

**Plant tissue culture and chemo-profiling of *Tinospora cordifolia*
and *Stevia rebaudiana* for therapeutic metabolite content analysis**

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Dedicated
to my
Parents, Brother and
Almighty



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STATEMENT

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

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

Date: January 27, 2022


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CERTIFICATE

It is certified that the work described in this thesis entitled '*Plant tissue culture and chemo-profiling of *Tinospora cordifolia* and *Stevia rebaudiana* for therapeutic metabolite content analysis*' by **Vartika Srivastava** for the award of degree of **Doctor of Philosophy** is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for the degree.

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‘It’s never too late to set another goal or to dream a new dream.’ – Les Brown

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--- Vartika Srivastava ---

ABSTRACT

Pursuit of natural flora has become essential for benefit of human health, however, from the usage of natural resources to their over-exploitation has led to the extinction of several indigenous plant species. The conventional conservation and propagation methodologies are unable to fulfil the demands of the soaring population. Consequently, plant tissue culture techniques are considered as best alternative strategy for large-scale propagation of the plant. It eliminates the seasonal and regional variability, provides yearly output, generates true-to-type elite lines and provides ease of physiological, metabolic and structural studies.

Hence, plant tissue culture technique was chosen for the present study and utilized for generation of elite plant lines of *Tinospora cordifolia* and *Stevia rebaudiana*. Amongst the rich biodiversity of North-east India, *T. cordifolia* has been evaluated as one of the high-valued medicinal plant. This herbaceous twiner grows well in tropical climates, and an essential ingredient of Ayurvedic and local treatments of North-East and other parts of India. On the other hand, *S. rebaudiana* was another chosen plant of study. Indigenous to South America, it has been amplified world-wide as a bio-sweetener with additional therapeutic values. Conventional mode via stem-cutting is the most feasible way of propagation, although, it is tedious, provides short supply of plants, and highly influenced by the environmental drifts.

The present study is an endeavour to develop improved micropropagation methodology for generation of true-to-type elite plant lines of *T. cordifolia* and *S. rebaudiana* via nodal segment cultures. At the end of 6 weeks, rate of shoot multiplication was 10-fold in *T. cordifolia*, while multiplication rate in case of *S. rebaudiana* was noticed to be 54.0-fold and 52.1-fold in Sr1 and Sr2 experimental plant lines. Genetic integrity of elite cell lines was appraised using inter simple sequence repeats (ISSR) microsatellite molecular markers as well as flow cytometry, stating the true-to-type nature of the in vitro plants.

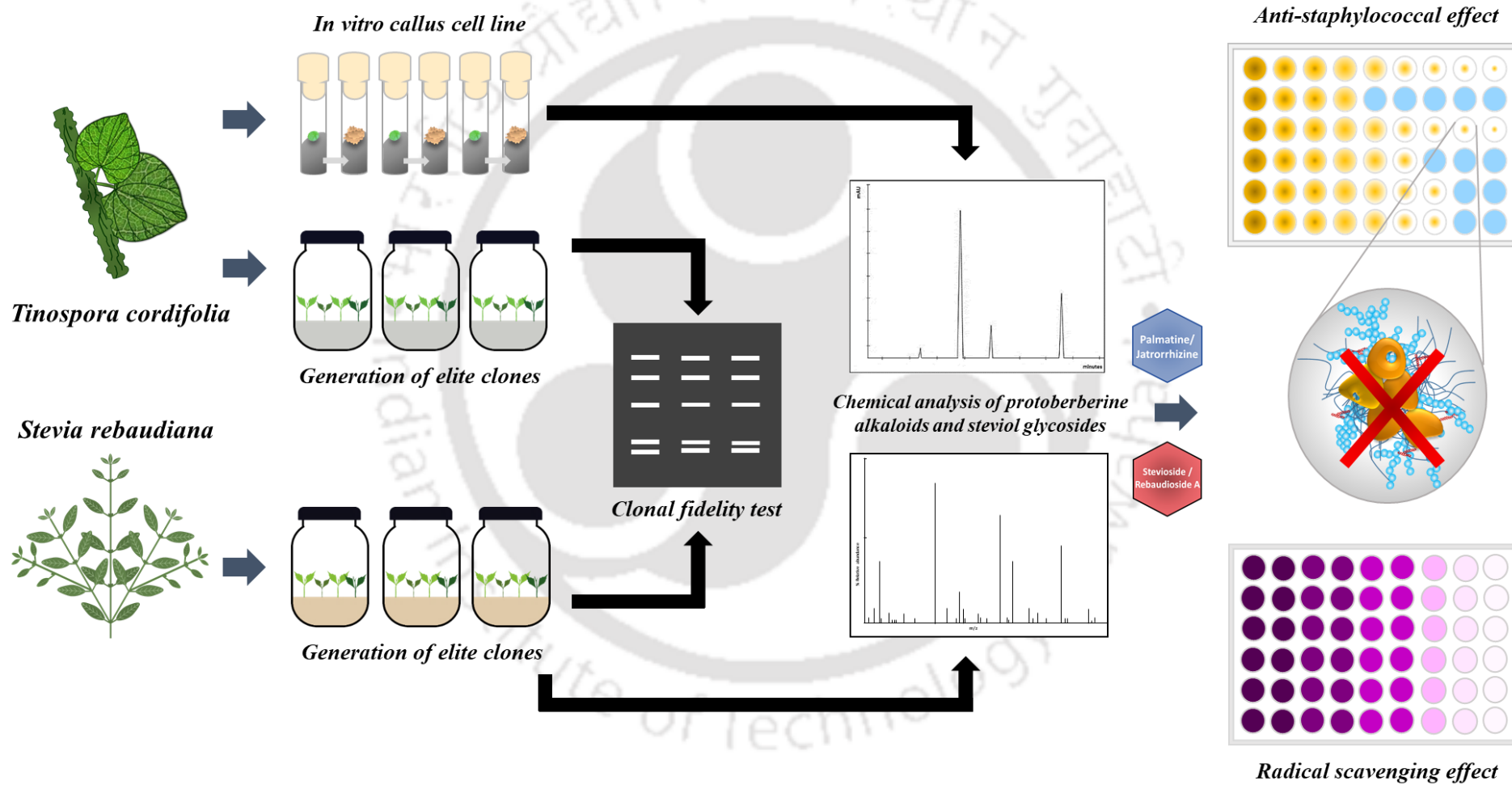
With respect to *T. cordifolia*, in vitro callus cell line was also developed using leaf explants, for estimation of protoberberine alkaloids, jatrorrhizine and palmatine. These alkaloids have profound medicinal effects, providing a vivid benefit to the therapeutic properties of the plant. Acid-base alkaloid extraction was employed for enhanced productivity of jatrorrhizine (10.9-fold) and palmatine (143.0-fold) with reduced phenolic contamination, from in vitro callus cultures in contrast to mother plant leaves. Besides, leaves of *S. rebaudiana* was chosen for

analyses of steviol glycosides, rebaudioside A and stevioside, being the reservoir for the same. With respect to the ratio of rebaudioside A to stevioside, as a significant factor for development of sweeter plant lines, in vitro propagated plants generate a vividly better ratio of rebaudioside A : stevioside as 2.1 : 1.7, while in the field grown mother plants, the ratio was 1.5 : 1.2.

Amidst therapeutic benefits of both the plant species, their role as anti-staphylococcal and antioxidant agent have also been elucidated in the study. In this context, the target bioactive secondary metabolites have been assessed qualitatively and quantitatively, against biofilm forming capabilities of *Staphylococcus aureus*, one of the key causative agents of diabetes. Furthermore, the study concludes with exploring anti-oxidative elucidations of protoberberine alkaloids, steviol glycosides and crude extracts in building effective future therapeutics.

Predominantly, the present research work proposed suitable methodologies in plant tissue culture, chemical and therapeutic analyses of *T. cordifolia* and *S. rebaudiana*. However, scale-up of protoberberine alkaloids and steviol glycosides via cell suspension culture and bioreactor shall be undertaken as future endeavours. Additionally, combination therapy of plant extracts or target bioactive compounds would also be interesting research prospective.

Graphical overview of thesis work



BACKGROUND AND MOTIVATION OF STUDY

The diverse and inbred usage of medicinal plants have been robust because of the roaring health fluxes globally. The natural resources have been greatly embezzled by the pharmaceutical industries, in order to fulfill the necessities of human life. However, researchers have left no stone unturned in discovering the potent natural bioactive metabolites as well as posing strategies for plant conservation. Among potent therapeutics plants, *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms and *Stevia rebaudiana* Bertoni, have been immensely utilized as a medicinal and commercial plants. The bioactive metabolites of these plants have been found effective against various ailments. According to the pharmaceutical features of both the plants, research and usage of plants' decoction and isolated compounds have been mostly in the field of diabetes. However, other ailments related to the occurrence or aggravation of the disease also need to be addressed upon. One of the related diseases includes invasive action of *Staphylococcus aureus* to a diabetic patient by aggravating insulin resistance. Moreover, the biofilm forming capacity of the microorganism efficiently influence the severity of infection and should be treated before it becomes acute or chronic in nature. In addition to this, rising concern of antibiotic resistance have possibly diverted the solution towards usage of plant-based drugs. Prevention of biofilm formation by target microbe, specifically, initial stage of biofilm formation has been the highlighting fact of study. However, the target bioactive metabolites were isolated from the in vitro generated cultures rather than utilizing the natural habitat of the plants. The study therefore includes establishment of true-to-type elite lines of both the medicinal plants, rendering a reproducible strategy for their conservation. In summary, the entire thesis has been divided into following five chapters:

Chapter 1 Highlights the introduction and literature review that describe the various perspectives of both the medicinal plants along with their tissue culture insights.

Chapter 2 Describes the experiments on establishment of true-to-type plantations of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms and its genetic integrity analyses.

Chapter 3 Deals with experiments on establishment of in vitro cell cultures of *T. cordifolia* (Willd.) Miers ex Hook. F. & Thoms and chemical analyses of bioactive protoberberine alkaloids, jatrorrhizine and palmatine, followed by their anti-staphylococcal and antioxidant studies.

Chapter 4 Illustrates the experiments on establishment of true-to-type elite plant lines of *Stevia rebaudiana* Bertoni and its clonal fidelity assessment. Further the chapter unfolds the chemical analyses of bioactive steviol glycosides, rebaudioside A and stevioside, from in vitro cultures followed by their anti-staphylococcal and antioxidant studies.

Chapter 5 Represents the conclusion and future prospects of the entire study.



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LIST OF ABBREVIATIONS

Abs	➤	Absorbance
ABTS	➤	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
AC	➤	Adenine-Cytosine
Acetyl CoA	➤	Acetyl coenzyme A
AFLP	➤	Amplified Fragment Length Polymorphism
AG	➤	Adenine-Guanine
AgNO ₃	➤	Silver Nitrate
AgNPs	➤	Silver Nanoparticles
ANOVA	➤	Analysis of Variance
ARF	➤	Auxin Response Factor
AS	➤	Ammonium Sulfate
AT	➤	Adenine-Thymine
ATP	➤	Adenosine triphosphate
BAP	➤	6-Benzylaminopurine
B-cell	➤	B-Lymphocytes Cells
Bcl-2	➤	B-Cell Lymphoma 2 Apoptosis Inhibitory Protein
C8161	➤	Cellosaurus Cell Line
CAPS	➤	Cleaved Amplified Polymorphic Sequences
CH	➤	Casein Hydrolysate
CHCl ₃	➤	Chloroform
Cl ⁻	➤	Chloride ion
CO ₂	➤	Carbon dioxide
CTAB	➤	Cetyltrimethylammonium Bromide
CuO	➤	Copper oxide
CuO-PEG	➤	Copper Oxide-Polyethylene Glycol
CuO-PVP	➤	Copper Oxide-Polyvinylpyrrolidone
CuSO ₄	➤	Copper sulfate
CV	➤	Coefficient of Variation
d	➤	Days
2,4-D	➤	2,4-Dichlorophenoxyacetic Acid
DKW	➤	Driver and Kuniyaki Walnut
DMSO	➤	Dimethyl Sulfoxide
DNA	➤	Deoxyribonucleic Acid

DPPH	➤	2,2-diphenyl-1-picrylhydrazyl
dsDNA	➤	Double-stranded deoxyribonucleic acid
DW	➤	Dry Weight
ECM	➤	Extracellular Matrix
EDTA	➤	Ethylenediaminetetraacetic Acid
EFSA	➤	European Food Safety Authority
eLtaS	➤	Insulin-Binding Protein Extracellular Domain
ER	➤	Endoplasmic reticulum
ESI-QTOF	➤	Electrospray-Ionisation Quadrupole Time-Of-Flight
FAO	➤	Food and Agriculture Organization
FAS	➤	Fatty Acid Synthase
FDA	➤	Food and Drug Administration
FDA	➤	Fluorescein Diacetate
FeNa ₂ EDTA	➤	Ethylenediaminetetraacetic Acid Ferric Sodium Salt
FESEM	➤	Field Emission Scanning Electron Microscopy`
FW	➤	Fresh weight
GA ₃	➤	Gibberellic Acid
GC-MS	➤	Gas Chromatography-Mass Spectrometry
Glc	➤	Glucose
Glut4	➤	Glucose Transporter Type 4
GRAS	➤	Generally Recognized as Safe
GSH	➤	Glutathione
h	➤	Hours
H ⁺	➤	Hydrogen ion
H ₂ O	➤	Water
H ₂ O ₂	➤	Hydrogen Peroxide
HCCA	➤	Alpha-cyano-4-hydroxycinnamic acid
HCl	➤	Hydrochloric acid
HgCl ₂	➤	Mercuric chloride
HILIC	➤	Hydrophilic Interaction Chromatography
HOCl	➤	Hypochlorous Acid
HPLC	➤	High-Performance Liquid Chromatography
IAA	➤	Indole-3-Acetic Acid
IBA	➤	Indole-3-Butyric Acid
IC ₅₀	➤	Half Maximal Inhibitory Concentration
IC ₉₉	➤	Concentration causing 99% inhibition
IL-1β	➤	Interleukin 1 Beta

2-iP	➤	Isopentenyl Adenine
ISSR	➤	Inter Sequence Specific Repeats
JECFA	➤	Joint FAO/WHO Expert Committee on Food Additives
K ⁺	➤	Potassium ion
KAH	➤	Kaurenoic Acid 13-Hydroxylase
KEGG	➤	Kyoto Encyclopaedia of Genes and Genomes
Kinetin	➤	6-Furfurylaminopurine
LAS	➤	Lateral Suppressor
LBD	➤	Lateral Organ Boundaries Domain
LPL	➤	Lipoprotein Lipase
LPS	➤	Lipopolysaccharide
LS	➤	Linsmaier & Skoog
MALDI-TOF	➤	Matrix Assisted Laser Desorption/Ionization – Time of Flight
MAPK	➤	Mitogen-Activated Protein Kinase
MBIC	➤	Minimum biofilm inhibition concentration
MCF7	➤	Michigan Cancer Foundation-7 (Breast Cancer Cell Line)
MEP	➤	Methylerythritol 4-Phosphate Pathway
MH	➤	Mueller Hinton
MIC	➤	Minimum Inhibitory Concentration
min	➤	Minutes
miRNAs	➤	MicroRNAs
MP	➤	Mother Plant
MRSA	➤	Methicillin-Resistant <i>Staphylococcus Aureus</i>
MS	➤	Murashige And Skoog
MTTC	➤	Microbial Type Culture Collection and Gene Bank
N	➤	Nitrogen atom
Na ⁺	➤	Sodium ion
NAA	➤	1-Naphthalene Acetic Acid
NaOH	➤	Sodium hydroxide
NCCS	➤	National Centre for Cell Science
Nd	➤	Nodal segment
NF-κB	➤	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NO	➤	Nitric Oxide
NPs	➤	Nanoparticles
ns	➤	not significant
NS2B-NS3	➤	Viral Serine Protease
O ₂ •-	➤	Superoxide radical

OD	➤	Optical Density
•OH	➤	Hydroxyl radical
ORAC	➤	Oxygen radical absorbance capacity
PBS	➤	Phosphate Buffer Saline
PCR	➤	Polymerase Chain Reaction
PEP	➤	Phosphoenolpyruvate
3-PGA	➤	3-Phosphoglycerate
PI	➤	Propidium Iodide
PPARs (P-24)	➤	Peroxisome proliferator-activated receptors
PPAR α	➤	Peroxisome Proliferator-Activated Receptor Alpha
PPAR γ	➤	Peroxisome Proliferator-Activated Receptor Gamma
PPP	➤	Pentose Phosphate Pathway
PTLC	➤	Preparative Thin Layer chromatography
PVP	➤	Polyvinylpyrrolidone
QC	➤	Quality control
R ²	➤	Correlation Coefficient
R _A	➤	Rebaudioside A
RAPD	➤	Random Amplification of Polymorphic DNA
RAX	➤	Regulator of Axillary Meristems
R _D	➤	Rebaudioside D
RINm5F	➤	Rat insulinoma clone m5F
R _M	➤	Rebaudioside M
ROS	➤	Reactive Oxygen Species
RP	➤	Reverse-Phase
(S)	➤	Left hand stereocenter
SCAR	➤	Sequence Characterized Amplified Region
SD	➤	Standard Deviation
secs	➤	Seconds
SH	➤	Schenk And Hildebrandt
SOD	➤	Superoxide Dismutase
SPE	➤	Solid-Phase Extraction
ssDNA	➤	Single-stranded deoxyribonucleic acid
ssRNA	➤	Single-stranded ribonucleic acid
T- cell	➤	T-Lymphocytes Cells
T47D	➤	Human Differentiated Epithelial Breast Cancer Cell Line
TCA	➤	Tricarboxylic Acid Cycle
TDZ	➤	Thidiazuron

TF	➤	Transcription Factor
TFA	➤	Trifluoroacetic Acid
THP-1	➤	Human Monocytic Cell Line
TLC	➤	Thin Layer Chromatography
TLR-2	➤	Toll-like Receptor 2
TNF- α	➤	Tumor Necrosis Factor Alpha
UDP	➤	Uridine diphosphate
UGT	➤	UDP-Glucosyltransferases
UGT74G1	➤	UDP-Glucosyltransferase 74G1
UGT76G1	➤	UDP-Glycosyltransferase 76G1
UGT85C2	➤	UDP-Glucosyltransferase 85C2
UHPLC	➤	Ultrahigh-Pressure Liquid Chromatography
UV	➤	Ultraviolet
WHO	➤	World Health Organization
WPM	➤	Woody Plant Medium
ZnO	➤	Zinc oxide
ZnO-PEG	➤	Zinc Oxide-Polyethylene Glycol
ZnO-PVP	➤	Zinc Oxide-Polyvinylpyrrolidone
α 2-receptor	➤	Alpha-2 Adrenergic Receptor

LIST OF UNITS

%	➤	Percent
m	➤	Meter
cm	➤	Centimeter
mm	➤	Millimeter
μm	➤	Micrometer
nm	➤	Nanometer
N	➤	Normal
M	➤	Molar
mM	➤	Milli molar
μM	➤	Micro molar
nM	➤	Nanomolar
psi	➤	Pound-force per square inch
L ⁻¹	➤	Per litre
g	➤	Gram
pg	➤	Picogram
ng	➤	Nanogram
mg g ⁻¹	➤	Milligram per gram
μmole L ⁻¹	➤	Micromole per litre
g L ⁻¹	➤	Gram per litre
mg L ⁻¹	➤	Milligram per litre
μg L ⁻¹	➤	Microgram per litre
mg mL ⁻¹	➤	Milligram per millilitre
μg mL ⁻¹	➤	Microgram per millilitre
ng mL ⁻¹	➤	Nanogram per millilitre
ng μL ⁻¹	➤	Nanogram per microlitre
t ha ⁻¹	➤	Tonne per hectare
mg kg ⁻¹	➤	Milligram per kilogram
g kg ⁻¹	➤	Gram per kilogram
μg mg ⁻¹	➤	Microgram per milligram
μL min ⁻¹	➤	Microliter per minute
L h ⁻¹	➤	Litre per hour
v/v	➤	Volume by volume
w/v	➤	Weight by volume

mT	➤	Milli tesla
lux	➤	Lumen per square metre
bp	➤	Base pairs
p	➤	Probability
secs	➤	Seconds
min	➤	Minutes
h	➤	Hours
d	➤	Days
ml	➤	Millilitre
amu	➤	Atomic mass unit
CFU mL ⁻¹	➤	Colony forming unit per millilitre
m/z	➤	Mass-to-charge
V	➤	Volt
rpm	➤	Rotation per minute
DW	➤	Dry weight
°C	➤	Degree Celsius
ppm	➤	Parts per million
μL	➤	Microliter
Da	➤	Dalton



Chapter 1

Introduction and Literature Review

1.1 GENERAL

The rich biodiversity of Mother Nature has been an exquisite gift to mankind and other life forms on earth. Utilization of flora for agriculture, feed or ethnomedicine, has been dated since centuries. The first reported manifestation of usage of medicinal plants for formulation of drugs, occurred five thousand years ago in Nagpur, India (Kelly 2009; Petrovska 2012). Thereafter, the journey of local and instinctive application of plants for battling and subsiding diseases is on a progressive way. From mild cold to chronic diseases, people have begun shifting their medication from synthetic drugs to natural therapies. Owing to this, industries and pharmaceutical companies have turned up their focus to plant-based drugs. Unfortunately, these growing needs of human civilization have bitterly affected the natural flora, causing a major reduction in their population, especially causing them to be endangered or extinct.

Medicinal plants are chemically diverse and credit their therapeutic potential to the secondary metabolites which provide vague influence on the growth and development of plant but considered as environment linkers and are produced during the course of environmental fluxes. They are not only defence providers but also exhibit added advantages, such as ultraviolet resistance, signalling molecules during pollination, providing stability during stressed conditions and other functions related to behavioural and physiological aspects of plant (Wink 2016). Nearly, more than 1,00,000 secondary metabolites have been discovered but analysing the soaring requirements of natural-based drugs, there have been an extensive research on discovery of novel and potent bioactive compounds (Verpoorte 1998).

1.2 SECONDARY METABOLISM

Secondary metabolites are distinct class of compounds forming an organized armour of plant system and the probable marker for taxonomic identification of different plant species. These molecules differ from the primary metabolites in several aspects such as their non-participation in the fundamental metabolism of the plant, occur in small population and limit themselves to specific cellular locations (Bennett and Wallsgrove 1994; Bourgaud et al. 2001). They are considered as complex molecules with low-molecular weight but with varied degree of actions. A vogue for the usage of natural products rose from the ancient era. However, evolution of secondary metabolism in plants originated through horizontal gene transfer via cyanobacteria, protobacteria or fungi during symbiosis (Wink 2016).

Apart from different functionalities of secondary metabolites, they are endorsed for the enormous and diversified medicinal properties of the plant. These compounds are classified into distinct classes, present in varied structural and functional forms, types and concentrations in different plant species. *Hereby, a question arises as how plant-based secondary metabolites differ from those obtained from microbes or insects?* Studies reveal that insect material are available in low amount, therefore purification of compounds would be insufficient from them. With respect to microbes, few metabolic pathways are expressed predominantly, such as polyketide pathway as compared to plants. However, if the target metabolite belongs to terpenoid or phenylpropanoid pathway, then plants represent the most suitable choice.

Secondary metabolites are end-products of responsive behaviour of plants for their survival against biotic and abiotic factors (Bennett and Wallsgrove 1994). Their synthesis varies on the basis of plant species, developmental stage, habitat, environment or pathogen attack. They are located at different cellular locations. Alkaloids accumulate themselves in phloem cells, pericycle, endodermis, epidermis and specialized mesophyll cells of specific plant species. They could also be located in the vacuoles, trichomes and other specialized structures like idioblasts. Phenolics are located in the plant cell wall in the form of lignins, providing support and protection to the plant. The largest class of secondary metabolite, terpenes, are biosynthesized in the cytosol and plastids (Wink and Roberts 1998). Being structural and functional diverse, these metabolites originate from crucial primary intermediates of plant primary metabolism and form a complex yet organized metabolic system. In this context, availability of resources in the form of inorganic and organic salts, have considerable impact on the qualitative and quantitative expression of secondary metabolites. Broadly, they are classified as terpenes, phenolic compounds and alkaloids. Other class of compounds include

glycosides, steroids, tannins and other aliphatic compounds. Broad biosynthetic pathway is described in **Figure 1.1**.

Limitations

Of the wide plethora of compounds, structure elucidation and bioactivities studies have been performed on limited set of metabolites, however, endless pursuit for discovery of novel compounds is still on the flux for manufacturing plant-based drugs. In this context, international export of medicinal plants and their products from India would reach 5 trillion from 60 billion dollars by 2050 (Government of India). Practicing continuous trend in export of plant-based drugs to developed countries and over-exploitation by the pharmaceutical industries would cause a downfall in the natural market due to extinction of medicinal plant species.

Against this backdrop, methods for obtaining these plants on a large-scale is mostly conventional and is dependent on seeding, grafting, layering, cuttings and other methods, which are labour intensive and time consuming and unable to meet the growing demands.

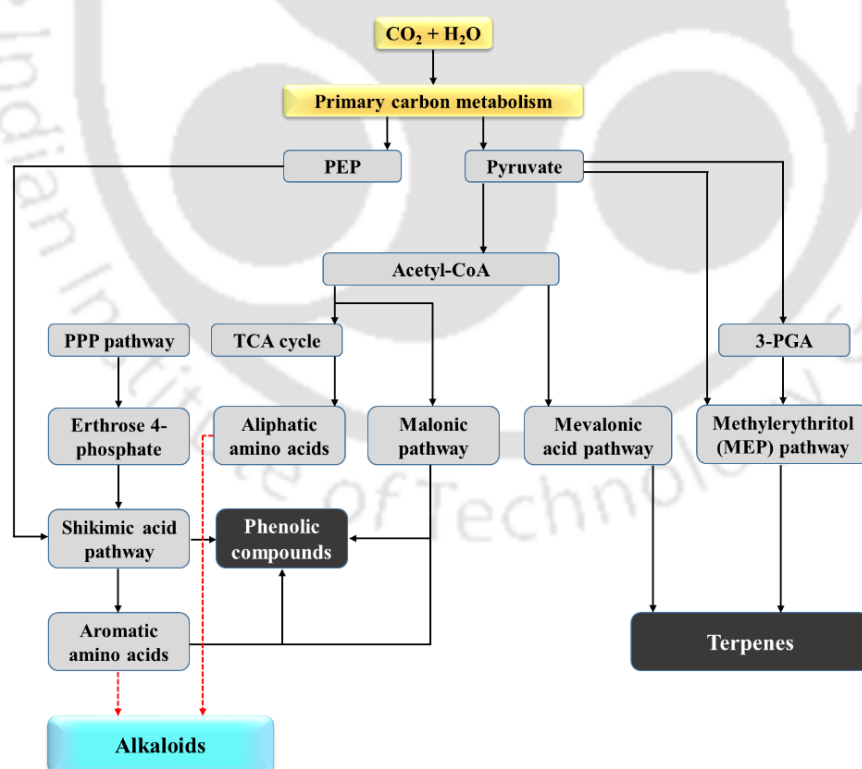


Figure 1.1: Biosynthetic pathways for broadly classified secondary metabolites (adapted from KEGG)

PEP: Phosphoenolpyruvate; **PPP:** Pentose phosphate pathway; **TCA:** Tricarboxylic acid cycle;
3-PGA: 3-Phosphoglycerate

Moreover, continuous climatic and environmental changes have led to severe modifications in plant growth, development and their metabolic profile. Entrepreneurs, research scientists and their technological advancements have initialized the large-scale propagation of medicinal plants with their sustainable use and their germplasm conservation. Among this, plant tissue culture technique is most feasible and effective way in achieving extensive plant propagation in short duration and space with desirable traits, along with down streaming of novel secondary metabolites.

1.3 PLANT TISSUE CULTURE: A GLOBAL ALTERNATIVE APPROACH

In the chronicles of events of plant tissue culture, it was Henri-Louis Duhumel du Monceau in 1756 who discovered the wound healing capacity of plants, followed by Haberlandt who initiated the tissue culture of isolated leaf tissues of *Lamium purpureum* and *Eichhornia crassipes* (Bhojwani and Razdan 1996; Thorpe 2007). Thereafter, a lineage walk from first callus culture of *Datura* in 1941 to development of single cell suspension culture of *Nicotiana glauca* by Muir in 1953, along with, in vitro large-scale propagation of plants via shoot-tip culture in 1960 by Morel, was a remarkable phase. The application of in vitro cell technology went well beyond micropropagation, and embraced all the in vitro approaches that were relevant or possible for the particular species, and the problems being addressed.

Over recent years, underlying molecular mechanisms behind redifferentiation and dedifferentiation have been gradually on an unfolded mode. It has been speculated that auxin induces channel of transcription factors where activation of LBD transcription factor (TF) by ARF7 and ARF19 TFs was carried followed by activation of E2Fa TF by LBD TF to hark back to S-phase of cell cycle (Berckmans et al. 2011). Kumari et al. (2017) in their study on *Solanum lycopersicum* revealed major changes during the reprogramming of cell in case of dedifferentiation and redifferentiation such as upregulation of carbohydrate, protein and fatty acid biosynthetic pathways, tricarboxylic acid cycle (TCA) and pentose phosphate pentose (PPP) cycle. On the contrary, metabolites with respect to cell wall lignification was downregulated signifying that the callus is soft, friable and less-rigid in nature as compared to organized cultures. In this context, secondary metabolite profile also varies during the course of re/de-differentiation. Few molecules are upregulated while yield of few molecules get subsided. Organized cultures pose added advantage in obtaining similar quantity of metabolites as their donor mother plants until they remain least affected by somaclonal variations. On the other way out, unorganized callus show variability in chemical profiling of metabolites,

nevertheless, biochemical alterations for scale-up of compounds is possible only by this methodology (Kefi 2018). Elicitation studies could also be performed as yield enhancement strategies of target secondary metabolites.

1.3.1 Micropropagation via axillary bud proliferation

Axillary bud proliferation via nodal segment culture is most widespread method of plant propagation for obtaining true-to-type elites of the plant. It involves development of shoot from pre-existing meristem and possess resistance to genetic changes even post-field transfer. Certain genes such as LATERAL SUPPRESSOR (LAS) and REGULATOR of AXILLARY MERISTEMS (RAX) genes, are responsible for initiation of meristematic potential of axillary meristem, followed by the its process of axillary bud formation. Auxin and cytokinin have their influential role in axillary bud activation involving upregulation along with export of auxin from the bud, cell cycle reactivation, thereby, triggering the bud break (Müller and Leyser 2011). Hence, obtaining disease-free clones, with higher rate of multiplication and regeneration potential via this methodology is relatively a rapid process and highly preferred on commercial scale.

However, inspite of obtaining true-to-type genetic clones, somaclonal variations were observed in various plant species. Somaclonal variations ascribes to various aspects, such as stress, effect of hazardous sterilants, media components including growth regulators, carbon source and even environmental factors such as temperature, light or humidity variations. Since auxins and cytokinin balance triggers the bud-break and shoot proliferation, it could indirectly force the cell to proceed to the next phase, bypassing the any repair mechanism, prompting variations at chromosomal level. Another probable reason could be epigenetic disparities with respect to transposable elements (Morrison et al. 1988).

Uncovering occurrence of somaclonal variations could be assessed by DNA based molecular marker-based techniques such as Inter Simple Sequence Repeats (ISSR), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Sequence Characterized Amplified Region (SCAR) or Cleaved Amplified Polymorphic Sequences (CAPS). Of these, RAPD analysis is a rapid technique involving amplification of repetitive DNA sequences of plant genome while ISSR deals with amplification of relatively small inter-sequence repeats between microsatellite nucleotide fragments. The later provides better resolution and reproducibility in genome mapping experiments. Both of the methods don't require prior knowledge of genome statistics of plant

or any organism (Bornet and Branchard 2001; Gostimsky et al. 2005). Cytological marker analysis is also one of the techniques used for assessment of genetic fidelity via flow cytometry. It allows to venture the total nuclear DNA content and ploidy determination of mother plant and regenerated in vitro plant samples. Genome size calculation is performed using the following formulae for examining the similarity between donor and in vitro regenerants species, where, $2C_a$ is value of 2C DNA content (pg) of experimental plant system, C_1 is average G_0/G_1 peak channel of experimental plant system, C_2 is average G_0/G_1 peak channel of known (standard) plant species and $2C_b$ is value of 2C DNA content of known (standard) plant species (Amaral-Silva et al. 2016).

$$2C_a = (C_1/C_2) * 2C_b$$

It is convenient for sample preparation and analysis. However, it has demerits like interference with other phenolic or related compounds takes place. Thus, multidisciplinary style needs to be followed for analysis of polymorphism or monomorphism in regenerated clones using aforementioned marker systems (Alizadeh and Singh 2009; Krishna et al. 2016).

1.3.2 *In vitro cell culture*

Plants are totipotent in nature and possess exceptional programming machinery for performing dedifferentiation and redifferentiation, based on environmental factors such as wounding. In the in vitro conditions, the culture environment including medium components favours formation of undifferentiated mass of cells of varying sizes and shapes. With a rapid growth, they behold the properties of stem cells and could be reprogrammed for development of organized structures (Delporte et al. 2012). During the early stages of callus development, storage reserves, proteins, carbohydrates and lipids increases. Primarily, post excision of tissue, penetration of ionic environment into the cell activates the biosynthetic pathways, thereby triggering cell division for the development of callus (Yeoman 1970).

Sequestration of secondary metabolites from organized cultures pose advantage over unorganized cultures in maintaining the metabolomic profile. Differences at sub-cellular level, such as lack of cell compartment could also be responsible for low yield of compounds in case of cell cultures. However, these changes are advantageous in ease and rapid sequestration and purification of compounds, followed by elicitation or scale-up approach (Kolewe et al. 2008). Additionally, there is no requirement for large cultivable land for whole plant system. Besides, both of the methodologies offer no seasonal and regional fluctuations and sustainable metabolite production without obstructing the floral diversity of nature.

1.4 *Tinospora cordifolia*

Woody lianas represent one of the most influential features of tropical and temperate forests habitat. Naïve in autonomous upright growth, they always need support to reach their maximum canopy and possess high canopy to stem ratio. Menispermaceae family which have originated 105-116 million years ago, generally constitutes lianas and dioecious vines and contributes to varying ethnobotanical uses (Anderson et al. 2005; Hoot et al. 2009; Kessler 1993; Wyka et al. 2013). Belonging to Menispermaceae family, *T. cordifolia* is a tropical woody liana and grows to a height of 700 m. Out of 40 species, only 4 species exist in India (Gaur et al. 2014). This woody liana shares a long history in serving the Ayurvedic and modern medicine, therefore, it is called as ‘heavenly elixir’. It is known as ‘Chakralakshanika’ and ‘Guduchi’ in Sanskrit and popularly known as ‘Giloy’, ‘Amritavalli’, ‘Amrita’, ‘Siddhilata’ and ‘Amarlata’ in different parts of India. The overall significance of these names corresponds in providing immunity, longevity and vitality to the body. Dwelling in the forests, it has been an essential folklore medicine for tribal and local people of different parts of India and other Asian countries (Upadhyay et al. 2010). The plant possesses diversified utilities of which the most utilized part is its stem followed by its root, leaf and fruit.

1.4.1 Botanical and functional aspects

1.4.1.1 Leaves

The leaves of the plant are simple, exstipulate, heart-shaped, alternately arrangement with long petiole while the lamina morphology reveals ovate, broad, membranous, sub-deltoid chordate and pubescent nature with seven-nerved reticulate venation (Sinha et al. 2004). The leaves possess hypoglycaemic (Wadood et al. 1992), hepatoprotective (Kavitha et al. 2011), antioxidant (Naik et al. 2014; Premanath and Lakshmidhevi 2010), hematological effect against lead toxicity (Sharma and Pandey 2010), anti-microbial (Mahesh and Satish 2008) and allelopathic effect (Raoof and Siddiqui 2012), while local people of Punjab use the leaves during ear pain or fever (Sinha et al. 2004). Besides, nanoparticles were also synthesized using leaf extracts and were found to possess pediculocidal and larvicidal effect (Jayaseelan et al. 2011), anti-bacterial and antioxidant effect (Selvam et al. 2017) with respect to synthesis of AgNPs while Udayabhanu et al. (2015) reported photocatalytic, antioxidant and anti-bacterial properties using cupric oxide NPs.

1.4.1.2 Roots

T. cordifolia possess long, fleshy, filiform aerial roots with primary structure to be tetra or penta-arched. It hangs from canopies and if the stem is cut and left hanging, it sends its aerial roots to the ground. Apart from stem, root is the most utilized part for cancer, fever, dysentery, diarrhoea and eye ailments among tribal and inhabitants of Gujarat, Orissa and Nagpur states of India (Sinha et al. 2004). The roots of the plant have been tested for its bioactivities against anti-histamine and bacterial infections, such as tonsillitis, sore throat, enteritis and urinary tract infections (Nayampalli et al. 1986; Zhao et al. 1991). Another group of researchers deciphered protective role of ethanolic root extracts against ulcerization and stress (Sarma et al. 1995a; Sarma et al. 1996). It also possesses hypolipidaemic, antioxidant and hypoglycaemic effects of aqueous and alcoholic extracts (Prince and Menon 1999; Prince and Menon 2000; Prince and Menon 2003; Prince et al. 2004). Curative effects of ethanolic root extracts against aflatoxin induced toxicity in mice was also investigated (Gupta and Sharma 2011).

1.4.1.3 Flower

Being dioecious in nature, the plants bear unisexual female flower in cluster with green dorsally convex sepals. However, male flowers are yellow or green in colour and occur in solitary. They are small with yellow dorsally concave sepals. The flowers exhibit racemes inflorescence and occur while the plant is leafless. They bloom during summers (Choudhary et al. 2013).

1.4.1.4 Fruits

Fruits of *T. cordifolia* develops twice in a year during January-February and June-July. They are of green (immature) and red (mature) colour, single seeded and fleshy. Seeds obtained from ripened fruit shows maximum germination rate as compared to partial ripened (yellow colour) and unripened (green colour) fruit (Warrier et al. 2008). There are a few literatures demonstrating the functional features of fruits. Khan et al. (2011) revealed the antioxidant property, nutraceutical and nutritional aspects of fruit extract. The reports revealed berberine and lycopene content to be 107 and 50.8 mg g⁻¹ while the nutritional content of *T. cordifolia* fruits is composed of potassium, ascorbic acid, tocopherols, niacin, protein, carbohydrate and lipids (unsaturated fatty acids). Moreover, in another report, the toxic effects of aqueous and petroleum ether extracts of *T. crispa* fruit against larvae of *Culex quinquefasciatus* post 72 h' of exposure, have been explored (Pal et al. 2016). This area still remains an area of study for further exploration of bioactivities of crude fruit extract and metabolomics.

1.4.1.5 Stem

Stem constitutes the most essential part of the plant. The plant bears succulent, soft and fleshy stem. Young stem is covered by thin cuticle with lenticels protrusions. Epidermis is composed of isodiametric cell with single parenchymatous layer. Cortex consists of huge amount of circular or oval starch grains. Vascular bundles are in bicollateral arrangement. Pith is small in size with high amount of starch grains. Unlike the previous, mature stem is hard with creamy-white bark with lenticels in rosette pattern. Secondary growth is observed due to single-layered vascular cambium. Secondary xylem and phloem are reported to be formed centripetally and centrifugally. Mature stem show vessel dimorphism signifying narrow and wide vessel elements. Secondary xylem is filled with abundant thin-walled parenchymatous cells, which contributes to the strength and flexibility during climbing (Patil and Malpathak 2018).

The preliminary studies on the use of stem in the form of water extract administered orally and intraperitoneally to albino rats and rabbits was reported by Pendse et al. (1977) and discovered the anti-inflammatory, analgesic and immunosuppressive effect. In 1978, Bedi uncovered the ethnobotany of plants of which stem decoction of *T. cordifolia* was used in fever by the aboriginal tribes of Ratan Mahal and nearby areas of Gujarat, India. Ever since, there have been several scientific reports focusing on the amelioration of immune system and suppression of deadly diseases by the plant. Stem decoction have been used by local and tribal people of India for recovery from several diseases such as fever, debility, asthma, snake bites, cough and cancer treatment in the regions of Uttar Pradesh, Gujarat, Rajasthan, Maharashtra, Haryana and Madhya Pradesh (Bedi 1978; Sinha et al. 2004). It provides a wide array of therapeutic applications in the fields of diabetes, cancer, ulcer, arthritis and proved to possess antioxidant and immunomodulatory properties (**Figure 1.2**).

