clusters of shoots in 70-80% of the cultures. On 18.6 μM K with 8.8 μM BA supplemented medium, an average number of 67 ± 1.45 shoots/ culture were formed. The liquid medium allows the close contact with the tissue, which stimulates and facilitates the uptake of nutrients and phytohormones, leading to better shoot and root growth. Data also showed that the periodic subculturing resulted in the formation of uncountable number of shoots in all the cultures without any decline in their proliferation. Role of liquid medium to induce multiple shoot organogenesis and plant regeneration is also reported in plants like *Cymbidium sinense* (Chiang *et al.*, 2010) and *Chrysanthemum indicum* (Eeckhaut and Huylenbroeck, 2011). Despite repeated efforts, rhizogenesis could not be induced on any of the media combinations tried.

5.1.3 Multiple shoot proliferation from nodal segments and shoot apices

Multiple shoot proliferation from nodal segments and shoot apices of *Stevia rebaudiana* was observed on MS medium containing BA in combination with either NAA or adenine sulphate. Best proliferation from nodal segments occurred on 8.8 μM BA with 2.7 μM adenine sulphate whereas shoot apices exhibited better shoot proliferation on 8.8 μM BA with 7.35 μM NAA supplemented medium. Uncountable numbers of multiple shoots were formed after subsequent subcultures without any decline in the growth thereafter. Earlier Himanshu *et al.*, 2006 and Kalpana *et al.*, 2010 have used combination of cytokinin BA with auxin NAA for inducing bud break, growth and multiple shoot proliferation from the nodal explants of *Stevia*. Sivaram and Mukundan, 2003; Debnath, 2008; Kalpana *et al.*, 2009; Naz, 2009 and Anbazhagan *et al.*, 2010 demonstrated the essentiality of BA with IAA for inducing forced axillary branching from shoot apices and nodal segments of *S. rebaudiana*. In agreement with the present study, there are numerous reports highlighting the stimulatory role of adenine sulphate in forced axillary branching in a number of medicinal plants like *Asparagus racemosus* (Bopana and Saxena, 2008), *Aloe vera* (Kalimuthu *et al.*, 2010) and *Anethum graveolens* (Jana and Shekhawat, 2011).

In *Tylophora indica*, MS containing BA as a lone growth regulator was quite effective in inducing bud break and multiple shoot proliferation from the nodal segments. However, MS medium supplemented with BA (13.2 μM) in conjunction with NAA (3.67 μM) and L-ascorbic acid (8.4 μM) gave the best proliferation rate. The effectiveness of cytokinin especially BA in promoting axillary shoot proliferation in many medicinal plants is well documented e.g. *Clerodendrum* sp. (Mao *et al.*, 1995), *Nymphoides* (Jenks *et al.*, 2000), *Ziziphus* (Sudershan *et al.*, 2001), *Rosa damascene* (Pati *et al.*, 2004), *Picrorhiza kurroa* (Chandra *et al.*, 2006), *Sida cordifolia* (Sivanesan and Jeong, 2007a), *Pentanema indicum* (Sivanesan and Jeong, 2007 b), *Beloperone plumbaginifolia* (Shameer *et al.*, 2009) and *Boscia senegalensis* (Khalafalla *et al.*, 2011). Superiority of BA for shoot induction and multiplication may be due to the ability of plant tissues to metabolize BA more readily or the ability of BA to induce production of natural hormone such as Zeatin within the tissue (Zaerr and Mapes, 1982).

Data under discussion is well supported by observations made by Sharma and Chandel, 1992 and Faisal *et al.*, 2007 who also employed similar set of media combinations for achieving plant

regeneration via enhanced axillary shoot proliferation from nodal segments of *T. indica*. Synergistic action of cytokinin with auxin in inducing axillary shoot proliferation has also been documented in a number of medicinal plants including *Hemidesmus indicus* (Patnaik and Debata, 1996), *Ceropegia candelabrum* (Beena *et al.*, 2003), *Psoralea corylifolia* (Anis and Faisal, 2005), *Vernonia amygdalina* (Khalafalla *et al.*, 2007), *Scrophularia takesimensis* (Sivanesan *et al.*, 2008), *Withania somnifera* (Sivanesan and Murugesan, 2008) and *Ocimum basilicum* (Daniel *et al.*, 2010).

5.1.4 Somatic embryogenesis

The development of embryoids under *in vitro* conditions demonstrates that the normal process of fertilization can be bypassed and the genome of the somatic cells can be triggered to recapitulate the ontogenetic pattern of normal embryogeny of the species under precise physical and chemical conditions. There are only two not very clear reports regarding somatic embryogenesis from the leaf explants in *Stevia rebaudiana*. Beshpalhok *et al.*, 1993 reported the formation of so called somatic embryos from leaf explants which latter developed root but failed to form shoots. Banerjee and Sarkar, 2010 reported the formation of somatic embryos from the leaf callus which developed into microshoots. However, despite our repeated efforts employing various growth regulators, embryogenesis could not be induced. One of the reasons for this could be that we might be working with unbalanced media composition or we have yet to discover the trigger hormone in this context.

In vitro propagation through somatic embryogenesis has been reported in various members of family Asclepiadaceae like *Hemidesmus indicus* (Sarasan *et al.*, 1994), *Ceropegia candelabrum* (Beena and Martin, 2003), *Leptadenia reticulata* (Sathyanarayana *et al.*, 2008) and *Cralluma stalagmifera* (Sreelatha and Pullaiah, 2010). There are only a few reports on the induction of somatic embryos in *Tylophora indica*, these too are of preliminary nature and no histological studies or detailed microscopic studies have been carried out to corroborate embryogenesis. In the present study, for the induction of somatic embryogenesis leaf callus was cultured on different concentrations of sucrose (1-5%) and 2, 4- D (4.87- 38.96 μ M) either alone or in combination with TDZ. 2, 4- D at the concentration of 19.48 μ M with 3 % sucrose was most effective in inducing somatic embryos in 95% of the cultures thereby showing the importance of both the components. A decrease or increase in the concentration of 2, 4-D and sucrose

concentrations adversely affected the embryogenesis. The capacity of an auxin generally 2, 4- D to induce embryogenic callus has also been reported previously in a number of plants like *Manihot glaziovii* (Joseph *et al.*, 2000), *Podophyllum peltatum* (Kim *et al.*, 2007), *Cnidium officinale* (Lee *et al.*, 2009) and *Dalbergia sissoo* (Singh and Chand, 2010). Likewise, the importance of higher sucrose level for inducing embryogenesis has also been demonstrated by Madakadze *et al.*, 1998 in *Pelargonium hortorum*, Sushmakumari *et al.*, 2000 in *Hevea brasiliensis* and Popova *et al.*, 2010 in *Coriandrum sativum*. Jayanthi and Mandal, 2001 induced embryogenic callus from leaf explants of *Tylophora indica* on MS containing 2, 4-D while Chaudhari *et al.*, 2004 reported BA to be most effective in inducing friable embryogenic calli from root explants of *T. indica*. Chandrasekhar *et al.*, 2006 reported 2, 4 D (1.5 μ M) along with TDZ (0.5 μ M) to be most effective hormone combination. Thomas, 2006 studied the effects of sugars, gibberellic acid and abscisic acid on somatic embryogenesis from the internodal explants of *T. indica*. Sahai *et al.*, 2010 reported high frequency somatic embryogenesis on BA and TDZ supplemented MS medium. As corroborated by Gill and Saxena, 1992, 1993, thidiazuron provides sufficient stimulus for the induction of somatic embryogenesis substituting for auxin or combined auxin and cytokinin requirements of embryogenesis.