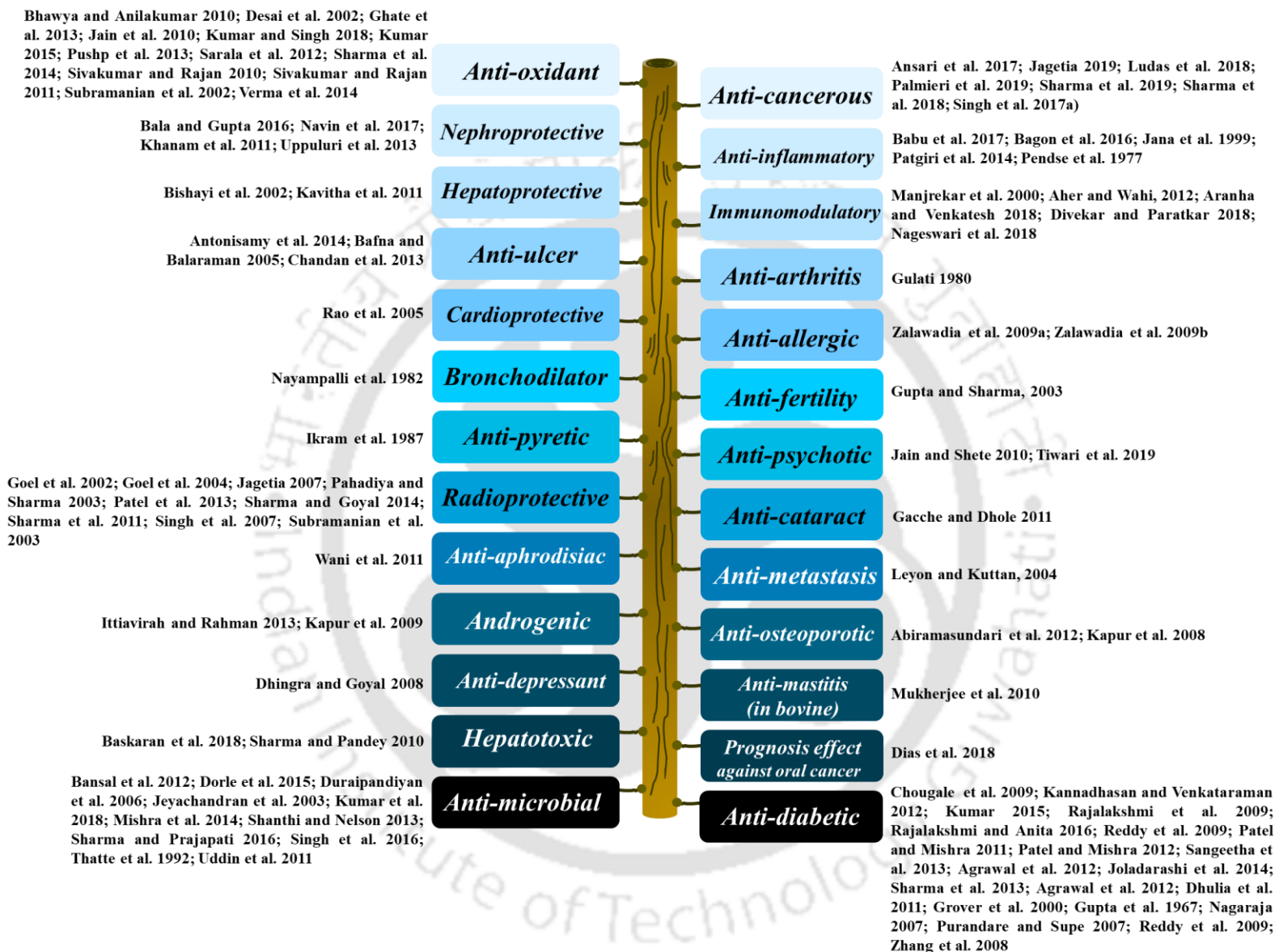


Figure 1.2: Therapeutic features of *T. cordifolia* stem

1.4.2 Secondary Metabolites

The characteristic functions of the plants are well contributed by array of secondary metabolites, each possessing their unique structure and functions. Occasionally, bioactivity is associated with efficacious nature of individual compounds or their cumulative effect. Aforementioned effects of *T. cordifolia* is due to presence of secondary metabolites, which categorize themselves into different classes such as terpenoids, alkaloids, phenolics, glycosides, steroids, diterpene lactones and aliphatic compounds.

1.4.2.1 Alkaloids

This class of secondary metabolites is accumulated in almost 20% of the plant species. These nitrogen-containing compounds with a heterocyclic ring are alkaline and non-peptidic in nature and are derived from aromatic amino acids and lysine (**Figure 1.3**). With an history of poisonous nature, they are an integral part of plants' defence system and a key ingredient of almost 60% of available medications (Khan et al. 2013). This is foremost approach of scientists and researchers for exploration of novel or potent alkaloids of plants' origin. Alkaloids are divided according to biosynthetic origin, chemical basis, pharmacological features and taxonomy, of which, former two categories are the most common and known categories. However, according to the recent mode of classification, they are sub-divided on the basis of heterocyclic and non-heterocyclic nature of compounds. Among the heterocyclic mode, isoquinoline alkaloids are commonly found in Menispermaceae family.

The biosynthetic pathway initiates from tyrosine to formation of dopamine and 4-hydroxyphenylacetaldehyde through a series of reactions. Thereafter, both of the molecules condense to form (S)-reticuline, which undergoes series of conversions to form (S)-tetrahydrocolumbamine. Further, the later molecule is directly converted to jatrorrhizine, which is further converted to berberine and palmatine (**Figure 1.4**). Moreover, indirect conversion of (S)-tetrahydrocolumbamine to palmatine involves formation of (S)-tetrahydropalmatine and (S)-canadine as intermediates in the formation of palmatine and berberine, respectively.

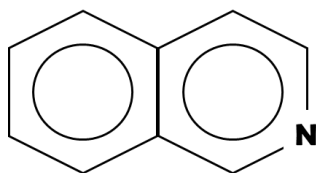


Figure 1.3: Basic ring structure of isoquinoline alkaloids

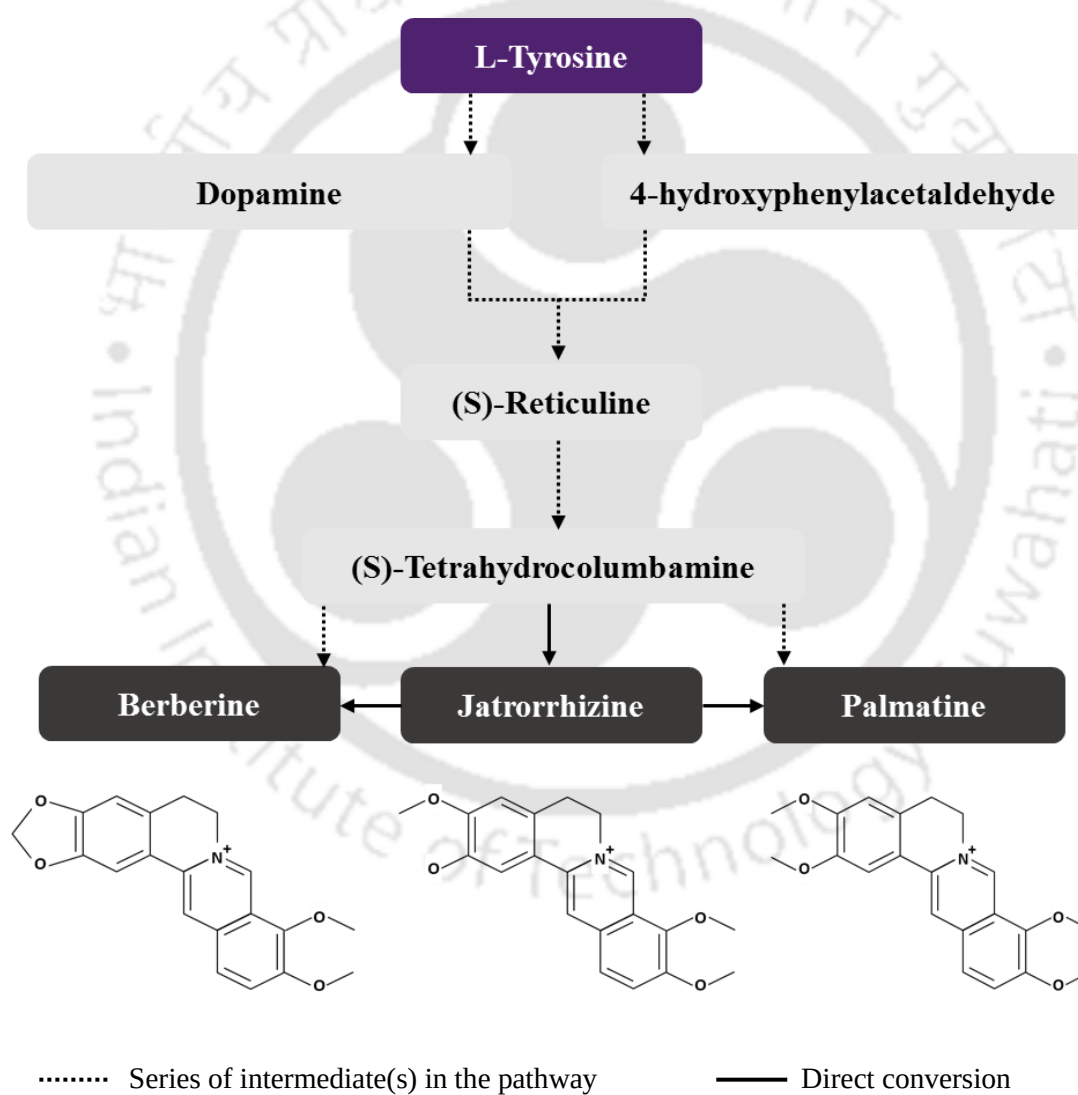


Figure 1.4: Biosynthetic pathway of important isoquinoline alkaloids (adapted from KEGG database)

1.4.2.1.A Protoberberine Alkaloids and their therapeutic applications

These quaternary molecules possess the characteristic $C=N^+$ polar bond and represents around 25% of the total alkaloids (Grycová et al. 2007). As per the available literature, the molecules target the organism by inhibiting DNA or protein biosynthesis, membrane permeability or oxidative phosphorylation. Of these molecules, berberine is one of the proficient natural product and key element of few commercially available drugs. Notably, it is highly effective alkaloid possessing anti-diabetic effect. It enhances the expression of insulin and Glut4 receptors, reduces the blood glucose level, lipid deposition in adipocytes, suppresses ROS generation and dipeptidyl peptidase IV (Al-masri et al. 2009; Ni et al. 2015). It is potent drug inhibiting multi-drug resistant pumps diminishing the cases of antibiotic resistance. As per literature, they are known to possess cytotoxic and anti-cancer effects. Barzegar et al. (2015) studied inhibition mechanism of berberine on T47D and MCF7 cancerous cell lines by apoptosis and cell cycle meddling. It is also found to potent in tumour inhibition by binding with Topoisomerase I and II. It prefers AT-rich DNA regions for effective binding (Mazzini et al. 2003). Besides, crude plant or alkaloid extracts comply different effects on our body in a multidirectional approach. Toxicity level and effect potential should be the focus of study of all the naturally occurring metabolites. Among the other forms of protoberberine alkaloids, there are a few literatures displaying the multi-functional properties.

A.1 Palmatine

Palmatine was initially isolated from *Jateorhiza palmate* (Gadamer 1902; Manske and Ashford 1954), while jatrorrhizine was isolated from *Mahonia philippinensis* (Castro 1932; Manske and Ashford 1954) and are widely present in Menispermaceae, Berberidaceae, Ranunculaceae, Gentianaceae, Piperaceae, Papaveraceae and Cactaceae families (da-Cunha et al. 2005). Inclusion of alkaloids render an effective defence to the plant against herbivores, microorganisms and viruses (Matsuura and Fett-Neto 2015). There are earlier literatures mentioning the applications of protoberberine alkaloids. Berberine, palmatine and sanguinarine were evaluated for providing chemical defence to the plant of which sanguinarine was effective against choline acetyl-transferase while palmatine and berberine were found to be potent against α_2 -receptor (Schmeller et al. 1997). In another study, palmatine was tested for its sedative activity in which catecholaminergic activity in the cortex and serotonin level in the brain stem was reduced unlike the enhanced serotonin level in the cortex in rats (Hsieh et al. 1993). Vasodilatory effect of palmatine was also inferred in a study in dose-dependent manner

along with reduction in the levels of free intracellular calcium in vascular smooth muscles in rats (Chang et al. 1999). The protoberberine alkaloid at an IC_{50} of 96 μM was also found to be effective against West Nile virus by suppressing its growth and NS2B-NS3 protease activity in dose-dependent manner (Jia et al. 2010). In a research related to galactosamine/lipopolysaccharide induced hepatic failures, mortality rates were subsequently decreased post treatment with palmatine. With respect to the anti-diabetic effect, palmatine was observed to enhance the activity of Glut-4 expression and peroxisome proliferator-activated receptor (PPAR α) unlike the expression of PPAR γ , fatty acid synthase (FAS) and lipoprotein lipase (LPL) (Sangeetha et al. 2013; Vasanthi and Kannan 2012). Inhibitory effect of palmatine isolated from *Coptis sinensis* was assessed against *Helicobacter pylori*. It was found to be better alternative to *Coptis japonica* extract and berberine in displaying antiulcer effect and posing growth inhibition of *H. pylori* (Jung et al. 2014). It was also tested as memory booster by hindering acetylcholinesterase activity in the brain (Dhingra and Kumar 2012). The alkaloid fraction extracted from *T. cordifolia* was analysed for their anti-glycaemic effect where, decreased gluconeogenesis and serum and blood glucose level with a simultaneous increase in insulin secretion from RINm5F cell lines (Patel and Mishra 2011). Anti-cancer potential of palmatine have also explored against induced skin cancer in Swiss albino mice. Statistical extraction methodology was applied for isolation of the alkaloid followed by its treatment in tumor reduction along with its reduction effect was assessed on levels of catalase, superoxide dismutase (SOD) and glutathione (GSH) (Ali and Dixit 2013). In another study, the target alkaloid was also experimented in breast cancer suppression by upregulation of tumour-suppressor microRNAs (miRNAs) (Hagiwara et al. 2015). Recent studies have evaluated the alkaloid to be highly potent against different types of cancer, such as rhabdomyosarcoma (Shinji et al. 2019), oral cancer (Qi et al. 2019), ovarian cancer (Johnson-Ajinwo et al. 2019) and pancreatic cancer (Muñoz et al. 2018). Its promising effects against Zika virus infection by disrupting its binding, entry as well as stability, has also been studied (Ho et al. 2019).

A.2 Jatrorrhizine

As stated, jatrorrhizine was primarily isolated from *Mahonia philippinensis*, however, with respect to *T. cordifolia*, it was successfully isolated from its roots, followed by its purification and characterization (Sarma et al. 1995b). Similar to other protoberberine alkaloids, it is correspondingly therapeutic in nature. In this context, the compound was found to be potent against diabetes as it was evaluated for lowering the blood glucose level and increase in levels of succinate dehydrogenase for successful occurrence of aerobic glycolysis (Yan et al. 2005).

Few other related works assessed the effect of the alkaloid on increasing the contraction of ileum muscles, influencing the treatment of gastrointestinal disorders (Yuan et al. 2011; Zhang et al. 2012). Later, anti-melanoma studies have been carried on where the compound successfully inhibited the proliferation of C8161 cell line in dose-dependent manner and inducing apoptosis at 320 $\mu\text{mole L}^{-1}$ (Liu et al. 2013). Enhanced lipid oxidation in liver along with simultaneous decrease in lipogenesis makes jatrorrhizine a better candidate for treatment of metabolic disorders (Yang et al. 2016). Besides, anti-inflammatory (Cho 2011), neuroprotective (Luo et al. 2011; Luo et al. 2017), neuraminidase inhibition (Wang et al. 2015) was also estimated. Amassing the literature, it is clear that the jatrorrhizine and palmatine are significant in therapeutic research, yet, the yield of these compounds is far less than their demands at medicinal and industrial levels. The most substituted way for figuring out higher production of compounds for ease access to the therapeutic potentials is isolation of compounds using plant cell cultures.

1.4.3 Micropropagation studies

An extensive, reproducible and economical propagation protocol is a pre-requisite for successful commercial production and conservation of *Tinospora*. In vitro large-scale propagation of *Tinospora* enumerates an efficient alternative for field grown mother plant. Thus, utilization of plants for extraction of desired bioactive compounds would be effortless for carrying forward the lineage of the use of *Tinospora* as traditional herb. The first report on the establishment of micropropagation protocol have been performed in Murashige and Skoog (MS; 1962) medium giving rise to 96% regeneration in three weeks by using 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA) for shoot regeneration. The authors also evaluated the effect of copper and zinc on morphogenetic changes in the plant (Kumar et al. 2003). In another study, micropropagation of *T. cordifolia* on Woody Plant Medium (WPM; Lloyd and McCown 1980) was conducted, in which, BAP was found to be most suitable for axillary bud proliferation while 6-furfurylaminopurine (kinetin) was better for shoot elongation. Rooting was achieved in Indole 3-acetic acid (IAA) supplemented half-strength MS medium (Raghu et al. 2006). Studies on micropropagation of *T. cordifolia* revealed the true-to-type large scale mode of propagation through nodal segment bearing axillary buds than the shoot-tip or inter-nodal segment culture (Sivakumar et al. 2014).

T. cordifolia constitutes lot of phenolics compounds which get exuded from the excised region of the explant. Several attempts have been made to reduce the oxidative browning effect and

to increase the survivability of the explant. Pre-treatment of explants in antioxidants, such as ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), silver nitrate (AgNO_3) and activated charcoal facilitate reduction in browning (Sivakumar et al. 2014). Apart from use of antioxidants, frequent transfer of cultures to fresh medium at short interval of time increases the rate of percent survivability of the cultures. This method is relatively simpler and doesn't affect the production of desired bioactive compounds. Soaking of explants in distilled water for 3-4 h expedited the leaching of phenolics into the medium, thus increasing the regeneration ability of plant (Gururaj et al. 2007). Among the available literature with respect to micropropagation, 16.5 number of shoots were obtained in high BAP concentration ($22.2 \mu\text{M}$) using shoot-tip explants. The authors used MS with 1 mg L^{-1} BAP and 0.6 mg L^{-1} GA_3 for shoot elongation resulting in 12.5 cm shoot length (Chatterjee and Ghosh 2016). It has been observed that rate of shoot multiplication decreases with subsequent subcultures/transfers, therefore, Panwar et al. (2018) speculated the effect of repetitive transfers and subcultures on the multiplication rate of in vitro shoots, of which higher multiplication rate was obtained in repetitive subcultures on modified MS medium supplemented with BAP and IAA. The other literature pertaining to the micropropagation studies are listed below in **Table 1.1**.

Table 1.1: Summary of updated work related to micropropagation studies of *T. cordifolia*

S.No.	Mode of propagation	Explant used	Medium	Rate of shoot multiplication per explant	Remarks	References
1.	Direct	Nodal segment	WPM + 8.8 μ M BAP	6.3-fold	-	Raghu et al. 2006
2.	Direct	Nodal segment	MS + 13.9 μ M kinetin + 11.4 μ M riacontanol	4.5-fold	-	Gururaj et al. 2007
3.	Direct	Nodal segment	MS + 6.9 μ M kinetin	-	-	Singh et al. 2009
4.	Direct	Nodal segment	MS+ 5 μ M BAP + 150 mg L ⁻¹ Glutamine	4-fold	-	Mishra et al. 2010
5.	Direct	Nodal segment	MS + 4.4 μ M BAP + 2.3 μ M kinetin	3-fold	Phytochemical analysis along with antibacterial assay was performed	Kumari 2012
6.	Direct	Nodal segment	MS + 6.9 μ M BAP + 8.2 μ M kinetin	4.8 -fold	Hypoglycaemic effect was examined	Sivakumar et al. 2014
7.	Direct	Nodal segment	MS+ 8.9 μ M BAP	4.8-fold	-	Sharma et al. 2015
8.	Direct	Shoot-tip	MS + 22.2 μ M BAP	16.5-fold	Cytological analysis of root-tips, cryopreservation as well as berberine estimation was performed	Chatterjee and Ghosh 2016
9.	Direct	Nodal segment	MS + 4.4 μ M BAP + 2.5 μ M 2-iP	7.9-fold	-	Mittal et al. 2017; Mittal and Sharma 2017
10.	Direct	Nodal segment	MS + 2.2 μ M BAP + 2.3 μ M kinetin	15.3-fold	-	Panwar et al. 2018
11.	Direct	Nodal segment	MS + 2 μ M BAP	3.8-fold	Selection of elite cell lines was performed on the basis of berberine content quantified using UV-visible spectrophotometry	Pillai and Siril 2019a

1.4.4 *In vitro* cell culture studies

Initial studies on callus and suspension cultures of *Tinospora* was successfully performed on MS medium supplemented with 0.2 mg L⁻¹ of kinetin and 1.0 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) generating a dark brown, loose and friable dedifferentiated cells (Chintalwar et al. 2003). Mostly, *in vitro* cultures of *Tinospora* featured the effect of BAP and NAA for initiation and multiplication of callus and suspension cultures and also reports for maximum alkaloid production. Rao et al. (2008) reported the maximum biomass yield along with maximum berberine production in MS supplemented with 19.8 µM NAA and 2.2 µM BAP. Later, the immobilization of callus cultures was also examined on the medium containing MS fortified with 1.0 mg L⁻¹ BAP and 2.0 mg L⁻¹ 2,4-D to enhance the production of secondary metabolites (Roja et al. 2005).

Plant suspension cultures began in 1963 by Muir (Bhojwani and Razdan 1996) and is now one of the crucial plant tissue culture techniques for scale-up of production of metabolites. In this regard, in a report, cell suspension cultures of *T. cordifolia* was successfully raised on Linsmaier & Skoog (LS; 1965) medium with 19.8 µM NAA and 2.2 µM BAP and kept at 100 rpm. It gave 5-14 times higher berberine yields than intact plants (Rao et al. 2008). Correspondingly, similar medium composition on MS medium generated cell suspension culture with high amount of protoberberine alkaloids. The content was enhanced by elicitation with fungal elicitors, *Piriformospora indica* and methyl jasmonate, chitin and chitosan (Kumar et al. 2017). Suspension culture also aids in monitoring the growth kinetics in homogenous population of plant cells and offer advantage of being a simplified model system for cellular and molecular process study of plants (Mustafa et al. 2011). The updated work on the cell culture studies have been mentioned in **Table 1.2**.

Table 1.2: Summary of updated work related to cell culture studies of *T. cordifolia*

S.No.	Explant used	Medium	Type of culture	Target metabolite yield	References
1	Stem	MS + 0.9 μ M kinetin + 4.5 μ M 2,4-D	Callus	Berberine 0.02% Jatrorrhizine 0.3%	Chintalwar et al. 2003
		MS + 0.9 μ M kinetin + 9.1 μ M 2,4-D	Suspension	Berberine 0.2% Jatrorrhizine 0.4%	
2	Stem	MS + 0.9 μ M kinetin + 4.5 μ M 2,4-D	Callus $\rightarrow$ Immobilized culture	Arabinogalactan 0.5% Berberine 0.01%	Roja et al. 2005
3	Leaves	MS + 4.5 μ M 2,4-D	Callus	-	Khalilsaraie et al. 2011
4	Leaves	MS + 16 μ M 2,4-D + 2 μ M kinetin	Callus	-	Bhalerao et al. 2013
5	Leaves, nodal and internodal stem	MS + 4.5 μ M 2,4-D + <i>Azadirachta indica</i> and <i>Acacia nilotica</i> methanol extracts	Callus	-	Patel and Ishnava 2014
6	Leaves	MS + 2.2 μ M BAP + 19.8 μ M NAA	Callus $\rightarrow$ Suspension $\rightarrow$ Elicitation with <i>P. indica</i> (endophytic fungus)	Jatrorrhizine (10.7 mg g ⁻¹ DW) Palmatine (4.4 mg g ⁻¹ DW)	Kumar et al. 2017
7	Stem	MS + 5 μ M NAA	Callus	Berberine (756 μ g g ⁻¹ DW) Berberine (1894 μ g g ⁻¹ DW) post tyrosine elicitation (40 μ M)	Pillai and Siril 2019b

1.5 *Stevia rebaudiana*

S. rebaudiana is 250-300 times sweeter than sucrose and is prioritized substitute for artificial sweeteners. The intake of the plant began since early 1960s and is well propagated in Japan, California, China, Thailand, Taiwan, Korea, Brazil, UK, Israel, Philippines, Canada, Hawaii, Ukraine and India (Brandle et al. 1998; Sivaram and Mukundan 2003). Researches have declared that excessive usage of sweeteners doesn't increase the risk of diabetes rather improper food habits with excessive sugar intake disturbs the lipid profile of the body (Basciano et al. 2005). A dietary monitoring by intake of natural sweeteners would thereby reduce the threats of the diseases.

1.5.1 *Botanical and functional aspects*

It is a perennial shrub and prefers long-day light for optimum growth. The leaves of the plant are simple, elliptical, opposite and sub-sessile and the trichomes are present on the epidermal surface of the leaves of both large and small sizes. Stem is woody, brittle and weak-pubescent at bottom while the roots are extensive, branched and fibrous in nature. It bears small, white, pentamerous and tubular flowers arranged in a non-uniform fashion. Flowering occurs from September to December (Northern Hemisphere) and from January to March (Southern Hemisphere) (Angelini et al. 2018). The nutritional aspects have initiated the propagation of plant as a crop in Japan in 1968. *Stevia* cultivation and its demand as a dietary supplement and natural sweetener got amplified over the years. It grows well at average temperature of 25°C under long-day conditions, thus favouring the vegetative growth of the plant. Vegetative phase is important for optimum growth and high yield of steviol glycosides. It grows properly in slightly acidic to neutral soil, which could be sandy, well-drained and organically rich in quality. It can tolerate mild frost but extremely sensitive to water logging, excessive moisture and reduced aeration. Growth of the plant also reduces when cultivated as monoculture and favours crop rotation (Angelini et al. 2018; Ramesh et al. 2006).

1.5.1.1 *Secondary metabolites*

The plant owes its sweetness and therapeutic properties to the diterpene steviol glycosides, which are the main secondary metabolite in leaves. Highest content of the main steviol glycosides is present in the leaves of the plant providing the sweet flavour to it. However, functional aspect of the plant is not limited here. The leaves of the plant as well as purified steviol glycosides pursue

therapeutic aspects including non-glycaemic in nature, anti-hyperglycaemic, hepatoprotective, anti-carcinogenic, immunomodulatory, anti-hypertensive, antioxidant, anti-inflammatory, anti-tuberculosis and antimicrobial (**Figure 1.5**). Rebaudioside A and stevioside along with steviol and steviolbioside have been reported to possess anti-diabetic effect. In vivo studies have reported that usage of stevioside have suppressed glucagon and blood glucose levels and enhanced insulin levels. Moreover, rebaudioside A and stevioside stimulates insulin secretion from mouse islets in glucose and calcium dependent manner by inhibiting ATP-sensitive K^+ channels. Holvoet et al. (2015) also mentioned that stevioside treatment enhanced the expression of nuclear receptor (PPARs) genes involved in the centralized regulation of glucose metabolism, fatty acid oxidation, lipid transport, and immune response.

Diabetes also increases the risk of having hypertension/blood pressure. Hypotensive, diuretic and natriuresis effect of stevioside and steviol on hypertensive as well as normal rats was successfully demonstrated by (Melis 1992a; Melis 1992b). It was hypothesized to be related to calcium influx mechanism. Later in a study, intravenous release of stevioside reduced the blood pressure of spontaneous hypertensive rat by inhibiting calcium influx in the blood vessels ultimately causing vasorelaxation (Chan et al. 1998; Lee et al. 2001). Obesity is also a known associated problem of diabetic patients. It is moreover related to increasing rate of epilepsy in the world. Therefore, an experiment was conducted to evaluate the anti-epileptic effects of rebaudioside A on pentylenetetrazol induced male rats, where 200 mg kg^{-1} rebaudioside A reduced the seizure spike percentage by 31.4% (Uyanikgil et al. 2016). Apart from the anti-glycaemic affects, the steviol glycosides are effective against other ailments as well. Studies proclaim the carcinogenic effects of the artificial sweeteners based on animal studies but the topic still remains in dispute. Nevertheless, natural sweeteners such as *Stevia* could be an optimum substitute, which doesn't cause but possess anti-carcinogenic property (Xili et al. 1992). In 2002, it was also reported that stevioside, rebaudioside A and C and dulcoside A inhibited the inflammatory ear edema in mouse and skin tumour formation (Yasukawa et al. 2002). Apart from stevioside, its aglycone, steviol was also found to be effective in inhibition of cyst formation and growth by 72.5% and 38.2%, respectively, during

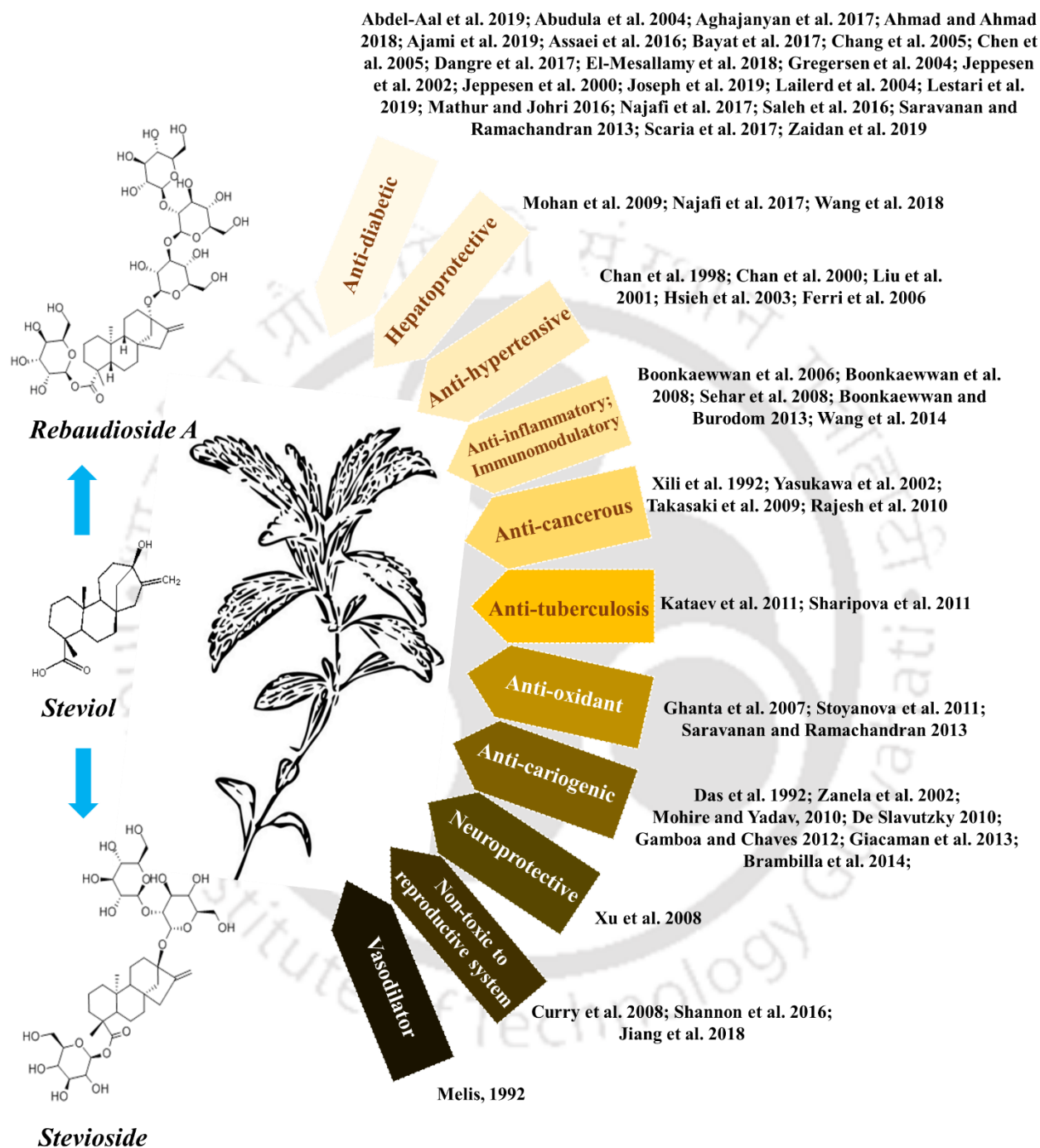


Figure 1.5: Functions of Stevia leaves and steviol glycosides

polycystic kidney disease. It also inhibited the cystic fibrosis transmembrane conductance regulator chloride channel, thus, repressing the forskolin-stimulated apical chloride content. Since, stevioside is degraded by the intestinal flora and converted to steviol, therefore, latter was the better choice of study on the polycystic in kidney (Yuajit et al. 2013).

There are reports based on the immunomodulating and anti-inflammatory effect of stevioside. Stevioside causes induction of inflammatory cytokines, $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and NO in unstimulated THP-1 (human monocytic) cells, thus enhancing innate immune system of the body. In another report, stevioside enhanced the B and T-cell mediated humoral and delayed type hypersensitivity response along with an increased phagocytic activity of the peritoneal macrophages (Sehar et al. 2008). Few literatures report the ill effects of steviol and crude extracts of *Stevia* leaves on the reproduction and fertility. Therefore, Curry et al. (2008) conducted an experiment in which purified rebaudioside A was administered to the male and female hamsters (F_0 , F_1 and F_2 generations) and reproductive studies related to changes in body weight, food intake, morphological changes in sperm concentration and motility, oestrous cycle, gestation period, mating performance and fertility were performed. Similarly, stevioside was also found to be safe in terms of growth and reproductive studies, even after supplementation of 2.5 g kg^{-1} of the isolated compound to male and female hamsters (Yodyingyuad and Bunyawong 1991). Recently, an experiment demonstrated that rebaudioside A, stevioside, and steviol disrupted the endocrine potential of the body, of which, steviol at 25000 ng mL^{-1} enhanced progesterone production by 5-fold (Shannon et al. 2016).

Action of steviol glycosides against tuberculosis was also studied. Stevioside and steviolbioside inhibited the 100% growth of *Mycobacterium tuberculosis* at 7.5 , $3.8 \text{ }\mu\text{g mL}^{-1}$ MIC, respectively and their respective derivatives showed the inhibitory activity within $5\text{--}20 \text{ }\mu\text{g mL}^{-1}$ (Kataev et al. 2011). In another study, isosteviol pre-treatment was given to the ischemia-reperfusion injured rat brain. Expression of Bcl-2 and superoxide dismutase (SOD) got increased. Apoptosis/necrosis of neurons, infiltration of neurocytes, lipid superoxidation, NF- κ B expression got reduced, conferring reduced brain injury and inflammation (Xu et al. 2008). Assessment of rebaudioside A and stevioside on the incidence of diarrhea was performed on the weaned pigs. Dosage of $191\text{--}213 \text{ mg kg}^{-1}$ (rebaudioside A) and 250 mg kg^{-1} (stevioside) was administered, which reduced the incidence of the disease as well high feed intake (supplementation of stevioside /rebaudioside A) improved the growth of weaned pigs. Apart from this, anti-cariogenic effect has also been examined in case

of *Stevia* leaf extract or steviol glycosides as sweetening agents. In this context, Brambilla et al. (2014) evaluated highest *Streptococcus mutans* biofilm formation in sucrose solution as compared to rebaudioside A and stevioside' solutions. Additionally, in vivo study was also conducted, in which *S. sobrinus* affected albino rats were fed with sucrose, rebaudioside A and stevioside of which the steviol glycosides were found to be non-cariogenic in nature (Das et al. 1992).

1.5.1.2 Factors governing steviol glycoside content

The main reservoir of steviol glycosides responsible for the composition and proportion of stevioside and rebaudioside-A contents in the leaves of the plant are factors, like harvest period, cultivar, climate, soil characteristics and proper storage conditions (Zeng et al. 2013). The most prominent phase of harvesting leaves is the onset of flowering for obtaining highest yield of metabolites (Pal et al. 2015). Authors have also stated that single harvest phase gives better yield of leaf biomass and compounds than multiple harvests, during a year (Moraes et al. 2013; Serfaty et al. 2013). Contrastingly, Midmore and Rank (2002) reported that multiple harvests provided better yield of biomass as compared to single harvest, provided replanting of the crop every year. In India, production of dry stem and leaf biomass was on average 2.5 t ha⁻¹ in the fourth year of planting, better than the initial three years (Megeji et al. 2005). Processing of the leaf biomass is also significant for determination of the highest yield of glycosidic content. According to the reports, drying of the leaves should be performed at ambient temperatures of 35-40°C for 24-48 h under proper air circulation. Dried leaves although enhances the phenolic content of the plant but it is rather easier for storage and commercialization of the leaves. Studies have been performed where steviol glycosides are extracted from the fresh leaves, providing less hindrance of the phenolics (Lemus-Mondaca et al. 2016).

1.5.1.3 Biosynthesis of steviol glycosides

Steviol glycosides are group of diterpene glycosides with glucose moieties attached to the basic aglycone non-glucosidic unit. Few of them possess rhamnose or xylose residues instead of glucose units. Biosynthesis of steviol glycosides share common precursor to gibberellic acid pathway. They are synthesized via methylerythritol 4-phosphate (MEP) pathway localized in the plastids of leaves (**Figure 1.6**). From the common pathway, kaurenoic acid 13-hydroxylase (KAH) produce steviol by hydroxylation of (-)-kaurenoic acid, in endoplasmic reticulum. Subsequent transfer of

glucose units is performed by plant UDP-glucosyltransferases (UGT) from UDP-glucose to steviolmonoside. There are three types of UGTs involved in the pathway, viz, UGT85C2, UGT74G1, UGT76G1, located in the cytosol. Further, steviol glycosides are translocated and accumulated in the vacuole. The probable transport from ER to vacuole remains unknown but

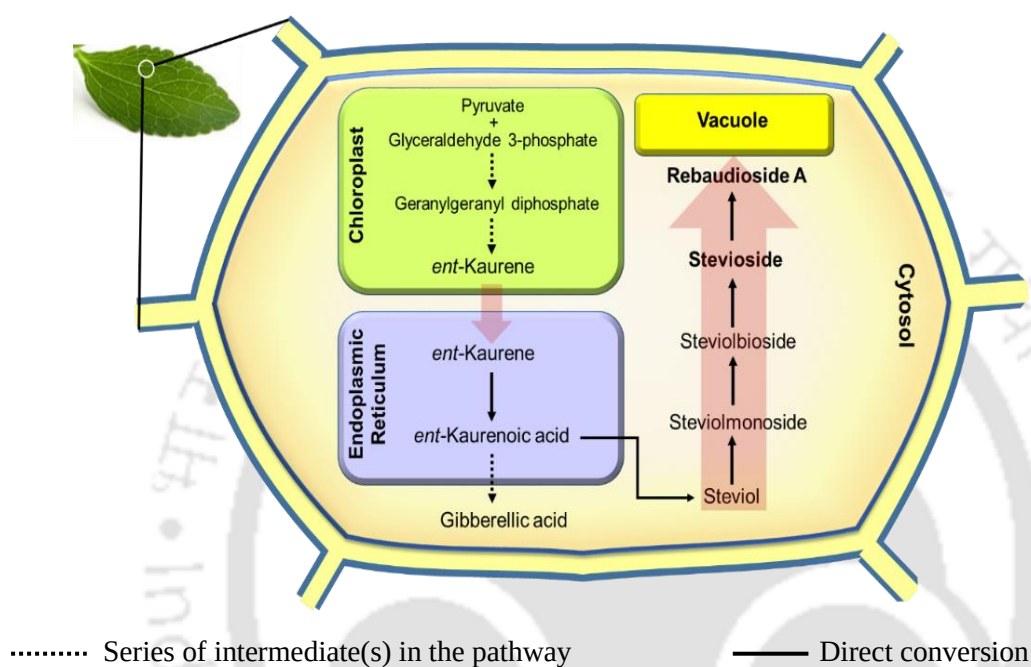


Figure 1.6: Biosynthetic pathway for the formation of steviol glycosides

hypothesized via vesicular trafficking, utilizing an electrochemical gradient or ATP for translocation of metabolites via vacuolar membrane (Brandle and Telmer 2007).