We identified a series of developmental stages like globular, heart, torpedo and cotyledonary in the callus cultures both histologically and through stereozoom microscopy. Some embryoids were however, restricted in their development at globular stage only and were unable to reach cotyledonary stage. Only 70% of the globular structures developed into cotyledonary embryos after 6-7 weeks of culturing which developed further into plantlets. Although auxin 2, 4- D plays an imperative role in the development of early staged somatic embryos, during their continual growth, however, the continuous presence of auxin in the medium blocks their further development. Therefore, in order to increase efficacy of development, the embryogenic callus at globular stage was transferred to basal MS medium with 3% sucrose only, which resulted in the development of embryos into plantlets in almost all the cultures. Jayanthi and Mandal, 2001 observed 80% germination after transfer of mature somatic embryos onto basal MS medium. Chaudhari *et al.*, 2004 also reported the formation of complete plantlets when somatic embryos were transferred at the globular or heart stage to MS medium without growth regulators.

5.1.5 Artificial seed production

In recent years, encapsulation technology has drawn much attention for the production of artificial seeds as it helps in minimizing the cost of micropropagated plants and can be used for the short-term conservation of germplasm of elite and endangered plants. Moreover, it also facilitates exchange and distribution of germplasm across different laboratories (Rai *et al.*, 2009 and Verma *et al.*, 2010). Mostly somatic embryos have been used for the production of synthetic seeds, however, in recent years, the possibility of encapsulating vegetative propagules such as axillary buds, shoot tips and nodal segments as an alternative for somatic embryos has also been explored (Mandal *et al.*, 2000; Chand and Singh, 2004; Rai *et al.*, 2008 a, b; Kumar *et al.*, 2010 and Singh *et al.*, 2010). Captivating the idea, authors tried encapsulating shoot tips of *Stevia rebaudiana* in sodium alginate and calcium chloride for synthesizing artificial seeds. Shoot tips generally yield better response than other non-embryogenic vegetative propagules which is due to greater mitotic activity in shoot tips (Verma *et al.*, 2010). Best gel complex was achieved with 2% sodium alginate and 2% CaCl₂ which resulted in the formation of firm, clear and isodiametric beads. A single report of artificial seed production by encapsulating shoot tips of *Stevia* in 3% sodium alginate and 2.5% calcium chloride solution was made recently by Andlib *et al.*, 2011 who further reported 70% germination response after 30 days of storage. In the present study, encapsulated shoot tips of *Stevia* could be stored at 4° C for 120 days but there was a gradual reduction in the conversion percentage and shoot regeneration when cultured on basal MS medium. The decline in the rate of plant recovery from stored vegetative propagules is attributed to a number of factors like oxygen deficiency in the gelled beads, its rapid drying and loss of viability caused by mechanical constraints or diffusional limitations (Bazinet *et al.*, 1992 and Ara *et al.*, 1999). The percentage response of growth also varied with the type of matrix used, sodium alginate prepared in full strength MS nutrient medium demonstrated significant superiority over the double distilled water with respect to shoot conversion and shoot development. Gelling matrix supplemented with MS salts served as an artificial endosperm, thereby providing nutrients to the encapsulated explant for the plant growth (Bapat and Rao, 1992). There are numerous reports highlighting the production of artificial seeds by encapsulating different vegetative propagules such as axillary buds in Mulberry (Bapat *et al.*,

1987), shoot tips in *Plantago asiatica* (Makowczynska and Andrzejewska, 2006), *Phyllanthus amarus* (Singh *et al.*, 2006) and *Simmondsia chinensis* (Kumar *et al.*, 2010).

In *Tylophora indica*, vegetative propagules like nodal segments have only been used for synthesizing artificial seeds (Faisal *et al.*, 2007). However, use of adventitious shoot buds in the present report clearly demonstrates that adventitious shoot buds are more efficient as a material in synthetic seed technology since a large number of synthetic seeds could be constructed. We describe for the first time, the successful encapsulation technology using *in vitro* raised adventitious shoot buds for the development of synthetic seeds. Encapsulation was also done using *in vitro* formed somatic embryos and the best gel complex for somatic embryos and adventitious shoot buds was 2% sodium alginate with 2% CaCl₂. Lower concentrations (1%, 1.5%) of sodium alginate resulted in the formation of fragile beads and higher concentrations (3%, 3.5%, 4%) favored the formation of hard beads which had a marked effect on the germination or conversion process later on. Encapsulation of somatic embryos and vegetative propagules using different concentrations of sodium alginate ranging from 1.5% to 6% has been reported in different plant species (Pintos *et al.*, 2008; Kumar *et al.*, 2010 and Singh *et al.*, 2010). In *T. indica*, there is only one report of synthetic seed production by encapsulating somatic embryos in sodium alginate and maximum frequency (65%) for conversion of encapsulated seeds into plantlets was investigated for 6 weeks only (Chandrasekhar *et al.*, 2006). There are no reports on the storability and morphogenetic response of these seeds beyond 6 weeks.

In the present study, synthetic seeds developed by encapsulation of somatic embryos and adventitious shoot buds could be stored up to 90 days at 4°C, although the percentage germination and conversion of these seeds into plantlets decreased gradually with increase in the storage duration. Similarly, decline in conversion frequency on storage of synthetic seeds has been reported in a number of plants. In *Spilanthes acmella*, encapsulated shoot tips stored at 4°C showed 50% conversion or germination after 60 days (Singh *et al.*, 2009). In *Dalbergia sissoo*, conversion percentage of encapsulated somatic embryo was 29.4% and 16.1% after 15 and 45 days respectively (Singh and Chand, 2010).

5.1.6 Rooting of Microshoots

Induction of root from the basal end of *in vitro* regenerated shoot is the crucial factor for the success of any micropropagation protocol. Auxins play a provital role in inducing roots at the base of microshoots, the importance of auxins such as IBA, IAA or NAA for rooting has already been studied among different members of family Asteraceae. In *Arctium lappa*, best rooting was observed on MS supplemented with IBA or IAA in combination with NAA (He *et al.*, 2006) whereas NAA alone was most effective in root formation in *Vernonia amygdalina* (Khalafalla *et al.*, 2007).

In the current study on *Stevia rebaudiana*, half and full strength MS media supplemented with NAA, IBA or IAA at varying concentrations were used for rooting of microshoots. The best rooting response (90%) was observed on auxin free half strength MS medium whereas addition of IBA to full strength MS medium also showed equally good results. Both the observations are well supported by references from the literature. Ali *et al.*, 2010 reported 96% root induction on hormone free medium whereas Sivaram & Mukundan, 2003; Debnath, 2008; Kalpana *et al.*, 2010 and Preethi *et al.*, 2011 declared half strength MS with IBA to be the best rooting medium. Slavova *et al.*, 2003 and Rafiq *et al.*, 2007, on the other hand, observed 99% and 81% rooting respectively when microshoots were cultured on full strength MS supplemented with NAA. The success of IBA in promoting efficient rooting has been reported in many medicinal plant species including *Cunila galoides* (Fracro and Echeverrigaray, 2001), *Azadirachta* sp. (Batra *et al.*, 2002) *Mentha* sp. (Shahzad *et al.*, 2002), *Chromolaena odorata* (Anyasi, 2011) and *Withania coagulans* (Valizadeh and Valizadeh, 2011).