The diterpene glycosides has a conservative aglycone non-glycosidic unit while the variable region consists of attached glycosidic moieties. Stevioside is the first steviol glycoside discovered and extracted from the leaves of the plant and is 250 times sweeter than sucrose. Further, other steviol glycosides were also discovered consisting of rebaudiosides' A-X of which rebaudioside A is the sweetest among all the steviol glycosides and is 300 times sweeter than sucrose. The sweet potency of the rebaudioside A is maximum, followed by rebaudioside M. Rebaudioside M doesn't possess any bitterness or licorice after taste as compared to rebaudioside A, however it is present in low amount in the plant. Being the key ingredients in food and pharmaceutical industries, the stability of the steviol glycosides is also crucial as they are degraded or decomposed into other form of steviol glycosides. Along with the sweetness, there is slight bitterness after taste, which is

contributed by the presence of essential oils, tannins, flavonoids and sesquiterpene lactones (Goyal et al. 2010; Soejarto et al. 1983). These glycosides are not metabolized in the alimentary tract and they further break into steviol and glucose by the large intestinal flora. Glucose is metabolized by bacterial flora in large intestine and steviol is converted to glucuronide in liver and are excreted out (Geuns et al. 2007; Renwick and Tarka 2008; Samuel et al. 2018; Sharma et al. 2009). Free steviol remnants get excreted out via faeces. Consumption of stevioside (administered orally) produce more steviol glucuronide than rebaudioside A.

(i) *Stevioside*

Stevioside is the most fundamental steviol glycoside contributing to the sweetness and therapeutic potential of the plant. It is 250-times sweeter than sucrose (Brandle et al. 1998; Crammer and Ikan 1986) and non-glycemic in nature (Gantait et al. 2015; Suzuki et al. 1977). Consumption of the stevioside or *Stevia* leaf powder has been since centuries. There is always a concern for safety prospects or toxicity studies of the parent metabolite and its transformed products, before being used as food additive. Therefore, there are several reports that mark the effects, metabolism and safety views of stevioside (Akashi and Yokoyama 1975; Geuns 2002; Samuel et al. 2018). Stevioside was tested on the grounds of mutagenicity, carcinogenicity, fertility and allergic studies and verified to be used as dietary food or additive. In 1994, US FDA approved the intake of steviol glycosides in the form of purified *Stevia* leaf extract, as dietary supplements with adequate daily amount of 7.9 mg kg⁻¹ body weight in humans of which, the major components should be rebaudioside A and stevioside. Further, European Food Safety Authority (EFSA) and Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2011 permitted them to be food additives with daily intake rate as 4 mg kg⁻¹ body weight. In terms of its metabolism, it is stated that stevioside is neither absorbed in the intestine, nor degraded by the digestive enzymes, rather, it is converted to its own aglycone, steviol by the bacterial microflora of the intestine in humans, rats, mice and pigs (Hutapea et al. 1997). According to its structure, it contains β-glycosidic linkages and human gut lack the enzymes for the degradation of such bonds (Geuns 2002). In another study, maximum traces of steviol were found in blood after 8 h of oral administration (Nakayama et al. 1986). Later, Cardoso et al. (1996) found the presence of steviol in the liver, kidney and intestine after intravenous administration with only <1.8% found in the heart, stomach, muscle and testes.

(ii) Rebaudioside A

Stevia is composed of several glycosides other than stevioside. Likewise, a new diterpene steviol glycoside, rebaudioside A, was discovered in Japan, with higher sweet potency than stevioside. It is comprised of four D-glucose units attached to the steviol, the aglycone (Kaneda et al. 1977). It is metabolized in the similar manner as stevioside. In another report, the authors experimented the toxicological behaviour and metabolism pattern of rebaudioside A in humans as compared to stevioside. They found the maximum radioactivity of steviol and minimum of isolated forms of steviol glucuronide, rebaudioside A and stevioside. Moreover, steviol along with rebaudioside A and stevioside was excreted out within 48 h via faecal matter (Roberts and Renwick 2008). Rebaudioside A is successfully approved by US FDA and EFSA to be used a dietary supplement or food additive. (JECFA 2005) confirmed non-genotoxic effects of rebaudioside A and stevioside on human intake. The dietary daily intake was proposed to be less than 6 mg kg⁻¹ per body weight for children and diabetic people. One of the *Stevia*-product, 'Rebiana', is commercialized in the market, which comprised of maximum content of rebaudioside A.

1.5.2 Micropropagation

The market value of the *Stevia* is already on a hike and demands proper method of propagation. Presently, the intake of leaves of the plant increased to 5000 metric tonnes per year. Conventionally, it could be propagated via stem-cuttings or seed. Fresh seeds sown at an ambient temperature of 25°C produces better germination rates. Long storage conditions, low temperatures and small seed size exaggerates loss in seed viability, negatively affecting its propagation (Ramesh et al. 2006; Randi and Felipe 1981; Shock 1982). Large-scale cultivation of the plant via stem-cuttings is rather better alternative, possessing high survival rate. Yet, direct planting of the cuttings led to their low survival, therefore, they were pre-treated with auxins, IAA/NAA at different concentrations, resulting into increase in their sprouting, average shoot length, number of leaves and number of roots (Ingle and Venugopal 2009; Khalil et al. 2014b; Smitha and Umesha 2012). In addition to this, Carneiro et al. (1997) examined the effect of different potting mixtures to attain maximum survival and growth of cuttings in green house and found maximum shoot fresh and dry weight while using mixture of sand clay, soil, laying hen manure (10% v/v) and lime. Stevioside content and growth was found to be better in plants propagated via stem-cuttings (Yadav et al. 2011). Nevertheless, vegetative propagation is highly labor intensive and time

consuming and offer great variability in the plant production and steviol glycosidic content. Therefore, plant tissue culture technique is the most suitable methodology curbing the limitations of other propagation methods.

Generation of true-to-type plantations via micropropagation, production of homozygous lines via anther culture and constant production of steviol glycosides through cell culture. Khalil et al. (2014a) reported micropropagation to be best the propagation method producing 85% survival than stem cuttings and seeds. Tissue culture in *S. rebaudiana* was pioneered by Handro et al. (1977) who initiated callus development using leaf and stem explants and also initiated micropropagation using nodal segments on MS basal medium. Thereafter, shoot organogenesis was established using leaf explants intervened by callus formation (Yang and Chang 1979). Following the advantages of the technology, several researchers have proposed variable protocols for large-scale propagation of *Stevia* via direct and indirect methodology (**Table 1.3**). Maximum rate of multiplication via direct mode of propagation, using nodal segment/shoot-tip explants, was reported to be 123 shoots per explant in 65 days (Thiyagarajan and Venkatachalam 2012). While indirect propagation method (via callus formation) resulted in maximum of 106 shoots per explant in 28 days (Khalil et al. 2014a). Micropropagation without callus formation offers generation of true-to-type plantations with least variability in their genetic and metabolic profile. However, long term culture poses somatic mutations, such as DNA methylation, chromosome rearrangements and other mutations types and affect the genetic integrity of the regenerants (Phillips et al. 1994). Therefore, to assess genetic relatedness among in vitro population, PCR-based molecular markers are the suitable techniques. Clonal fidelity analysis was carried in *Stevia* using RAPD and ISSR (Das et al. 2011; Hassanen and Khalil 2013; Khan et al. 2016; Lata et al. 2013a; Modi et al. 2012; Singh et al. 2017c; Singh et al. 2014; Soliman et al. 2014). Former DNA fingerprinting methodology offer amplification of multiple loci of a genome with single arbitrary primer sequence, while the later technique is based on amplification of inter-specific microsatellite loci.

1.5.3 In vitro cell culture studies

Since the discovery of *Stevia* as a natural sweetener, there have been continuous research on large-scale propagation of the plant as well as extraction of known and unknown steviol glycosides. Initial study for regeneration of callus from *S. rebaudiana* was performed by Handro et al. (1977) using leaf and stem explants. The authors emphasized on the application of $800 \mu\text{g L}^{-1}$ 2,4-D than

IAA in addition to any cytokinin (BAP, kinetin, zeatin, each 200 $\mu\text{g L}^{-1}$). Isolation of stevioside from callus and suspension cultures was first initialized using 0.18 mg L^{-1} IAA by Komatsu and co-workers in 1976 (Ferreira and Handro 1988b). Moreover, Hsing et al. (1983) reported extraction of rebaudioside A and stevioside from leaf generated callus. The authors obtained maximum of 19.1% of rebaudioside A and 36.4% stevioside and at 38th day of culture, which decreased till 74th day (8.2% rebaudioside A and 18.2% stevioside). Addition of casein hydrolysate (0.5 and 2 g L^{-1}) resulted in decrease in dry weight of callus unlike yield of rebaudioside A and stevioside improved to 28.8% and 30.38%, respectively, even after 74th day. Till date, there are several literatures that have successfully generated callus or cell suspension cultures and have also estimated the steviol glycoside content in them. Among them, Ahmad et al. (2018) elucidated high rebaudioside A (13.1 mg g^{-1} DW) and stevioside (79.5 mg g^{-1} DW) content in adventitious roots at higher NAA concentration. In addition to this, changes in pH profile on steviol glycosidic content was also monitored. Maximum yield of steviol glycosides and varietal improvement is the central idea of the global research. However, the plant is prone to seasonal and regional fluctuations, micropropagation and extraction of steviol glycosides could be the best alternative and could be easily mass propagated in temporary immersion bioreactors. However, monitoring the biochemical and physiological characteristics of the plant, cell culture studies could be useful.

Table 1.3: Summary of updated work related to micropropagation and steviol glycosidic analysis in *S. rebaudiana*

S.No.	Mode*	Explant used	Rate of shoot multiplication per explant	Target steviol glycoside/s	Other significant finding/s	References
1.	Direct	Nd	15.0-fold in 50 d	-	-	Yang et al. 1981
2.	Indirect	Leaf	50.0-fold in 60 d	-	-	Ferreira and Handro 1988a
3.	Direct	Stem-tip	40.0-fold in 50 d	-	-	Tamura et al. 1984
4.	Direct	Shoot apex	11.2-fold in 40 d	Rebaudioside A and stevioside (3.6% in in vitro leaves as compared to 4.6% in in vivo leaves)	-	Sivaram and Mukundan 2003
	Direct	Nd	10.0-fold in 40 d		-	
	Indirect	Leaf	8.0-fold in 40 d	5.8% glycosidic content in in vitro callus	-	
5.	Direct	Nd	23.4-fold in 42 d	10.7 mg g ⁻¹ stevioside in regenerants than 12.0 mg g ⁻¹ as in mother plants	-	Hwang 2006
6.	Direct	Nd	8.7-fold in 42 d	-	-	Ahmed et al. 2007
7.	Direct	Nd	8.3-fold in 21 d	-	Total carbohydrates and proteins and compared with other variety	Rafiq et al. 2007
8.	Direct	Nd	39.0-fold in 28 d	-	Anti-microbial activity of in vivo and in vitro leaf extracts was analyzed	Debnath 2008
9.	Direct	Leaf	4.9-fold in 49 d	-	-	Sreedhar et al. 2008
10.	Direct	Shoot-tip	-	-	Varying rate of production was observed at different concentrations of MS basal medium and macro-salts	Ibrahim et al. 2008a
11.	Direct	Shoot-tip	73.6-fold in 42 d	-	Effect of growth regulators on shoot development was observed	Ibrahim et al. 2008b

12.	Indirect	Nd	12.0-fold in 28 d	-	-	Karim et al. 2008
13.	Direct	Leaf	37.4-fold in 28 d	-	Supplementation of CuSO ₄ to the medium increased shoot-bud proliferation rate	Jain et al. 2009
14.	Direct	Nd	17.6-fold in 28 d	-		
15.	Indirect	Leaf	14.0-fold in 28 d	-	-	Janarthanam et al. 2009
16.	Indirect	Stem and leaf callus	4.6-fold in 28 d	-	-	Patel and Shah 2009
17.	Indirect	Leaf	20.8-fold in 21 d	-	Shoot organogenesis from somatic embryos occurred	Banerjee and Sarkar 2010
18.	Direct	Shoot-tip	16.2-fold in 40 d	-	-	Anbazhagan et al. 2010
	Direct	Nd	14.0-fold in 40 d	-	-	
	Direct	Leaf	2.4-fold in 40 d	-	-	
19.	Direct	Nd	16.4-fold in 28 d	-	Addition of CuSO ₄ aided in increase in shoot-bud development	Kalpana et al. 2010
	Direct	Leaf	27.2-fold in 28 d	-		
20.	Direct	Shoot-tip	28.0-fold in 28 d	-	Shoot multiplication in case of shoot-tip with inverted polarity was also analyzed	Giridhar et al. 2010
21.	Direct	Shoot-tip	11.3-fold in 35 d	-	Clonal fidelity assessment through ISSR marker was performed	Das et al. 2011
22.	Direct	Root-tip	-	Absence of steviol glycosides in roots	Roller bottle system was established for scale-up of adventitious roots	Reis et al. 2011
23.	Direct	Shoot-tip	8.9-fold in 42 d	-	Cryogenic storage methodology was also developed	Shatnawi et al. 2011
24.	Indirect	Flower	21.6-fold in 35 d	-	Ultrastructural analysis of pollen grains was studied	Ahmad et al. 2011
25.	Direct	Nd	21.4-fold in 28 d	-	Effect of different carbohydrate sources (glucose, fructose, maltose and sucrose) on multiple shoot regeneration was analyzed	Preethi et al. 2011a
26.	Indirect	Leaf	8.5-fold in 28 d	-	-	Preethi et al. 2011b

27.	Direct	Nd	4.3-fold in 14 d	-	-	Laribi et al. 2012
28.	Direct	Nd	3.4-fold in 21 d	-	-	Mehta et al. 2012
29.	Direct	Nd	7.2-fold in 28 d	-	Clonal fidelity using RAPD was performed	Modi et al. 2012
30.	Direct	Nd	123.0-fold in 65 d	-	Frequency of shoot-bud proliferation increased with subsequent three subcultures	Thiyagarajan and Venkatachalam 2012
31.	Direct	Nd	4.3-fold in 21 d	-	-	Taleie et al. 2012
32.	Direct	Nd	14.8-fold in 30 d	-	-	Tiwari et al. 2013
33.	Direct	Microshoots	-	2.9 mg g ⁻¹ stevioside	-	Dey et al. 2013
34.	Direct	Shoot-tip	22.0-fold in 20 d	-	Effect of polyamines in enhancing shoot proliferation was studied	Guruchandran and Sasikumar 2013a
35.	Indirect	Leaf	12.0-fold	-	-	Guruchandran and Sasikumar 2013b
36.	Direct	Shoot-tip	43.9-fold in 42 d	-	Ex-vitro acclimatization followed by clonal fidelity assessment via RAPD, ISSR, AFLP and isozyme analysis was performed	Hassanen and Khalil 2013
37.	Direct	Nd	-	-	-	Javad et al. 2013
		Shoot-tip	35.4-fold	-		
38.	Direct	Nd	60.3-fold in 28 d	3.4% rebaudioside A and 5.4% stevioside	Leaf anatomy and gaseous exchange features was compared between mother and in vitro plants	Lata et al. 2013b
	Direct	Nd	53.2-fold in 28 d	3.3% rebaudioside A and 5.3% stevioside	ISSR marker analysis was examined	Lata et al. 2013a
39.	Direct	Nd	62.0-fold in 55 d	-	Effect of adenine sulfate on shoot growth was analyzed	Khan et al. 2014a
40.	Direct	Nd	8.0-fold in 42 d	-	ISSR profiling of the regenerants was carried	Khan et al. 2014b

	Direct	In vitro leaves	15.0-fold in 42 d	Rebaudioside A (3.1%) Stevioside (3.4%)	Direct regenerants were subjected to <i>Agrobacterium</i> mediated transformation for varietal improvement; ISSR profiling of the regenerants was carried	
	Indirect	In vitro leaves	5.0-fold in 42 d	Rebaudioside A (3.2%) Stevioside (6.4%)	ISSR profiling of the regenerants was carried	
41.	Direct	Nd	11.0-fold in 21 d	9.2% stevioside in in vitro leaves	-	Singh and Dwivedi 2014
42.	Indirect	Nd	20.0-fold in 30 d	9.2% stevioside in in vitro leaves	ISSR clonal fidelity analysis was performed for shoots derived from direct and indirect methods	Singh et al. 2014
43.	Direct	Nd	3.4-fold in 35 d	-	ISSR marker analysis was performed to check the genetic fidelity of the regenerants	Soliman et al. 2014
44.	Direct	Nd	22.7-fold in 21 d	-	Method was optimized for production of synthetic seeds using shoot-tip explants	Nower 2014
45.	Indirect	Leaf	106.0-fold in 28 d	-	Other propagation methods, such as seed germination and stem cuttings were also experimented	Khalil et al. 2014a
46.	Direct	Nd	7.8-fold in 28 d	-	-	Razak et al. 2014
47.	Direct	Nd	8.0-fold		-	Pawar et al. 2015
		Shoot-tip	6.0-fold			
48.	Direct	Nd	2.0-fold in 21 d	Rebaudioside A was found to be 5.0% and 4.7%, while stevioside was found to be 6.7% and 6.9% in in vitro leaves and seedlings	-	Yücesan et al. 2016a
49.	Direct	Nd	2.0-fold in 21 d	11.7% rebaudioside A and 4.8% stevioside in regenerants	Synthetic seed methodology was also developed	Yücesan et al. 2016b

50.	Direct	Nd	10.7-fold in 28 d	14.7 and 15.1 mg g ⁻¹ DW stevioside in in vitro and ex vitro leaves	Elicitation by alginate, salicylic acid, methyl jasmonate, chitosan, casein hydrolysate, pectin, yeast extract for micropropagation and stevioside production	Bayraktar et al. 2016
51.	Direct	Nd	44.6-fold in 40 d	-	Clonal fidelity using RAPD markers was analyzed	Khan et al. 2016
52.	Direct	tTCL of hypocotyls	31.2-fold in 42 d	-	-	Ramírez-Mosqueda et al. 2016
53.	Direct	Nd	14.4-fold in 28 d	12.2 mg g ⁻¹ rebaudioside A and 34 mg g ⁻¹ stevioside in in vitro plants	-	Röck-Okuyucu et al. 2016
		Shoot-tip	16.2-fold in 28 d			
54.	Direct	Nd	4.0-fold in 21 d	Total steviol glycosidic content found to be 40.1 mg g ⁻¹ DW	Scale-up via temporary immersion bioreactor was experimented	Vives et al. 2017
55.	Direct	Nd	4.9-fold in 28 d	4.2 rebaudioside A and 2.1% stevioside at 10 ppm of CuO nanoparticles	Synthesis and characterization of CuO nanoparticles and its effect on in vitro culture and steviol glycoside production was studied	Javed et al. 2017
56.	Direct	Nd	7.3-fold in 21 d	-	Clonal fidelity using ISSR markers was analyzed	Singh et al. 2017b
57.	Direct	Nd	4.5-fold in 28 d	Rebaudioside A (4.7%) Stevioside (1.9%)	Supplementation of ZnO, ZnO-PEG, ZnO-PVP, CuO, CuO-PEG, CuO-PVP nanoparticles to shoot organogenesis medium, enhanced the overall yield of steviol glycosides	Javed et al. 2017
58.	Direct	Nd	-	Rebaudioside A (2.9%) Stevioside (38.9%)	Elicitation of salicylic acid enhanced production of steviol glycosides and analyzed the upregulation and downregulation of respective genes	Tahmasi et al. 2017

59.	Direct	Nd	-	270 mg g ⁻¹ rebaudioside A 740 mg g ⁻¹ stevioside and in in vitro regenerants	Effect of nitrogen and phosphate on shoot growth and steviol glycoside content was analyzed	Magangana et al. 2018
60.	Direct	Shoot-tip	32.7-fold in 30 d	-	-	Beniwal et al. 2018
61.	Direct	Shoot-tip	9-fold in 60 d	-	-	Zarei et al. 2018
62.	Indirect	Leaf	6-fold in 30 d	-	GC-MS analysis of ex vitro and 1-year field transferred in vitro shoots was done	Solanki et al. 2018
63.	Direct	Shoot-tip	5.3-fold in 30 d	-	Effect of MS and Schenk and Hildebrandt (SH; 1972) medium was studied	Bhingradiya et al. 2018a; Bhingradiya et al. 2018b
64.	Direct	Nd	12-fold in 30 d	-	Effect of magnetic field (50 and 100 milli Tesla) was examined on the growth pattern	Madhavan and Anand 2019
65.	Direct	Nd	8.2-fold in 30 d	-	-	Rezaie et al. 2018
66.	Direct	Nd	3.7-fold in 21 d	19.4 mg g ⁻¹ DW stevioside	Effect of elicitors, methyl jasmonate and salicylic acid was tested for enhanced shoot growth, stevioside production and oxidative defense system	Moharramnejad et al. 2019
67.	Direct	Nd	11.8-fold in 28 d	-	-	Thilakarathne et al. 2019
68.	Direct	Nd	-	-	Data was recorded in terms of average shoot length and number of branches	El-Liel et al. 2019
69.	Direct	Nd	-	-	Data was recorded in terms of average shoot length	Javed et al. 2019
70.	Direct	Shoot-tip	3.3-fold in 63 d	-	Role of different types of algae filtrations was evaluated for micropropagation of the plant, instead of MS medium and other growth hormones	Emara et al. 2019
71.	Indirect	Leaf	-	-	Plant regeneration was attempted; Volatile compounds analysis via GC-MS was performed using in vitro callus	Zahri et al. 2019

72.	Direct	Nd	19-fold in 30 d	-	Identification of rebaudioside A and stevioside was performed using HPLC	Tufail et al. 2019
73.	Indirect	Internodal segment	8.7-fold in 30 d	-	-	Asmono et al. 2020

Note – ‘**’ Mode of propagation; Nd: Nodal segment; d: days; DW: dry weight.



1.6 THE THERAPEUTIC EXPLORATION AGAINST *STAPHYLOCOCCUS AUREUS* AND ANTI-OXIDATIVE POTENTIAL OF THE TWO MEDICINAL PLANTS

According to the pharmaceutical features of both the plants, research and usage of plants' decoction, the isolated compounds have been mostly in the field of diabetes. However, other ailments related to the occurrence or aggravation of the disease also need to be addressed upon. One of the related ailments includes invasive action of *Staphylococcus aureus*, especially in Type I diabetic patients, leading to recalcitrant diabetic foot. Rich and Lee (2005) demonstrated that hyperglycaemic conditions caused reduced leucocyte oxidative burst in case of bacterial infection, along with the increase in antibiotic resistance in the microorganism. Several cases have been reported of increased bacterial infection at the site of surgery, as well as, in case of obese patients with decreased blood supply and oxygen deficiency, bacterial killing by neutrophils has reduced (Scully et al. 2017). However, the mechanism of infection was still unknown. Hence, Liu et al. (2018) speculated that chronic *S. aureus* infection could lead to insulin resistance, thus, causing impaired glucose intolerance. It blocks glucose uptake after binding of eLtaS (insulin-binding protein extracellular domain), secreted by *S. aureus*. In addition to this, Ortines et al. (2018) found that *S. aureus* attack caused skin tissue necrosis, causing foot ulcers in diabetic patients and delayed wound healing.

Consequently, the *S. aureus* infection in diabetic or non-diabetic individuals is unsafe as it causes insulin intolerance in former and increases the future risks of diabetes in a healthy individual. *S. aureus* is well-known gram-positive bacteria, that easily invades the body of weak-immunized individual and mainly attacks the skin or upper respiratory tract. The biofilm forming capacity of the microorganism efficiently influence the severity of infection and should be treated before it becomes acute or chronic in nature. Biofilm are considered to be complex bacterial communities that develop post-colonization of planktonic bacterial cells on biotic or abiotic surfaces. They possess structured machinery of extracellular matrix (ECM), exopolysaccharides, proteins and extracellular DNA, providing a solitary protective environment for their growth and sustenance in hostile surroundings. Generally, biofilms follow three stages of formation: (a) *Initial attachment*; (b) *Cell aggregation and multilayer formation*; (c) *Maturation and detachment of planktonic cells for new life cycle*. Therefore, it reduces the susceptibility of anti-microbials to 10-1000 folds and till date, no promising anti-biofilm drugs have been into clinical trials (Galdiero et al. 2019; Mohammed et al. 2018). Moreover, development of antibiotic resistance in *S. aureus* is also major concern of their

sustained infection, hence, the researchers highly demand an amalgamation of potent antimicrobial agents that could inhibit and kill the bacteria and biofilm, and plant-based formulations or drugs could be a remarkable solution.

With respect to *T. cordifolia*, being an essential part of ethnomedicine, several researchers hypothesized the anti-microbial effect of stem and leaf crude extracts against *S. aureus* (Bonvicini et al. 2014; Jeyachandran et al. 2003). Besides, berberine have been experimented for possessing biofilm inhibition potential against *S. epidermis* and *Enterococcus faecalis*, and inhibited the expression of *sortase A* and *esp*, enzymes that mediates the attachment, adhesion and virulence of *E. faecalis* (Chen et al. 2016). Similarly, the alkaloids have been documented for enhancing the bactericidal activity of known antibiotics against methicillin resistant *S. aureus*, by inhibiting the formation of amyloid fibril formation, a component of biofilm extracellular matrix (Chu et al. 2016). Rich in all classes of secondary metabolites, especially production of phenolic compounds, *T. cordifolia* possess immense antioxidant property with respect to the crude plant extract (Bhawya and Anilakumar 2010; Ilaiyaraja and Khanum 2011; Jain et al. 2010; Jayaprakash et al. 2015; Polu et al. 2017; Sivakumar et al. 2010; Sivakumar and Rajan 2010; Sivakumar and Rajan 2011; Subramanian et al. 2002; Upadhyay et al. 2013). Moreover, phenolic compounds are well-known for their major contribution to the antioxidant properties of the plant, however, little is known in case of alkaloids. Few researchers have explored the evaluation of antioxidant potential of alkaloid(s) in different plant species such as *Guatteria hispida* (Costa et al. 2010), *Stephania rotunda* (Gülçin et al. 2010), *Aconitum laeve* (Shaheen et al. 2005); *Catharanthus roseus* (Tiong et al. 2013). The primary effect is the presence of hydrogen ion or electron donor in the secondary metabolite that effectively reduces the free radicals. Specifically, known protoberberine alkaloids have been isolated from other plant species such as *Mahonia* or *Coptis* sp., of which berberine proved to possess higher anti-oxidative potential (Jung et al. 2009; Misik et al. 1995; Rackova et al. 2007). In addition to this, antioxidant potential of crude extract of in vitro raised callus culture of *T. cordifolia* still remains an unknown fact.

With respect to *S. rebaudiana*, containing steviol glycosides, possessing ≥ 3 units of glucose or other sugar derivatives, the plant extends several therapeutic purposes especially in the treatment of diabetes. Alike, fighting microbial borne ailments also render a challenge since it could also pose favourable effects in enhancing microbial growth. However, several researchers have demonstrated the anti-microbial effect of the plant or isolated metabolites. Stevioside was found to inhibit lipopolysaccharides (LPS) of gram-negative bacteria, thus,

triggering the synthesis of proinflammatory cytokines (Boonkaewwan et al. 2006), along with reduced inflammation and apoptosis of epithelial cells infected with *S. aureus* (Wang et al. 2014). Stevioside also found to be effective against dental caries development due to *S. mutans* invasion (Brambilla et al. 2014). Thus, interpretation of their antimicrobial and anti-biofilm effect could be an interesting fact, which would rather help in analysing their potential as potent food additive. A dietary food intake of leaf powder or isolated steviol glycosides should also be examined for their antioxidant potential since reactive free radicals forms the basis for chronic ailments. In this regards, leaf powder of *S. rebaudiana* have been explored for its free radical scavenging activity (Karimi et al. 2014; Kim et al. 2011; Periche et al. 2015; Shivanna et al. 2013; Shukla et al. 2012). However, the authors considered phenolic compounds, as contributors of antioxidant potential. Amidst this, Stoyanova et al. (2011) demonstrated the ROS scavenging nature of stevioside via hydrogen radical, superoxide radical and hydrogen peroxide assay. Alike, Saravanan and Ramachandran (2013) postulated rebaudioside A as potent antioxidant as it was experimented to enhance the activities of both enzymatic (glutathione peroxidase, catalase and superoxide dismutase) and non-enzymatic antioxidants (vitamin C, E and glutathione) in diabetic rats. Therefore, apart from phenolic compounds, these diterpene steviol glycosides also extend their role in restraining possibilities of oxidative stress in body.

Objectives of the study

Post-synopsizing the updated literature, it would be interesting to unfold few known as well as unknown features of the two elite medicinal plants, *T. cordifolia* and *S. rebaudiana*. Therefore, the present study selected these two essential anti-diabetic plants, which are globally known for their medicinal and commercial values. The former is bitter woody liana while latter is natural bio-sweetener and dietary food additive. Accessing their individual benefits initially was the main focus of study, which could be utilized in amalgamation in the near future, for added-on benefits to natural therapeutics.

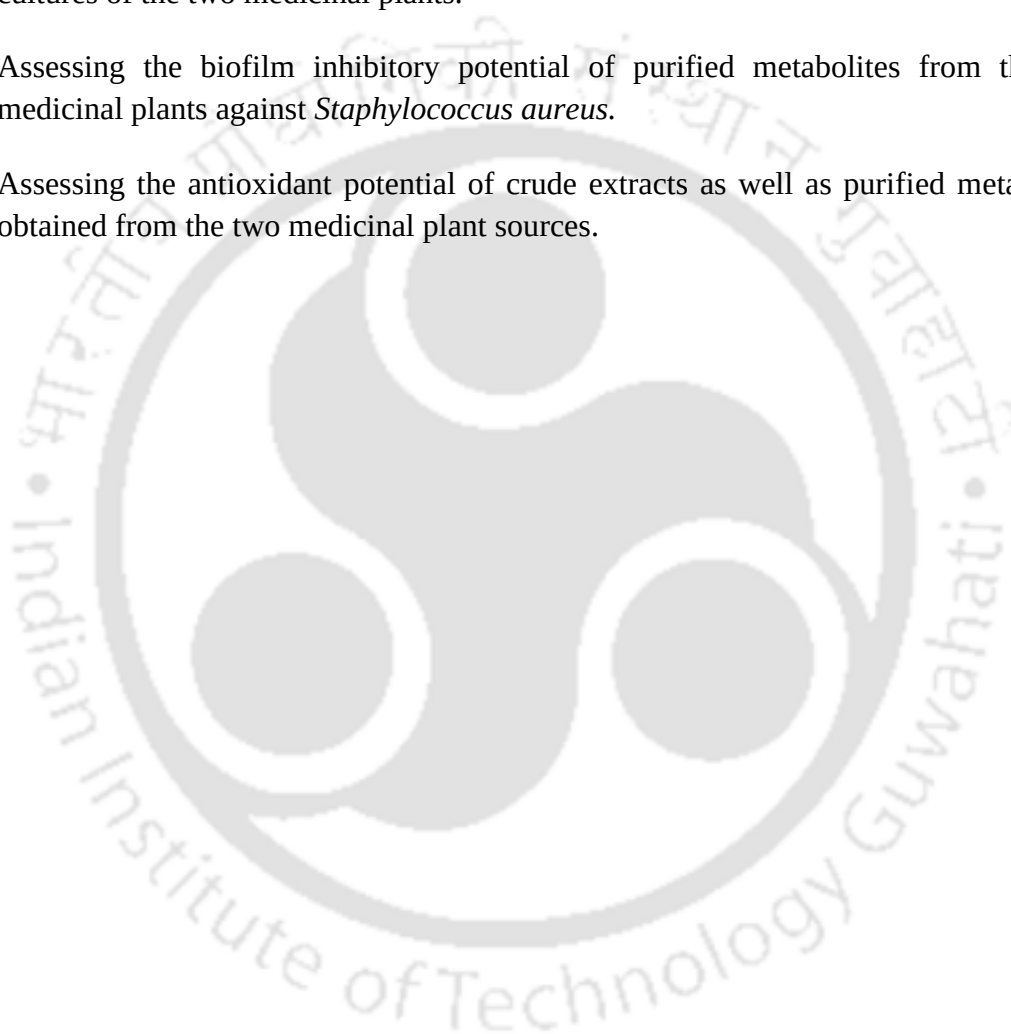
T. cordifolia hold a promising therapeutic value, which could rather drive it towards over-exploitation. It is widely grown deciduous plant, mainly propagated via stem-cuttings. However, seasonal limitations and conventional mode of propagation limits its therapeutic supply and also force it towards verge of extinction. Henceforth, **Chapter 2**, features upon establishment of reproducible micropropagation methodology for large-scale production of true-to-type elite plants of *T. cordifolia*. This also provides a valuable solution to the annual

supply of plants with no seasonal dependency and fluctuations. The elite regenerants underwent genetic fidelity investigation via ISSR molecular markers as well as by flow cytometry. Further, **Chapter 3** highlights upon the development of callus culture using leaf explants and extraction of bioactive protoberberine alkaloids, jatrorrhizine and palmatine from leaf derived callus, since none of the literature reported their presence in the leaf except Kumar et al. (2017). As per the available literature, yield of these compounds are relatively lower, than other plant species of Menispermaceae family. Hence, our research was focussed on enhancing the yield proficiency of target protoberberine alkaloids in in vitro cultures, under controlled environment. Subsequently, the purified alkaloids were evaluated against biofilm forming prospective of *S. aureus* and their antioxidant analysis.

Chapter 4 diverge the study towards *S. rebaudiana* and its commercially important steviol glycosides - rebaudioside A and stevioside. According to the unconditional usage and supply of the plant, production rate of the plant needs to be heightened. The stereotype propagation methods via seeds suffers low viability, leading to low germination rate, while, stem-cuttings fail to fulfil the endless demand of the plant. A reproducible micropropagation technology strikes the way out of these restrictions and provide year-around supply of the plant with no seasonal and regional variations. Another key aspect of the chapter pertains to extraction and purification of steviol glycosides from in vitro leaves of *S. rebaudiana*. The present scenario circulates around generation of high yielding plant lines of *S. rebaudiana* with high rebaudioside A to stevioside ratio and higher rate of multiplication of tissue cultured plants. For this purpose, two elite plant lines of *Stevia*, were chosen for the study and grown in the climatic conditions of North-east India. Post chemical analysis, the steviol glycosides were also experimented for their anti-microbial effect against biofilm forming ability of *S. aureus*. Consequently, the target secondary metabolites were also determined for the scavenging probabilities of free radicals, thereby, eliminating chances of oxidative stress in the body.

Specific objectives

- ❖ Establishment of in vitro cultures and micropropagation of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms and *Stevia rebaudiana* Bertoni.
- ❖ Extraction, purification and quantification of bioactive metabolites from in vitro cultures of the two medicinal plants.
- ❖ Assessing the biofilm inhibitory potential of purified metabolites from the two medicinal plants against *Staphylococcus aureus*.
- ❖ Assessing the antioxidant potential of crude extracts as well as purified metabolites obtained from the two medicinal plant sources.



Chapter 2

In vitro micropropagation of *Tinospora cordifolia* and clonal fidelity assessment of in vitro regenerants

ABSTRACT

The herbal medicines are in widespread use and often utilized in different combinations. They are drawn from different plant species with wide medicinal properties and promise enhancement of the span and quality of life. One such plant species is *Tinospora cordifolia*, which belongs to Menispermaceae family. The plant is a woody climber, which aids in enhanced immunity and rejuvenates the body for fighting diseases, such as diabetes, cancer, malaria, cholera, tuberculosis, allergies and other infections. An efficient in vitro tissue culture system is required to be established, which could generate a high yield of true-to-type plants, throughout the year, free from environmental variations. The present chapter, therefore, enumerates upon the establishment of micropropagation methodology for large-scale clonal propagation of *T. cordifolia*. The best response obtained on Murashige and Skoog (MS) medium fortified with 10 μ M 6-benzylaminopurine (BAP), where 100% bud proliferation was observed in 4 days. The medium promoted multi-nodal, long shoots which grew up to an average height of 10.3 cm with an average number of nodes per shoot as 10.1 at the end of 6 weeks. Occasionally, the shoots reached up to a maximum height of 18 cm. It was possible to obtain 10-fold shoot multiplication at the end of 6 weeks. However, after subsequent two cycles of multiplications by nodes, taken from in vitro shoots at the end of the growth cycle, on the fresh medium of same composition, the medium did not support axillary shoot growth beyond 2 cm. Therefore, these small shoots were transferred to elongation medium. Elongation medium, consisted of MS₁ (ammonium nitrate and potassium nitrate concentrations reduced to half strength) + 5 μ M BAP + 3 μ M IAA + 100 mg L⁻¹ ammonium sulfate (AS), proved to be effective in producing an average of 7.8 cm long shoot with an average of 8 nodes per shoot at the end of 6 weeks. Hence after, regularly, the shoots were multiplied via single nodal segment cultures on MS + 10 μ M BAP medium and elongation was achieved on MS₁ + 5 μ M BAP + 3 μ M IAA + 100 mg L⁻¹ AS medium. The rooting of the shoots was achieved on MS + 1 μ M IBA with 90% success. The plantlets were hardened in the field conditions with 80% survival rate. Clonal fidelity was also evaluated using precise DNA-based studies, such as, inter-specific sequence repeats (ISSR) molecular markers, which revealed 96.3% monomorphism among the regenerants and the mother plant. Further confirmation in the ploidy level was qualitatively analyzed using flow cytometry. The investigation also inferred the genome size of *T. cordifolia* to be 1.5×10^9 bp.

Keywords: Clonal fidelity, Flow cytometry, ISSR molecular markers, Micropropagation, *Tinospora cordifolia*.

2.1 INTRODUCTION

The significance of *T. cordifolia*, a member of Menispermaceae family, has been from the traditional era to the age of modern medicines and research. Therefore, this liana species enumerates most of the economic services, such as therapeutic agents, succulent products and also as cattle feeds (Krishna et al. 2008; Sinha et al. 2004). The annual demand of the *Tinospora* stem was around 10,000 tonnes, a decade ago (Warrier et al. 2008). Propagation via stem cuttings is a general conventional approach since the method is economical, rapid and least prone to insect attacks, unlike seeds that suffer from low viability, poor seed set and germination rate, and are susceptible to pest attacks too. However, stem-cuttings are not able to accomplish the prerequisites of the pharmaceutical industries and the main reason is that the metabolite profiling undergoes seasonal and regional variations. Being a tropical plant, it prefers warm climate and hence, the plant remains dormant during winters. Moreover, the conventional methods are labor intensive and time consuming.

Further, *T. cordifolia* is known to contain a stockpile of different types of alkaloids with varied therapeutic applications. Apart from this, it has high concentrations of phenolic compounds, terpenoids, polysaccharides and proteins. These metabolites enhance the medicinal value of plant decoction or commercial medicines, increasing their utilization by the pharmaceutical industries. Owing to the high demand of this plant to be used as raw material by the pharmaceutical industry, a suitable methodology should be developed for its proper conservation, usage and large-scale propagation. Plant tissue culture technique provides the most effective solution for conservation of plant biodiversity as well as the availability of germplasm to meet the demand from pharmaceutical companies and industries. The current chapter evaluates the in vitro propagation of this plant using nodal segment cultures, wherein, seasonal aspects on shoot-bud survival and proliferation have been experimented. The effect of various plant growth regulators, individually or in combination, and media components were also studied to achieve the high rate of in vitro shoot proliferation in *T. cordifolia*. Possible methodologies were also examined for elongation of in vitro shoots. Later, the genetic fidelity of in vitro regenerants were quantitatively and qualitatively examined using inter-specific sequence repeats (ISSR) microsatellite molecular marker and flow cytometry.

2.2 MATERIALS AND METHODS

2.2.1 Micropropagation and clonal fidelity assessment of true-to-type elite lines of *T. cordifolia*

2.2.1.1 Media preparation

Initiation of nodal segment cultures were performed on MS medium consisting of macronutrients, micronutrients, iron, vitamins and plant growth regulators. The MS medium components mentioned in **Table 2.1** are stock concentrations, where, major salts are 20X concentrated, while minor salts, vitamins and iron components are 200X concentrated. During media preparation, the salt solutions were accordingly diluted. Sucrose as carbon source at 3% concentration and 100 mg L⁻¹ myo-inositol were freshly added. The medium pH was adjusted to 5.8 using 1N HCl and 1N NaOH post volume make-up with distilled water. Agar (0.8%) was added as solidifying agent and boiled. The melted medium (20 mL) was poured in each glass test-tube (25 mm dia × 150 mm height) of 50 ml capacity. The test-tubes were closed with cotton-plugs and autoclaved at 121°C/15 psi for 20 min.