In our study on *Tylophora indica*, among different auxins tried, IBA was observed to induce good rooting response. However, the highest frequency (90%) of rooting was achieved on auxin free half strength MS medium alone. It is new observation since till date, either IBA or IAA are reported to be optimal for rooting in regenerated shoots of *T. indica* (Bera and Roy, 1993; Thomas and Philip, 2005; Faisal *et al.*, 2006 and Verma *et al.*, 2010). Similarly, beneficial effects of low salt MS medium for root initiation *in vitro* grown shoots have also been reported by other systems like *Adhatoda vasica* (Nath and Buragohain, 2005), *Plumbago rosea* (Gopalakrishnan *et al.*, 2009) and *Grammatophyllum scriptum* (Abbas *et al.*, 2011).

5.1.7 Acclimatization of micropropagated plants

In vitro propagation system provides an alternative method for the rapid production of plants but its ultimate success depends upon the successful transfer and establishment of these plants in the field conditions. Plants produced under *in vitro* conditions under controlled high humidity, diffused light and constant temperature need to be acclimatized because transferring of these plants from *in vitro* to *ex vitro* conditions is the most traumatic experience for them. Direct transfer of tissue culture raised plants to field conditions is not possible due to high mortality rate as the plants kept under controlled environmental conditions have heterotrophic mode of nutrition and uncontrolled loss of water. It is therefore necessary to transfer the plants to field through various hardening stages to increase the survival percentage.

In vitro raised plants of both *Stevia* and *Tylophora* were carefully rescued from the vessels and were initially transferred to the culture bottles containing moist cotton covered with perforated plastic covers to maintain higher relative humidity and were kept for a period of 15 days under growth room conditions. In this period, the plants developed an efficient root system, built up new leaves and became photosynthetically active. Plantlets initially acclimatized on moist cotton exhibited better survival in the subsequent acclimatization process. This step of hardening is well supported by observations of Gill *et al.*, 2004 who hardened rooted plantlets of *Saccharum officinarum* on moist cotton in open test tubes before their transfer to the soil.

Type of potting mixture used during acclimatization is one of the important factors determining the survival percentage of the plants under *ex-vitro* conditions. *In vitro* raised plantlets of *Garcinia indica* showed 76% survival rate when hardened on cocopeat as a potting mixture (Chabukswar *et al.*, 2005). Press mud cake mixed with soil was most cost effective and eco-friendly potting medium for obtaining sturdy banana plantlets (Vasane and Kothari, 2006). An efficient one step hardening technique for tissue culture raised orchid seedlings was reported on chips of charcoal, bricks and decayed wood as an alternate substratum (Deb and Imchen, 2010). Vermicompost was shown to be the most suitable planting substrate for hardening which ensured high frequency survival (96%) of regenerated plants of *Tylophora indica* (Rani and Rana, 2010).

In the present study, a successful attempt has been made to acclimatize the tissue culture raised plants of *Stevia rebaudiana* and *Tylophora indica* using various potting mixes. Plantlets of *S. rebaudiana* were successfully hardened off on potting mixture of soil: vermicompost where they showed 80% survival rate whereas plantlets of *T. indica* were acclimatized on potting mixes consisting of soil, vermicompost and biofertilizers (*Azotobacter* and *Pseudomonas*) in different combinations. Among all the combinations tried, soil: vermicompost: *Azotobacter* (N₂ fixer): *Pseudomonas* (phosphate solubilisers) in equal ratios showed the highest percentage survival (92%) upon transplantation of plants to the field conditions followed by soil: vermicompost (1:1) which registered 88% survival (Kaur *et al.*, 2011 d).

Use of biofertilizers along with basic potting mixture also exhibited better shoot growth and number of leaves formed per plant and reduced the mortality rate by 4-5 % on transplantation of the plants to the field. The present results are in accordance with the reports in banana where better response of biofertilizers treatment over other treatments was reported (Vasane and Kothari, 2006). It is obviously due to the complementarity in the function of two biofertilizers, *Azotobacter* in N₂ fixation and *Pseudomonas* in phosphate solubilisation, both contributing to the growth promoting characteristics of biofertilizers. Although the inclusion of biofertilizers in the soil: vermicompost treatment gave the best results in terms of plant growth and percentage survival (92%), the soil: vermicompost treatment was also closer to it. Therefore, if we take cost factor into consideration, soil: vermicompost treatment is most cost effective and is almost in par with the biofertilizers supplemented potting mixture in terms of survival percentage of plants (88%). Therefore, for subsequent hardening process, soil: vermicompost potting mixture was used. The acclimatized plants showed well-developed shoot and root systems with large number of secondary and tertiary branches and all the plants are thriving very well in field conditions.

5.2 Extraction and characterization of major secondary metabolites from *in vitro* and *in vivo* plants.

5.2.1 Extraction of secondary metabolites from *Stevia rebaudiana*

The broad distribution of nutrients and phytochemicals provides a basic rationale for their use as supplements in improving health of individual and population (Sankhala *et al.*, 2005). *Stevia* is nutrient rich and contains substantial amount of proteins, fats, carbohydrates and other important

nutrients (Viana and Metivier, 1998 and Savita *et al.*, 2004). The bioactive constituents (in terms of primary metabolites) present in the medicinal plants, add to characteristic properties like odor, pungencies and color of plant, while secondary metabolites give the particular plant its culinary and medicinal virtue (Sofowara, 1995; Evans, 2002 and Nalawade and Tsay, 2004).

In the current study, a comparative analysis of primary and secondary metabolites from leaf, stem and roots of *in vitro* raised plants of *Stevia rebaudiana* was conducted using standard protocols. So far, there is no data on comparative screening of primary and secondary metabolites from different vegetative parts of tissue culture raised plants of *Stevia rebaudiana*. The phytochemical evaluation of *in vitro* plant parts in the present study depicted that leaves contained highest amount of various primary metabolites like total sugars (3%), reducing sugars (1.9 %), sucrose (2.16 %), starch (1.26 %), proteins (0.96 %) and lipids (0.94 %) as compared to other plant parts. Stem contained second highest amount of total sugars (0.41%), reducing sugars (1.40 %) and sucrose (1.80 %) whereas the amount of starch, proteins and lipids were almost comparable to the root parts. Tadhani and Rema, 2006 made a similar observation with the leaves of *in vivo* plants of *Stevia* and showed that they possess high content of proteins, carbohydrates and other chemical constituents like tannins, saponins as well as palmitic and linolenic acids. Similar study for the analysis of phytochemicals from stem and root parts was conducted in another member of family Compositae i.e. *Vernonia amygdalina* by Eyong *et al.*, 2011. We observed a similar trend for secondary metabolites with highest amount of sterols (1.72 %), phenols (3.90 %) and flavonoids (1.74) present in leaves, followed by stem having 1.54 % sterols and 0.97 % flavonoids and roots contained second highest amount of phenols (2.20 %). Abou-Arab and Abu-Salem, 2010 made a comparative study of leaves of *in vivo* plants and callus cultures of *S.rebaudiana* and reported higher amounts of phenols (2.4%) and flavonoids (1.8 %) in the leaves.

The accumulation of major secondary metabolite- stevioside had been reported from all the parts of *Stevia* plant with highest amount present in the leaves (Rajab *et al.*, 2009). This report is in agreement with the earlier data published by Zaidan *et al.*, 1980 who reported substantially higher amounts of steviol glycosides in *Stevia* leaves. The present work was framed with an idea to develop a simple and efficient method for determining the major secondary metabolite-

stevioside from the raw leaves harvested at different times of the year. The pre-separation method for the extraction consisted of processing of leaf samples by drying them under shade conditions to remove excess moisture content, which also reduced the particle size and made the samples homogenous for testing. The method utilized series of steps, which successively removed impurities as well as undesirable components for the required purpose. The grounded samples were extracted using variety of solvents having different polarities such as petroleum ether, methanol, ethanol, butanol, diethyl ether and hexane. Different workers have used mixtures of solvents for extraction of sweet tasting glycosides from *Stevia rebaudiana* like hot and de-ionized water (Kitada *et al.*, 1989; Bovanova *et al.*, 1998 and Vanek *et al.*, 2001), water, ethanol, ethyl acetate and cyclohexane (Nikolova-Damyanova *et al.*, 1994), chloroform and methanol (Kolb *et al.*, 2001) and a combination of n-butanol, n-hexane and methanol (Choi *et al.*, 2002). Ibrahim *et al.*, 2007 used chloroform soluble fractions of the methanol extract of *Stevia rebaudiana* leaves to isolate five labdane diterpenoids viz. austroinulin, iso-austroinulin, sterebin E, sterebin E acetate and sterebin A acetate.

Among all the different extraction protocols followed in the present study, the best results were obtained with solvent extraction using petroleum ether followed by extraction with methanol and then diethyl ether (used to remove green colour of the extract). The plant material at each stage was treated with a solvent to ensure substantial removal of stevioside from the material and the number of times each step was repeated was regulated in order to gain the maximum quantitative extraction of metabolite. Further, the colorless extract was concentrated in a rotary flash evaporator at 50-60° C to produce the desired stevioside. The concentrate was resuspended in methanol for further purification using different chromatographic techniques. Kolb *et al.*, 2001 has compared the efficiency of method of solvent extraction of stevioside over the soxhlet method used by Makapugay *et al.*, 1984. Many techniques have been proposed for determining stevioside from the leaves of *S. rebaudiana*. Liu *et al.*, 1997 obtained stevioside with extraction efficiency of 88% whereas Yoda *et al.*, 2003 recovered only 50% of the original stevioside using simple supercritical fluid extraction method (SCFE) whereas Pasquel *et al.*, 1999 used same technique of SCFE for the extraction of non-glycoside fractions of *Stevia* leaves. Mizukami *et al.*, 1982 quantified stevioside by a chemical method following enzymatic hydrolysis, whereas Sakaguchi and Kan, 1982 quantified total glycoside content by means of gas chromatography

after acid hydrolysis. Nishiyama and Alvarez, 1992 and Hearn and Subedi, 2009 employed near infrared reflectance spectroscopy to extract sweet diterpene glycosides from 64 samples (with 4-13% stevioside) and 33 samples (with 64.09% stevioside) respectively. HPTLC has been frequently employed to chromatograph the extract as the technique is fast (15-20 samples/hr), minimum little clean up is required and substantial information is obtained from the chromatograph like quantitative estimation, multi wavelength scan, photodocumentation and post chromatographic derivatization. There have been only a few studies concerning the estimation of stevioside using HPTLC (Jaitak *et al.*, 2008 and Shirwaikar *et al.*, 2011). The expected results of this technique are presented as numerical limits or as a range or discretely observable results and hence considered acceptable for intended use.

5.2.2 Characterization of major secondary metabolite- stevioside

Determination of sweet tasting stevioside in the plant material has been done using various chromatographic separation methods such as high performance liquid chromatography (HPLC), thin layer chromatography and spectroscopic methods like near infrared spectrometry, VIS spectrometry etc (Nishiyama and Alvarez, 1992 and Hirata *et al.*, 2002).

In the present study, to have a complete assessment of stevioside estimation, harvesting of leaf samples was done from *in vitro* and *in vivo* leaf samples after three, four, five, eighteen and thirty months intervals. Extract was initially analyzed using thin layer chromatography, column chromatography, preparative TLC followed by high performance liquid chromatography (HPLC) and high performance thin layer chromatography analysis (HPTLC). Preliminary investigation for the optimization of ideal solvent system was done on TLC followed by other chromatographic techniques. Similarly, TLC in combination with optical densitometry and HPLC was carried out by Nikolova-Damyanova *et al.*, 1994 and Hirata *et al.*, 2002 for stevioside estimation. Liquid chromatography/Electrospray ionization mass spectrometry was also used for stevioside extraction by Pol *et al.*, 2007, Rajasekaran *et al.*, 2008 and Jackson *et al.*, 2009. In the present study, leaf samples collected at different intervals and chromatographed on thin layer glass plates using solvent system of chloroform: methanol: water (7:3:1) showed the presence of stevioside in different tracks when compared with standard R_f values. Kedik *et al.*, 2003 and Inamake *et al.*, 2010 used similar solvent system of chloroform: methanol: water in the ratios of

10:5:1 and 25:65:4 respectively on TLC plate to isolate stevioside from the leaves of *S.rebaudiana*.

Ahmed and Dobberstein, 1982a developed HPLC method for determination of eight diterpene glycosides with satisfactory resolution on two protein columns in series after extraction with chloroform. Separation by means of HPLC has been achieved using silica gel (Nikolova-Damyanova *et al.*, 1994), hydroxyapatite (Kasai *et al.*, 1987), hydrophilic (Hashimoto *et al.*, 1978) and size exclusion (Ahmed and Dobberstein, 1982a, 1982b), amino bonded columns (Kinghorn *et al.*, 1984; Makapugay *et al.*, 1984; Striedner *et al.*, 1991 and Liu and Li., 1995). However, we used C₁₈ column with a mobile phase of methanol: water (8:2) as developed by Bovanova *et al.*, 1998 and Kedik *et al.*, 2003 and scanned at wavelength of 210 nm as the optimum operating condition for chromatographic analysis of stevioside. Preliminary chromatographic estimation of thirty months old plant samples from *in vitro* and *in vivo* plants carried out using HPLC showed the presence of stevioside peak in both the test samples.

The choice of solvent for extraction is a crucial step in metabolite profiling and fingerprinting studies and may highly depend on the biological material and the metabolite of interest. Varieties of solvents having different polarities were used in sequential treatment that concluded with HPTLC. Extraction with two different solvent systems namely ethyl acetate: methanol: n-hexane and chloroform: methanol: water favored the quantification of stevioside, however, best results in terms of highest yield of stevioside were obtained with chloroform: methanol: water (7:3:1). Similarly, solvent system consisting of chloroform: methanol: water was used for obtaining secondary metabolite (alkaloids) from various medicinal plants including *Cortex phellodendri*, *Semen strychni*, *Sophora flavescens*, *Datura metel* and Green tea (Yuan *et al.*, 2001). Jaitak *et al.*, 2008 used ethyl acetate: ethanol: water (80:20:12) whereas Shirwaikar *et al.*, 2011 employed ethyl acetate: methanol: water (75:15:10) for the HPTLC based identification of stevioside from *Stevia rebaudiana*. We carried out multiwavelength scan of the test samples and an optimum operating wavelength of 210 nm was selected. It is assumed that since the compound does not possess an appropriate chromophore, therefore it favors the detection at low wavelength (Choi *et al.*, 2002).

To the best of author's knowledge and from cited literature, this is perhaps the first report where a comparative study has been made regarding the amount of stevioside present *in vitro* raised leaf samples taken from 3, 4 and 5 months old plants of *S. rebaudiana*. Highest amount of stevioside (68 µg/ml) was detected in 3 months old sample, followed by 4 months and 5 months old plant samples that contained 52.7 µg/ml and 44.1 µg/ml of stevioside respectively. The variations in the stevioside content may be contributed to different levels of glycoside synthesis at different ages of the plant. Rajasekaran *et al.*, 2007 extracted eight predominant sweet diterpene glycosides in the various plant parts of *S.rebaudiana* and reported highest content of 64.80 g steviolbioside kg⁻¹ in one month old green house leaves and 0.99 g rebaudioside A kg⁻¹ *in vitro* leaves by HPLC analysis. A survey of pertinent literature shows that a number of factors such as method of cultivation, type of organ and biomass yield (Nipovim *et al.*, 1998 and Geuns, 2003), environmental conditions (Wilkens *et al.*, 1996) and genetic variations (Zangerl and Bazzaz, 1992) are responsible for the variations in the secondary metabolite content in the plant harvested at different durations. Apart from these factors, day length sensitivity is another factor associated with the amount of secondary metabolite content. Bondarev *et al.*, 2003 found that different plant organs of *S.rebaudiana* contained different amounts of SGs, which declined in the order leaves, flowers, stem, seeds and roots. Madan *et al.*, 2010 reported higher amounts of steviol glycosides when *S.rebaudiana* plants were grown under long day conditions.