Table 2.1: MS basal medium stock composition

Stock components	Amount (L ⁻¹)
MAJOR (20X)	
Ammonium nitrate	33 g
Potassium nitrate	38 g
Calcium chloride dihydrate	8.8 g
Magnesium sulfate heptahydrate	7.4 g
Potassium dihydrogen phosphate	3.4 g
MINOR (200X)	
Potassium iodide	0.16 g
Boric acid	1.24 g
Manganese sulfate tetrahydrate	4.46 g
Zinc sulfate heptahydrate	1.72 g
Sodium molybdate dihydrate	50 mg
Copper sulfate pentahydrate	5 mg
Cobalt chloride hexahydrate	5 mg
IRON (200X)	
Iron (II) sulfate heptahydrate	5.56 g
Sodium EDTA dihydrate	7.46 g
VITAMINS (200X)	
Inositol	0.1 g
Nicotinic acid	0.1 g

Pyridoxine HCl	0.1 g
Thiamine HCl	0.02 g
Glycine	0.4 g

Table 2.2: List of plant growth regulators utilized for media preparation

Growth regulators	Manufacturer
AUXINS	
2,4-Dichlorophenoxy acetic acid (2,4-D)	Sigma, USA
1-Naphthaleneacetic acid (NAA)	Sigma, USA
Indole 3-acetic acid (IAA)*	Sigma, USA
Indole 3-butyric acid (IBA)	Sigma, USA
CYTOKININS	
6-Benzylaminopurine (BAP)	Sigma, USA
6-Furfurylaminopurine (kinetin)	Sigma, USA
OTHER SUPPLEMENTS	
Casein hydrolysate (CH)	HiMedia, India
Sucrose	Merck, India

*Filter sterilized (non-autoclavable)

2.2.1.2 Surface sterilization and culture initiation

Clonal propagation of elite *T. cordifolia* plant (Accession Number GUBH 79866, Gauhati University, Guwahati, India), was undertaken using nodal segment explants. To establish the in vitro cultures, fresh, diseased-free shoots of *T. cordifolia* were collected from IIT Guwahati campus, at different seasons in a year. After removing leaves, the nodal segments of the stems containing single axillary bud were used as explants. They were thoroughly washed in distilled water for 4-5 times to remove soil and other superficial contaminants and soaked in distilled water for 4-5 h to facilitate the phenolics leaching out. The explants were further washed with 0.3% (v/v) Tween-20 for 20 min with gentle agitation, followed by thorough wash with distilled water for 4-5 times till the foam disappears. The explants were then washed with mixture of 0.1% (w/v) mercuric chloride and 0.4% (v/v) Tween-20 for 3 min. Under aseptic conditions of laminar-air-flow, the nodal segments were then surface sterilized with 70% ethanol for 1 min and 0.1% (w/v) mercuric chloride for 4 min. Each step was accompanied by a thorough wash with sterile distilled water for 4-5 times.

2.2.1.3 Axillary bud proliferation

Post-sterilization, the nodal segments were given a fresh oblique cut to prepare an explant. They were then inoculated on MS medium supplemented with BAP, kinetin and casein hydrolysate (CH) at different concentrations, combinations and ratios; MS Basal medium devoid of any growth regulator was used as control. Responses in terms of percent cultures showing bud-break, time-taken required for the bud-break, shoot length, number of nodes per shoot and rate of shoot multiplication were recorded weekly till 6th week from the time of inoculation with respect to aseptic cultures. The cultures were incubated at standard culture room conditions under 1000-1600 lux of light under photoperiod of 16 h light /8 h dark, temperature at 25±2°C and 50-60% of relative humidity.

2.2.1.4 Multiplication and elongation of shoots

Shoot multiplication was achieved routinely in vitro via axillary shoot proliferation by taking individual nodes from fully grown in vitro shoots at the end of each growth cycle of 6 weeks and inoculated on fresh medium of same composition. The nodal explants, taken from in vitro developed shoots, supported only small shoot proliferation from the axillary buds. Therefore, these small shoots were transferred for elongation on different set of medium to achieve the significant growth of shoots. The shoot elongation medium constituted modified MS basal medium, such as ½ MS (only major salts reduced to half strength), ¼ MS (only major salts reduced to one-fourth strength), and MS₁ and MS₂ medium where concentrations of only ammonium nitrate and potassium nitrate were reduced to half and one-fourth, respectively, to obtain long, multinodal shoots. The media were variously supplemented with BAP, IAA and ammonium sulfate (AS) at varying proportions, and the cultures were incubated at 25±2°C, under photoperiod of 16 h light /8 h dark and 50-60% of relative humidity. The increase in shoot length was recorded at regular intervals until the end of growth cycle of 6 weeks. At the end of each passage, the terminal 2 cm long part of each in vitro shoot was utilized for shoot elongation by inoculating it to fresh elongation medium while remaining portion of each shoot was again cut into single node segments and utilized for next passage of shoot multiplication. By following this approach, large-scale shoot multiplication was established.

2.2.1.5 Rooting and hardening

The terminal 4 cm portions of in vitro grown, elongated shoots were taken for rooting. The excised shoots were cultured on MS, ½ MS (only major salts reduced to half-strength) and ¼ MS (only major salts reduced to one-fourth strength) media supplemented with IBA at 0.5 µM

or 1 μM concentrations. The rooted plantlets were taken out from the test-tubes, washed with distilled water to remove the medium and transferred to plastic pots containing sterile soil, sand and coco peat (1:1:1) for hardening at green house conditions first. The potted plants were sprinkled periodically for a week with $\frac{1}{4}$ concentration of MS major salts. The established plantlets were then transferred to the field conditions for acclimatization.

2.2.1.6 Data analysis

The experiments are based on completely randomized design and they all are performed in sequenced manner.

For surface sterilization and culture initiation, single nodal segments bearing single axillary bud was considered as single replicate. While, for rooting, terminal 4 cm long in vitro shoot was used as single replicate. The parameters were calculated using two-way ANOVA and the significant difference between the mean values was found via Tukey's multiple comparison test at $p \leq 0.05$. Similarly, for elongation purpose, 2 cm in vitro developed single shoot was considered as a single replicate and the data was analyzed using one-way ANOVA. In each experimental design, 18-replicates were raised per treatment in 25 mm $\times$ 150 mm (dia $\times$ height) glass test-tubes containing 20 mL of the medium. The experiments were repeated at least three times. Values for all experiments are represented as mean $\pm$ standard deviation.

2.2.1.7 Clonal fidelity assessment

A) ISSR marker analysis

The leaves were taken randomly from ten different regenerated shoots to analyze their genetic integrity with respect to the mother plant. Genomic DNA was isolated using cetyl trimethylammonium bromide (CTAB) methodology for PCR analysis using universal ISSR markers of UBC series. DNA quantification was performed using Nano-spectrophotometer prior to assessment studies.

i) *DNA isolation*: Extraction of the total genomic DNA was performed using modified CTAB methodology (Doyle and Doyle 1990; Inglis et al. 2018). Randomly 10 leaf samples from in vitro shoots were chosen and 150-200 mg of the leaf tissues were taken for the study. The samples were grinded in liquid nitrogen in chilled mortar and pestle and macerated in sorbitol wash buffer (100 mM Tris-HCl pH 8.0, 5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.35 M sorbitol, 3% polyvinylpyrrolidone (PVP-30) and freshly added 1% β -mercaptoethanol), followed by vigorous vortexing. The samples were centrifuged at 5000 rpm for 10 min. The

supernatant was decanted and the sorbitol pre-wash step was repeated thrice for effective removal of mucilaginous and polysaccharides contamination. Thereafter, the samples were re-suspended in the pre-warmed CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 3% CTAB, 20 mM EDTA, 3 M NaCl, 3% PVP-30 and freshly added 1% β -mercaptoethanol) and vortexed properly. The samples were incubated at 65°C for 60 min with every 10 min' inversion for proper mixing. Post-incubation, the samples were cooled down to room temperature for 5 min, followed by addition of equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each samples with vigorous shaking. The samples were centrifuged at 12500 rpm for 15 min. Carefully, the upper aqueous layer was collected without hampering the debris. Equal volume of chloroform:isoamyl alcohol (24:1) was added to each samples for effective removal of phenol from the sample, followed by centrifugation at 12500 rpm for 15 min. Upper aqueous layer was collected, followed by addition of sodium acetate 3 μ L of sodium acetate (15 mM dissolved in 75% ethanol) and 1 mL of ice-cold isopropanol for precipitation. The samples were inversely mixed and incubated at -20°C for 60 min. Following this, the samples were centrifuged at 14000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol twice, followed by centrifugation of samples at 12500 rpm for 10 min each. Ethanol was decanted and the samples were air-dried for complete removal of ethanol. The DNA samples were re-suspended in TE buffer (10 mM Tris-HCl; pH 8.0 and 1mM EDTA; pH 8.0) and treated with 0.1 mg mL⁻¹ of DNase-free RNase and incubated at 37°C for 60 min. Finally, the DNA samples were incubated at 4°C for routine experiments and at -20°C for long-term storage. DNA samples were quantified using Nanospectrophotometer (Implen, NP80) at 260 nm while DNA integrity was checked on 1% agarose gel.

ii) PCR amplification: Genetic integrity of the in vitro samples with respect to the mother plant was assessed using inter-specific sequence repeats (ISSR) molecular markers. Reaction mixture (20 μ L) for the PCR amplification consisted of 25 ng of template DNA, 2X PCR master mix (New England Biolabs, USA), consisting of 10 mM Tris-HCl, 1.5mM MgCl₂, 50mM KCl, 0.2 mM dNTPs and Taq DNA polymerase (25 units/mL), 200 nM of primers and nuclease-free water and was set-up for 35 PCR cycles. The conditions used for amplification began with initial denaturation at 95°C for 4 min and denaturation at 95°C for 30 secs, followed by annealing at 42°C for 50 secs. Extension was carried on at 72°C for 2 min, followed by final extension at 72°C for 10 min. The PCR was held at 4°C for cooling the amplified products. The amplified DNA products were run on 1.5% agarose with 100 bp ladder (Invitrogen, USA)

and visualized at gel documentation system (Biorad, USA). Data was analyzed on the basis of only scorable bands of in vitro and mother plant amplified products.

iii) *Data analysis*: Manual scoring of individual resolved bands of in vitro regenerants and the mother plant have been scored as '1' and '0' on the basis of their presence and absence in the gel for the documentation of monomorphic and polymorphic percentage among regenerants and the mother plant.

B) Flow cytometry

The flow cytometric analysis was performed according to Hazarika and Chaturvedi (2013). The nuclear suspension of leaves from in vitro shoots were prepared by chopping the leaf samples with sharp razor blade in ice-cold conditions supplemented with chilled nuclear isolating buffer (pH 7.5), which maintains the integrity of the isolated nuclei and facilitates its staining (**Table 2.3**). The buffer was filtered using 0.22 µm nylon 6,6 membrane filter, prior usage along with the addition of 1/10th volume of β-mercaptoethanol. The homogenate was further sieved via 30 µm nylon net filter for removal of debris. To this, 50 µL of filtered RNase and PI (50 µg mL⁻¹ each) was added and kept for incubation for 15 min, followed by their DNA content analysis using Ploidy Analyzer (BD Accuri C6) against PI fluorescence intensity measurements. The instrument was run with DNA QC particles (6 and 8-bead standards) for linearity check and DNA analysis. Sample was run at slow flow rate (14 µL min⁻¹). Nearly, 40000 to 50000 nuclei were analyzed for each sample followed, by their analysis on the basis of G₁ value as per following formulae.

$$\text{Sample 2C DNA content} = \frac{\text{sample } G_1 \text{ peak mean}}{\text{standard } G_1 \text{ peak mean}} \times \text{standard 2C DNA content (pg DNA)}$$

Table 2.3: List of components of nuclear isolation buffer

Components	Concentration
Tris-Cl	0.2 M
MgCl ₂ .6H ₂ O	4 mM
EDTA Na ₂ .2H ₂ O	2.5 mM
NaCl	86 mM
Sodium metabisulfite	10 mM
Triton X-100	1.5% (v/v)
Polyvinyl pyrrolidone (PVP-30)	2% (w/v)
2-mercaptoethanol	1/10 th volume
pH	7.5

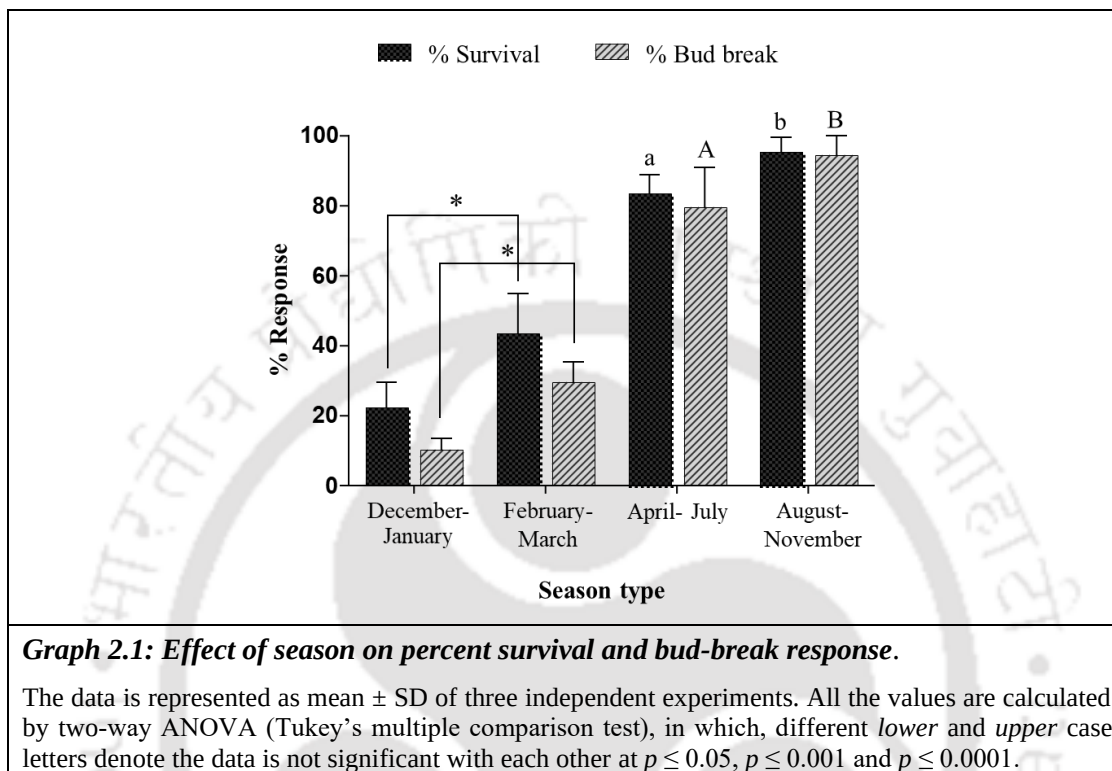
2.3 RESULTS AND DISCUSSION

2.3.1 Surface sterilization and culture initiation

To obtain true-to-type plantations of *T. cordifolia*, surface sterilized single nodal segment, bearing axillary bud, was utilized to establish the in vitro cultures. As the plant is rich in phenolics, soaking the nodal segments in distilled water for 4-5 h was proved beneficial to remove phenolic contents and thereby, reducing the rate of contamination. Similar strategy was followed by some other authors in plant like *Acacia nilotica* (Dewan et al. 1992) to reduce the rate of contamination and to increase the survival rate of the nodal segments, prominently. Panwar et al. (2018) established cultures of nodal explants of *T. cordifolia* by soaking the material in antioxidant solution, such as ascorbic acid and citric acid.

Woody plants are highly susceptible to cyclic climatic changes that modulates the period of bud dormancy and bud-break. The rate of contamination and degree of phenolic leach out also varies with respect to the climatic changes (Martini et al. 2013; Yordanov et al. 2014). Therefore, in the present study, the nodal segments were collected at different time intervals in a year for observation of seasonal effect. As seen in **Graph 2.1**, maximum aseptic cultures (95.4%) were established during August to November (Autumn season), which also induced highest bud-break (94.4%). Summer season from April to July, showed lower percentage of aseptic culture establishment (83.3%) and bud-break (79.6%). The cultures initiated during Spring season (February to March) showed bud-break only in 29.6% cultures and very low percentage (43.5%) of success rate in bud survival. Shoot growth was also slow during this time, which coincides with excessive phenolic leaching from the explants leading to bud necrosis immediately after the emergence of leaf primordia. Extreme cold condition (December to January) was found to be inappropriate for culture initiation as the axillary buds remain dormant during this phase and cultures suffered fungal and bacterial contamination. Generally, microbes invade the buds during this season while they tend to infect other plant parts during summer and spring season (Holb et al. 2004). In conclusion, the nodal segment cultures were recurrently raised during summer and autumn season only. Whenever there was higher phenolic leach out, the inoculated nodal segments were transferred to fresh medium regularly. Effect of explanting season have been studied by Panwar et al. (2018) where the authors reported spring and autumn season to be better for establishment of nodal segment cultures of *T. cordifolia*. Authors also suggested that phenolic exudation is one of the major source of hindrance in in vitro explantation process, apart from microbial contamination. Singh and

Chaturvedi (2010) also reported highest amount of aseptic cultures establishment accompanied with maximum bud-break response in spring season in *Spilanthes acmella*. In another report, autumn season was found to be better for establishment of *Bambusa nutans* (Mudoi et al. 2014).



2.3.2 Axillary bud proliferation

Finally, the sterile nodal explants were inoculated on MS medium supplemented with various combinations of growth regulators as mentioned in **Table 2.4**. MS basal medium without any growth regulator (control) did not support axillary bud proliferation. Supplementation of cytokinins, BAP and kinetin, to the basal medium initiated the process of axillary bud proliferation. Earlier published reports also suggested the usage of BAP and kinetin for bud-break, multiplication and elongation of shoots of *T. cordifolia*. Gururaj et al. (2007) stated that MS medium with higher concentration of kinetin at 13.9 μM along with triacontanol at 11.4 μM , as optimum for 100% axillary bud proliferation in 45 days. While Panwar et al. (2018) evidenced that MS medium with 8.8 μM BAP promoted bud-break in 87.3% cultures in 30 days with an average of 3.8 shoots, each with 2.5 cm length. Mittal et al. (2017) also found highest response (93%) on MS + BAP (4.4 μM) supplemented medium in *Tinospora* in 21 days, leading to average 3.9 shoots, as well as found average 7.9 shoots on MS + BAP (4.4 μM) + 2-isopentenyl adenine (2iP; 2.4 μM) medium composition.

In the present study, single node segment was used as explant (**Figure 2.1A**). The bud-break from axillary bud, present at the node, occurred in minimum of 4 days and variably extended to 15 days depending on media composition (**Table 2.4**). Of the combinations tested, MS medium supplemented with 10 μM BAP either alone or in combination with 250 mg L^{-1} CH and also a lower concentration of BAP at 5 μM along with 12 μM kinetin + 250 mg L^{-1} CH favored early bud-break in ~ 4 days. Additionally, these media combinations were found superior over other media combinations in terms of parameters, like percent bud-break, rate of shoot multiplication, shoot length and number of nodes per shoot (**Table 2.4; Graph 2.2 and Graph 2.3**). Overall, the best response obtained on MS + 10 μM BAP medium, where 100% bud proliferation was observed in 4 days (**Figure 2.1B**). This medium promoted multi-nodal, long shoots, which grew up to an average height of 10.3 cm with number of nodes per shoot as 10.1 (**Figure 2.1C-E**). Occasionally, the shoots reached up to a maximum height of 18.0 cm. It was possible to obtain 10-fold rate of shoot multiplication at the end of 6 weeks, which was highest among all treatments. As per previous reports, Pillai and Siril (2019a) obtained 3.8 rate of multiplication (with an average of 2.9 shoots) at MS + 8.8 μM BAP using nodal explants till 4th subculture. On the contrary, Chatterjee and Ghosh (2016) noticed higher number of shoots (16.5 shoots) using shoot-tip culture on MS + 22.2 μM BAP in 45 days.

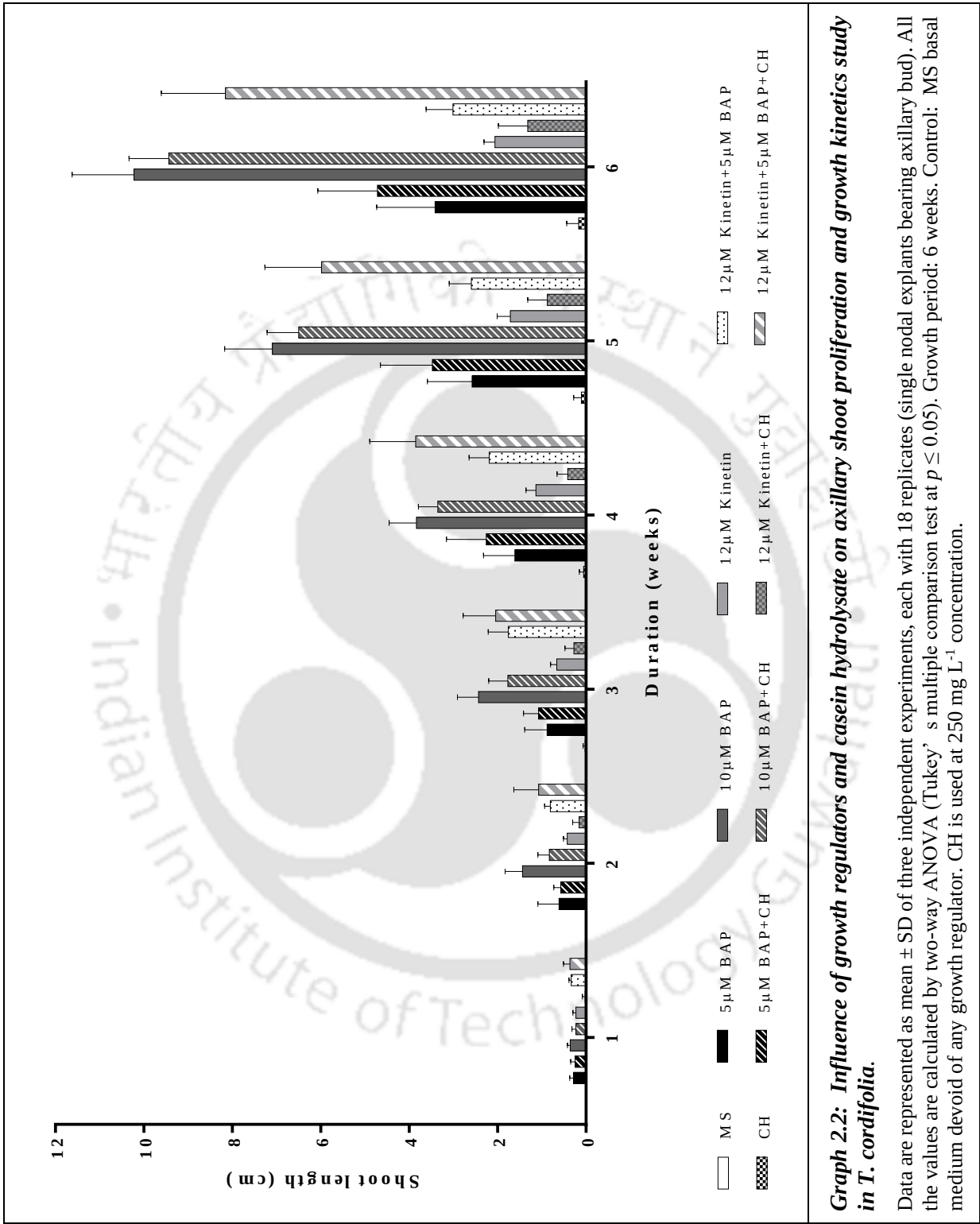
Increase in BAP concentration beyond 10 μM was observed to be detrimental for shoot proliferation using nodal segment culture. Similar observation was reported by Raghu et al. (2006), where the authors obtained multiple shoot proliferation (6.3 shoots per explant) in 4 weeks duration in *T. cordifolia*. Likewise, Pillai and Siril (2019a) also found that BAP-fortified MS medium is better over kinetin supplemented medium for axillary bud proliferation from nodal segment cultures of *T. cordifolia*. Incorporation of CH at 250 mg L^{-1} to the basal MS medium initiated the bud-break but did not support much shoot proliferation. While presence of kinetin alone in the medium led to increase in petiole length and expanded leaves but the shoots remained weak and stunted. The growth pattern of the shoots was also investigated at different treatments. According to **Graph 2.2**, the growth cycle of shoots from nodal segments was observed to be of 6 weeks. Beyond that, the shoots exhibited leaf and shoot-tip necrosis and survival percentage decreased.

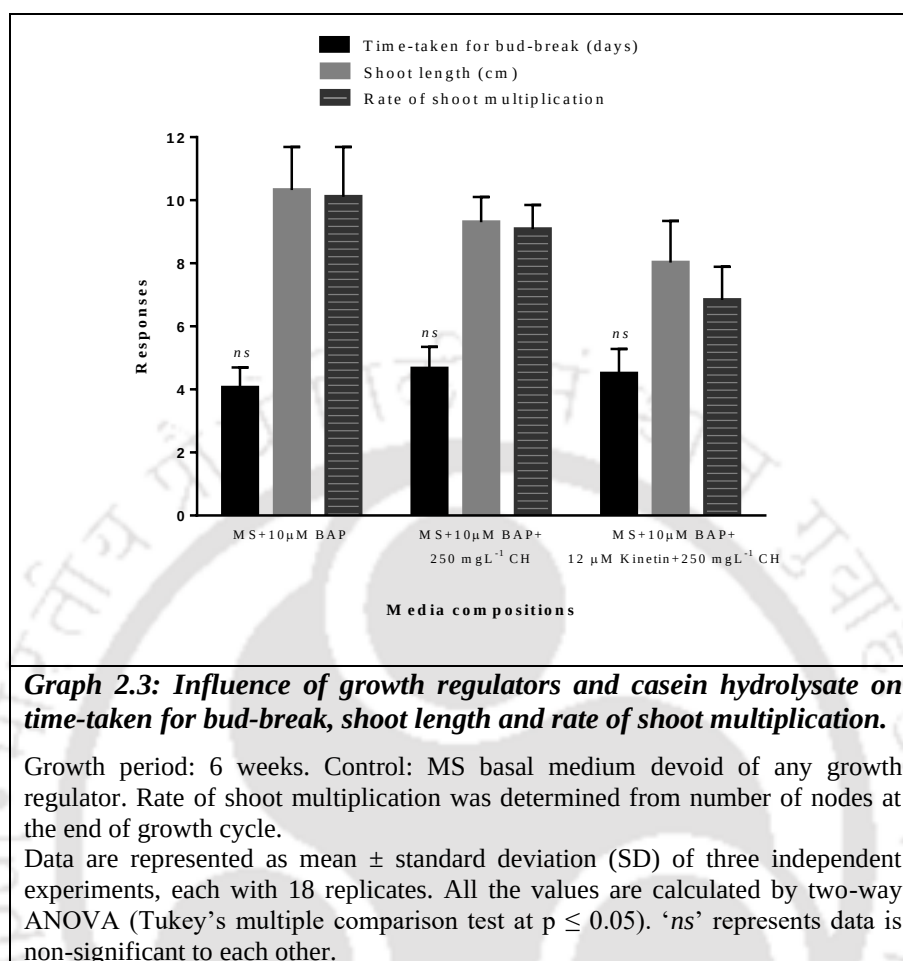
Table 2.4: Influence of growth regulators and casein hydrolysate on axillary shoot proliferation in *T. cordifolia*.

S.No.	PGR concentrations in μM		CH (mg L^{-1})	% Bud break	Time taken for bud-break (days)	Shoot length (cm)	*Rate of shoot multiplication
	BAP	Kinetin					
1.	0.0	0.0	0.0	0.0	0	0.0	0.0
2.	0.0	0.0	250	55.6 ± 6.1	15	0.2 ± 0.1	1.0 ± 0.3
3.	5.0	0.0	0.0	55.6 ± 0.0	8	3.5 ± 1.1	3.2 ± 1.3
4.	5.0	0.0	250	100 ± 0.0	7	4.8 ± 1.2	4.4 ± 1.2
5.	10.0	0.0	0.0	100 ± 0.0	4	10.3 ± 1.4	10.1 ± 1.6
6.	10.0	0.0	250	100 ± 0.0	5	9.3 ± 0.8	9.1 ± 0.8
7.	0.0	12.0	0.0	88.8 ± 3.1	7	2.1 ± 0.3	1.6 ± 0.5
8.	5.0	12.0	0.0	88.8 ± 0.0	9	3.0 ± 0.6	2.8 ± 0.6
9.	0.0	12.0	250	66.7 ± 3.2	9	1.2 ± 0.5	1.0 ± 0.5
10.	5.0	12.0	250	100 ± 0.0	5	8.0 ± 1.3	6.8 ± 1.1

Data are represented as mean $\pm$ standard deviation (SD) of three independent experiments, each with 18 replicates (single nodal explants bearing axillary bud). All the values are calculated by two-way ANOVA (Tukey's multiple comparison test at $p \leq 0.05$). MS basal medium devoid of any growth regulator is used as control. PGR is plant growth regulators; CH is casein hydrolysate.

*Rate of shoot multiplication was determined from number of nodes obtained at the end of growth cycle of 6 weeks.





2.3.3 Elongation and mass multiplication of shoots

In case of *T. cordifolia*, multiplication of shoots is a crucial aspect. It could be possible by two-ways, either by subculture of single node bearing axillary bud or by repetitive transfer of small shoots at the end of growth cycle to fresh medium of same composition. The later strategy on repetitive transfer method is quite beneficial to avoid phenolic exudation in the medium due to cutting as happens during nodal explant preparation. Panwar et al. (2018) experimented multiplication of *Tinospora* by the above two methodologies and obtained high rate of shoot multiplication as 15.3-fold from subculture method using nodal segments.

In the present study, both the above-mentioned multiplication techniques were employed. After 6 weeks of growth cycle, the single node segments from in vitro shoots were subcultured again on the fresh medium of same composition, MS + 10 μ M BAP (**Figure 2.1F**). However, after subsequent two cycles of multiplications by nodes, taken from in vitro shoots at the end of the growth cycle, on the fresh medium of same composition, the medium did not support axillary shoot growth. The pre-existing axillary bud grew into small shoot within a week. However, the

shoot growth was slow and they did not grow beyond 2 cm. After two weeks, most of the shoots turn brown and could not survive. Therefore, in another approach, the experiments were conducted where different salt concentrations of MS basal medium were investigated to achieve long, multinodal shoot. These small shoots were transferred to shoot elongation medium constituting modified MS basal medium, such as $\frac{1}{2}$ MS (only major salts reduced to half strength), $\frac{1}{4}$ MS (only major salts reduced to one-fourth strength), and MS₁ and MS₂ medium where concentrations of only ammonium nitrate and potassium nitrate were reduced to half and one-fourth, respectively. The media were variously supplemented with BAP alone or in combination with IAA and ammonium sulfate (AS) at varying proportions. MS salt composition variously affected the shoot elongation. When major salts reduced to half ($\frac{1}{2}$ MS) and one-fourth ($\frac{1}{4}$ MS) strength in MS medium, no significant increase in shoot length was observed. However, reduced concentration of ammonium nitrate and potassium nitrate in MS medium significantly promoted the length of the shoot. Of the treatments, MS₁ + 5 μ M BAP + 3 μ M IAA + 100 mg L⁻¹ AS medium, promoted the shoot length effectively (**Table 2.5**). On this medium, 7.8 cm long shoot was obtained carrying an average of 8 nodes per shoot at the end of 6 weeks (**Figure 2.1G**). Hence after, regularly, the shoots were multiplied via single nodal segment cultures on MS + 10 μ M BAP medium and elongation was achieved on MS₁ + 5 μ M BAP + 3 μ M IAA + 100 mg L⁻¹ AS medium.

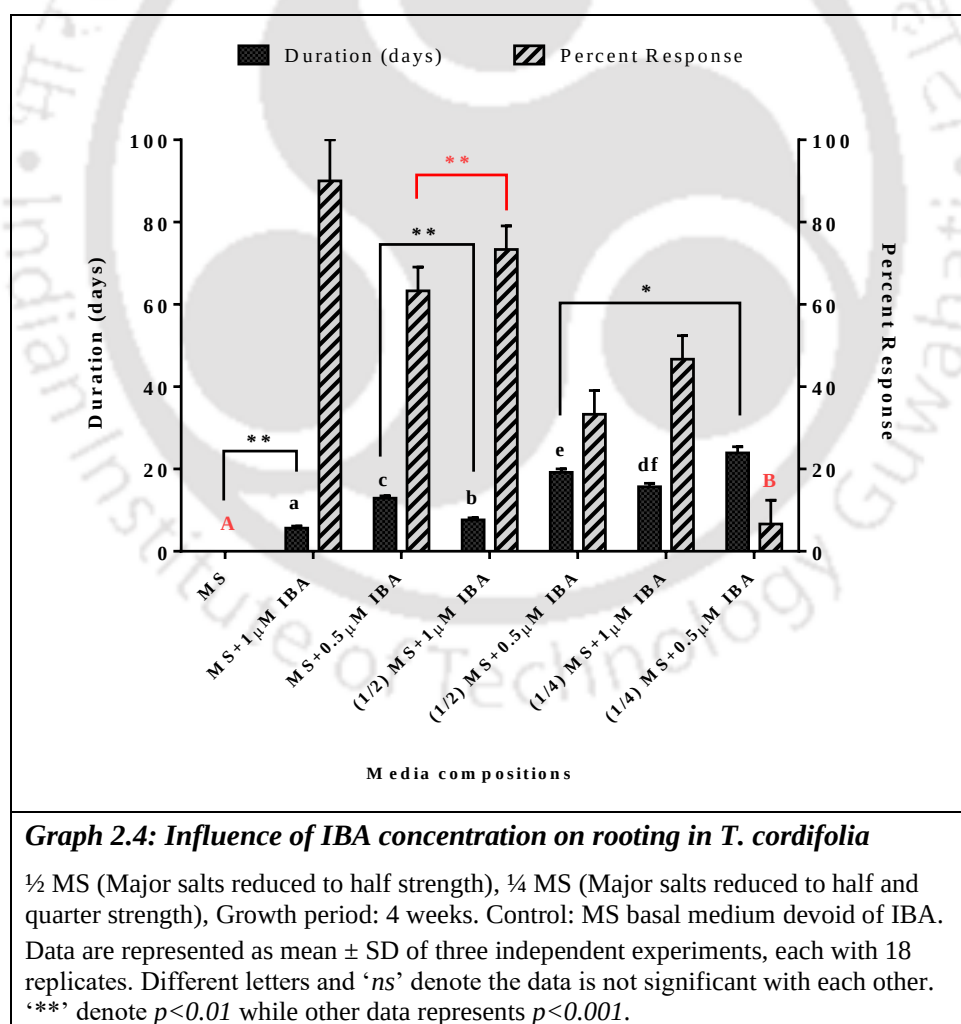
Table 2.5: Influence of growth regulators on elongation of in vitro shoots in *T. cordifolia*

S. No.	Media compositions	Shoot length (cm)
1.	MS	1.5 ^a ± 0.4
2.	MS + 5 μ M BAP	2.6 ^{ab} ± 0.3
3.	MS + 3 μ M IAA	2.3 ^{ac} ± 0.4
4.	MS + 5 μ M BAP + 3 μ M IAA	3.0 ^{acde} ± 0.4
5.	$\frac{1}{2}$ MS + 5 μ M BAP + 3 μ M IAA	3.1 ^{acdfg} ± 0.4
6.	$\frac{1}{4}$ MS + 5 μ M BAP + 3 μ M IAA	1.8 ^{acd} ± 0.4
7.	MS + 5 μ M BAP + 3 μ M IAA + AS (100 mg L ⁻¹)	2.2 ^{acd} ± 0.3
8.	MS ₁ + 5 μ M BAP + 3 μ M IAA	3.8 ^{afh} ± 0.6
9.	MS ₁ + 5 μ M BAP + 3 μ M IAA + AS (100 mg L ⁻¹)	7.8 ^a ± 0.5
10.	MS ₂ + 5 μ M BAP + 3 μ M IAA + AS (150 mg L ⁻¹)	4.9 ^{afi} ± 0.8

$\frac{1}{2}$ MS and $\frac{1}{4}$ MS where major salts reduced to half and quarter strength. MS₁ and MS₂: modified MS₁ and MS₂ medium, where, (ammonium nitrate and potassium nitrate reduced to half and one-fourth, respectively). Growth period: 6 weeks. Control: MS basal medium devoid of any growth regulator. Data is represented as mean ± SD of three independent experiments, each with 18 replicates. Different letters denote the data is not significant with each other. '*' denote $p < 0.05$, '**' denote $p < 0.01$ and other data represents $p < 0.001$.

2.3.4 Rooting and transplantation

The terminal 4 cm long portion from elongated shoots was transferred for rooting on various MS media compositions consisting of MS (full, half and one-fourth strength of major salts) along with IBA (**Graph 2.4**). The earliest signs of rooting were visible in 6 days on MS medium supplemented with 1 μ M IBA, where 90% of the shoots rooted (**Figure 2.1H**). Therefore, MS + 1 μ M IBA was found to be distinctly better and promoted the development of roots directly at the base of shoot. Half-strength of MS (major salts reduced to half strength) with 1 μ M IBA was also effective in rooting of shoots in 75% cultures and the roots appeared in 8 days. After 30 days, the in vitro plantlets were transferred for hardening, first to the green house conditions and then gradually to the field conditions, which leads to 80% survival rate of shoots (**Figure 2.1I**). Among the reported literature, IBA was the most effective auxins for in vitro and ex vitro rooting of shoots (Gururaj et al. 2007; Mittal et al. 2017; Pillai and Siril 2019a).



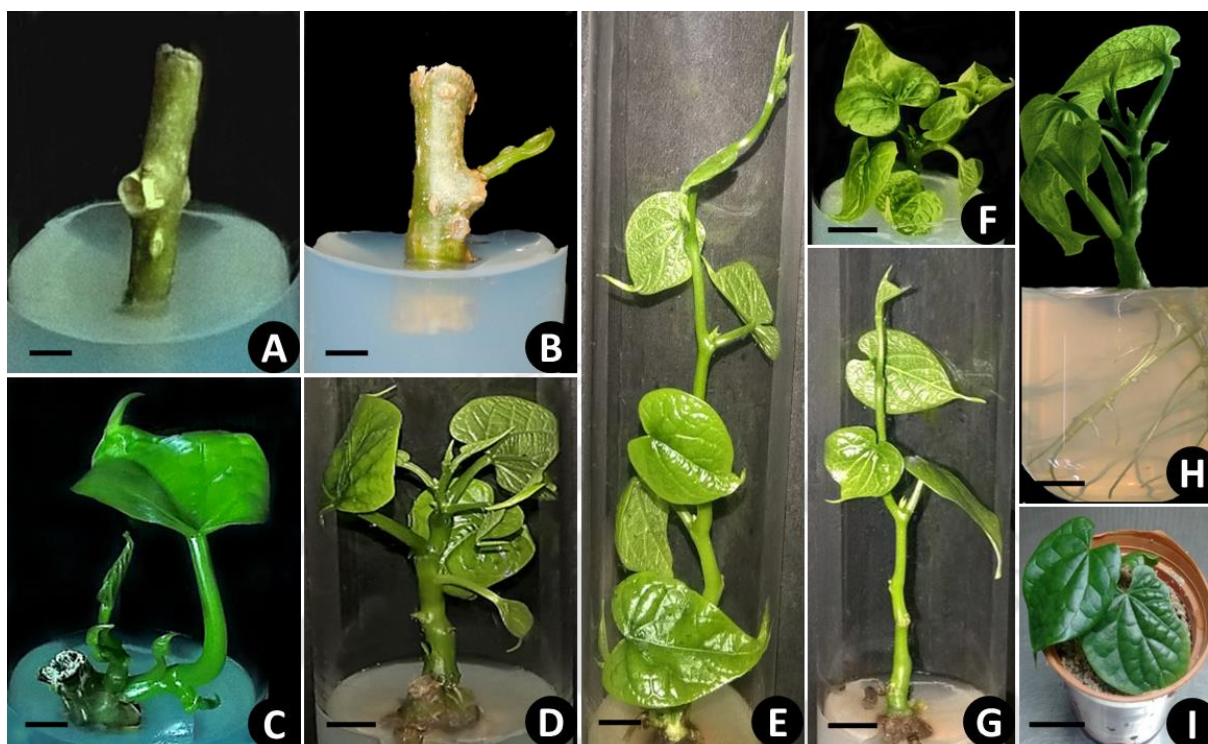


Figure 2.1: Schematic representation of micropropagation protocol for *T. cordifolia* through nodal segment culture, where,

- (A)** A nodal segment inoculated on MS + 10 μ M BAP (Bar=0.8 cm),
- (B)** Same as A, an axillary bud-break after 4 days of inoculation (Bar=0.7 cm),
- (C)** Same as A, two-week-old culture showing shoots from the axillary bud (Bar=0.5 cm),
- (D)** Same as A, after four weeks, showing fully grown shoot (Bar=1 cm),
- (E)** Same as A, six-week-old nodal segment culture showing multinodal long shoot (Bar=0.5 cm),
- (F)** Two-week-old culture of nodal segment, taken from in vitro shoot, grown on MS + 10 μ M BAP medium for shoot multiplication. A short shoot is developed at node (Bar=0.9 cm),
- (G)** A small shoot from F, inoculated on elongation medium on MS₁ + 5 μ M BAP + 3 μ M IAA + 100 mg/L AS, showing significant growth of shoot after 6 weeks (Bar=0.8 cm),
- (H)** A shoot rooted on MS + 1 μ M IBA, after 4 weeks (Bar=1 cm),
- (I)** A hardened plant in soil mixture in pot after 4 weeks (Bar=2.5 cm).