Another aim of the present study was to characterize the distribution of stevioside in intact *in vivo* and *in vitro* *Stevia* plants during different stages of ontogeny. During ontogeny, a gradual increase in stevioside content was observed in the leaves of both *in vivo* and *in vitro* plants and this process lasted upto the onset of flowering which usually occurs before the third year of plantation. Maximum amount of stevioside (94.9 and 52.6 µg/ml) was found in thirty months old *in vitro* and *in vivo* plants followed by eighteen months old *in vitro* and *in vivo* plants having 86.8 and 48.0 µg/ml of stevioside respectively when chromatographed on HPTLC. The results correlate well with those of Bondarev *et al.*, 2003 who clearly showed that variation in the metabolite content is associated with the age and phase of development of plant. In the present investigation, plants of *Stevia* harvested just prior to flowering showed higher levels of glycosides as is also suggested by various reports that during ontogeny the steviol glycoside (SGs) content gradually increases up to the budding phase and the onset of flowering and then it

gradually declines (Bondarev *et al.*, 2003). Rajasekaran *et al.*, 2008 reported very low stevioside content *in vitro* leaves as compared to the *ex vitro* leaves. In contrast, we report higher amounts of stevioside *in vitro* leaves as compared to *in vivo* leaves. Similarly, in *Catharanthus roseus*, higher levels of alkaloids vindoline, catharantine, vinblastine and vincristine were obtained from the roots of tissue cultured plants than intact plants (Ataei-Azimi *et al.*, 2008). A perusal of literature also revealed higher yield of active compounds *in vitro* regenerated plants of *Picea glauca* (Fowke *et al.*, 1994); *Taxus* sp. (Han *et al.*, 1999) and *Oroxylum indicum* (Gokhale and Bansal, 2010).

The highest stevioside yielding samples i.e. from thirty months old plants were column chromatographed using gradient system where the solvent composition changed during the course of elution. The elution was started with a non-polar solvent i.e. n-hexane on a polar stationary phase of silica gel so that compounds are eluted based upon their polarities to achieve a desired and effective separation. Among the different column fractions showing stevioside peaks, fraction showing maximum peak area of stevioside was selected and purified using preparative TLC and HPTLC. The selected sample yielding stevioside peak with similar R_f values was scrapped and dissolved in polar solvent like methanol. The test sample on analytical scale with loadings on prep-TLC showed good separations. For the final purity of isolated compounds, the process was further performed on prep-HPTLC. The purified fraction obtained was elucidated structurally for stevioside using NMR analysis. The ¹H NMR of stevioside was recorded on Bruker spectrophotometer (Model AVANCE DPX-400 MHz). Inamake *et al.*, 2010 interpreted the structure of stevioside using Varian mercury 300 NMR instrument and D₂O as solvent. We showed the presence of three sugar moieties in the stevioside structure which were confirmed at δ 5.24, δ 4.44 and δ 4.34 and are well supported by observations of Chaturvedula and Prakash, 2011. Interpretation of basic groups in the isolated compound confirmed the presence of stevioside.

5.2.3 Extraction of secondary metabolites from *Tylophora indica*

Vegetative parts of *in vitro* raised *Tylophora indica* were investigated for the phytochemical screening of primary and secondary metabolites. The phytochemical analysis revealed that total sugars (0.23%), reducing sugars (1.39%), sucrose (1.70%) and proteins (0.61%) were highest in

leaves, whereas starch (3.20%) and lipids (2.40%) were highest in the root. Earlier Kaushik *et al.*, 2010 have reported maximum amount of proteins and lipids in the leaves of *T. indica*, whereas highest amount of carbohydrates and starch was found in the stem. As far as secondary metabolites are concerned, maximum amount of sterols (2.2%) were found in leaves whereas maximum flavonoids (4.0%) were contained by roots. Meera *et al.*, 2009 also analyzed the alcoholic and aqueous extracts of leaves of *Tylophora indica* for the presence of flavonoids, alkaloids, proteins, saponins and carbohydrates. Similarly, Rathinavel and Sellathurai, 2010 and Kumar *et al.*, 2011 reported presence of alkaloids, sterols, saponin, terpenoids and tannins in the leaf extracts of *T. indica*. In the present study, almost comparable amounts of phenols were detected in both leaves and roots (0.98%). Presence of phenolic compounds add to multiple pharmacological effects such as antibacterial, anti-inflammatory, antiallergic and antiviral and flavonoids are mostly responsible for providing pigmentation of flowers, fruits and leaves (Sarin, 2005).

Although a number of reports are available regarding micropropagation of *Tylophora indica*, a major aspect that needs to be explored is the extraction of secondary metabolites from the cell cultures and different vegetative parts of *in vitro* regenerated plants. The leaves, stem and roots of this medicinally important plant contain several active alkaloids including tylophorine, tylophorinine and tylophorinidine (Mulchandani *et al.*, 1971; Gellert, 1982 and Chandrasekhar *et al.*, 2006). Several protocols are available for isolation of major alkaloid tylophorine from different vegetative parts of *T. indica* using various solvent systems and techniques (Rao and Brook, 1970; Jain *et al.*, 2007 and Reddy *et al.*, 2009).

Among the different extraction protocols followed for the present study, the highest percentage of tylophorine was obtained using extraction method of Rao and Brook, 1970 with slight modifications. The different plant parts were processed using cold extraction in methanol: acetic acid followed by further treatment with ethyl acetate, chloroform and methanol since the alkaloids are readily extractable in neutral to slightly basic solutions or organic solvents. The final step for the extraction process involved the removal of solvent in rotary flash evaporator operated at 50°C so that the substance of interest could be purified further. Similarly, acid extraction was employed in *Echium vulgare* for extracting pyrrolizidine alkaloids (Betteridge *et al.*, 2005) and in *Catharanthus roseus* for extracting alkaloids like vindoline and catharanthine (Verma *et al.*,

2007). In *Tylophora mollissima*, method of cold extraction was adopted to extract caffeine, tylophorine and tylophorinine (Viswanathan and Pai, 1985).

5.2.4 Characterization of major secondary metabolite- tylophorine

In *T. indica*, different plant alkaloids have been estimated using various techniques such as infrared spectroscopy (Vishwanathan and Pai, 1985), high performance liquid chromatography (Chaudhari *et al.*, 2005) and direct-injection electrospray ionization mass spectrometry (Verma *et al.*, 2007). Ratnagiriswaran and Venkatachalam, 1935 had isolated two alkaloids i.e. tylophorine and tylophorinine from *Tylophora asthmatica* (syn. *T. indica*), which were separated by fractional crystallization of the mixed salts. Similar studies for isolation of alkaloid tylocrebrine from *Tylophora crebriflora* were carried out by Gellert *et al.*, 1962. Viswanathan and Pai, 1985 reported chemical examination of *Tylophora mollissima* and yielded caffeine as major alkaloid and tylophorine and tylophorinine as minor alkaloids using techniques like ultraviolet, infrared and mass spectroscopy. Govindachari, 2002 isolated tylophorine, tylophorinine, tylophorinidine, septicine and isotylocrebrine from *T. asthmatica* by chromatography on alumina and reported the structure of tylophorinine and tylophorinidine with X- ray study. HPTLC based analysis for the hepatoprotective activity of the methanolic leaf extracts of *T. indica* was also carried by Mujeeb *et al.*, 2009 using toluene: chloroform: ethyl acetate (1:5:3) saturated with 10% acetic acid as a mobile phase. Comparative study conducted on the leaves and roots to quantify the amount of tylophorine using technique of high performance thin layer chromatography (HPTLC) is one of the first reports presented by us.

In the first experiment, a comparative analysis of tylophorine was carried out from the leaves of *in vitro* plants, which were raised through various micropropagation techniques. Leaf explants were cultured on different combinations of MS medium for plant regeneration through intervening callus stage and directly through the formation of nodular meristemoids. Similarly, root callus mediated plant regeneration of *Tylophora indica* was also achieved and eventually from all these plants, leaves were harvested for the extraction and comparative analysis of tylophorine. Tylophorine content analysis of all the samples revealed the presence of tylophorine and the maximal content of tylophorine (80µg/ml) occurred in the plants raised through leaf

callus while root callus regenerated plants yielded minimal amount (35µg/ml) of tylophorine (Kaur *et al.*, 2011 a, c).

Since roots are known to contain highest amounts of tylophorine, in another set of experiment, a comparative analysis of tylophorine was made from roots of twelve and twenty four months old *in vitro* and *in vivo* plants. Preliminary screening for the presence of tylophorine was done on TLC using various combinations of solvent systems. However, best results were observed on toluene: ethyl acetate: diethyl amine in ratio of 7:2:1 with tylophorine peaks at retention time of 0.64. Quantitative analysis of the root extracts on HPTLC using same solvent system as optimized for TLC showed that the roots of *in vitro* raised plants under both the age groups yielded more amount of tylophorine as compared to *in vivo* plants. Chaudhari *et al.*, 2005 using HPLC technique revealed the presence of tylophorine from the root-regenerated plants of *T. indica* but the amount of tylophorine was lower than field plants. However, they reported tylophorine content of transformed root clones 1.2 -1.5 times higher than the roots of non-wild plants, which has been attributed to the genetic transformation induced autonomous growth of transformed roots.

In the present study, root samples of twenty-four months old *in vitro* raised and *in vivo* plants yielded 90.75 and 59.40 µg/ml of tylophorine whereas twelve months old *in vitro* and *in vivo* plants contained 88.34 and 57.47 µg/ml of tylophorine respectively. The crude root extract of twenty four months old *in vitro* raised plant yielding maximum amount of tylophorine were further column chromatographed on silica gel with increasing polarities of mixture of chloroform and methanol. Abe *et al.*, 1995 obtained mixture of alkaloids from leaves and stems of *T. indica* by means of column chromatography and prep TLC. Reddy *et al.*, 2009 chromatographed methanolic extract of leaves of *Tylophora indica* on neutral alumina and eluted the column with increasing order of solvents like petroleum ether and benzene. In the current chromatographic analysis, all the fractions with uniform R_f values were pooled together when chromatographed on TLC and HPTLC using toluene: ethyl acetate: diethyl amine (7:2:1) and the selected fraction XI showing highest peak area for tylophorine was selected and analyzed on preparative TLC using the same solvent system. Use of same solvent system for preparative TLC was made for the isolation of triterpenoid glycoside from the bark of *Terminalia arjuna* by Pattnaik *et al.*,

2007. Both leaves and roots of *in vitro* raised plants of *T. indica* contained tylophorine and highest content of tylophorine in roots suggests that roots serve as a main organ for both synthesis and primary accumulation of tylophorine.

Quantitative yield of pure fractions of tylophorine in the present study, were obtained through preparative TLC, which were structurally analyzed through NMR analysis on Bruker spectrophotometer operated at 400 MHz. The ^1H NMR signal when compared with the standard tylophorine showed signals with similar values. The results are well in conformity with the NMR spectra of tylophorine as described by Zeng and Chemler, 2008. The basic interpretation of the current data through comparison with the standard showed that the final product obtained was alkaloid tylophorine. In *Phyllanthus amarus*, similar steps were adopted for the isolation of securinega type alkaloids using column chromatography and preparative TLC (Houghton *et al.*, 1996). Similarly, a group of 35 different plumeran indole alkaloids were isolated from the *Aspidosperma* genera using ^1H and ^{13}C -NMR spectra (Guimaraes *et al.*, 2012).

5.3 To develop protocol for mass extraction of secondary metabolites

5.3.1 Secondary metabolites in callus/suspension cultures of *S.rebaudiana*

In vitro culture technique has been widely accepted as a potential alternative for the production of valuable plant metabolites (Bourgaud *et al.*, 2001). Several reports of stevioside production in the cell cultures have been published from time to time (Kotani, 1980; Tamura, 1984; Brandle *et al.*, 1998 and Starratt *et al.*, 2002), however, there is still a need to standardize an efficient protocol for the enhanced production of metabolites in the cultures in order to meet commercial competitiveness. Although a number of reports are available regarding micropropagation of *S. rebaudiana*, the reports of stevioside biosynthesis in cultures are highly variable and callus has not yet been shown to be a stable producer of stevioside. The production of stevioside in callus cultures was reported by Komatsu *et al.*, 1976 while Handro *et al.*, 1977 could detect no sweetness in cultures maintained and subcultured for an extended period of time. Further, Hsing *et al.*, 1983 reported 50% decrease in stevioside production from day 38 to day 74, thereby showing the influence of growth intervals of callus on the content of stevioside.

The present investigation was carried out to ascertain if the callus and suspension cultures of *Stevia rebaudiana* do retain the ability for synthesis of main sweetener-stevioside. A comparative study of stevioside accumulation in callus and suspension cultures derived from stem and leaves of *S. rebaudiana* was made. Callus cultures were established from leaf and stem explants on Murashige and Skoog's medium supplemented with various growth regulators and maximum callus biomass from leaf and stem explants occurred on 2, 4-D (19.48 μM) + K (4.65 μM) and NAA (29.4 μM) + K (4.65 μM) supplemented medium respectively. The best-harvested cell mass was processed for extraction of active principle using HPTLC. The maximum amount of stevioside (44.37 $\mu\text{g/ml}$) was found in leaf callus and stem callus yielded 26.9 $\mu\text{g/ml}$ of stevioside when chromatographed on HPTLC using ethyl acetate: methanol: n-hexane (2:2:5) as developing solvent. Other solvent systems used yielded less amount of stevioside. Earlier studies have indicated the use of water: methanol and water: methanol: chloroform for stevioside extraction (Vanek *et al.*, 2001 and Sivaram and Mukundan, 2003). Further, when compared with the stevioside content (94.9 $\mu\text{g/ml}$) of *in vitro* leaves, the amount of stevioside detected in callus tissue was comparatively less. Rajasekaran *et al.*, 2008 reported the occurrence of maximum stevioside content in the leaves of intact plant which was several times higher than those of *in vitro* plants, callus and suspension cultures. On the other hand Sivaram and Mukundan, 2003 reported maximum glycoside content of 5.8% in the leaf callus of *Stevia rebaudiana* raised on MS + BA (8.87 μM) + IBA (9.80 μM) which was greater when compared to the glycoside content of the leaves (3.6%). Hsing *et al.*, 1983 reported increase in the stevioside content of callus tissue with the supplementation of medium with 2g/l casein hydrolysate (CH) and it was four times to that of leaf samples taken from field grown plants. Nevertheless, our study clearly indicate that the callus tissue derived from *Stevia rebaudiana* retain the ability to biosynthesize stevioside under *in vitro* conditions.