2.3.5 Clonal fidelity assessment

Gradual changes in the cell programming from adaptation of explant to artificial chemical and physical parameters, restoration of cell division, effect of growth regulators or additives during different stages of micropropagation, loss of photosynthetic ability in case of indirect organogenesis, age and other factors, lead to genetic and epigenetic changes (Krishna et al. 2016). Therefore, DNA-based molecular markers pose efficient remedy in determining the presence of somaclonal variation generated due to any of the above-mentioned parameter, during the course of in vitro culture. For this, the leaves of the 6-months old regenerants were assessed for clonal fidelity assessment using ISSR microsatellite marker and flow cytometry.

2.3.5.1 Clonal fidelity assessment via ISSR marker analysis

Method optimization for DNA extraction: DNA extraction from *T. cordifolia* was challenging task since the plant is highly rich in phenolic compounds, polysaccharides and proteins, which are common contaminations of downstream processing of nucleic acids. Therefore, the current DNA extraction protocol pertaining mainly to the sorbitol pre-wash was critical step in removal of polysaccharides and phenolic compounds. The gelatinous polysaccharides, if not properly removed causes turbidity in the final extracted DNA, troublesome in reproducible PCR amplifications and pipetting, while the phenolic compounds, such as tannins bind irreversibly to the DNA, displaying final brown DNA pellet, causing improper primer binding during PCR amplification. In addition to this, PVP along with β -mercaptoethanol reduced the phenolic content in the process. Reduction in protein levels was carried on with the addition of phenol-chloroform-isoamyl alcohol, thus, making the procedure more economic, however, phenol being toxic in nature, proper precautions need to be undertaken. A thorough wash with chloroform-isoamyl alcohol successfully removed the residual effects of phenol. Finally, the purified DNA with least amount of contamination was checked for its quality and quantity for further PCR reactions. Similarly, few researchers reported the extraction of high-quality nucleic acid from varied plant samples rich in phenolics, cambium and herbarium samples (Inglis et al. 2018; Sahu et al. 2012).

ISSR marker analysis: Microsatellite regions represent sensitivity towards the aforesaid characteristic changes. Therefore, ISSR markers of UBC series were chosen for analysis of genetic stability of the in vitro regenerants. Leaves from 10 randomly chosen in vitro shoots were utilized for the study and compared to its mother plant. Genomic DNA at optimized concentration at 25 ng μL^{-1} were used for the entire experimental setup. Higher DNA

concentration gave extremely bright overlapped bands. Similarly, annealing temperature was set up at 43°C post-optimization, for all the primers. According to **Table 2.6**, ‘AG’ dinucleotide motifs yielded a greater number of bands with better resolution and clarity.

Scoring of the amplified sequence products were analysed the mother plants and its respective regenerants for their clonal fidelity assessment. A total of 800 bands were amplified using 8 ISSR primers, with 9.37 mean number of amplified bands per primer (**Figure 2.2A, B, C, D**). The range of amplification varied from minimum of 300 (UBC-809) to 3000 bp (UBC-834). Percent monomorphism was observed to be 96.33% while there was 3.67% of polymorphic bands among the regenerated clones and mother plant leaves.

Table 2.6: List of ISSR primers along with their sequence, annealing temperature, amplified bands per primer and monomorphic percentage of randomly selected in vitro regenerants of *T. cordifolia*

S. No.	UBC series	Primer (5'-3')	Annealing temperature (°C)	No. of scorable bands	% monomorphism	Range of amplification (bp)
1	807	(AG) ₈ T	42.0	9	95.6	700-2000
2	808	(AG) ₈ C	43.8	11	100	500-1600
3	809	(AG) ₈ G	43.2	11	100	300-2000
4	811	(GA) ₈ C	41.8	13	95.4	450-2300
5	817	(CA) ₈ A	45.3	10	96.0	500-1600
6	820	(GT) ₈ Y	45.7	4	90.0	600-2300
7	826	(AC) ₈ C	47.8	7	95.7	400-2000
8	834	(AG) ₈ YT	44.2	10	98.0	700-3000
Total				75	96.3	300-3000

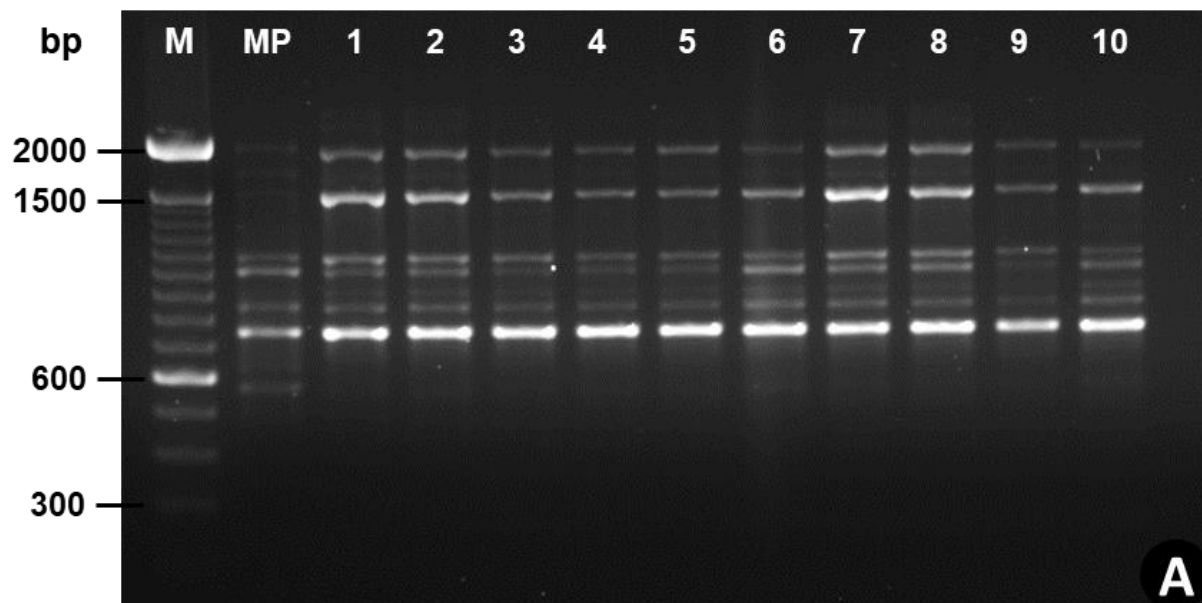


Figure 2.2 (A): ISSR marker analysis of mother plant and randomly selected in vitro clones of *T. cordifolia* using UBC-807 primer

Lane M: 100 bp ladder (Invitrogen), lane MP: Mother plant and lane 1-10: In vitro raised clones

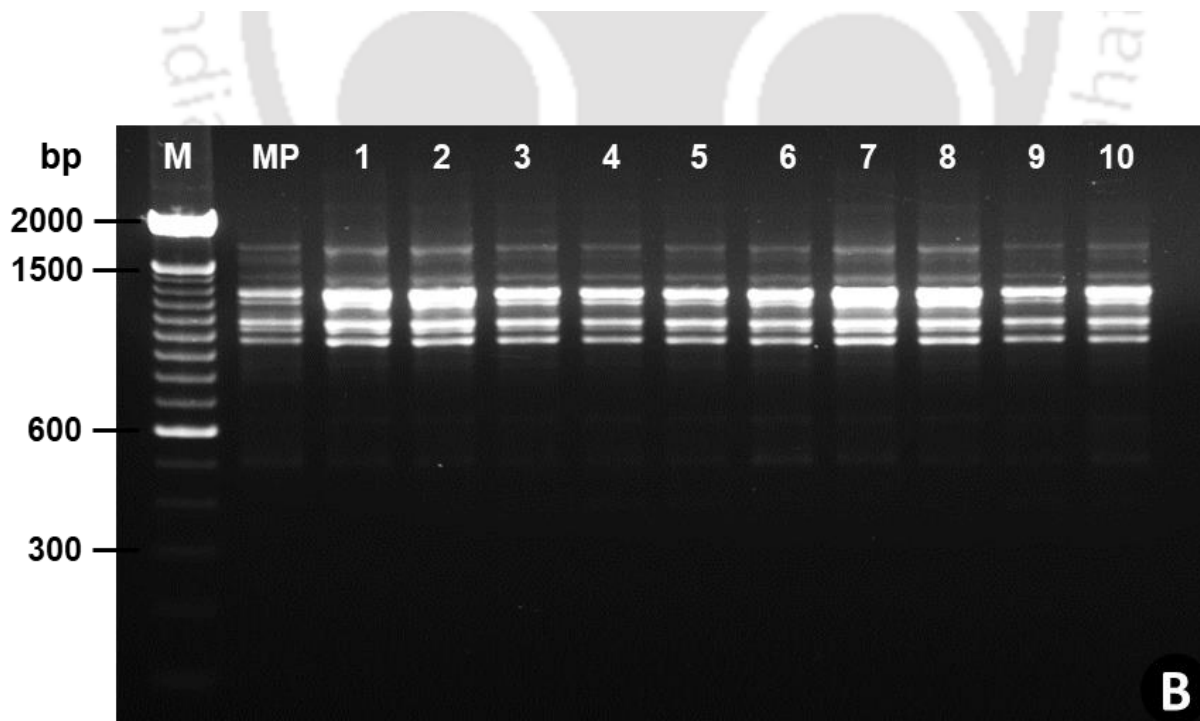


Figure 2.2 (B): ISSR marker analysis of mother plant and randomly selected in vitro clones of *T. cordifolia* using UBC-808 primer.

Lane M: 100 bp ladder (Invitrogen), lane MP: Mother plant and lane 1-10: In vitro raised clones

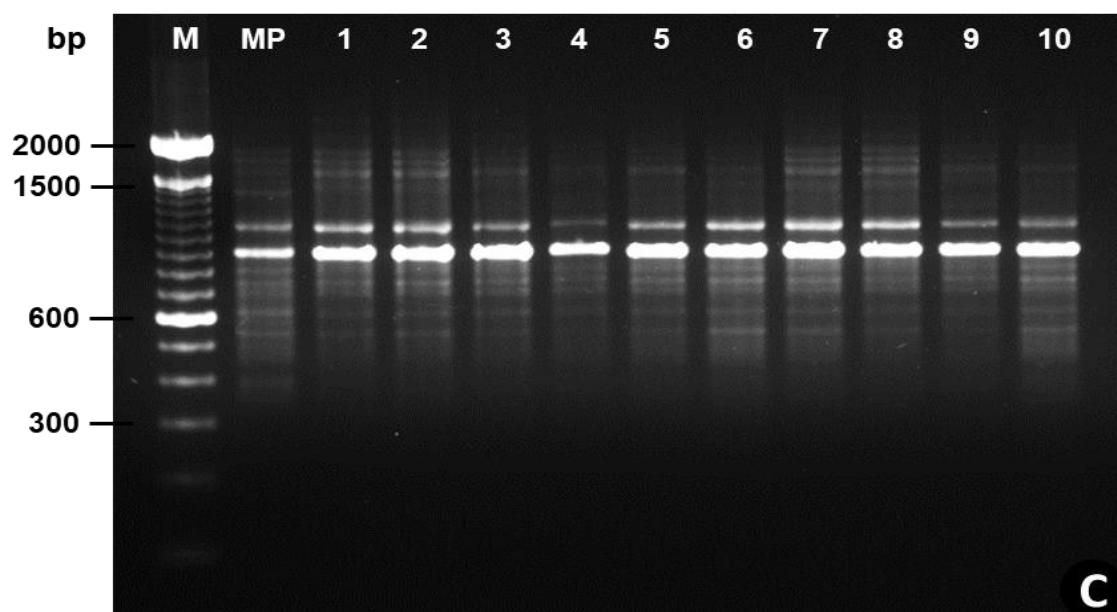


Figure 2.2 (C): ISSR marker analysis of mother plant and randomly selected in vitro clones of *T. cordifolia* using UBC-809 primer.

Lane M: 100 bp ladder (Invitrogen), lane MP: Mother plant and lane 1-10: In vitro raised clones

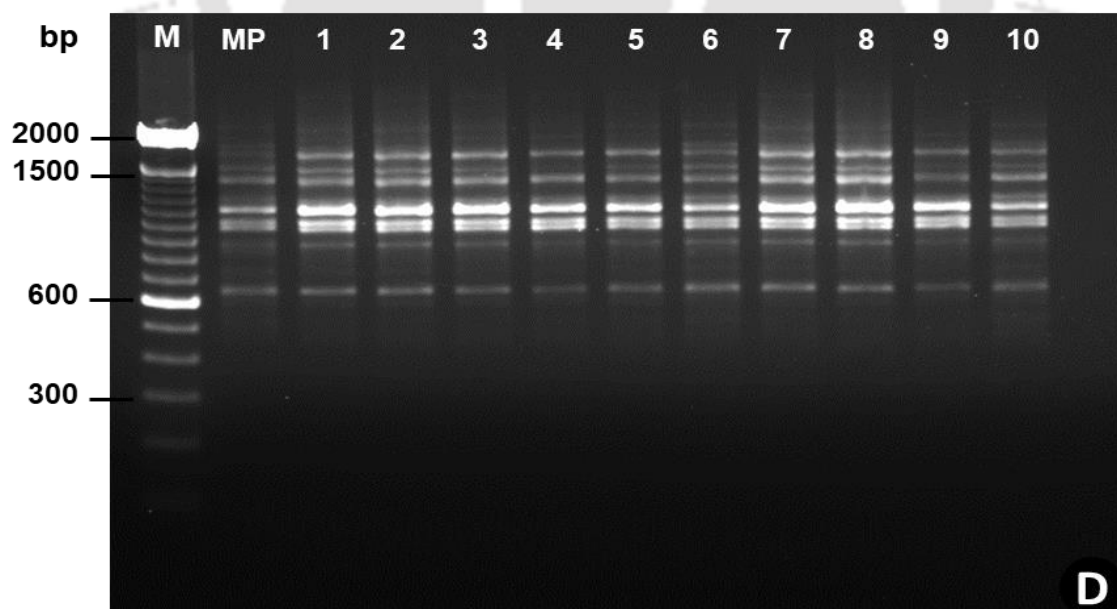


Figure 2.2 (D) ISSR marker analysis of mother plant and randomly selected in vitro clones of *T. cordifolia* using UBC-817 primer.

Lane M: 100 bp ladder (Invitrogen), lane MP: Mother plant and lane 1-10: In vitro raised clones

2.3.5.2 Genome size estimation using flow cytometry

The frequent usage of plant growth regulators such as BAP and 2,4-D cause changes at the chromosomal level in certain plant species, thus, posing variations in their ploidy (Krishna et al. 2016). Apart from molecular marker techniques, flow cytometry has emerged as valuable technique for evaluation of genetic integrity of in vitro regenerants during their long-term culture. In addition to this, the choice of the reference plant standard has been a concern in flow cytometry because of variations in plant species, cultivar, explant type, secondary metabolite content and environmental conditions. It should possess a few key attributes, such as wide availability, cytologically stable, low secondary metabolite content, easy to grow and the genome size should be closure to the target plant species (Suda and Leitch 2010). In this thesis, fresh leaves of *Vigna radiata* was chosen as reference plant with known genome size (1.20 pg genome size; Marie and Brown (1993). Similar to the marker studies, the qualitative flow cytometric analysis also showed similarity in the ploidy level of mother plant and the in vitro clones as mentioned via the mean G_1 peak channel positions of mother plant and it's in vitro regenerants (Table 2.7). Being diploid, their stability during the course of micropropagation, seems unchanged. The average coefficient of variation (CV%) was observed to be below 5% displaying the accuracy of the experiment.

Table 2.7: Relative DNA content and genome size estimation of mother plant and in vitro micropropagated clones of *T. cordifolia*

S. No.	Sample type	Mean G_1 channel peak position*	Relative nuclear DNA content (pg)	CV%*	2C genome size (bp)
1.	Mother plant	110620 $\pm$ 1792.8	1.6	2.3 $\pm$ 0.1	1.5 $\times$ 10 ⁹
2.	In vitro plant	110237 $\pm$ 1395.5	1.5	3.8 $\pm$ 0.1	1.5 $\times$ 10 ⁹

* The values represented as mean $\pm$ SD of three independent experiments.

According the updated literature on the plant, the genome size of *T. cordifolia* was unknown, therefore, the present study primarily estimates the genome size of mother plant, to be 1.56 pg (1.5 $\times$ 10⁹ bp), using *V. radiata* as reference standard. Similarly, the in vitro leaves also showed similar genome size of 1.55 pg (1.5 $\times$ 10⁹ bp), using mother plant as reference standard (Figure 2.3).

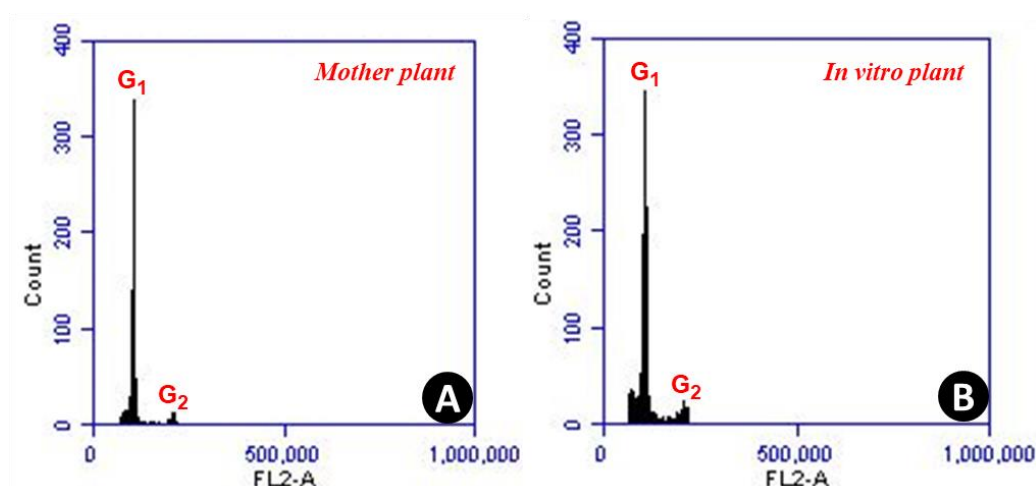


Figure 2.3: Clonal fidelity assessment via flow cytometry,

(A) Flow cytometry of mother plant of *T. cordifolia* (G₁ channel position: 110620),

(B) Flow cytometry of in vitro plant of *T. cordifolia* (G₁ channel position: 110237).

Note: The histograms containing y-axis represents number of events (counts) and x-axis signifies relative nuclear DNA content.

The current nuclease isolation methodology drove optimally in minimizing the phenolic leaching during the extraction, since the modified buffer composition was fortified with 2% PVP and β -mercaptoethanol (1/10th), which acted as reducing agents. These chemicals are known to form hydrogen bonds with the polyphenols and keep them in their reduced state, thus preventing them to irreversibly bind to DNA (Jedrzejczyk and Sliwinska 2010; Sadhu et al. 2016). Optimum incubation time for DNA staining was kept to be 15-20 min. Beyond the optimum time, degradation in intact nuclei could be observed posing variations in mean G₁ channel position. Aliyu (2012) suggested that initial weight (fresh/dry) of the samples should also be optimized because of variation in the secondary metabolite profile which tend to interfere during PI staining. Large-scale data analysis and ease of skilled manpower which is a key requirement for cytological analysis, makes flow cytometry quite beneficial for the researchers for estimation of DNA content, ploidy stability and genome size during micropropagation and other breeding experiments. Comparison of ploidy stability of in vitro regenerants to their mother plants has also been performed in *Solanum lycopersicum* L. (Alatar et al. 2017), *Mucuna bracteata* (Aziz et al. 2018), *Bacopa monnieri* L. (Faisal et al. 2018), *Solanum trilobatum* L. (Shilpha et al. 2014).



Chapter 3

**Identification and quantification of
therapeutic protoberberine alkaloids
from in vitro callus cultures of
Tinospora cordifolia and their effect on
Staphylococcus aureus biofilm formation**

ABSTRACT

Tinospora cordifolia is a well-known medicinal plant with numerous therapeutic potentials and also helps in building up the immune system of the body. Understanding the properties, it becomes essential to establish the in vitro organized/unorganized cultures of *Tinospora* that could provide constant source of raw material for the production of bioactive metabolites, without affecting the plants' natural habitat. The current chapter elucidates the establishment of in vitro callus cultures, utilizing leaf explants, on Murashige and Skoog (MS) medium fortified with 3 μ M 6-benzylaminopurine (BAP) and 10 μ M 1-naphthalene acetic acid (NAA). Subsequently, protoberberine alkaloids, jatrorrhizine and palmatine, were identified from leaves (organized tissue) and calluses (unorganized tissue) via High Performance Liquid Chromatography (HPLC). Phenolic compounds are predominant in the plant and are the major contaminants during extraction process. Therefore, the specific alkaloid extraction process was followed for analysing the isolation profiles of the target alkaloids. Characterization of compounds was done using Mass spectrometry. Jatrorrhizine and palmatine were quantified as 435.9 and 567.5 μ g mg^{-1} , respectively, from in vitro callus cultures. Afterwards, the purified alkaloids (jatrorrhizine and palmatine) were assessed for their activity against biofilm forming capability of *S. aureus*. The two compounds, jatrorrhizine and palmatine, resulted into 78.3% and 87.0%, reduction of *Staphylococcus aureus* activity, respectively. The organism, *S. aureus*, was chosen for the study, since it is well-known in colonizing the diabetic patients and enhances insulin resistance in them. The chapter also enumerates the antioxidant competence of crude callus extract (87.1%) and leaf extract (76.7%) as well as purified, palmatine with 75.6% free radical scavenging activity. The finding will provide better perception of their usages in future therapeutics.

Keywords: Antioxidant, Biofilm, DPPH assay, HPLC, Jatrorrhizine, Protoberberine Alkaloids, Palmatine, *Staphylococcus aureus*.

3.1 INTRODUCTION

The alkaloidal vitality in the world and their extraction from medicinal plants began in early 1900s through the discovery of morphine. Numerous findings exist proclaiming their versatile effects, which includes environmental interfacing and defence against herbivores as well as immunomodulating human body to fight against diseases (Bisset 1992; Raffauf 1970). These nitrogen containing alkaline compounds form salts with acids and have been derived from shikimic acid pathway. Phosphoenolpyruvate (PEP) and erythrose 4-phosphate in a series of reactions forms phenylalanine, which sequentially forms benzyltetrahydroisoquinoline nucleus, an important intermediate in the formation of alkaloids (Barbosa-Filho et al. 2000). The schematic biosynthetic reaction pathway is represented in **Figure 3.1**.

Protoberberine alkaloids, like jatrorrhizine and palmatine, are nitrogen containing tetracyclic compounds, belongs to isoquinoline group of alkaloids with tyrosine as their biosynthetic precursor (Grycová et al. 2007). Besides berberine, the other types of protoberberine alkaloids, such as jatrorrhizine and palmatine, have been successfully isolated from several plant species belonging to Menispermaceae, Berberidaceae, Ranunculaceae, Papaveraceae, Rutaceae, Annonaceae families (da-Cunha et al. 2005). The site of biosynthesis and accumulation of alkaloids could be same or different depending upon the plant species and their developmental stage. In case of later, translocation occurs via xylem, phloem or by apoplastic space. Intracellularly, they could be synthesized in other organelles, such as mitochondria or chloroplasts (γ -coniceine), vesicles (berberine) or cytoplasm, but, finally they get accumulated in vacuoles (Roberts 1981; Sato et al. 1990).

The immeasurable benefits of these alkaloids also attribute in *T. cordifolia*, the woody liana of Menispermaceae family. Micropropagation methodology as proposed in **Chapter 2**, contributes to its large-scale propagation and conservation strategy, however, turning the table of extraction of these protoberberine alkaloids from plant cell cultures, provides an advantage to preserve the biodiversity of plant. Unlike, there could be chances of higher yield of target compounds in the mother plant and the regenerated in vitro cell cultures. Several notable works mentioned higher yield of secondary metabolites in in vitro cell cultures as compared to the mother plant.

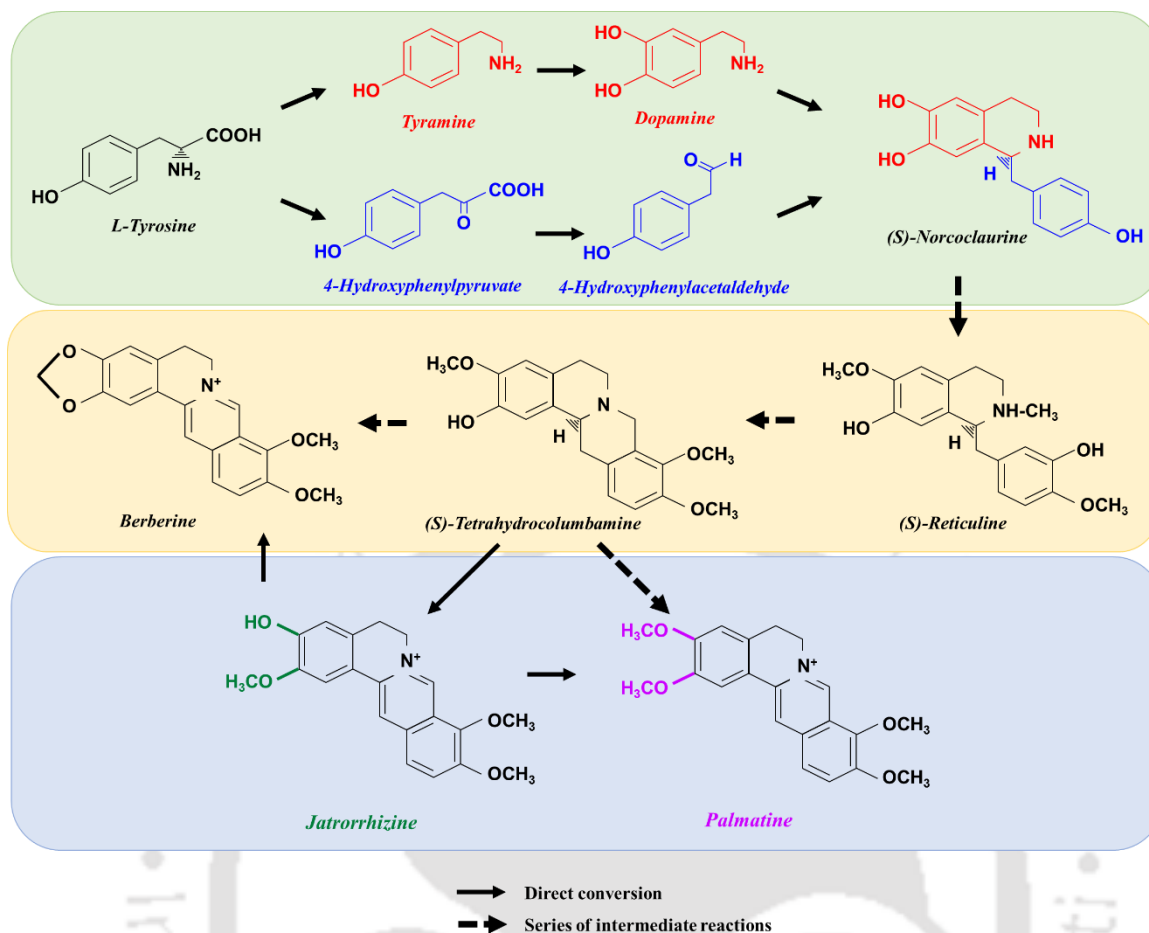


Figure 3.1: Schematic representation of biosynthetic pathway of jatrorrhizine and palmatine

Rajendran et al. (2017), also reported the better yield of spilanthol (N-alkylamide) in flower induced callus cultures (2.2 mg g^{-1}) as compared to flower-head (0.8 mg g^{-1}) of mother plant of *Spilanthes paniculata*. Similarly, betulinic acid (triterpenoid) was absent in mother plant but was quantified as 2.1% (DW) in leaf derived in vitro callus cultures (Srivastava and Chaturvedi 2010). On the contrary, literature also mentions the low yield of metabolites in the plant cell cultures due to shear-sensitive cells, slow growth rate and lack of knowledge of biochemical and physiological pathways, (Dörnenburg and Knorr 1995). Despite, there have been substantial efforts in improvement of metabolite productivity, thereby, refining the economy and usage of plant-based drugs. It has been suggested that increase in product yield corresponds to the culture conditions employed for the cell culture growth, apart from selection of high yielding cell line. The effect of macronutrients, micronutrients, carbon source, growth hormones, precursors and elicitors have varied role in bringing plausible changes in the metabolite profile.

In *T. cordifolia*, although stem is the most utilized part of the plant, there are a few reports that have used leaf explants for regeneration of callus (Bhalerao et al. 2013; Khalilsaraie et al. 2011; Rao et al. 2008). However, none mentioned the presence of protoberberine alkaloids from in vitro cell cultures of *T. cordifolia*. Later, our research group successfully established the in vitro callus cultures of the plant using leaf explants on BAP and NAA fortified MS medium, and quantified two major protoberberine alkaloids, jatrorrhizine and palmatine (Kumar et al. 2017). The present chapter brings forth the effect of different media compositions on raising in vitro cell cultures from leaf explants. Further, the morphological analysis of callus cells during the process of oxidative browning and quantification of protoberberine alkaloids, such as, jatrorrhizine and palmatine, were also documented from leaf-induced callus cultures.

In analysing the healing effects of the plant, the literature reveals that the utmost usage of the *Tinospora* plant is for treatment of diabetes and related infirmities. One of the related ailments includes, invasive action of *Staphylococcus aureus*, especially in Type-I diabetic patients, showing a recalcitrant diabetic foot ulcers with delayed healing that occurs by the action of alpha-toxin, causing tissue necrosis and impaired glucose tolerance (Ortines et al. 2018). In addition to this, effect of antimicrobial agents seems diminishing due to development of antibiotic resistance in the microorganism. Thus, better therapeutics need to be developed and plant-based bioactive compounds are profound alternative for the same. However, as per the updated literature, biofilm inhibition potential of jatrorrhizine and palmatine have not been experimented so far, and the present study uncovers the inhibitory roles of both the protoberberine alkaloids.

Experimenting various sectors of therapeutic properties, understanding the medicinal benefits of other prime alkaloids is also necessary, since, crude plant or alkaloid extract is an amalgamation of all types of secondary metabolites, owing to the cumulative effects of any therapeutic plant-based drug. Among the key protoberberine constituents of Menispermaceae family, berberine possess several therapeutic features, and antioxidant evaluation of the compound is one of them (Pandey et al. 2018; Shirwaikar et al. 2006). There are no reports for the interpretation of antioxidant property of jatrorrhizine and palmatine. The present study documents the promising antioxidant property of the two alkaloids and also of the crude extracts of *T. cordifolia*, for better insight of the plant therapeutics.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and cell culture

The leaf samples were collected from the plant growing in the IIT Guwahati campus during the months from July to September to raise in vitro callus cultures. The leaves were gently washed with distilled water for 4-5 times to remove soil and other superficial contaminants. They were then soaked in distilled water for 4-5 h to facilitate the phenolics leaching out. Subsequently, the explants were rinsed with 0.3% (v/v) Tween-20 for 20 min with gentle agitation, followed by thoroughly washing with distilled water for 4-5 times until the foam disappeared. Under aseptic conditions, the explants were first surface sterilized with mixture of 0.1% (w/v) mercuric chloride and 0.3% (v/v) Tween-20, for 3 min, and finally with 0.1% (w/v) mercuric chloride for 4 min. Aforementioned steps were accompanied by a thorough wash with sterile distilled water, 4-5 times. Leaf-discs explants of 5 mm diameter were prepared using cork-borer and inoculated on the medium for callus induction.

3.2.2 Media optimization for callus induction

Leaf-discs were transferred to media treatments comprising of MS basal medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), 6-furfurylaminopurine (kinetin), 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA), either individually or in combinations, at different concentrations. Sucrose (3%) was added as carbon source and medium pH was set at 5.8 using 1 N HCl/NaOH and autoclaved before inoculation. Glass test-tubes (25 mm dia × 150 mm height) of 50 ml capacity was used for the experiments, in which 20 mL of the medium was poured in each test-tube. Eighteen leaf-discs explants per medium composition were raised and the experiments were repeated thrice. The cultures were incubated under culture room conditions of 1000-1600 lux of light, temperature at 25±2°C and 50-60% of relative humidity. The frequency of callus formation was calculated by recording the number of explants exhibiting callusing versus the total number of explants. MS medium devoid of any growth regulators was used as a negative control.

3.2.3 Maintenance of in vitro callus and their morphological analysis

The induced callus was successfully maintained on MS medium supplemented with BAP and NAA. Rich in phenolics, the callus was initially subcultured at regular intervals, every 3-weeks, aiming for reduction in browning of callus. With subsequent passages, the browning decreased and fresh, light-brown friable callus was chosen for target protoberberine alkaloid analysis.

With respect to the growth of callus, morphological studies were conducted for analyzing the size and shape of cells using fluorescence microscopy. The cells for analyses, initially taken on 2nd day of inoculation, followed by harvesting at weekly intervals. They were washed with 1X phosphate buffer saline (PBS) buffer (pH 7.4) twice for removal of medium and for proper dispersal of cells. It was thereby stained by 2 μ L addition of 1% fluorescein diacetate (FDA) and 10 μ g mL⁻¹ of propidium iodide (PI). The image analysis was performed individually to avoid overlapping effects and also combination of dyes using Fluorescence Inverted Microscope (Nikon, Japan) at blue (Excitation wavelength: 450 to 495 nm) and green filter (Excitation wavelength: 510 to 560 nm) for analysis of FDA and PI-stained cells, respectively.

3.2.4 Estimation of dry cell weight

Friable, proliferated calluses, after a period of 3 weeks, were harvested for alkaloidal analysis. It was oven-dried at 35 $\pm$ 2°C for 4-5 days, till it attained a constant weight. Low temperature was maintained for minimizing loss of thermolabile compounds. The dried calluses can be stored for longer duration at -20°C, to prevent any kind of degradation. Similar methodology was performed using leaves from mother plant.

3.2.5 Extraction and chemical analysis of protoberberine alkaloids

3.2.5.1 Preparation of crude extracts

The dried calluses and leaves (each 5 g) were taken and pulverized into fine powder using porcelain mortar-pestle or mixer grinder. The dried powders were soaked in methanol (Emplura grade, Merck, India) for overnight at room temperature under continuous shaking. The extract was further sonicated at 35% amplitude (pulse of 5 secs on/off cycle) and filtered using 90 mm Whatman filter paper (Whatman plc, UK). The debris was re-suspended in 10 mL of fresh methanol, re-extracted and pooled till the color of the re-extract lightened. The pooled filtrate was concentrated to dryness under vacuum at 50°C using rotary evaporator (IKA, Staufen, Germany). Dry weight of the crude extracts was estimated, prior to chemical analysis. For HPLC analysis, the samples were re-dissolved in methanol (HPLC grade, Merck, India) and filtered using 0.22 μ m of nylon 6,6 membrane (Axiva Siche Biotech, New Delhi, India).

3.2.5.2 Preparation of alkaloid-rich fractions

The crude extracts of in vitro calluses and leaves from mother plant were subjected to additional methodology for extraction of alkaloids. They were processed in hydro-methanolic solvent consisting of 3% HCl and methanol in 80:20 ratio; pH $\leq$ 2.0, with continuous stirring for 2-3

h. The debris was settled on the magnetic surface, while the hydro-methanolic decoction was subjected to fractionation with equal volume of chloroform. This step was repeated thrice and the chloroform fractions (**Ila**) were pooled. The remaining hydro-methanolic decoction was turned to a basic pH (9-10) with liquid ammonia. Thereafter, the basic hydro-methanolic portion was again fractionated with equal volume of chloroform (**I Ib**) thrice. The chloroform fractions were made to paste by evaporating chloroform at 50°C while the basic hydro-methanolic portions were freeze-dried (-100°C) using lyophilizer. The fractions were

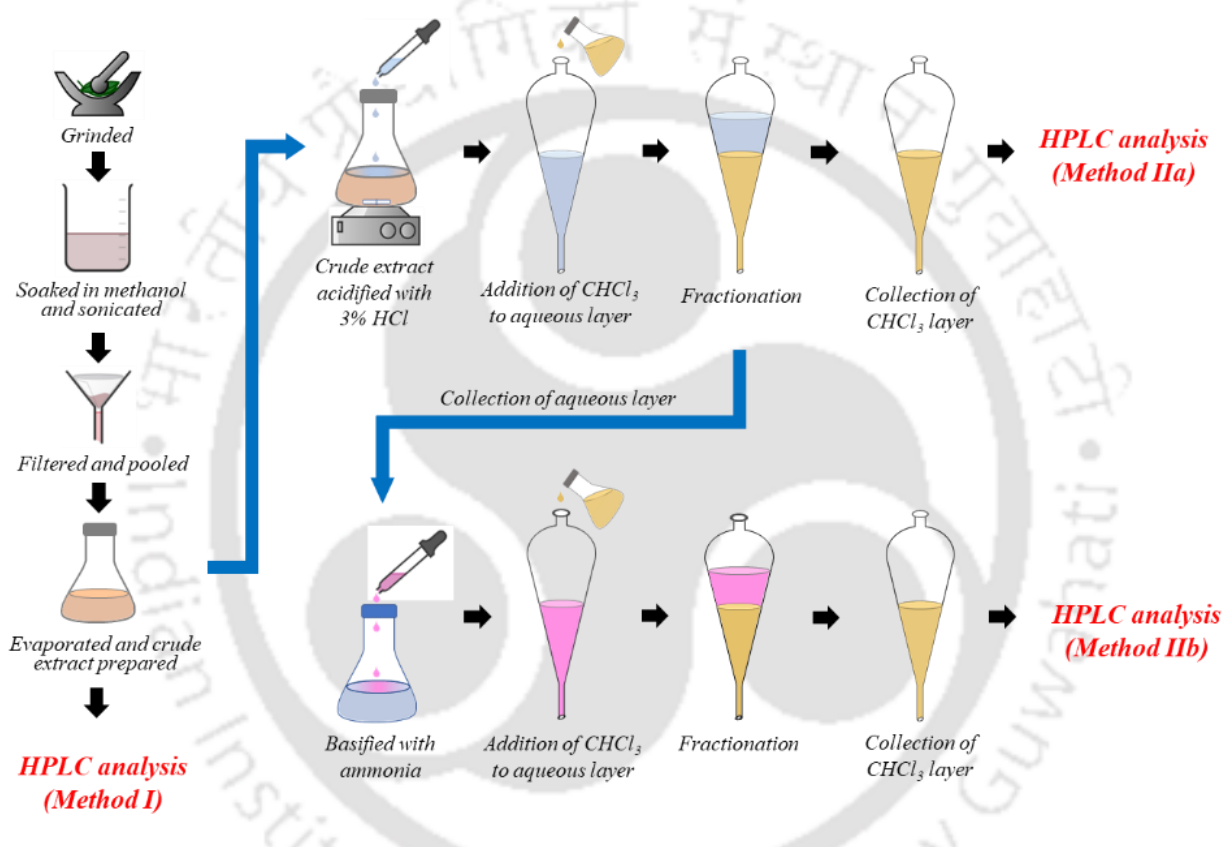


Figure 3.2: Schematic representation of extraction of alkaloids followed for HPLC analysis

Note- Method I: crude extract preparation; Method II: Alkaloid extraction methodology; *Ila*: Chloroform I fraction; *I Ib*: Chloroform II fraction

accurately weighed and re-dissolved in HPLC grade methanol, filtered via 0.22 µm nylon 6,6 filter for analytical analyses. Before proceeding with the purification of alkaloids, a quick screening on the presence of target alkaloids was also performed via preparative thin layer chromatography (PTLC) utilizing callus extract obtained from method *Ila*.

3.2.5.3 Sample preparation and method optimization for HPLC

The respective crude extracts (leaf and in vitro callus) and fractionated extracts (*Ila*, *Iib*) was filtered using 0.22 μm nylon 6,6 filter and 10 μL of each sample was used as injection volume. The HPLC analysis was performed using methodology from Kumar et al. (2017) with slight modifications, using Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan), equipped with quaternary pump, UV detector and 100 μL injection loop. The solvents were filtered using 0.45 μm nylon 6,6 filter and degassed before use. The column was thoroughly washed with the solvents and equilibrated before the onset of the analysis. The samples were analyzed at 50°C in BDS Hypersil C-18 column (250×4.6×5 μm ; Thermo Fisher Scientific, USA). Mobile phase comprised of A= MilliQ with 0.03% formic acid and B= Acetonitrile (20:80) at a flow rate of 0.6 mL min^{-1} and monitored at 345 nm wavelength.

3.2.5.4 Preparation of standard solutions and linearity check

Standards stock solution (1 mg mL^{-1}) of jatrorrhizine and palmatine (Sigma-Aldrich, Japan) were prepared and were serially diluted in range at 3.12 $\mu\text{g mL}^{-1}$ to 500 $\mu\text{g mL}^{-1}$ concentrations for calibration studies. HPLC chromatograms generated were represented as *y axis* = absorbance (mAU) and *x axis* = retention time (min). Each sample and standards were run in triplicates and the data was integrated and acquired via Lab Solutions software (Shimadzu, Kyoto, Japan). Calibration curve was generated for the quantification of compounds along with the computation of correlation coefficient value (R^2). The coefficient of variation ($\text{CV}\% = \text{standard deviation} / \text{mean} \times 100$) was calculated for analysis of system and method suitability for compound analysis. An optimum sample should possess CV less than 15%

3.2.5.5 Quantification and purification of protoberberine alkaloids

The chromatogram peaks for jatrorrhizine and palmatine were identified by comparing the retention time with their respective standards. They were quantified using straight-line equation as generated by the calibration curve. The HPLC purified eluents of both the compounds were pooled and concentrated at 45°C using SpeedVac (Thermo Fisher Scientific, USA) for further confirmation of the compounds.

3.2.5.6 Validation of protoberberine alkaloids using Mass-spectrometry

The concentrated purified compounds were re-dissolved in HPLC grade methanol for mass spectrometry analysis using matrix assisted laser desorption/ionization – time of flight (MALDI-TOF; Bruker-Autoflex Speed, USA) with 355 nm nitrogen laser pulse. alpha-Cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix (blank) for MALDI, and the spectrum

was collected in the positive mode and averaged for 100 shots. The mass spectrometry data was obtained in scan mode range of 100-1000 amu.

3.2.6 Inhibition test for biofilm formation of *S. aureus*

3.2.6.1 Microbial strain and culture conditions

S. aureus strain (MTCC 737; procured from NCCS, Pune) was used for the biofilm inhibition studies. It was cultivated in Mueller Hinton (MH) medium by continuous stirring, overnight, at 180 rpm and at 37°C temperature. Prior to analysis, the Optical density (OD) of the bacterial culture was adjusted to 0.5 ($\sim 1 \times 10^8$ CFU mL⁻¹) by following the McFarland standard method. Then 4 μ L ($\sim 6 \times 10^5$ CFUs) of the microbial culture was added to each well containing the purified compounds of jatrorrhizine and palmatine. The experiments were conducted in triplicates and represented as mean $\pm$ SD.

3.2.6.2 Determination of Minimum Inhibitory Concentration (MIC)

The HPLC purified alkaloids, jatrorrhizine and palmatine, dissolved in 2% DMSO (Merck, India), at serial dilution concentration range of 3.12 μ g mL⁻¹ to 1000 μ g mL⁻¹ were used for the study. Kanamycin (20 μ g mL⁻¹) were used as positive control, while 2% DMSO was used as negative control. MIC determination was performed using broth dilution method in 96-well microtiter plate format. The plates were incubated for 24 h and 48 h incubation at 37°C. The microbial growth was measured via microplate reader (Tecan, Switzerland) at 600 nm. The MIC was determined on the basis of no visible growth (clear wells) after 24 h of treatment.

3.2.6.3 Biofilm inhibition assay

Each purified alkaloid (3.12 μ g mL⁻¹ - 1000 μ g mL⁻¹) of 100 μ L volume was added to the wells, individually, along with overnight grown microbial culture ($\sim 6 \times 10^5$ CFU mL⁻¹). The mixture was incubated for 24 h and 48 h duration at 37°C. Thereafter, the supernatant was discarded and the wells were washed with distilled water thrice to remove the planktonic bacterial cells. Then the remaining surface-associated bacterial biofilm in the well was stained with 0.1% safranin (Sigma-Aldrich, Japan), for 30 min. Following this, the wells were rinsed with distilled water to remove excess dye. Finally, biofilm bound safranin was released using 30% acetic acid and the absorbance of the supernatant was measured at 530 nm. Minimum Biofilm Inhibitory Concentration (MBIC), after safranin staining was calculated as:

$$\text{Inhibition\%} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / (\text{OD}_{\text{control}}) \times 100$$

3.2.6.4 Microscopic assessment of biofilm via light and fluorescence microscopy

Biofilm inhibition methodology, as mentioned in **section 3.2.6.3**, was performed and the compounds at their MBIC were used as biofilm inhibition concentration. After incubation, the remaining surface-associated bacterial biofilm in the well was stained with 0.01% acridine orange (Sigma-Aldrich, Japan). The microscopic studies were carried out in 24-well plate and observed under bright-field and fluorescence microscopy using inverted fluorescence microscope under blue light filter (Nikon TiS, Japan). All the image as analyzed using NiS elements (version 4.5).

3.2.6.5 Surface morphological evaluation of *S. aureus* after alkaloidal treatment

The effect of target compounds (at their MBIC) on the morphological, structural changes of *S. aureus* and its biofilm formation was observed using field emission scanning electron microscope (FESEM). The microorganism was co-incubated with purified compounds in 96-well microtiter plate, as mentioned in **section 3.2.6.3**. The small pieces of coverslips were placed in the incubation mixture in each microtitre well for the adherence of bacteria on it. After 48 h of incubation, the cover-slips were taken out, washed with distilled water, thrice, to remove the planktonic bacterial cells. The coverslips with the bacterial colonies growing over it were kept for fixation in 2.5% glutaraldehyde for 5-6 h. Subsequently, they were washed with distilled water and the adherent cells over the cover slips were dehydrated gradually in ethanol series (10% to 90%) by dipping the coverslips for 15 min in each series. After air-drying, the cover-slip adhered cells were eventually spur-coated with gold before analysis and scanned using FESEM (Zeiss, Germany).

3.2.7 Assessment of antioxidant potential of extracts and purified alkaloids using DPPH radical scavenging assay

Crude extracts of leaf and in vitro calluses, along with purified alkaloids, jatrorrhizine and palmatine of 100 μL volume were added in 96-well plate in serially diluted concentrations ($3.12 \mu\text{g mL}^{-1}$ to $1000 \mu\text{g mL}^{-1}$). To this, 0.13 mM of DPPH reagent (Sigma-Aldrich, Japan) dissolved in HPLC grade methanol was added to each well, pipetted well, wrapped in foil and incubated in dark for 1 h. Ascorbic acid (Sigma-Aldrich, Japan) was used as positive control while the DPPH reagent without any treatment was used as negative control. The reading was measured at 515 nm via microplate reader (Tecan, Switzerland). Each sample was run in triplicates while experiment was repeated thrice. Linear trend line was generated for each sample for the determination of IC_{50} (concentration at which 50% of DPPH radicals are

neutralized) and IC₉₉ values (concentration at which 99% of DPPH radicals are neutralized), using straight line equation. The radical scavenging percentage was calculated as:

$$\% \text{ DPPH scavenging} = (\text{Abs}_{\text{DPPH}}) - (\text{Abs}_{\text{Sample}}) / (\text{Abs}_{\text{DPPH}}) \times 100$$

3.2.8 Statistical analysis

For experiments related to callus induction, it was completely randomized designed and all the experiments were conducted in sequenced manner. Each leaf-disc was considered as single replicate, inoculated in 25 mm × 150 mm (dia × height) test-tubes containing 20 mL of the medium. In each experimental design, 18-replicates were raised per treatment, while the experiment was repeated thrice. The investigated parameters were analyzed using two-way Analysis of Variance (ANOVA) using Graph Pad software 6 (Graph Pad, California, US). The significant difference between the mean values was found via Dunnett's multiple range test at $p < 0.01$. For chemical and anti-staphylococcal analysis, observations were made on the basis of three separate experiments and the values are represented as mean ± standard deviation (SD). Lastly, the antioxidant effect was statistically analyzed using one-way ANOVA and the significant difference between the mean values was found via Tukey's multiple comparison test at $p < 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Media optimization for callus induction from leaf explants

The venturing usage of growth regulators is essential for dedifferentiation and redifferentiation process. In the present study, distinctive and amalgamation of growth regulators (BAP, kinetin, 2,4-D and NAA) in different ratios were used for induction of callus from leaf explants (**Table 3.1**). Callus initiation did not occur from leaf-discs inoculated on MS basal medium (Control). Supplementation of growth regulators to basal medium began the process of callus development, originating from the margins and midrib region of leaf-discs (**Figure 3.3B**). Usage of 2,4-D, kinetin and BAP, individually, peculiarly led to only ≤50% callus initiation. Combination of 2,4-D and kinetin improved the callus induction up to 74% but led to light-yellow, friable callus, which turned brown on subsequent subcultures and exhibited slow growth. Chintalwar et al. (2003) documented callus development from inter-nodal segments, which was dark-brown but friable in nature on semi-solid and liquid medium. The present study demonstrated the usage of NAA for commencement of callusing, where higher concentrations (10, 15 and 20 μM) were promising, while 5 μM of NAA was futile in providing callus

response. Cytokinins, when used alone, was found to be less effective in callus development compared to when combined with an auxin. In conclusion, among all the tested media compositions, MS + 3 μ M BAP + 10 μ M NAA gave 100% response for callus induction within 15 days (**Figure 3.3C-F**). During initial subcultures, the calluses were hard and dark brown in texture. Regular subculture of calluses at short intervals of every 21 days favoured decrease in browning of callus, which later resulted into massively grown, whitish-brown and friable calluses (**Figure 3.3F**). Another medium, MS + 11 μ M BAP + 5 μ M NAA also showed proliferated callus in more than 92% cultures but the calluses were moderately hard in nature. Similarly, Kumar et al. (2017) performed subculturing of leaf-induced calluses after 3 weeks, which was further utilized for raising cell suspension cultures. Later, the cells, harvested on 16th day from suspension culture, notifying the highest biomass concentration. Unlike, Rao et al. (2008) raised leaf-induced in vitro cell cultures of *T. cordifolia* and inferred maximum biomass on 30th day of cell suspension culture while maximal productivity of berberine was obtained on 25th day. In the present study, the cells were harvested in the exponential phase (21st day of subculture), since the calluses-initiated browning after 4 weeks. Similar harvested calluses were utilized for extraction of target alkaloids.

Table 3.1: Influence of growth regulator combinations at varying levels on callus formation in *T. cordifolia*.

S. No.	PGR concentrations in μM				Percent of explants forming callus [§]	Duration (days)
	2,4-D	Kinetin	BAP	NAA		
1.	0.0	0.0	0.0	0.0	0.0 $\pm$ 0.0	0
2.	2.5	0.0	0.0	0.0	0.0 $\pm$ 0.0	0
3.	5.0	0.0	0.0	0.0	25.9 $\pm$ 6.4	20
4.	7.5	0.0	0.0	0.0	33.3 $\pm$ 11.1	18
5.	0.0	2.5	0.0	0.0	0.0 $\pm$ 0.0	0
6.	0.0	5.0	0.0	0.0	0.0 $\pm$ 0.0	0
7.	0.0	5.0	0.0	0.0	48.2 $\pm$ 6.4	20
8.	2.5	2.5	0.0	0.0	63.0 $\pm$ 6.4	10
9.	2.5	5.0	0.0	0.0	26.0 $\pm$ 6.4	15
10.	2.5	7.5	0.0	0.0	74.1 $\pm$ 6.4**	15
11.	5.0	2.5	0.0	0.0	63.0 $\pm$ 13.0	15
12.	5.0	5.0	0.0	0.0	51.8 $\pm$ 6.4	20
13.	5.0	7.5	0.0	0.0	63.0 $\pm$ 6.4	15
14.	7.5	2.5	0.0	0.0	18.5 $\pm$ 6.4	20
15.	7.5	5.0	0.0	0.0	18.5 $\pm$ 12.8	15
16.	7.5	7.5	0.0	0.0	70.4 $\pm$ 6.4**	9
17.	0.0	0.0	1.0	0.0	0.0 $\pm$ 0.0	0
18.	0.0	0.0	3.0	0.0	51.8 $\pm$ 6.4	25
19.	0.0	0.0	5.0	0.0	44.4 $\pm$ 11.1	25
20.	0.0	0.0	7.0	0.0	25.9 $\pm$ 6.4	25
21.	0.0	0.0	9.0	0.0	29.6 $\pm$ 6.4	25
22.	0.0	0.0	11.0	0.0	48.2 $\pm$ 6.4	25
23.	0.0	0.0	0.0	5.0	0.0 $\pm$ 0.0	0
24.	0.0	0.0	0.0	10.0	66.6 $\pm$ 0.0	20
25.	0.0	0.0	0.0	15.0	70.4 $\pm$ 6.4**	20
26.	0.0	0.0	0.0	20.0	74.1 $\pm$ 6.4**	20
27.	0.0	0.0	3.0	5.0	51.8 $\pm$ 6.4	25
28.	0.0	0.0	3.0	10.0	100 $\pm$ 0.0	15
29.	0.0	0.0	3.0	15.0	81.5 $\pm$ 6.4 ^{ns}	18
30.	0.0	0.0	11.0	5.0	92.6 $\pm$ 6.4 ^{ns}	20

Control: MS basal medium devoid of growth regulator. PGR: Plant growth regulators. Growth period is 4-weeks.

[§] The values represented as mean $\pm$ SD of three independent experiments. The values have been statistically analysed using two-way ANOVA (Dunnett's multiple range test), wherein, '**' signifies $p \leq 0.01$, 'ns' signifies $p > 0.05$, and other values represents $p \leq 0.0001$.

Reduction in browning of calluses was also observed by Srivastava and Chaturvedi (2010), where, the authors adopted repeated-subculture methodology and, thereby avoided the use of additives in the medium, which sometimes have ill-effects on cell biomass and secondary metabolite production.

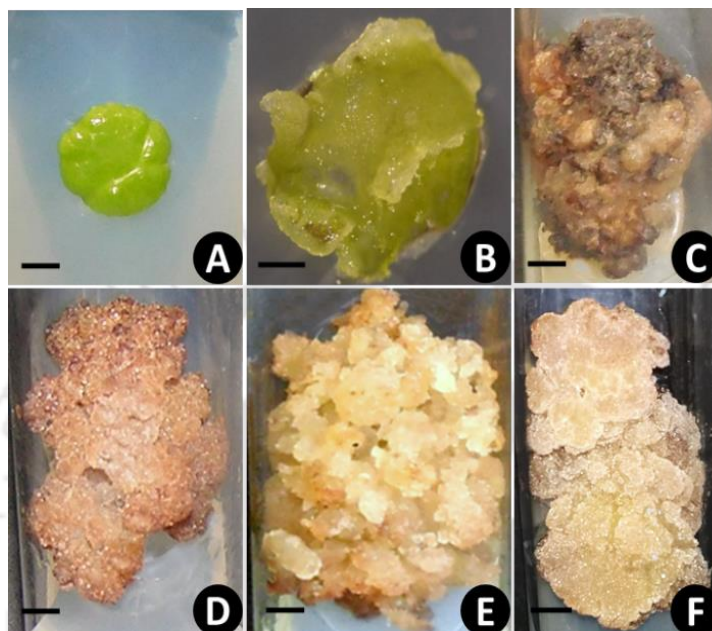


Figure 3.3: Establishment of in vitro callus culture of *T. cordifolia*, where,

(A) Leaf-disc inoculated on MS medium supplemented with 3 μ M BAP and 10 μ M NAA (Bar=0.4 cm),

(B) Leaf-disc at culture after two weeks, showing callus initiation from the margin of leaf-disc (Bar=1.8 cm),

(C) 30-week-old leaf-disc culture, showing proliferated, compact, brown callus after 10 passages, each of 21 days (Bar=0.4 cm),

(D) 60-week-old culture showing proliferated and friable brown callus after 20 passages, each of 21 days (Bar=0.3 cm),

(E) 90-week-old culture showing proliferated, friable, light-brown callus after 30 passages, each of 21 days (Bar=0.6 cm),

(F) A 120-week-old culture showing proliferated, friable and light-brown callus after 40 passages, each of 21 days (Bar=0.9 cm).

3.3.2 Morphological and viability analysis of in vitro calluses

When organized plant parts, excised from the plant, are redefined for dedifferentiation under right composition of nutrients, the cells undergo at varying degree of developmental re-programming and the in vitro environment plays a major role. In addition to this, the plants

with higher secondary metabolite content produces them on higher scale during the stress conditions provided in vitro, such as temperature, light intensity, growth regulator combinations, medium mineral composition and gaseous exchange in the culture vessel. Generally, phenolic compounds are produced in chloroplasts and transported via endoplasmic reticulum to vacuole for storage (Zaprometov and Nikolaeva 2003). Stress conditions during culture can disrupt localization of phenolic compounds leading to their release in the cytoplasm and subsequent oxidation. Subsequently, the oxidised phenolics synthesized brown color pigments, such as quinones, causing browning of the tissue and medium. Increased lignification is also one of the factors attributing to stress induced response. This hinders the process of nutrient uptake by the cells leading to slow growth, poor regeneration potential, tissue blackening and death. Similarly, increase rate of phenolics leach out in *T. cordifolia* attributes to the decreased process of nutrient uptake.

In the present study, morphological studies of in vitro calluses were conducted during subsequent growth period. Fresh friable calluses was subcultured on the medium (**Figure 3.4A**), which gradually turned brown after subculturing process. Thereafter, development of fresh and friable cells from the base of brown calluses tissue was visible (**Figure 3.4B**). Further,

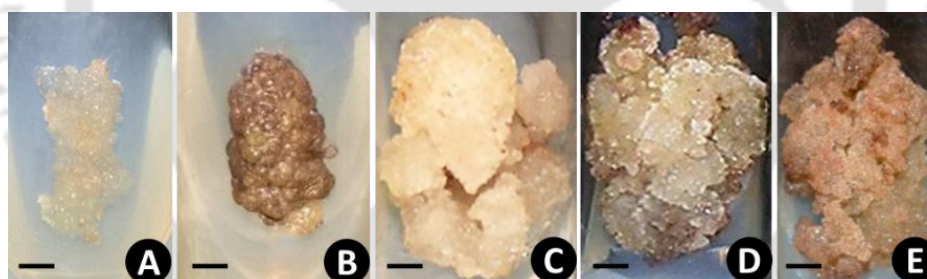
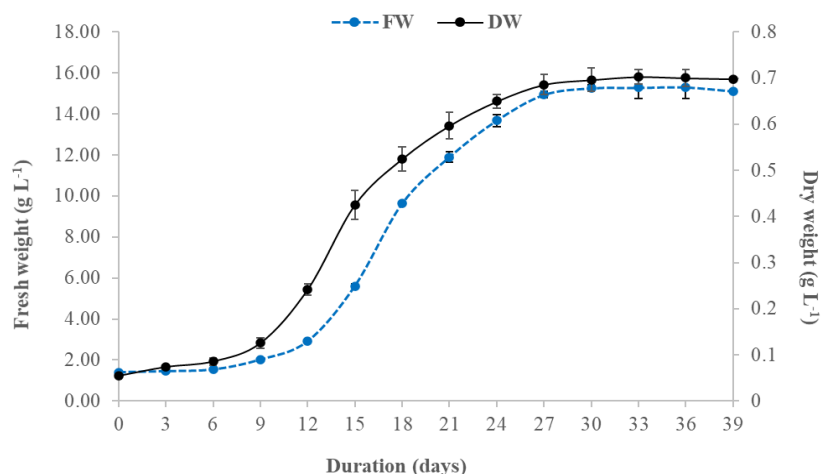


Figure 3.4: Progressive view of in vitro callus, where,

- (A) In vitro callus, just after subculturing on fresh medium (Bar=0.6 cm),
- (B) Callus tissue showing oxidative browning during 1st week of subculture and emergence of fresh cells at the base (Bar=0.6 cm),
- (C) In vitro callus, after 2nd week, with increased mass of whitish-friable cells (Bar=0.6 cm),
- (D) In vitro callus after 3rd week, showing whitish-friable cells with onset of browning at outermost layer of callus (Bar=0.3 cm),
- (E) Increased browning of callus during the 4th week of culture (Bar=0.7 cm).

proliferation enhanced displaying white friable callus cells during 2nd week (**Figure 3.4C**) and 3rd week (**Figure 3.4D**). However, end of 3rd week marked the onset of browning in the outermost layer of the callus tissue. The browning increased further in the 4th week of culture

(Figure 3.4E). The biomass growth curve of the calluses (Graph 3.1) is indicating that the calluses on subculture remained in the lag phase till 6th day. Then the cell biomass increased until 27th day, following which the stationary phase started.



Graph 3.1: Biomass growth curve analysis of in vitro grown calluses of *T. cordifolia* on semi-solid medium, MS + 3 μ M BAP + 10 μ M NAA.

The ultrastructural study using FESEM analysis was also conducted for better visualization of friable callus in which cluster of cells were visible along with the heterogeneous layer of mucilage-like extracellular matrix (ECM) (Figure 3.5), which is responsible for cell-cell adhesion, cell signalling and growth. The ECM layer could be verified by the presence of ruptured layer and crack formation. This is possible due to electron beam damage during the

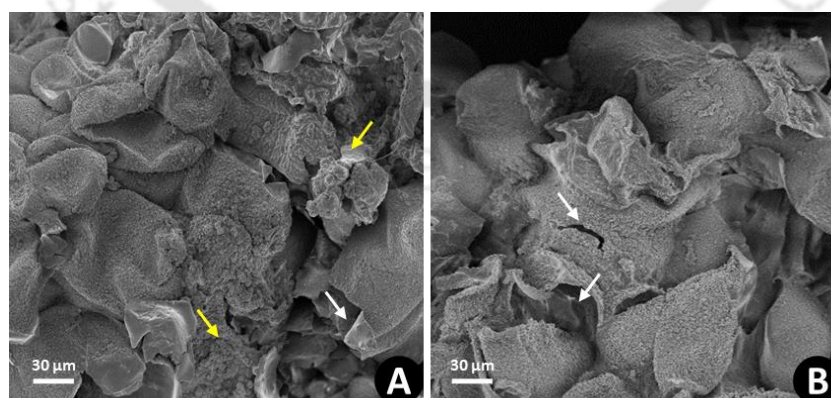


Figure 3.5: FESEM imaging showing ultrastructural surface morphology analysis of in vitro generated calluses (3-week-old) of *T. cordifolia*, along with presence of ECM aggregates and membranous layer. The arrows represent the presence of ECM (White arrows shows structural damage of ECM during the analysis) (Bar=30 μ m).

analysis. Few authors have also reported the sample preparation method, including dehydration, desiccation and drying, to be the causes of artefacts, such as presence of high amount of water content in the friable callus and ECM matrix, loss of which, alters the texture and geometry of cells (Laukkanen et al. 2000; Ming et al. 2019; Popielarska-Konieczna et al. 2010).

The microscopic insight of the changes in cells has also been evaluated for viability analysis during the course of growth cycle. With respect to the morphology, the cells exhibited different shapes and sizes commonly, spherical, circular and isodiametric with nucleus at the cell's periphery as visualized under fluorescence microscope. As described in Materials and Methods section, these cells were later stained with combination of dyes, FDA and PI, for further analyses on their viability using Fluorescence Inverted Microscope at blue (FDA Excitation wavelength: 450 to 495 nm) and green (PI Excitation wavelength: 510 to 560 nm) filters. As seen in **Figure 3.6**, in a group of cells, only viable cells were visualized as green exhibiting metabolic active cells. It clearly represented the viability of the intact cells since the non-polar dye (FDA) fluoresces green upon cleavage by the esterase enzyme in cell's cytoplasm. Under-stressed cells or cells with membrane disintegration, during the process of subculture or browning, have been visualized as red indicating PI uptake. However, it could be clearly envisaged that another population of brown cells with phenolic leach out was unable to uptake FDA indicating them as metabolic inactive, however, their integrated membrane prevented them for PI uptake either. According to this, oxidative browning led to formation of thickened cell walls and lignification, preventing the uptake of FDA, soon after it was subcultured (**Figure 3.6A, B, C, D**). However, presence of few metabolically active cells in the subcultured calluses initiated the formation of fresh, friable and white cells after a week of subculture (**Figure 3.6E, F, G, H**). Exponential phase showing extensive cell division from 9th day till 27th day as per dry weight (DW) analysis as in **Graph 3.1 (Figure 3.6I, J, K, L)**. However, towards the end of the exponential phase, the outermost cells of calluses began turning brown due to nutrient limitations. On 4th week of culture, extensive browning of callus started and cells could not take up either of the stain due to lignification of cell wall (**Figure 3.6M, N, O, P**). After the exponential phase, there was the onset of stationary phase. Therefore, the cells were harvested on 21st day, before the onset of browning to avoid phenolic hindrance in alkaloids' quantification studies. Similarly, Rao et al. (2008) estimated the maximum biomass production at 36th day of subculture while berberine was found to be maximum on 24th day in the cell suspension cultures of *T. cordifolia*.

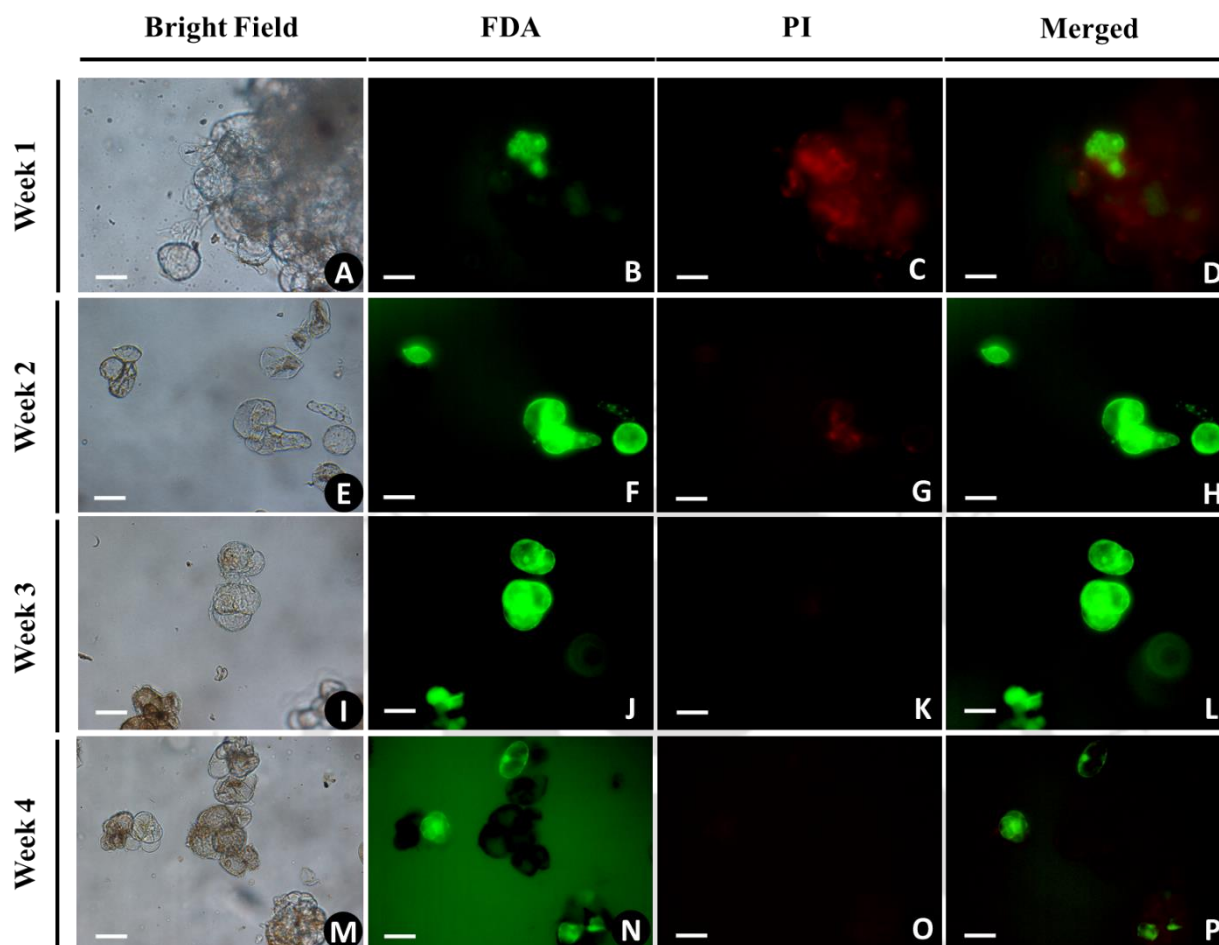


Figure 3.6: Morphological analysis of in vitro developed calluses of *T. cordifolia* during 1st, 2nd, 3rd and 4th week of culture

(A, E, I, M) represents bright field visualization of callus cells (Bar=10 mm),

(B, F, J, N) shows callus cells visualized at blue filter showing FDA-stained metabolic active cells (Bar=20 mm),

(C, G, K, O) represents calluses stained with PI visualized under green filter (Bar=20 mm),

(D, H, L, P) show the callus cells representing merged format of FDA and PI-stained cells (Bar=10 mm).

3.3.3 Method optimization for extraction of alkaloids

Mother plant leaves and in vitro calluses were harvested, dried and processed for crude extract preparation. Alkaloids need to be isolated in their true-form without any artefacts. Therefore, methanol was chosen for extraction of crude extracts, since it dissolves both salt and free-forms of alkaloids. However, the yield of alkaloids was observed to be low with lots of phenolic contamination in crude extract. Hence, the crude extracts were subjected to alkaloid extraction with the help of acid-base procedure as described in Materials and Methods section. Acidic

treatment to the crude extracts rendered the dissolution of polar compounds into the aqueous phase, leaving the non-polar aggregation of compounds on the surface of the magnet used for stirring the solvent. Zaidi et al. (2014) proposed that in the presence of magnetic field, the non-polar molecules orient their positive and negative charges, leading to their coagulation and aggregation, thus, could be useful in separation of impurities. The alkaloids show higher stability in low pH and the H^+ ions provide an ease in the movement and release of alkaloids (Verma et al. 2007). Thereafter, the remaining acidic portion is filtered and fractionated with chloroform, which dissolved the weak basic alkaloids. Basification of the aqueous fraction led to the removal of an extra H^+ bound to N-atom, facilitating the complete release of alkaloids (strong basic alkaloids). The method optimization resulted in extraction yield for crude methanolic extract (**method I**) and alkaloid rich fractions (**method IIa and IIb**) as elaborated in Materials and Methods section. A quick screening of callus extract obtained from method IIa, performed via preparative thin layer chromatography (PTLC), confirmed the presence of both the alkaloids, jatrorrhizine and palmatine (**Figure 3.7**).

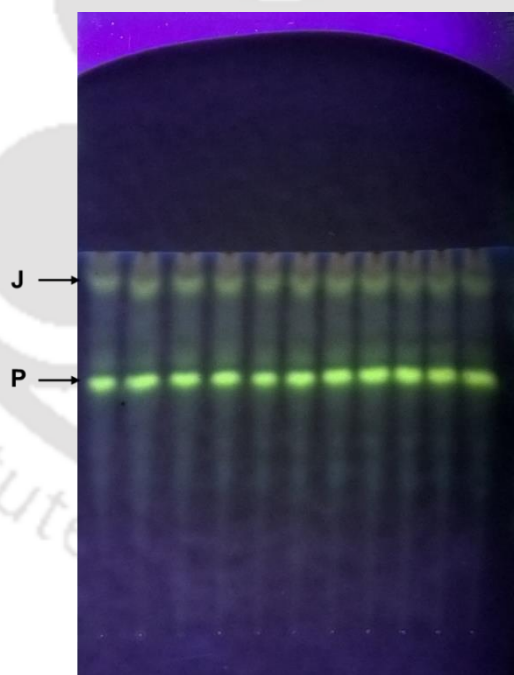


Figure 3.7: PTLC analysis of callus extract (method IIa) run in solvent system of butanol: glacial acetic acid: water (14:3:4), showing presence of jatrorrhizine (J) and palmatine (P)

3.3.4 Identification of protoberberine alkaloids in different extraction fractions

3.3.4.1 Method optimization for HPLC

Jatrorrhizine and palmatine were successfully identified and quantified using reverse-phase chromatography technique (RP-HPLC) in which solvent A was devised to be MilliQ with 20 mM ammonium acetate and 0.03% formic acid as polar solvent and acetonitrile as solvent B. The compounds were separated in isocratic mode while the alkaloid peaks were monitored at 345 nm wavelength. The methodology was adopted from Kumar et al. (2017), where the authors successfully found separation of both the alkaloids and quantified them as well. The compounds got separated on the basis of their polarity difference. When the reference standards of alkaloids were run in HPLC, jatrorrhizine eluted earlier at retention time of 6.8 and palmatine at 7.4 min (**Figure 3.8**). Corresponding elutions were observed in case of leaf and in vitro calluses samples (**Figure 3.9**). In the reports of Chintalwar et al. (2003), jatrorrhizine and berberine were obtained in cell suspension cultures of *T. cordifolia* with no detection of palmatine. Rao et al. (2008) obtained maximum berberine content in suspension cultures of *T. cordifolia*. Therefore, the presence of palmatine in callus and leaf extracts were reconfirmed in the present study. The HPLC eluent from callus and leaf extracts were collected between 7.3-7.4 min, concentrated and run in thin layer chromatography (TLC) along with the reference standards of palmatine and berberine, individually as well as their mixtures in equal proportions (**Figure 3.10**). The TLC analysis confirmed that the two alkaloids, berberine and palmatine, have different retention factor (R_f -value) and there was no co-elution of similar alkaloids. Further, MS analysis also verified the elution of target alkaloids. This study, reports for the

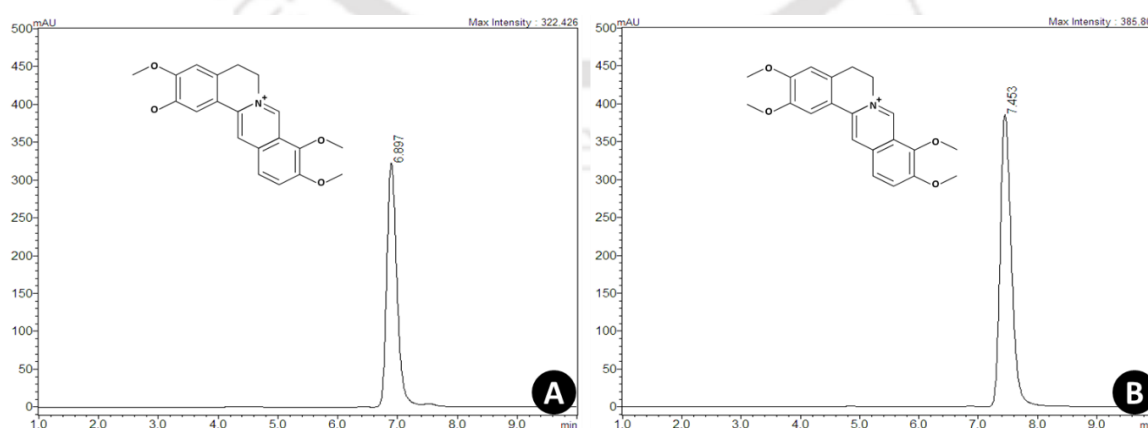


Figure 3.8: HPLC chromatograms of protoberberine standards

(A) Jatrorrhizine standard (retention time: 6.8 min),

(B) Palmatine standard (retention time: 7.4 min).

first time, the simultaneous presence of jatrorrhizine and palmatine in callus and leaf extracts of *T. cordifolia*.

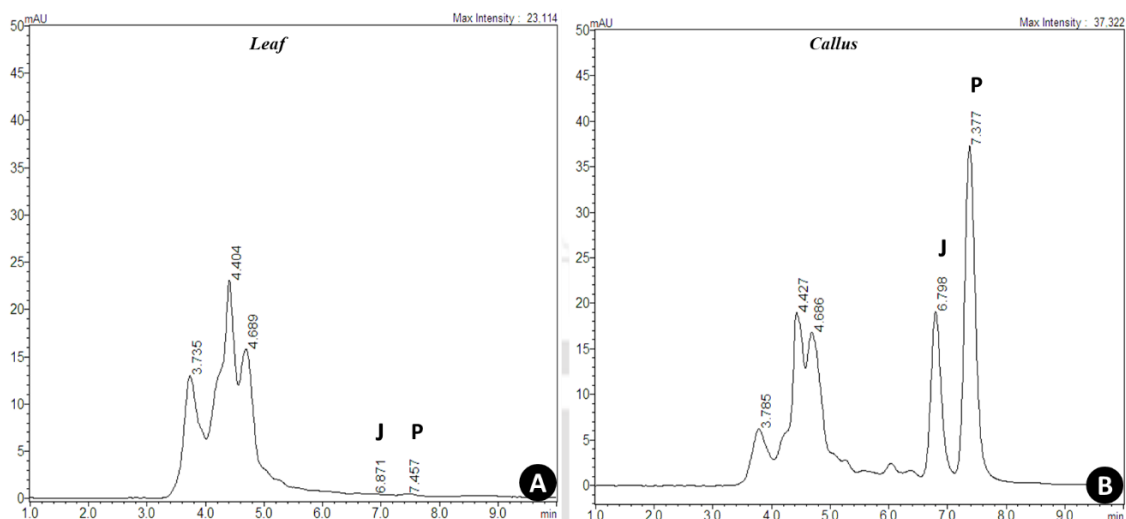


Figure 3.9: HPLC chromatograms of leaf and in vitro callus extracts, processed via extraction method II

(A) Leaf extract from mother plant showing jatrorrhizine (J) at retention time of 6.8 and palmatine (P) at 7.4 min,
(B) In vitro calli extract showing jatrorrhizine (J) at retention time of 6.7 and palmatine (P) at 7.3 min.



Figure 3.10: TLC analysis in solvent system of butanol: glacial acetic acid: water (14:3:4) for the confirmation of palmatine in purified HPLC eluent, where,

B: Berberine chloride standard (R_f 4.6),

P: Palmatine chloride hydrate standard (R_f 4.1),

B+P: Mixture of standards of berberine and palmatine (1:1),

P_{HPLC}: Purified HPLC eluent of palmatine (7.37 min).

3.3.4.2 Linearity and Precision

HPLC method development was crucial for separation and identification of compounds. The linearity check was performed for generation of calibration graph for the quantification of the compounds along with precision studies in which the standards were run in triplicates within same day and on alternate days. Calibration graph was generated for jatrorrhizine and palmatine according to the serial dilution concentrations mentioned in Materials and Methods section. The straight-line equation was generated, where both compounds had regression coefficient (R^2) values >99%, thus, confirming a linear calibration curve. With the help of the equation, both the alkaloids, jatrorrhizine and palmatine, were quantified in the respective samples.

In the present study, coefficient of variation (CV%) for intra-day was analyzed to be 0.3% and 0.1% for jatrorrhizine and palmatine, respectively, while, inter-day precision was calculated as 1.7% and 1.3%, for jatrorrhizine and palmatine, respectively (**Table 3.2**). In addition to this, callus extract (method *Ila*) was spiked with known amounts of jatrorrhizine and palmatine standards, for the confirmation of compounds. The mixture was then run in HPLC, where spiked area of peaks arrived at 6.8 and 7.4 min, for jatrorrhizine and palmatine, respectively, which is similar to the unspiked samples and thereby, reconfirming their presence (**Figure 3.11**).