Suspension cultures were prepared by transferring leaf and stem calli to liquid MS media supplemented with 2, 4-D (19.48 μM) + K (4.65 μM) and NAA (29.4 μM) + K (4.65 μM) respectively. Cultures were incubated at $25 \pm 2^\circ \text{C}$ at 120 rpm and were monitored after every 3 days. Growth curve was plotted and it was observed that period of maximum biomass yield was achieved from day 12th to 24th and 15th - 27th in leaf and stem suspension cultures respectively. Similar to previous reports of Khiet *et al.*, 2006 in *Passiflora edulis* and Gopi and Vatsala, 2006

in *Gymnema sylvestre*, the suspension cultures in the present approach showed lag phase followed by exponential and stationary phases. Not much change was observed in terms of biomass after 24th and 27th day in leaf and stem cultures and this phase was referred to as stationary phase. Once stationary phase is reached, growth is arrested and secondary compounds are more actively synthesized (Bourgaud *et al.*, 2001). Extract at this stage was used for the chromatographic analysis. The HPTLC profile of both the extracts clearly showed that the amount of stevioside was higher than the one detected in callus cultures under similar operating conditions. Leaf suspension extract contained 69.40 µg/ml while stem suspension contained 57.69 µg/ml of stevioside. The presence of stevioside in cell cultures provides many opportunities for further investigation into large-scale production of stevioside by studying and standardizing different physical and chemical parameters. Cell suspension cultures as compared to callus cultures constitute a good biological material that allows the recovery of large quantities of active ingredients. Further, it enables scale up of secondary products on pilot scale using bioreactors. Production of shikonin by Tabata and Fujita, 1985 was one of the first successful attempt of an industrial scale up process using bioreactor technologies. The accumulation of active metabolites in cultured cells at a higher level than those in native plants through optimization of cultural conditions has been observed in a number of plants. For example, rosmarinic acid by *Coleus blumei* (Ulbrich *et al.*, 1985), berberin by *Coptis japonica* (Matsubara *et al.*, 1989), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991) and ginsenosides by *Panax ginseng* (Choi *et al.*, 1994) were accumulated in much higher amounts in cultures than in intact plants.

5.3.2 Secondary metabolites in callus/suspension cultures of *Tylophora indica*

Plant cell culture is an effective alternative way for large-scale production of secondary metabolites such as alkaloids, flavonoids and diterpenoids in *Ailanthus altissima*, *Torreya nucifera* (Sierra *et al.*, 1992 and Orihara *et al.*, 2002), sanguinarine in *Papaver somniferum* (Tyler *et al.*, 1989), cerpegin in *ceropegia juncea* (Nikam and Savant, 2009), phyllanthusol in *Phyllanthus acidus* (Duangporn and Siripong, 2009) and steroidal alkaloids in *Solanum lyratum* (Kuo *et al.*, 2011). In *Tylophora indica*, not many reports are available for the extraction of alkaloids from the callus or suspension cultures. Alkaloid productivity has been studied in stem and root callus cultures of *T. indica* where four secondary metabolites such as β-amyrin, β-

sitosterol, stigmasterol and campesterol were reported but phenanthroindolizidine alkaloids were not detected (Benzamine and Mulchandani, 1973). Benzamine *et al.*, 1979 studied alkaloid productivity in tissue cultures and regenerated plants and were the first to detect alkaloids in callus cultures. Chaudhari *et al.*, 2005 obtained tylophorine from the suspension and callus cultures of nine different transformed root clones of *T. indica*. They observed that in liquid medium, transformed root cultures exhibited vigorous growth and accumulated tylophorine at much higher levels than the non-transformed roots.

In the current investigation, compact callus masses from leaf explants were obtained when cultured on NAA (29.0 μM) + K (4.65 μM) supplemented medium while root explants favored IBA (24.6 μM) + BA (13.2 μM) supplemented medium. High biomass yielding callus cultures were analyzed chromatographically to purify secondary metabolite tylophorine. Leaf callus cultures contained 24.46 $\mu\text{g/ml}$ of tylophorine while root callus cultures yielded 26.42 $\mu\text{g/ml}$ of tylophorine when chromatographed in solvent system of toluene: ethyl acetate: diethyl amine (7:2:1) on high performance thin layer chromatography. To the best of author's Knowledge, this is the first report where leaf and root calli and suspension cultures were analyzed for the presence of tylophorine using HPTLC. In order to purify useful secondary metabolites effectively from cell cultures, scientists have employed various chromatographic techniques such as high performance liquid chromatography and high performance thin layer chromatography for determination of indole alkaloids from callus cultures of *Rauwolfia serpentina* and hairy root cultures of *R. Vomitoria* (Klyushnichenko *et al.*, 1995), high performance liquid chromatography for isolation of anticancerous alkaloids from callus cultures of *Catharanthus roseus* (Ataei-Azimi *et al.*, 2008) and thin layer chromatography for purification of alkaloid cerpegin from callus cultures of *Ceropegia juncea* (Nikam and Savant, 2009).

For establishing a finely dispersed cell suspension, leaf and root calli raised on respective media combinations were transferred to liquid media. The growth of cultures was monitored after an interval of 3 days. In the growth cycle, increase of biomass in leaf and root suspension cultures was maximum between day 12th-24th and 15th -27th respectively and thereafter it became stationary. Cultures were harvested at stationary phase and analyzed for the amount of major metabolite-tylophorine. HPTLC analysis of extracts showed that highest amount (34.71 $\mu\text{g/ml}$)

of tylophorine was yielded by root suspension cultures, whereas leaf suspension extract contained 28.30 µg/ml of tylophorine. Our study revealed higher amount of tylophorine in suspension cultures as compared to callus cultures. The results are consistent with the observation by Chaudhari *et al.*, 2005 who reported higher amount of tylophorine in the suspension cultures of *T. indica* than one obtained from callus cultures. Similar report was observed in *Vernonia cinerea*, where 5 weeks old callus cultures yielded 750 µg/g of alkaloid whereas maximum alkaloid contents of 1.15 mg/g was obtained from 20 day old suspension cultures (Maheshwari *et al.*, 2007). Presence of higher concentrations of asiaticoside in the suspension cultures as compared to callus cultures was observed by Nath and Buragohain, 2005 in *Centella asiatica*. Suspension cultures not only favor the recovery of large quantities of metabolites but also help in tracking the biosynthetic pathways responsible for the synthesis of valuable metabolites (Dougall, 1981). Moreover, as the demand for plant-based raw material is ever increasing, therefore, the present study employing *in vitro* culture technology is an alternative towards the production of valuable metabolites.

The HPTLC analysis of samples from *in vitro* raised plants, callus and suspension cultures revealed that the amount of tylophorine was maximum in *in vitro* plants followed by suspension and callus cultures. The current results are well in accordance with that of Zayed *et al.*, 2006, who reported higher tropane alkaloid content in the roots of regenerated plants than cell cultures of *Datura innoxia*. It is opined that production of secondary metabolites is generally higher in differentiated tissues as compared to callus cultures and also the levels of metabolites varied with the state of differentiation in the cultured tissues (Madhusudhan and Ravishankar, 1996). Moreover, the metabolites in the intact plant have a natural biosynthetic pathway and mechanism responsible for their production, which is normally disrupted during dedifferentiation state of the tissue (Dornenburg and Korr, 1995).