Table 3.2: Linearity and Precision studies for jatrorrhizine and palmatine

Protoberberine alkaloid	Retention time (min)	Calibration curve ^a			Coefficient of variation (CV%)	
		Y-intercept	Slope	R^2	Intra-day	Inter-day
Jatrorrhizine	6.8	46983	72439	0.99	0.3	1.7
Palmatine	7.4	18414	79918	0.99	0.1	1.3

^a Values represented as mean $\pm$ standard deviation ($n=3$)

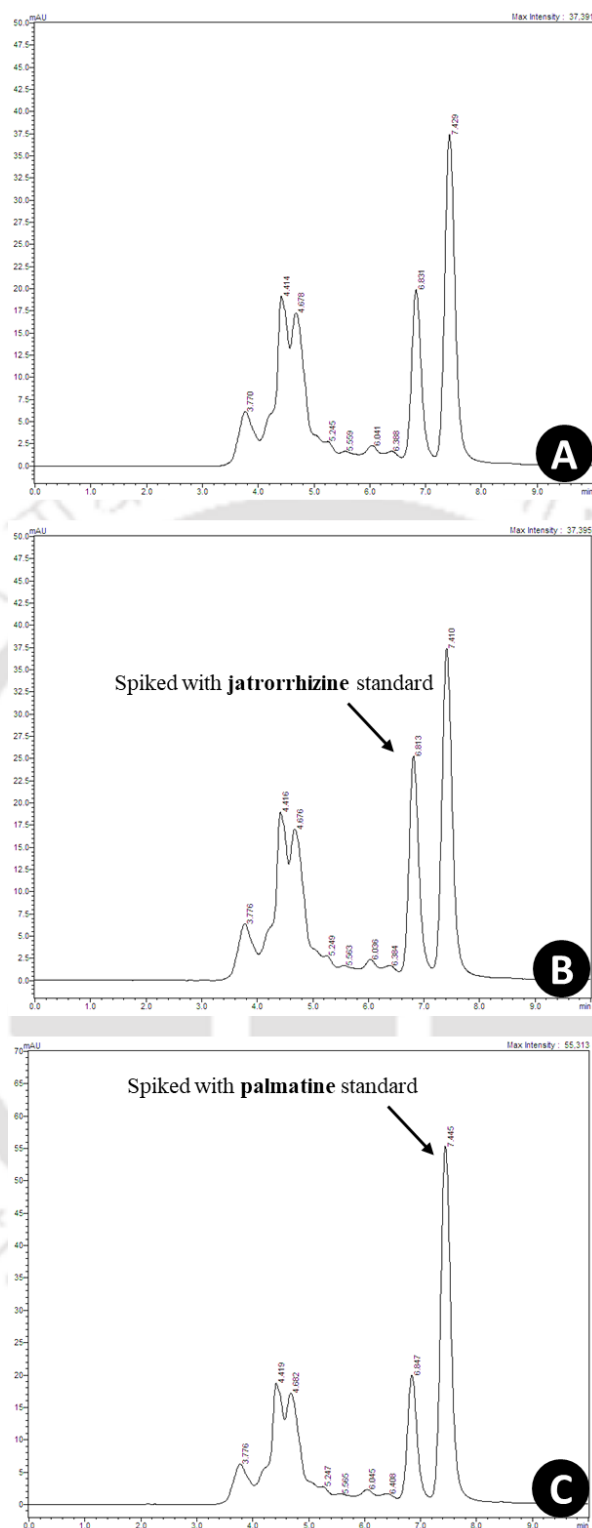


Figure 3.11: HPLC chromatograms showing confirmation of jatrorrhizine and palmatine via spiking analysis, where,

- (A)** Callus extract (method IIa),
- (B)** Extract from A spiked with known amount of jatrorrhizine standard,
- (C)** Extract from A spiked with known amount of palmatine standard.

3.3.5 Confirmation of alkaloids via Mass spectrometry

The HPLC eluent peaks of standards of both the alkaloids (retention time of 6.8 and 7.4 min) were collected, concentrated and validated using MALDI-TOF, where, mass values of 338.10 m/z and 352.14 m/z correspond to jatrorrhizine and palmatine, respectively, as shown in **Figure 3.12**. Similarly, HPLC peak eluents of both the compounds were collected from in vitro calluses extract (method II), concentrated it and subjected for MS analysis. According to **Figure 3.13**, mass value of 338.11 m/z signifies presence of jatrorrhizine with fragmentation of 324.09 m/z ($[M]^+ - CH_4 + 2H^+$). Similarly, palmatine was denoted as 352.14 m/z with fragmentation of 338.11 m/z value ($[M]^+ - CH_4 + 2H^+$) in **Figure 3.14**. Analyzing the samples in the positive mode led to the addition of protons to the fragments as well.

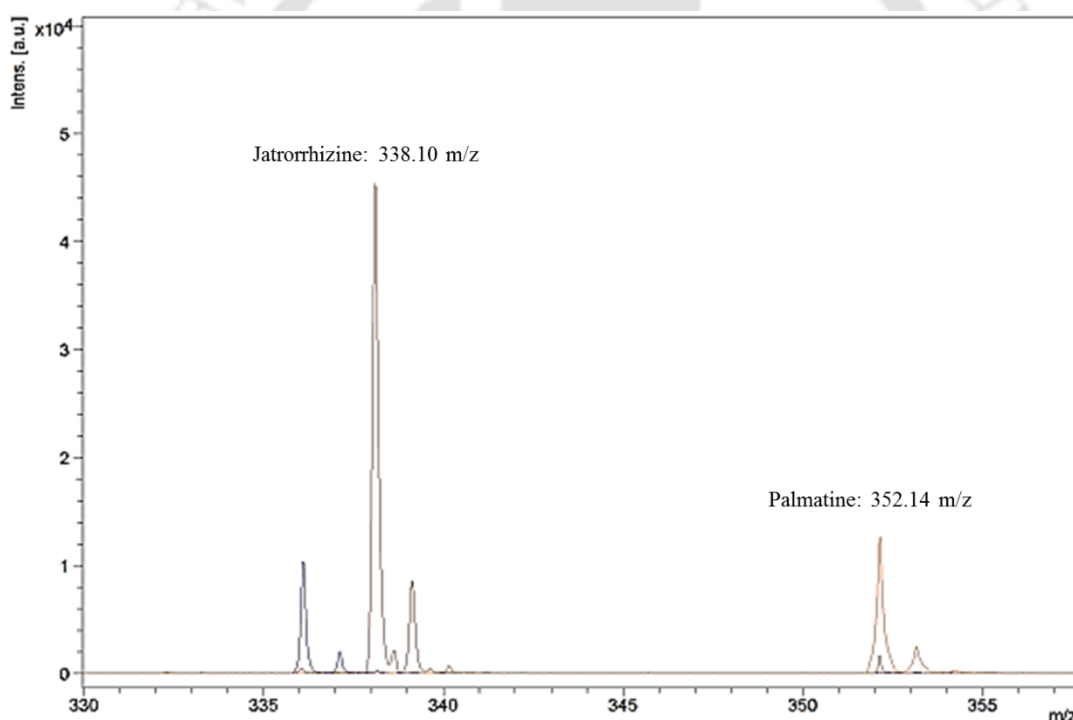


Figure 3.12: Mass spectrometric analysis of standards of target alkaloids showing jatrorrhizine at 338.10 m/z and palmatine at 352.14 m/z values, respectively.

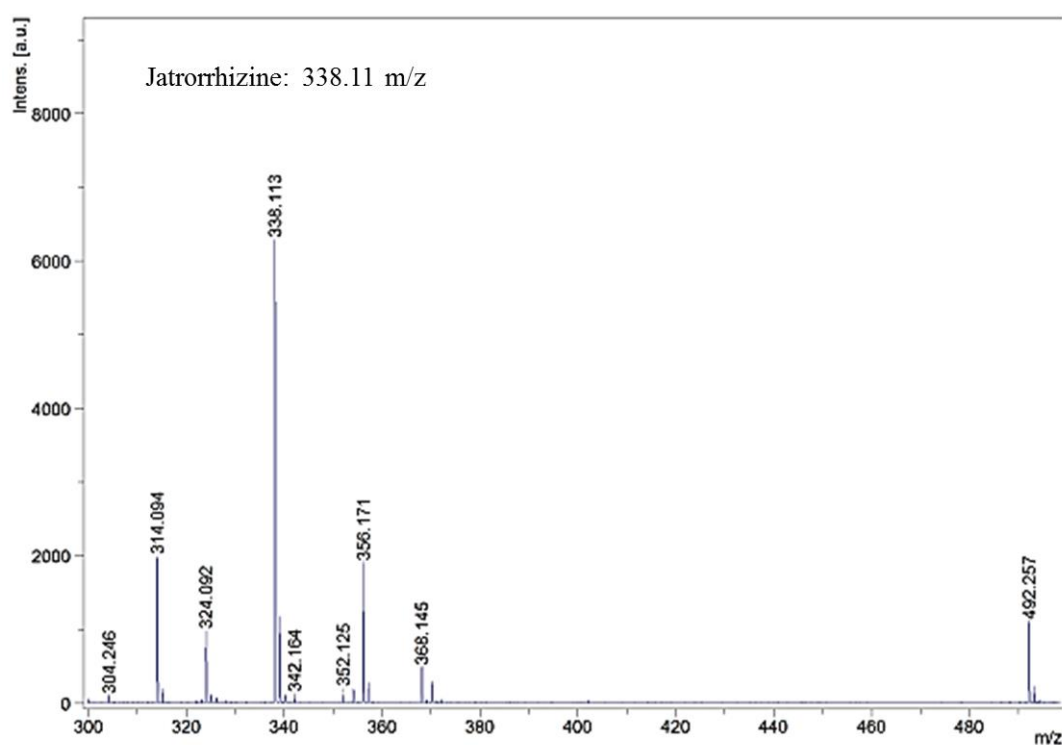


Figure 3.13: Mass spectrometric analysis of *in vitro* callus extract of *T. cordifolia* showing jatrorrhizine at 338.11 m/z value with fragmentation at 324.09 m/z value.

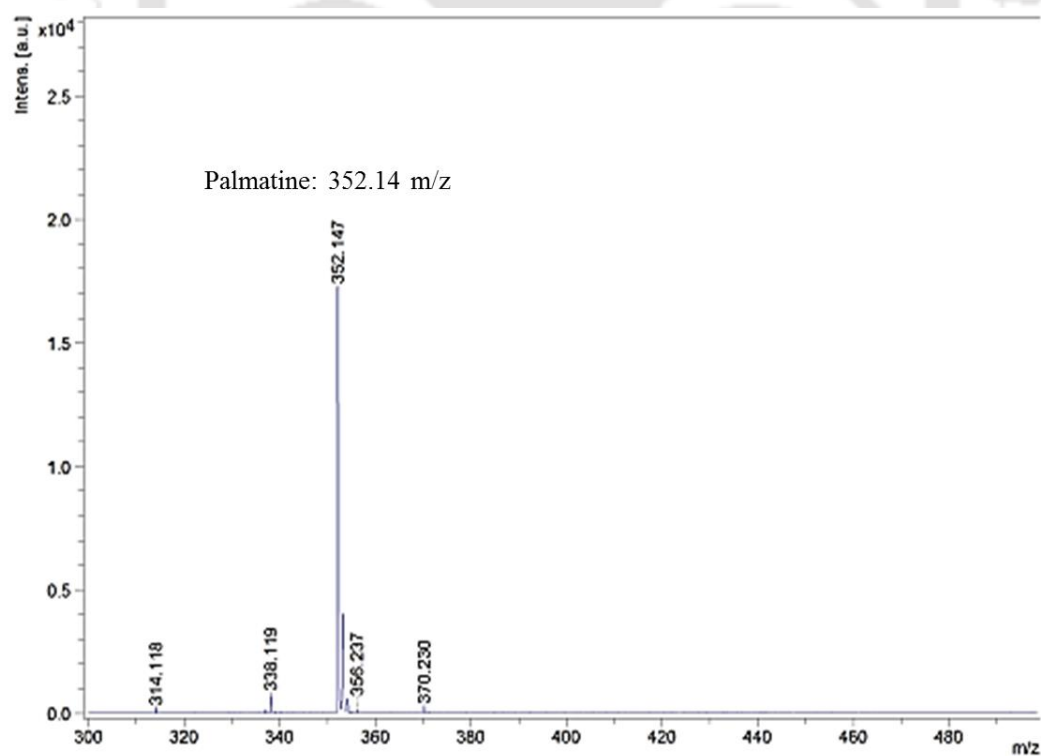


Figure 3.14: Mass spectrometric analysis of *in vitro* callus extract of *T. cordifolia* showing purified palmatine at 352.14 m/z value with fragmentation at 338.23 m/z ($336+2H^+$) value.

3.3.6 Quantification studies

On application of optimized method, jatrorrhizine and palmatine were identified along with evaluation of linearity of calibration graph and precision of the experiment. In the present study, comparative assessment of the alkaloids were proposed according to the extraction methodologies *I* and *II*, in both in vitro callus extracts (**Figure 3.15**) and methanolic leaf extracts (**Figure 3.16**). Consequently, Application of method *II* rendered the reduction of highly polar phenolic compounds and enhanced the elution of target alkaloids. Many researchers comply the co-occurrence or co-elution of berberine and palmatine. Therefore, a confirmatory TLC analysis was performed to ensure the presence of particular alkaloid (see **section 3.3.4.1** and **Figure 3.10**).



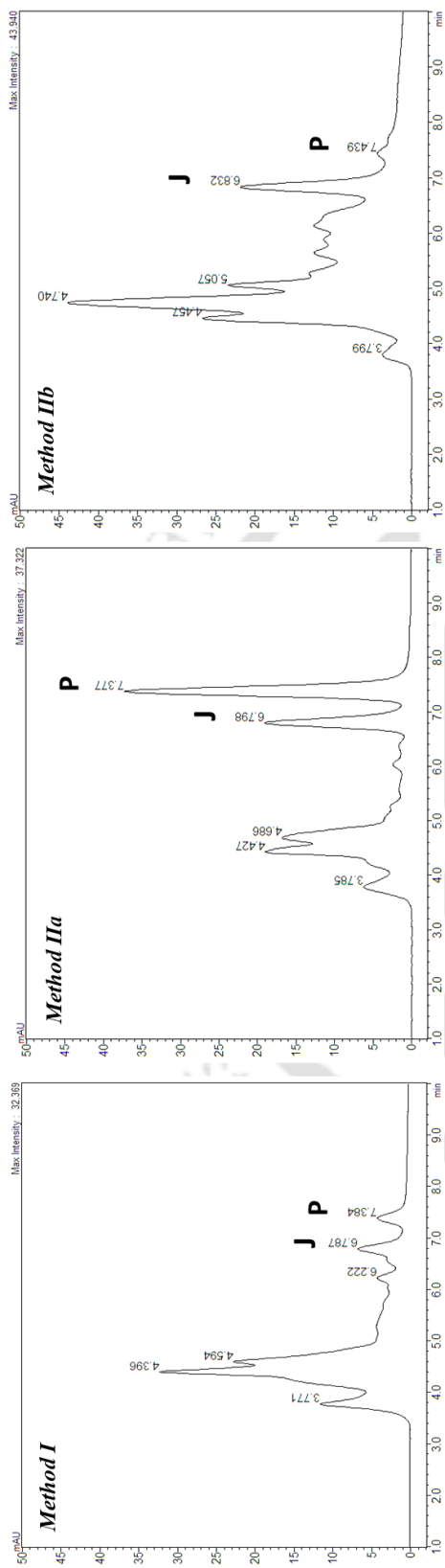


Figure 3.15: HPLC chromatograms of in vitro callus extracts showing presence of Jatrorrhizine (J) and Palmatine (P)

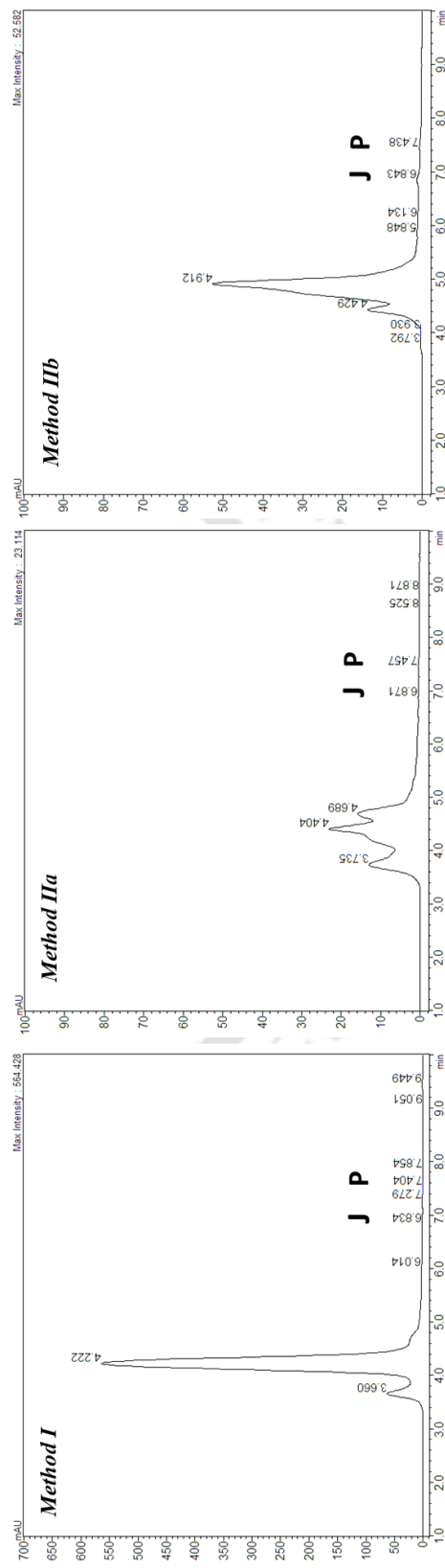
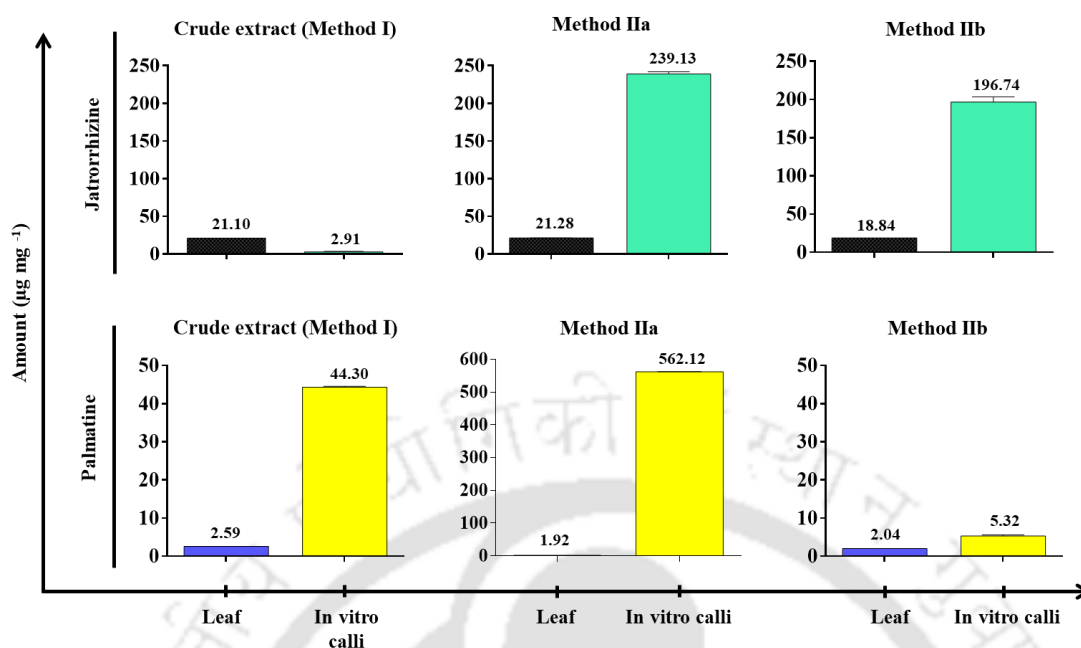


Figure 3.16: HPLC chromatograms of leaf extract showing presence of Jatrorrhizine (J) and Palmatine (P)



Graph 3.2: Quantification of alkaloids per se extraction methodologies

According to **Graph 3.2**, jatrorrhizine was found to be $21.1 \mu\text{g mg}^{-1}$ and $2.9 \mu\text{g mg}^{-1}$ in the crude extracts (Method I) of leaf and in vitro calluses, respectively. On the other hand, method II slightly influenced the yield enhancement, releasing 21.3 and $18.8 \mu\text{g mg}^{-1}$ of the alkaloid from leaf extract in method IIa and IIb, respectively. However, enormous increase in jatrorrhizine content was observed from in vitro calluses when method IIa and IIb of extraction was followed, which released $239.1 \mu\text{g mg}^{-1}$ and $196.7 \mu\text{g mg}^{-1}$ of jatrorrhizine.

Similarly, another target alkaloid, palmatine, was also detected and quantified as $2.6 \mu\text{g mg}^{-1}$, $1.9 \mu\text{g mg}^{-1}$ and $2.0 \mu\text{g mg}^{-1}$ from method I (crude extract), method IIa and method IIb, respectively. Yield of palmatine ($44.3 \mu\text{g mg}^{-1}$) was better in crude extracts of in vitro calluses as compared to jatrorrhizine. Interestingly, highest yield of palmatine was noticed from method IIa ($562.0 \mu\text{g mg}^{-1}$) and the remaining palmatine ($5.32 \mu\text{g mg}^{-1}$) was eluted in method IIb.

Table 3.3: Quantitative assessment of jatrorrhizine and palmatine

Source	Compound	Method I ($\mu\text{g mg}^{-1}$)	Method II* ($\mu\text{g mg}^{-1}$)
Mother plant	Jatrorrhizine	21.1 ± 0.03	40.1 ± 0.1
	Palmatine	2.6 ± 0.03	3.9 ± 0.1
In vitro calluses	Jatrorrhizine	2.9 ± 0.5	435.9 ± 9.6
	Palmatine	44.3 ± 0.2	567.5 ± 0.6

*Method II include quantification values of *Ila* and *Ilb*

In conclusion, methodology *II* was found superior in extracting the target quaternary protoberberine alkaloids, jatrorrhizine and palmatine, from the leaf and in vitro calluses. The alkaloids were hypothesized to be stable in acidic aqueous extract and being semi-polar in nature, hence, easily get extracted in chloroform fraction. However, among the two, jatrorrhizine being more polar, therefore, partially get extracted from method *Ila* and remaining compound got extricated by method *Ilb*. Both the compounds were not detected in basified aqueous extract, signifying the complete withdrawal of target alkaloids. Maximum yield of alkaloids was retrieved from in vitro calluses as $435.9 \mu\text{g mg}^{-1}$ and $567.5 \mu\text{g mg}^{-1}$ of jatrorrhizine and palmatine, respectively, as mentioned in **Table 3.3**. The present study illustrates dual benefits in providing higher component yield from in vitro calluses over parent plant, with an added advantage of optimized alkaloid extraction procedure, that supported better extraction of metabolites from in vitro calluses. Similar observation has been mentioned in other literatures. Berberine has been reported 10% (DW) in suspension culture of *Coptis japonica* and 0.01% (DW) in mother plant (Matsubara et al. 1989). With respect to *Panax ginseng*, ginsenoside (steroid glycosides) was found to be 4.5% (DW) in intact plant while the 27% (DW) was being produced in in vitro callus cultures (Bhojwani and Razdan 1996; Misawa 1994).

The current experimental analysis also throws light upon the fact that the quaternary alkaloids are produced in higher concentration in in vitro cell lines, as influenced by the growth hormones, medium composition and other physical and chemical factors. Furuya et al. (1983) devised the higher alkaloidal content in in vitro callus of *Dioscoreophyllum cumminsii* (0.3-0.9% DW) as compared to intact plants (0.02 to 0.03% DW), with major component to be jatrorrhizine. Similarly, 20.4 mg of jatrorrhizine was purified from stem-induced callus of *Nandina domestica* (Ikuta and Itokawa 1988). Nakagawa et al. (1986) examined the higher

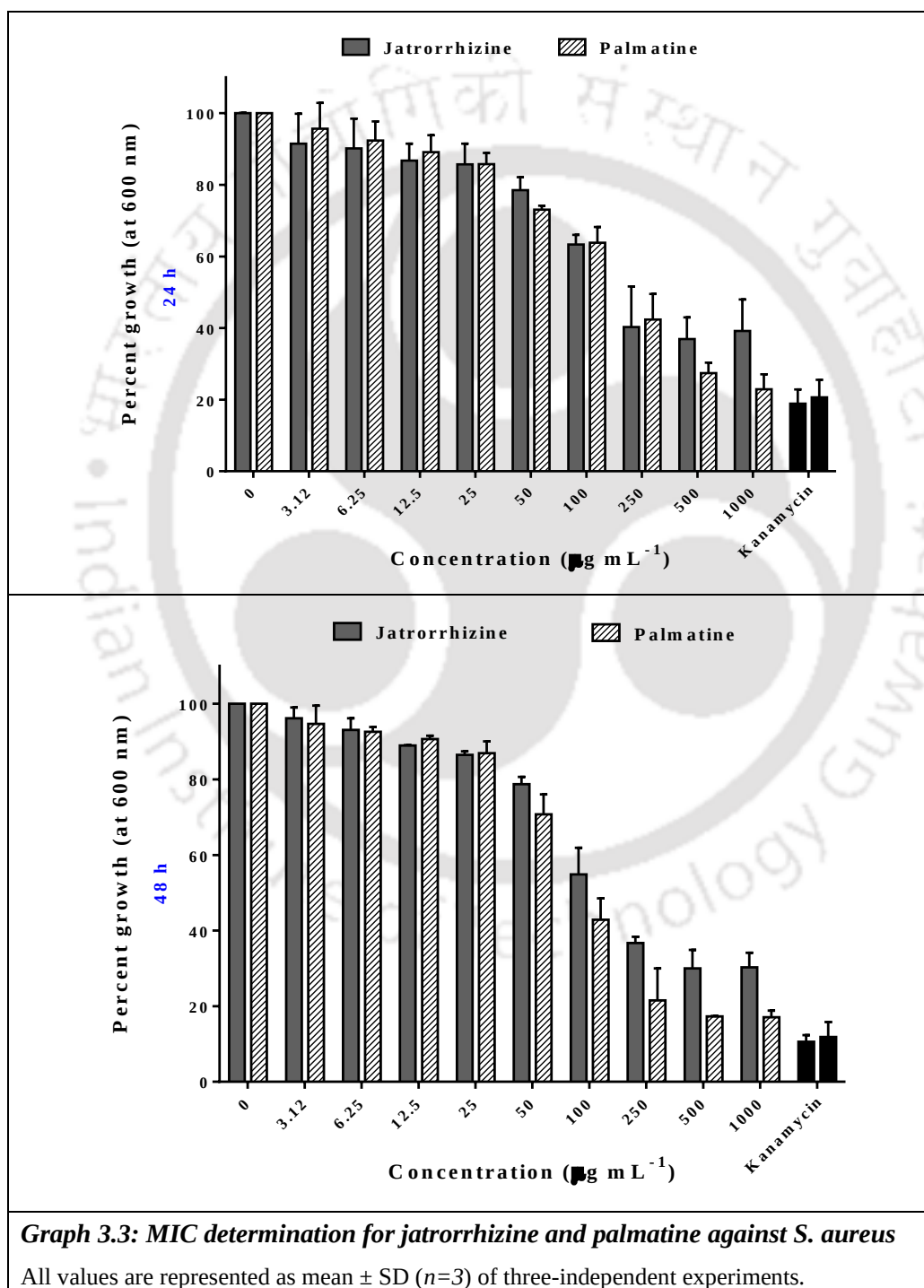
berberine productivity (20 mg/30 ml medium) from cell suspension culture of *Thalictrum minus* in Linsmaier and Skoog (LS) medium added with 60 μM NAA and 10 μM BAP. Moreover, berberine was observed to be leached out in the medium and reduction in the cell growth was also noticed. Nair et al. (1992) evaluated the enriched effect of NAA on higher berberine content. Another report also documented the synthesis and accumulation of berberine by gibberellic acid (GA_3) treated cells of *C. japonica* (Hara et al. 1988). In *T. cordifolia*, berberine content was reported to be 5-14-fold higher than intact plants and the authors suggested the growth hormones, such as BAP, NAA and 2,4-D, medium pH, carbon-source and cell-aggregate size to be responsible candidates for higher berberine production in in vitro cell lines (Rao et al. 2008). Additionally, in a recent study, the authors successfully retrieved 756.3 $\mu\text{g g}^{-1}$ DW of berberine from stem-induced in vitro callus and further enhanced the berberine yield to 1894 $\mu\text{g g}^{-1}$ DW, after 40 μM of tyrosine (precursor) treatment (Pillai and Siril 2019b).

3.3.7 Inhibition of biofilm formation by *S. aureus*

3.3.7.1 MIC determination

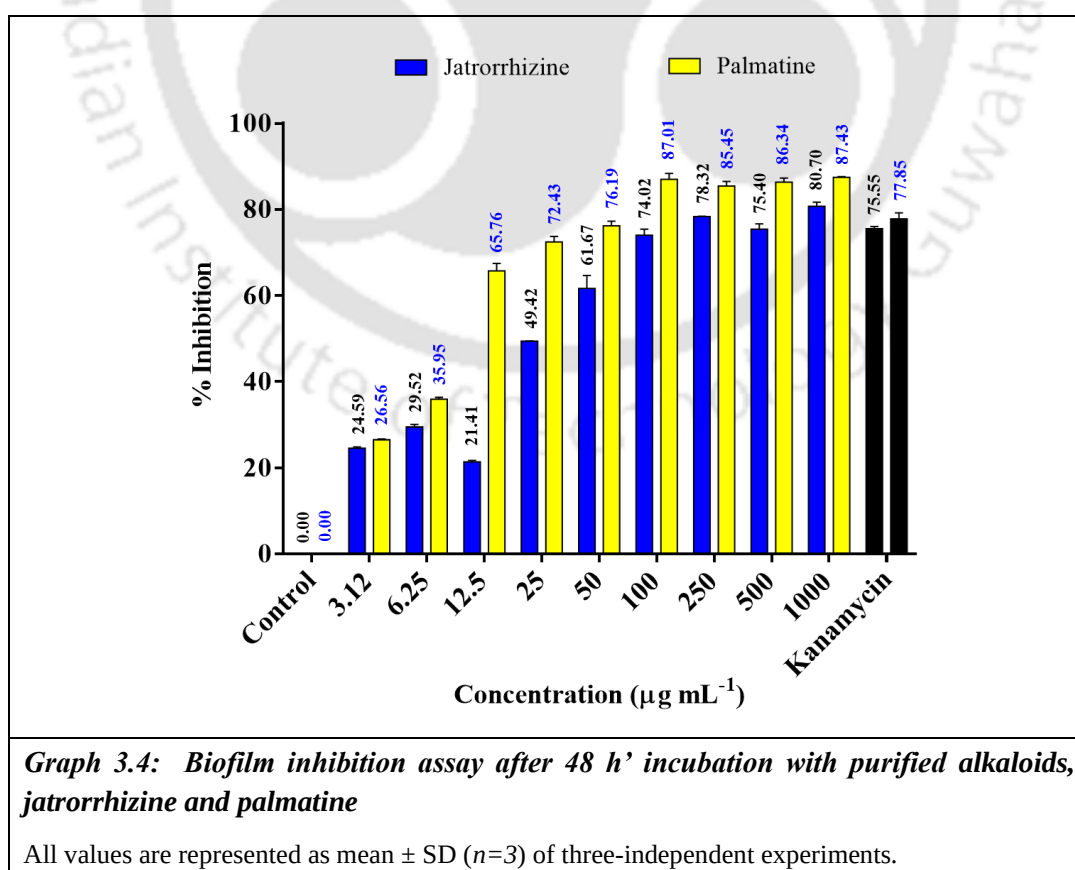
The susceptibility of jatrorrhizine and palmatine was examined on the growth reduction of planktonic cells of *S. aureus*. The anti-bacterial assessment was carried out for 24 h and 48 h duration to analyze the chances of re-occurrence especially in case of biofilm forming bacteria. MIC determination at lowest concentration of compound displaying no visible growth of bacteria by unaided eyes, depicts, 250 $\mu\text{g mL}^{-1}$ at 48 h' treatment for both jatrorrhizine and palmatine, respectively. The alkaloids were also effective at higher concentrations of 500 $\mu\text{g mL}^{-1}$ and 1000 $\mu\text{g mL}^{-1}$ in inhibiting bacterial growth at 24 h and 48 h of incubation (**Graph 3.3**). Lower concentrations $<50 \mu\text{g mL}^{-1}$ were ineffective in providing anti-bacterial efficacy. Similar broth dilution methodology was also adopted by other researchers, which is a reproducible and rapid technique and better for large-scale sample analysis (Saikia and Chaudhary 2018; Wang et al. 2009). The present study has also monitored the optimum range of concentrations that should not generate the resistant, non-treatable organisms (Wiegand et al. 2008). Kanamycin was used as positive control, which is a broad-spectrum aminoglycoside antibiotic, effective against gram-positive and gram-negative microbes. It binds to 30S ribosomal subunit and inhibits protein synthesis. It also causes fissures in the bacterial cell membrane. The antibiotic has been reported to cause inhibitory effects against *S. aureus* and induced biofilm. In a study by Yang et al. (2017), kanamycin significantly inhibited *S. aureus*

biofilm at all sub-MIC concentrations, and down-regulated the genes associated with biofilm formation (*fnbA*, *rbf*, *lrgA*, and *cida*). According to a meta-analysis performed by Deyno et al. (2017), highest level of *S. aureus* resistance was observed in case of amoxicillin > penicillin > ampicillin > tetracycline > methicillin > erythromycin, whereas, kanamycin was found to have least resistance. In another study, White et al. (2003) found 92% inhibition rate of kanamycin against avian *S. aureus* isolates, while it showed 8% resistance at MIC₉₀ (128 µg mL⁻¹).



3.3.7.2 Biofilm inhibition assay and MBIC determination

Consequently, the biofilm forming capability of *S. aureus* was tested at similar concentration of target alkaloids. Biofilm are considered to be the mature and huge bacterial community, adhered to surfaces and supported by exopolysaccharides, proteins and nucleic acids and interconnecting via signaling molecules (Mohammed et al. 2018). Safranin dye which effectively binds to the glycosaminoglycan, component of extracellular matrix (ECM), was used for analyzing the biofilm forming capability of the bacteria (Kiviranta et al. 1985). As depicted in **Graph 3.4**, inhibition percentage of *S. aureus* was calculated as 87.0% by palmatine at MBIC of 100 $\mu\text{g mL}^{-1}$ at 48 h treatment, while jatrorrhizine exhibited 78.3% inhibition at MBIC of 250 $\mu\text{g mL}^{-1}$ at 48 h treatment. Summarization of the biofilm inhibition assay revealed that palmatine significantly decreased the formation of biofilm in dose-dependent manner at concentration $\geq 12.5 \mu\text{g mL}^{-1}$. However, alike palmatine, jatrorrhizine was equally effective in initiating the biofilm inhibition at concentrations $\geq 25 \mu\text{g mL}^{-1}$ in similar mode. The study was effectively visualized by the safranin coloration which is directly proportional to the biofilm formation. Thus, one of the criteria of biofilm formation by means of its inhibition was achieved by the target protoberberine alkaloids.



3.3.7.3 Qualitative analysis of biofilm inhibition by fluorescence assay

The reduction in the biofilm formation was verified qualitatively using fluorescence microscopy. Acridine orange dye has been considered as biofilm mass indicator as it binds to ssDNA, dsDNA and ssRNA as well as nucleic acid of ECM. In addition to this, it stains viable or non-viable cells of biofilm (Stiefel et al. 2016). As visible in **Figure 3.17**, the biofilm is clearly visible in control (untreated) bacterial cells under bright field and fluorescent dye stained, representing the increased biovolume of *S. aureus* within 48 h'. This duration was undertaken to monitor any increase in growth of resistant bacteria beyond 24 h', although the former time duration was better in displaying the outcome. Upon treatment with purified alkaloids at their MBIC concentration, evidence of densely populated biofilm was not visible except a few discrete bacterial colonies are seen. Among the two alkaloids, palmatine was found to be more effective in biofilm inhibition than jatrorrhizine. Thus, it was clear from fluorescence microscopic visualization that biofilm formation was significantly declined with purified protoberberine alkaloids.

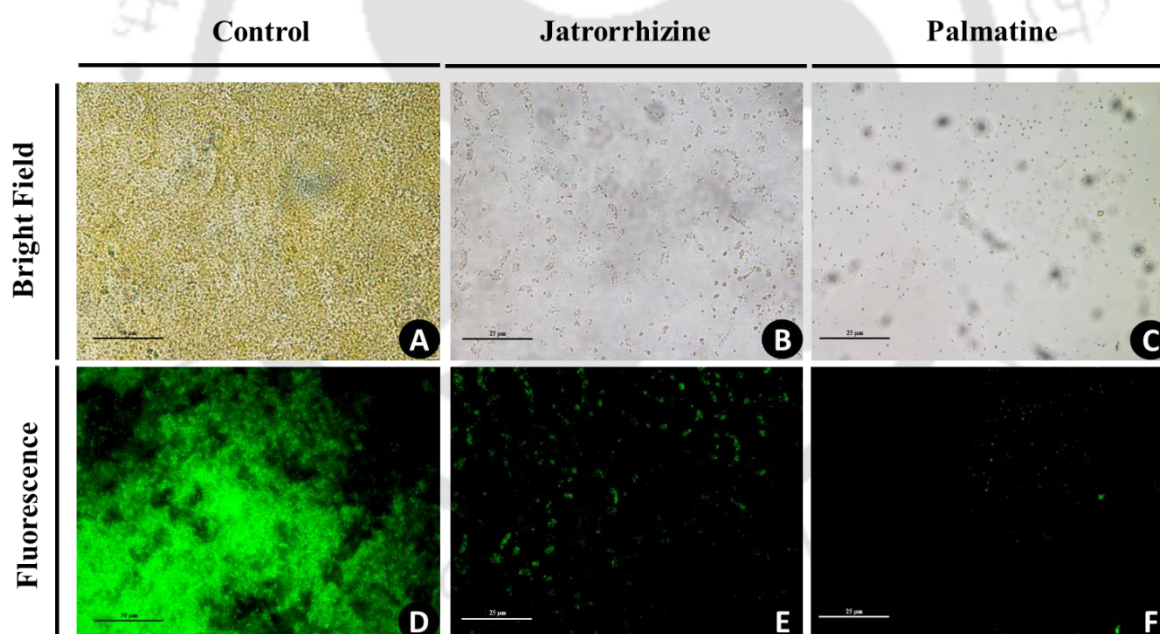


Figure 3.17: Fluorescence assay for assessing biofilm inhibition upon purified alkaloidal treatment,

(A, D) depicts densely populated, aggregated clusters of *S. aureus* (Bar=50 µm),

(B, E) represents jatrorrhizine treated bacterial cells with clustered colonies with no signs of biofilm formation (Bar=25 µm),

(C, F) shows palmatine treated bacterial cell line in minimal discrete colonies (Bar=25 µm).

3.3.7.4 Surface morphological evaluation using FESEM

One of the reasons behind the reduction in biofilm formation after alkaloid treatment and also their effects on planktonic cells could be assessed via FESEM ultrastructural analysis. Upon treatment at their respective MBIC concentration (Jatrorrhizine: $250 \mu\text{g mL}^{-1}$ and palmatine: $100 \mu\text{g mL}^{-1}$), abnormal structural changes, such as membrane deformation or damage, pore formations, leakage of cytoplasmic content, could be clearly observed on discrete or aggregated bacterial cells, as compared to the untreated bacterial cells which showed smooth and uninjured surface (Figure 3.18). The alkaloids were found to be effective in showing the unusual or distressed morphology in the aggregated stage of *S. aureus*. Previous inhibitory analyses were well-correlated and supported by the fact of membrane disruption as one of the inhibitory actions of target alkaloids. Similar observations have also been described in berberine treated methicillin resistant *S. aureus* (MRSA) (Chu et al. 2016), *Bacillus subtilis* (Prince et al. 2016), *E. coli* (Saikia and Chaudhary 2018), *S. epidermis* (Wang et al. 2009).