CONCLUSION

1. The present work which is an attempt to investigate the dedifferentiation and redifferentiation responses of cells of various organs of *Stevia rebaudiana* and *Tylophora indica* to varied and diverse chemical milieu corroborates the concept of totipotency and supports the contention that every cell of the plant body is totipotent and can express its hidden morphogenetic potentialities under proper nurture conditions.
2. *Tylophora indica* exhibited high degree of propensity for plant regeneration through different methods of micropropagation viz. forced axillary branching, *de novo* adventitious shoot formation and somatic embryogenesis.
3. A successful plant propagation system through multiple shoot proliferation and *de novo* adventitious shoot formation directly from different explants and through intervening callus phase was achieved in *Stevia rebaudiana*.
4. A wide spectrum of differentiation from calli of different organ origin of *T. indica* was observed in the form of xylogenesis, rhizogenesis, caulogenesis and differentiation of embryoids.
5. The detailed studies to corroborate embryogenesis in *T. indica* were carried out both histologically and through stereozoom microscopy depicting embryos at different developmental stages.
6. Synthesis of artificial seeds using different propagules like somatic embryos and shoot buds in *T. indica* and shoot tips in *S. rebaudiana* was achieved which opens a way for mass clonal propagation of these two species and also facilitates short term conservation and exchange of germplasm. It is especially significant in case of *Stevia rebaudiana* where somatic embryogenesis is not well established.
7. Comparative study of secondary metabolites in *in vitro* regenerated plants and *in vivo* plants at different age intervals was made to quantify the amount of metabolites yielded by plants at different phases of growth.
8. Optimised solvent system comprising of chloroform: methanol: water (7: 3: 1) yielded highest amount of stevioside in leaf extracts of thirty months old *in vitro* raised plants while *in vivo* plants yielded lesser amounts of stevioside.
9. Highest amount of tylophorine was obtained from the roots of twenty four months old *in vitro* plants of *T. indica* as compared to *in vivo* plants of same age when chromatographs were developed using toluene: ethyl acetate: diethylamine (7: 2: 1).

10. Suspension cultures yielded higher amounts of secondary metabolites as compared to callus cultures but these were less than the one obtained from *in vitro* regenerated plants. The results of the present work indicate that *in vitro* raised plants of *S. rebaudiana* and *T. indica* respectively may be ideal candidates for further research in the natural plant based pharmaceutical products.

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ANNEXURE- I

Composition of Murashige and Skoog's medium (1962)

Major Elements	
Salt	Concentration (mg/l)
NH ₄ NO ₃	1650
KO ₃	1,900
CaCl ₂ . 2 H ₂ O	440
MgSO ₄ .7 H ₂ O	370
KH ₂ PO ₄	170
Na ₂ EDTA	37.31
Minor Elements	
H ₃ BO ₃	6.2
MnSO ₄ .4 H ₂ O	22.3
ZnSO ₄ .7 H ₂ O	8.6
KI	0.83
Na ₂ MoO ₄ .2 H ₂ O	0.25
CuSO ₄ .5 H ₂ O	0.025
CoCl ₂ .6 H ₂ O	0.025
FeSO ₄ . 7 H ₂ O	27.81
Organic supplements	
Glycine	2.0
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.5

Sucrose (20 mg/l)

Stock solutions of major elements (8 X), minor elements (100 X) and vitamins (10 X) were prepared in distilled water. All the components of stock I, II and III were weighed separately, dissolved one by one to avoid precipitation in three different conical flasks.

Stock solutions of auxins and cytokinins

For the preparation of stock solutions of auxins (4X), 40 mg of salt was dissolved in few drops of alcohol and the final volume was made 100 ml with distilled water. Similarly, stock solutions of cytokinins (2X) were dissolved in 0.1 N HCl and the final volume was adjusted to 100 ml with distilled water. All the stock solutions were stored at -4°C , mixed in desired proportions only before use.

Publications in International/National Journals

1. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2011. Extraction of tylophorine from *in vitro* raised plants of *Tylophora indica*. Journal of Medicinal Plants Research. 5 (5): 729-734 (IF = 1.05).
2. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2011. Establishment of efficient protocol for micropropagation from stem explants of *Tylophora indica*-an important medicinal plant. African Journal of Biotechnology. 10 (36): 6928-6932 (IF = 0.57).
3. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2011. Establishment of an efficient and reproducible protocol for the micropropagation of *Tylophora indica* and extraction of tylophorine from *in vitro* raised plants. International Journal of Biotechnology and Bioengineering Research. 2 (2): 297–306.
4. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2011. Optimization of potting mixture for hardening of *in vitro* raised plants of *Tylophora indica* to ensure high survival percentage. International Journal of Medicinal and Aromatic Plants. 1(2): 83-88.
5. Manju Anand, **Harmanjit Kaur**, and Dinesh Goyal, 2012. HPTLC based analysis of tylophorine from cultures and in vitro regenerated plants of *Tylophora indica*- and important medicinal plant. International Journal of Environmental Science and Development (Revised manuscript submitted).

Manual Book Published

1. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., June 2009. Business Development in Plant Tissue Culture Technology. Excel Publishing House, New Delhi. (Sponsored by Department of Science and Technology, Govt).

National/ International Conferences

1. Manju Anand, **Harmanjit Kaur** and Dinesh Goyal., 2008. Rapid micropropagation of *Tylophora indica*- an important medicinal plant through

- axillary bud proliferation. 11th Punjab Science Congress, Thapar University, Patiala. A 134: 26.
2. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2008. “*In vitro* plantlet regeneration from leaf explants of *Tylophora indica*- An important medicinal plant, 11th Punjab Science Congress, Thapar University, Patiala. AP-133:101.
 3. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2009. “Sweet Diterpene Glycosides from *Stevia rebaudiana*”. National conference on Recent advances in Chemical and Environmental Sciences (RACES-2009). M.M.M. College Patiala. 88: 96.
 4. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2009. High efficiency adventitious shoot bud formation and plant regeneration from root segments of *Tylophora indica*-an important medicinal plant. 12th Punjab Science Congress, Punjab Agricultural University, Ludhiana. AP-11: 35.
 5. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2009. “Plant regeneration from leaf explants of *Tylophora indica*- an important medicinal plant”. BIOTECH-2009, Punjabi University, Patiala. A-4: 64.
 6. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2009. Extraction of secondary metabolites from *Stevia rebaudiana*- an important medicinal plant. National Symposium on Green Chemistry. Thapar University, Patiala. 41:57.
 7. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2009. *In vitro* cloning of *Tylophora indica*- an important medicinal plant. Plant Propagation, Conservation and Modification. IHBT, Palampur. 30
 8. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2010. Extraction of tylophorine using HPTLC technique from *in vitro* regenerated plants from root explants of *Tylophora indica* - an important medicinal plant. National Conference on Emerging Trends in Biopharmaceuticals: Relevance to Human Health. Thapar University, Patiala. 70
 9. **Harmanjit Kaur**, Manju Anand and Dinesh Goyal., 2011. Production of artificial seeds from *in vitro* raised somatic embryos and shoots buds of *Tylophora indica*- an important medicinal plant. 14th Punjab Science Congress, SLIET, Longowal. AOP-08: 48-49.

10. Manju Anand, **Harmanjit Kaur** and Dinesh Goyal., 2012. A micropropagation system for *Tylophora indica* and extraction and purification of tylophorine from cultures and in vitro regenerated plants. International Conference on Biotechnology and Food Engineering (ICBFE-2012), Dubai. U.A.E. International Proceedings of Chemical, Biological and Environmental Engineering. 41, 2012 ISBN; 978-981-07-3001-7.pg 14-17.