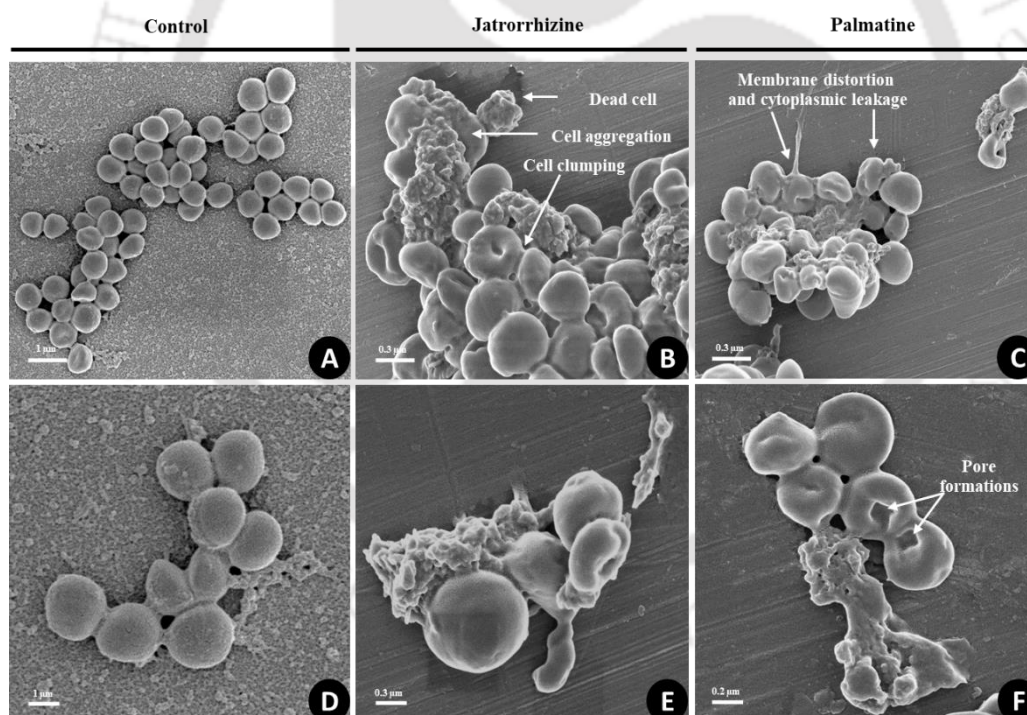


Figure 3.18: Ultrastructural morphological analysis of treated and un-treated *S. aureus*,

(A, D) Bacterial cells without any treatment (Control) (Bar=1 μm),

(B, E) Jatrorrhizine ($250 \mu\text{g mL}^{-1}$) treated bacterial cells showing the altered surface morphology in both discrete or aggregated bacterial population (Bar=0.3 μm),

(C, F) Palmatine ($100 \mu\text{g mL}^{-1}$) treated bacterial cells depicting membrane distortion as well as cytoplasmic leakage (Bar=0.3 and 0.2 μm).

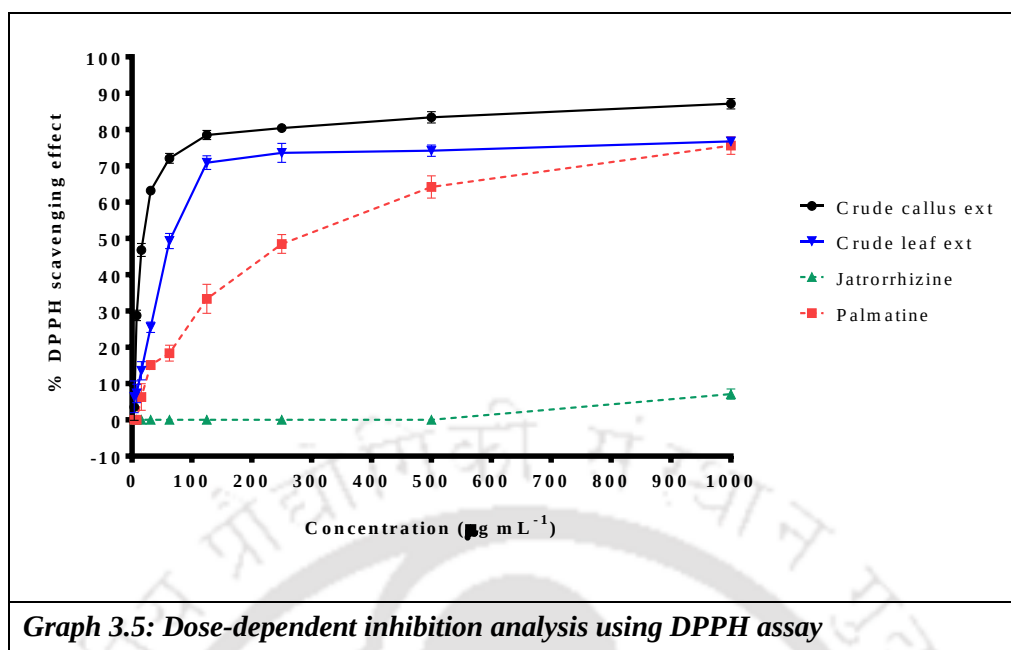
3.3.8 Assessing the antioxidant potential via DPPH assay

Reviewing the antioxidant potential, plants have the inbuilt capacity for prevention against reactive oxygen species (ROS), which is majorly enzymatic and non-enzymatic based approaches. Of these, the latter includes the antioxidant effect due to secondary metabolites. Among the secondary metabolites, phenolic compounds bear the maximum responsibility of possessing this protective potential. Among the antioxidant-rich compounds, ascorbic acid represents significant scavenger of free radicals generated from DPPH reagent. In the present study, it was used as positive control and demonstrated highest scavenging effect of 93.6% and IC_{50} value of $16.9 \mu\text{g mL}^{-1}$ (Table 3.4). Consequently, the DPPH radical scavenging effects of crude extracts of leaf and in vitro callus extracts were assessed in dose-dependent way, of which the latter represented promising effects in curbing DPPH free radicals by instantly neutralizing them into non-radical form (Graph 3.5). The highest antioxidant activity was found in callus extract as 87.1% with IC_{50} value of $18.4 \mu\text{g mL}^{-1}$. Comparing this, the crude leaf extract exhibited 76.7% activity with IC_{50} value of $313.3 \mu\text{g mL}^{-1}$. The purified compounds were also evaluated for their antioxidant activity. Palmatine demonstrated a promising alkaloid-based scavenger with highest inhibition of 75.6% with IC_{50} value of $497.7 \mu\text{g mL}^{-1}$, thereby, contributing an essential part to the antioxidant potential of crude callus extract. However, jatrorrhizine showed negligible antioxidant activity.

Table 3.4: DPPH scavenging activity of crude extracts and purified alkaloids displaying IC_{50} and IC_{99} values

Sample	Maximum effect (%)	IC_{50} ($\mu\text{g mL}^{-1}$)	IC_{99} ($\mu\text{g mL}^{-1}$)
Ascorbic acid	93.6 ± 0.6	16.9	787.4
Crude callus extract	87.1 ± 1.4^a	18.4	975.5
Crude leaf extract	76.7 ± 0.4^b	313.3	1077.8
Purified jatrorrhizine	7.1 ± 1.4^{ab}	8159.5	16062.7
Purified palmatine	75.6 ± 2.4^c	497.7	1142.4

Values represented as mean $\pm$ SD ($n=3$). The values sharing the same letter do not differ significantly ($p < 0.05$) according to one-way ANOVA (Tukey's multiple comparison test).



The crude extracts of *T. cordifolia* with an amalgamation of non-enzymatic compounds (majorly phenolics, alkaloids, terpenoids) and antioxidant enzymes (catalase, glutathione peroxidase, glutathione reductase, superoxide dismutase), proved a highly probable candidate for ROS scavenging effects. This is one of the fundamental concept in prevention of chronic diseases, such as cancer, cardiovascular and liver disorders, diabetes, ulcer, arthritis and others (Liao and Yin 2000). Previous studies on the usage of medicinal plants or extracted compounds have been found exemplary in exhibiting the ROS generated superoxide radical ($O_2^{\cdot-}$), hypochlorous acid (HOCl), hydroxyl radical ($\cdot OH$) or hydrogen peroxide (H_2O_2) (Kasote et al. 2015; Wink 2015). Although these free radicals are highly reactive and merges with other molecules for stabilization, generating a series of unstable reactions, but plants bear the capability of curbing their oxidizing potential mostly by donation of hydrogen.

T. cordifolia has been proven for its antioxidant effect contributing to its immense therapeutic potential. An arabinogalactan polysaccharide from *Tinospora* stem showed protection against iron-induced lipid peroxidation (Subramanian et al. 2002). In a recent study, the authors speculated undigested stem proteins (trypsin and α -chymotrypsin) and enzyme hydrolysates, which strongly exhibited antioxidant potential via DPPH, ABTS (2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and Fe^{2+} chelation assay and could be an essential food supplement (Pachaiappan et al. 2018). Other potent bioactive compounds and crude plant extracts of *T. cordifolia* have been tested for the antioxidant potential (Bhalerao 2012; Kumar et al. 2018; Pushp et al. 2013; Raghu and Rao 2016; Sarala et al. 2012; Sengupta et al. 2009) but none of them have determined the vital effects of leaf derived in vitro callus.

Chapter 4

**Identification and quantification of steviol
glycosides from true-to-type elite plant
lines of *Stevia rebaudiana* and their effect on
Staphylococcus aureus biofilm formation**

ABSTRACT

Stevia rebaudiana, one of the commercially acclaimed plant, has been widely known as natural bio-sweetener. Across the globe, there is an urge for large-scale propagation of this plant, especially after being considered as GRAS (Generally Recognized as Safe) in 2008 and 2009, as food additives. The amplified usage of the herb has attracted the food industries for its commercial production with approved ~95% steviol glycosidic content. In vitro micropropagation approach would provide higher plant yield in shorter duration, with superior productivity of steviol glycosides, round the year. Therefore, the present study focused on large-scale propagation of *Stevia* plants by in vitro means using single node explants in two experimental plant lines, Sr1 and Sr2. The axillary buds, present at the node, proliferated in ~3 days on modified Driver Kuniyuki Walnut (DKW₁ with Murashige and Skoog (MS) iron and vitamin supplements) medium supplemented with 5 µM 6-benzylaminopurine (BAP). An average of 3-4 shoots per node were obtained, which grew up to a height of 2.4 cm in Sr1 and Sr2 plant lines, at the end of 4 weeks. Further, multiplication and elongation of shoots were obtained by pretreating the cluster of shoots with 0.5 µM gibberellic acid (GA₃) for 7 days, before transferring them on modified DKW₁ medium + 5 µM BAP + 0.05 µM indole-butyric acid (IBA). At the end of 6 weeks of growth cycle, multiple, multinodal, branched and elongated shoots were obtained in both the experimental plant lines, Sr1 and Sr2. The shoots attained a height of 7.1 cm with 10.0 number of shoots and 5.4 number of nodes per in vitro shoot in Sr1 plant line. Similar outcome was also observed in the Sr2 plant line where an shoot length of 6.8 cm and 10.8 number of shoots with 4.8 number of nodes per in vitro shoot were obtained. By following the protocol mentioned here, the higher rate of shoot multiplication was achieved as 54.0-fold and 52.1-fold per nodal explant, in Sr1 and Sr2 plant lines, at every 6 weeks. Rooting of shoots initiated in 9 days on MS + 0.5 µM IBA in 88.9% and 94.4% of cultures of Sr1 and Sr2 lines, respectively. Further, the rooted plants were hardened in greenhouse and transferred for acclimatization in field. The following protocol represents a reproducible, economic and productive method for procuring higher plant yield. The in vitro developed lines were evaluated for their genetic integrity tests via Inter Simple Sequence Repeats (ISSR) molecular marker analysis and flow cytometry. From ISSR analysis, 98.0% and 95.2% monomorphism was observed among the in vitro regenerated clones and mother plant of Sr1 and Sr2 lines. The true-to-type nature of in vitro regenerants was also confirmed qualitatively by measuring the relative DNA content via flow cytometric analysis. Further in the chapter, identification, quantification and characterization of rebaudioside A (R_A) and

stevioside (S) was executed via High Performance Liquid Chromatography (HPLC) and Mass spectrometry. The content of rebaudioside A in in vitro plant leaves was analyzed as 10.7% and 8.7% in Sr1 and Sr2 plant lines, which was higher than that present in the mother plant leaves as 5.8% and 5.7% in Sr1 and Sr2 plant lines, respectively. Besides, a marginal increase in stevioside content was found in in vitro Sr1 plant line as 5.2% and in Sr2 plant line as 5.1%, as compared to 3.8% and 5.0% in mother plants of Sr1 and Sr2 plant lines, respectively. The purified rebaudioside A and stevioside were found to be effective as 65.8% and 70.0%, respectively, in restraining the biofilm formation by *Staphylococcus aureus*. The biofilm inhibition was qualitatively confirmed by fluorescence microscopy, while field emission scanning electron microscopy (FESEM) study displayed the morphological effects of steviol glycosides on *S. aureus*. The chapter concluded with interpretation of antioxidant features, exhibiting free radical scavenging effects of purified rebaudioside A and stevioside as 47.8% and 50.0%, respectively, while the crude extracts of *S. rebaudiana* demonstrated 79.6%-82.7% reduction in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals.

Keywords: Antioxidant analysis, Clonal fidelity, Flow cytometry, ISSR markers, Micropropagation, *Staphylococcus aureus*, *Stevia rebaudiana*.

4.1 INTRODUCTION

Over the past few years, there is an increased hike of health promoting non-chemical-based sweeteners and food additives. Discovered in 1964, *Stevia rebaudiana* has emerged as one of the promising alternative natural bio-sweetener. Among the secondary metabolites, such as phenolics, terpenoids, alkaloids, the major group of bioactive compounds present in the plant include steviol glycosides, which are non-caloric in nature and are responsible for the sweetness of the plant. These diterpene glycosidic contents are synthesized and accumulated mainly in leaves, making it a source explant for extraction (Angelini et al. 2018). However, several reports define the presence of glycosides in stem, flowers and roots as well, although in lower amounts (Rajasekaran et al. 2007; Ramesh et al. 2006). Besides, *Stevia* has been found potent against diabetes, cancer, tuberculosis, hypertensive and inflammatory diseases, and possess hepatoprotective, antimicrobial and immunomodulatory effects (Ahmad and Ahmad 2018; Boonkaewwan et al. 2006; Ferri et al. 2006; Kataev et al. 2011; Takasaki et al. 2009; Wang et al. 2018).

The nutritional aspects have initiated the propagation of plant as a crop in Japan in 1968. *Stevia* cultivation and its demand as a dietary supplement got amplified over the years utilizing leaves up to 5000 metric tonnes per year and motivating the researchers for large-scale production of the plant. Seed propagation is difficult due to low germination rate, low viability and long storage conditions. Nevertheless, stem-cuttings could be the possible means of plant propagation, although, it is time-consuming and prone to seasonal variations that affects the productivity of the plant and steviol glycosidic content. The method also requires technical guidance without which the eminence of the plant is compromised. With respect to the quality and quantity, usage of micropropagation technology could delimit the chances of steviol glycosidic flux and enhance the supply of the plant on a commercial scale. Khalil et al. (2014b) reported micropropagation to be best the propagation method producing 85% survival than stem cuttings and seeds. There are numerous findings on micropropagation of *S. rebaudiana* by direct, using nodal segment or shoot-tip explants, and indirect means via callus formation as illustrated in **Table 1.3**. However, its scale-up and systematic propagation method is still awaited that needs to be made available to the farmers and plant growers. Some of the findings have mentioned about the large-scale propagation protocols as well (Khalil et al. 2014b; Khan et al. 2014a; Lata et al. 2013a, b; Thiyagarajan and Venkatachalam 2012). Of these reports, only Lata et al. (2013b) specified the quantification of rebaudioside A (3.4%) and stevioside (5.4%) from tissue culture derived plants, although, usage of Thidiazuron (TDZ) in their

micropropagation protocol increased the overall cost of the method. Apart from this, being a commercial plant, the central idea has always been to generate superior varieties, rich in rebaudioside A content, bringing forth lots of patented reports in this aspect.

Further, the link between diabetes and *Staphylococcus aureus* infection was a prompting step for choosing the assessment of the steviol glycosides against it, although, the crude extracts of the plant is known to possess anti-staphylococcal and non-cariogenic effects. Capable of anti-diabetic potential, the plant also possesses antioxidant property and have been significantly proven by some reports. A few literatures also evaluated these bioactivities in extracts from in vitro cell culture or shoots (Ahmad et al. 2011; Dey et al. 2013; Dwivedi et al. 2016; Hajihashemi and Ehsanpour 2014). Mostly, the published reports demonstrated the anti-oxidative effects due to presence of secondary metabolites, exclusively, phenolic compounds. However, none of the reports appraised the endowment of steviol glycosides to it.

The discussion in the previous paragraphs have adequately revealed that *Stevia rebaudiana* holds an important place in global scenario owing to its economic and medicinal properties. However, the benefits claimed in the previous reports cannot be utilized until the limitations on scaling operations on plant productivity and steviol glycosidic production are addressed. Therefore, the present study emphasized upon the development of reproducible and economical, large-scale micropropagation methodology of *S. rebaudiana* using two *Stevia* plant lines, Sr1 and Sr2, collected from two different sources of Guwahati, India. Additionally, clonal fidelity assessment was performed via ISSR molecular marker and flow cytometry to assert the true-to-type nature of the in vitro regenerants. Above all, steviol glycosidic contents were quantitatively evaluated leading to confirmation of rebaudioside A-rich in vitro elite plant lines. Hence, the target steviol glycosides were measured for their biofilm inhibitory or anti-bacterial potential by qualitative and quantitative means. The chapter also demonstrates upon the antioxidant potential of crude extracts as well as isolated bioactive steviol glycosides obtained from in vitro regenerated cultures, via DPPH assay.

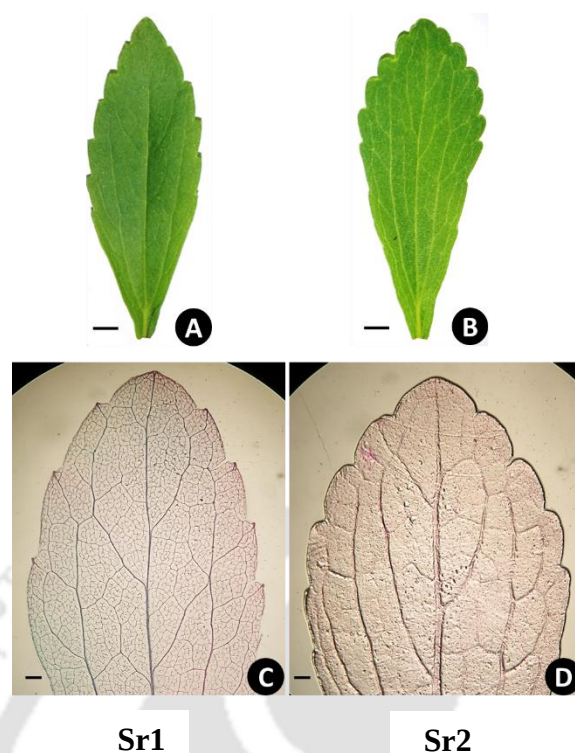


Figure 4.1: Experimental plant lines of *S. rebaudiana*

4.2 MATERIALS AND METHODS

4.2.1 Micropropagation and clonal fidelity assessment of true-to-type elite lines of *S. rebaudiana*

4.2.1.1 Media preparation

Initiation of nodal segment cultures were performed on Murashige and Skoog (MS; 1962), and Modified Driver and Kuniyuki Walnut (DKW₁; 1984) basal media (**Table 4.1**) consisting of macronutrients, micronutrients, iron and vitamins in stock concentrations similar to MS basal medium stock components mentioned in **Table 2.1**. DKW stock A, C, D, E were 100X concentrated, while stock B was 50X concentrated. They were accordingly diluted before adding them in the final media preparations. In modified DKW medium, henceforth, abbreviated as DKW₁, the vitamins and iron components were replaced by MS vitamins (200X concentrated) and iron constituents (200X concentrated). Myo-inositol (100 mg L⁻¹), a vitamin component, and sucrose, as carbon source, were added freshly at 3% concentration in the basal media during media preparation. Additionally, the basal media were fortified with plant growth regulators, such as 6-benzylaminopurine (BAP), 6-furfurylaminopurine (kinetin) and indole 3-butyric acid (IBA) either individually or in various combinations and the final volume of the

media was made up using distilled water. The pH of the medium was adjustment to 5.8 using 1N HCl and 1N NaOH. Agar (0.8%) or phytigel (0.2%) was added as solidifying agent in the medium. The medium was then poured in the required glass jars (60 mm dia × 120 mm height; 350 mL capacity) or test tubes (25 mm dia × 150 mm height; 50 mL capacity), closed and autoclaved at 121°C/15 psi for 20 min.

Table 4.1: Modified DKW basal medium stock composition*

Components	Amount (L ⁻¹)
STOCK A (100X)	
Ammonium nitrate	141.60 g
Calcium nitrate tetrahydrate	196.80 g
Zinc nitrate hexahydrate	1.70 g
STOCK B (50X)	
Potassium sulfate	77.95 g
STOCK C (100X)	
Magnesium sulfate heptahydrate	74.00 g
Manganese sulfate monohydrate	3.35 g
Copper sulfate pentahydrate	0.025 g
STOCK D (100X)	
Calcium chloride dihydrate	14.90 g
STOCK E (100X)	
Potassium dihydrogen phosphate	26.50 g
Boric acid	0.48 g
Sodium molybdate dihydrate	0.038 g
STOCK MS VITAMINS* (200X)	
Inositol	0.1 g
Nicotinic acid	0.1 g
Pyridoxine HCl	0.1 g
Thiamine HCl	0.02 g
Glycine	0.4 g
STOCK MS IRON* (200X)	
Iron (II) sulfate heptahydrate	5.56 g
Sodium EDTA dihydrate	7.46 g

* Indicating modified DKW basal medium (DKW₁) constituting DKW basal medium + MS vitamins + MS iron. DKW Iron supplements (3.38 g L⁻¹ Iron (II) sulfate heptahydrate and 4.54 g L⁻¹ Sodium EDTA dihydrate) were replaced with MS Iron supplements (5.56 g L⁻¹ Iron (II) sulfate heptahydrate and 7.46 g L⁻¹ Sodium EDTA dihydrate). DKW vitamins completely lack pyridoxine HCl, while, glycine and thiamine hydrochloride are present in 0.2 g L⁻¹ stock concentration, as compared to MS vitamins.

4.2.1.2 Surface sterilization and culture initiation

The experiments were initiated using Sr1 and Sr2 plant lines of *S. rebaudiana* (Accession number: GUBH 21653, Gauhati University, Guwahati, India), which were distinguished by morphological observations (**Table 4.2**). The parent plants of both the lines were maintained successfully at green house facility at IIT Guwahati, from where the young and disease-free shoots were taken for initiation of nodal segment cultures in vitro. After removing the leaves, nodal segments containing opposite axillary buds were used as explants. The explants were thoroughly washed in distilled water for 4-5 times to remove soil and other superficial contaminants. The explants were further washed with 5 drops of Tween-20 in distilled water, with gentle agitation for 15 min. After a thorough wash with sterile distilled water for 4-5 times, the explants were surface sterilized with 0.1% (w/v) mercuric chloride for 5 min. The explants were ready for inoculation after 4-5 times wash with sterile distilled water.

Table 4.2: Comparative analysis on morphological features of the experimental plant lines of *S. rebaudiana*

Characteristics	Sr1 line	Sr2 line
Plant height	50-60 cm	70-80 cm
Flowering period	September to December & April to June	September to December & April to June
Leaf shape and arrangement	Cuneate	Elliptic
Leaf base	Attenuate	Attenuate
Leaf tip	Acute	Acute
Leaf venation	Longitudinal	Longitudinal
Leaf margin	Serrated	Crenulated
Leaf length* (cm)	4.9 ± 0.3	6.3 ± 0.4
Leaf width* (cm)	1.9 ± 0.2	2.2 ± 0.2

* Mean ± SD of 24 samples of mature leaves.

Note: With respect to the leaf-margin, they are not well-developed in young leaves of Sr2 as compared to the Sr1 line. In addition to this, the stem of the former line is sturdy in both, young and full-grown mature plant, however, it is feeble in case of Sr1 line of *S. rebaudiana*.

4.2.1.3 Initiation of nodal segment cultures

The sterilized nodal segment explants were given a fresh oblique cut at both the ends before inoculating them on MS and DKW₁ media. The media were variously supplemented with BAP and kinetin at various concentrations. Basal MS and DKW₁ media without any growth regulator were used as controls. Responses in terms of percent cultures showing bud-break, time-taken required for the bud-break and shoot length were recorded till 4th week from the

time of inoculation. The cultures were incubated at standard culture room conditions, under 1000-1600 lux of light at photoperiod of 16 h light/ 8 h dark, temperature at $25\pm 2^{\circ}\text{C}$ and 50-60% relative humidity.

4.2.1.4 Multiplication and elongation of shoots

Multiple shoots obtained on $\text{DKW}_1 + 5\ \mu\text{M BAP}$ were again transferred, after 4 weeks, to the fresh medium of same composition. It again resulted into few more small shoots to the clusters and with little elongation of a few shoots. To achieve significant growth of the shoots, GA_3 at $0.5\ \mu\text{M}$ was added to the medium for the shoots to grow in clusters. After one week, the clusters of these GA_3 pre-treated shoots were transferred to $\text{DKW}_1 + 5\ \mu\text{M BAP} + 0.05\ \mu\text{M IBA}$ medium. At the end of growth cycle of 6 weeks, multiple, multinodal, elongated shoots were obtained, which were cut into solitary nodal segments and transferred to fresh medium $\text{DKW}_1 + 5\ \mu\text{M BAP} + 0.05\ \mu\text{M IBA}$ for further shoot multiplication in the glass vessel (60 mm dia $\times$ 120 mm height; 350 mL volume). In both the in vitro plant lines (Sr1 and Sr2), rate of shoot multiplication was calculated on the basis of average number of shoots $\times$ average number of nodes per nodal explant containing two opposite axillary buds, at the end of the multiplication cycle (growth period) of 6 weeks.

4.2.1.5 Rooting and hardening

Terminal ~ 4 cm portions of in vitro grown, elongated shoots were taken for rooting. The shoots were inoculated on MS and $\frac{1}{2}$ MS (only major salts reduced to half strength) basal media, each supplemented with IBA at 0.5 or $1\ \mu\text{M}$ concentrations. At the end of the growth cycle of 4 weeks, the rooted plantlets were carefully taken out from the test-tube and washed with sterile distilled water to remove the medium and transferred to plastic pots containing sterile soil and sand in 2:3 ratio for hardening at green house conditions.

4.2.1.6 Data analysis

A completely randomized experiments were designed in which 24-cultures or replicates were raised for each treatment, while all the experiments were repeated thrice. Single nodal explants having two axillary buds (as single replicate) were cultured in 25 mm $\times$ 150 mm (dia $\times$ height) glass test-tube containing 20 mL of the medium, for initiation of cultures. Similarly, terminal 4 cm long in vitro shoot was used as replicate, inoculated in glass test-tube of same capacity. The response for culture initiation and rooting were calculated using two-way ANOVA and the significant difference between the mean values was found via Tukey's multiple comparison

test at $p \leq 0.05$. Multiplication and elongation were performed in glass-vessels (60 mm dia $\times$ 120 mm height; 350 mL capacity) and each glass-vessel consisted of six in vitro nodal explants. Data pertaining to estimation of shoot length, number of nodes and rate of shoot multiplication was analyzed using one-way ANOVA. Values for all experiments are represented as mean $\pm$ standard deviation.

4.2.1.7 Clonal fidelity assessment

A) ISSR marker analysis

The leaves from randomly selected regenerated shoots were utilized for the study. Genomic DNA was isolated using cetyltrimethylammonium bromide (CTAB) methodology for PCR analysis using Inter Simple Sequence Repeats (ISSR) molecular markers of universal UBC series. DNA quantification was performed using Nano-spectrophotometer prior to assessment studies.

i) *DNA isolation*: The young leaf samples from in vitro regenerants and mother plants of Sr1 and Sr2 plant lines were obtained for their DNA extraction and immediately stored in liquid nitrogen. The samples were grinded in chilled mortar pestle using liquid nitrogen and the powder was transferred to sorbitol wash buffer (100 mM Tris-HCl pH 8.0, 5 mM Ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.35 M sorbitol, 3% Polyvinylpyrrolidone-30 (PVP-30) and freshly added 1% β -mercaptoethanol) and vortexed thoroughly, for elimination of polysaccharides. After the pre-wash step, the samples were extracted using CTAB methodology as followed in **Chapter 2 section 2.2.1.7Ai**. The DNA obtained were quantified using nanospectrophotometer (Implen, NP80) at 260 nm while DNA integrity was checked on 1% agarose gel.

ii) *PCR amplification*: Genetic integrity of the in vitro samples with respect to the mother plant was assessed using ISSR markers. Reaction mixture (20 μ L) for the PCR amplification consisted of 25 ng of template DNA, 2X PCR master mix (New England Biolabs, USA), consisting of 10 mM Tris-HCl, 1.5mM $MgCl_2$, 50mM KCl, 0.2 mM dNTPs and Taq DNA polymerase (25 units/mL), 200 nM of primers and nuclease-free water and was set-up for 35 PCR cycles. The conditions used for amplification began with initial denaturation at 95°C for 3 min and denaturation at 95°C for 20 secs, followed by annealing for 50 secs (Annealing temperature was primer specific as mentioned in **Table 2.5**). Extension was carried on at 72°C for 2 min followed by final extension at 72°C for 10 min. The PCR was held at 4°C for cooling

the amplified products. The amplified DNA products were run on 1.5% agarose with 100 bp ladder (Invitrogen, USA) and visualized at gel documentation system (Biorad, USA). Data was analyzed on the basis of only detectable scorable bands of in vitro plants and mother plants' amplified products.

iii) *Data analysis*: Manual scoring of individual resolved detectable bands of in vitro regenerants and the mother plant have been scored as '1' and '0' on the basis of their presence and absence in the gel for the documentation of monomorphic and polymorphic percentage among regenerants and the mother plant.

B) Flow cytometry: Fresh leaf samples (~50 mg) were taken from the in vitro regenerants and mother plant for the isolation and analysis of nuclear content. The samples were finely chopped in filtered nuclear isolation buffer under ice-cold conditions, using sharp razor blade (**Table 2.3**). Further, each sample was collected and filtered using 30 µm nylon net filter. They were incubated with RNase and PI and incubated for 15 min. The analysis was performed following the methodology described in **Chapter 2 section 2.2.1.7B**. Nearly, 40000 to 50000 nuclei were analyzed for each sample followed by their analysis on the basis of G_1 value as per following formulae.

$$\text{Sample 2C DNA content} = \frac{\text{sample } G_1 \text{ peak mean}}{\text{standard } G_1 \text{ peak mean}} \times \text{standard 2C DNA content (pg DNA)}$$

4.2.2 Extraction and chemical analysis of Sr1 and Sr2 elite plant lines of *S. rebaudiana*

4.2.2.1 Extraction methodology

Fresh leaves (~5 g) from mother plants were collected, in the month of April, and washed with distilled water for removal of soil and other surface contaminants. Similarly, the leaves from 1-month old, fully grown, elongated in vitro shoots were collected and utilized for the study. The samples were grinded in mortar and pestle with methanol (analytical grade) as solvent. The samples were further filtered through Whatman filter paper (GE healthcare, UK). The filtrate was collected while the residue underwent maceration with fresh methanol solvent thrice under room temperature. All the methanol filtrate was pooled and further fractionated using n-hexane to remove the non-polar portion of the extract. This process was repeated five times. Finally, the methanolic layer was collected and concentrated to dryness under vacuum at 50°C using rotary evaporator. The extract was re-dissolved in methanol and stored at -20°C for HPLC analysis.

4.2.2.2 Sample preparation and method optimization for HPLC

The methanolic leaf extracts were filtered through 0.22 μm nylon 6,6 membrane filter (Pall Corporation, New York, USA), prior to HPLC analysis and 10 μL of each sample was used as injection volume. The HPLC analysis was performed using Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with quaternary pump, UV detector and autosampler (100 μL). The samples were analyzed at 25 $^{\circ}\text{C}$ in Shimpack GIST C-18 column (250 $\times$ 4.6 $\times$ 5 μm ; Shimadzu Corporation, Kyoto, Japan). Mobile phase comprised of solvent A= MilliQ with 0.05% formic acid and solvent B= Acetonitrile in 20:80 at a flow rate of 0.6 mL min^{-1} . The column was washed and equilibrated with the solvent system, before the onset of the analysis. The peaks were monitored at 205 nm wavelength. This analysis was repeated thrice and each sample and standards were run in triplicates and the data was integrated and acquired via Lab Solutions software version 5.0 (Shimadzu, Kyoto, Japan).

4.2.2.3 Linearity and method precision

Standards stock solution of rebaudioside A and stevioside were prepared (each 1 mg mL^{-1}) in methanol. They were serially diluted and aliquots of standard stock solutions were prepared from 0.0312 mg mL^{-1} to 0.5 mg mL^{-1} , for calibration studies. HPLC chromatograms generated were represented as *y axis* = absorbance (mAU) and *x axis* = retention time (min). Each standard was run in triplicates and at least once before analyzing the samples in HPLC. Linear regression equation was generated ($y=mx+c$, where *y* is area and *x* is concentration) for each standard, along with R^2 (coefficient of determination). Intra and inter day HPLC analysis was also conducted, where, the samples were run in triplicates on similar day as well as on alternate days, for assessing the reproducibility of the method. The precision studies were calculated in the form of coefficient of variation (% CV) which is stated as (standard deviation / mean $\times$ 100). An optimum sample should possess CV less than 15%.

4.2.2.4 Mass spectrometric studies

Identification of rebaudioside A and stevioside was further characterized through mass spectrometry coupled with electrospray ionization using quadrupole time-of-flight detector (MS-ESI-QTOF). The standards and the samples were analyzed in both positive and negative mode. However, better ionization was observed in negative mode. Purified HPLC eluents of rebaudioside A (4.3 min) and stevioside (5.01 min) were collected from the samples. Respective eluents were pooled, concentrated and analyzed via mass spectrometry along with the crude extracts. The conditions for the analysis include desolvation gas flow rate at 450 L h^{-1} .

¹, cone gas flow rate at 50 L h⁻¹ and capillary voltage at 2500 V. Mass-to-charge ratio was obtained in full scan mode from 100 to 1500 *m/z* values and the spectra were studied and obtained using MassLynx software (4.1).

4.2.3 Inhibition test for biofilm formation of *S. aureus*

4.2.3.1 Microbial strain and culture conditions

For biofilm inhibition studies, *S. aureus* strain (MTCC 737; procured from NCCS, Pune) was used. It was cultivated in Mueller Hinton (MH) medium by continuous stirring, overnight, at 180 rpm and at 37°C temperature. Prior to analysis, optical density (OD) of the bacterial culture was adjusted to 0.5 ($\sim 1 \times 10^8$ CFU mL⁻¹) by following the McFarland standard method. Then 4 μ L ($\sim 6 \times 10^5$ CFUs) of the microbial culture was added to each well containing the purified compounds of rebaudioside A and stevioside. The experiments were conducted in triplicates and represented as mean $\pm$ SD.

4.2.3.2 Determination of Minimum Inhibitory Concentration (MIC)

MIC determination was performed using broth dilution methodology in 96-well microtiter plate format. The HPLC purified steviol glycosides, rebaudioside A and stevioside, were dissolved in 2% DMSO (Merck, India), and diluted in serial diluted concentration range of 3.12 μ g mL⁻¹ to 1000 μ g mL⁻¹. Kanamycin (20 μ g mL⁻¹) were used as positive control, and 2% DMSO being the negative control. The plates were incubated for 24 h and 48 h incubation at 37°C. Thereafter, microbial growth was assessed at 600 nm via microplate reader (Tecan, Switzerland). The MIC was determined on the basis of no visible growth (clear wells) atleast after 24 h of treatment.

4.2.3.3 Biofilm inhibition assay

Purified rebaudioside A and stevioside (3.12 μ g mL⁻¹ - 1000 μ g mL⁻¹), each of 100 μ L volume was added to the wells individually, along with overnight grown microbial culture ($\sim 6 \times 10^5$ CFU mL⁻¹). The mixture was incubated for 24 h and 48 h duration at 37°C. Thereafter, biofilm inhibition assay was performed as described in **Chapter 3 section 3.2.6.3**. The absorbance was recorded at 530 nm and Minimum biofilm inhibition concentration (MBIC) after safranin staining was calculated as:

$$\text{Inhibition\%} = (\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}) / (\text{OD}_{\text{control}}) \times 100$$

4.2.3.4 Microscopic assessment of biofilm via light and fluorescence microscopy

Biofilm inhibition methodology, as mentioned in **Chapter 3 section 3.2.6.3**, was performed and the compounds at their MBIC were used as biofilm inhibition concentration. The microscopic studies were carried in 24-well plate. After incubation, the remaining surface-associated bacterial biofilm in the well was stained with 0.01% acridine orange (Sigma-Aldrich, Japan). The microscopic studies were carried out in 24-well plate and observed under bright-field and fluorescence microscopy using inverted fluorescence microscope under blue light filter (Nikon TiS, Japan). All the image as analyzed using NiS elements (version 4.5).

4.2.3.5 Surface morphological evaluation of *S. aureus* after steviol glycoside treatment

The plausible action of rebaudioside A and stevioside on the morphological, structural changes on *S. aureus* and its biofilm formation was qualitatively visualized using field emission scanning electron microscope (FESEM). The microorganism was co-incubated with purified compounds in 96-well microtiter plate. The methodology on analysis was followed as described in **Chapter 3 section 3.2.6.5**. According to this, 4 μL ($\sim 6 \times 10^5$ CFUs) of the microbial culture was incubated with steviol glycosides at their MBIC concentration. The bacterial colonies were fixed in 2.5% glutaraldehyde for 5-6 h. Subsequently, they were washed with distilled water and dehydrated gradually in ethanol series by passing the sample in each series for 15 min. After overnight air-drying the sample, they were spur-coated with gold and analyzed using FESEM (Zeiss, Germany).

4.2.4 Assessment of antioxidant potential of extracts and purified steviol glycosides using DPPH radical scavenging assay

The HPLC purified rebaudioside A and stevioside as well as the crude extracts of in vitro and ex vitro plant leaves of the two *Stevia* line, Sr1 and Sr2 were assessed for antioxidant potential. The 100 μL sample was added in 96-well plate in serially diluted concentrations ($3.12 \mu\text{g mL}^{-1}$ to $1000 \mu\text{g mL}^{-1}$). To this, 0.13 mM of DPPH reagent (Sigma-Aldrich, Japan) dissolved in HPLC grade methanol was added to each well, pipetted well, wrapped in foil and incubated in dark for 1 h. Ascorbic acid (Sigma-Aldrich, Japan) was used as positive control while the DPPH reagent without any treatment was used as negative control. The reading was measured at 515 nm via microplate reader (Tecan, Switzerland). Each sample was run in triplicates while experiment was repeated thrice. The radical scavenging percentage was calculated as:

$$\% \text{ DPPH scavenging} = (\text{Abs}_{\text{DPPH}} - (\text{Abs}_{\text{Sample}})) / (\text{Abs}_{\text{DPPH}}) \times 100$$

In addition to this, linear trend line was generated for each sample for the determination of IC₅₀ (concentration at which 50% of DPPH radicals are neutralized) and IC₉₉ values (concentration at which 99% of DPPH radicals are neutralized), using straight line equation.

4.2.5 Statistical analysis

For chemical and anti-staphylococcal analysis, observations were made on the basis of three separate experiments and the values are represented as mean $\pm$ standard deviation. Lastly, the antioxidant effect was statistically analyzed using one-way ANOVA and the significant difference between the mean values was found via Tukey's multiple comparison test at $p < 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Surface sterilization and initiation of cultures

The single node segments, bearing two opposite axillary buds, were utilized to raise true-to-type plantations of *Stevia rebaudiana* (family Asteraceae). Establishment of nodal segments was carried out during the summer season (March to July). The nodal explants, surface sterilized with Tween-20 and 0.1% HgCl₂, generated 72.2% aseptic cultures. Other reports also concluded that mercuric chloride (0.1%) is the most effective surface sterilization agent to establish in vitro cultures of *Stevia* (Pawar et al. 2015; Singh et al. 2017). The extremely high/low temperature, high humidity and weather fluctuations in different time of the year have critical impact on the overall growth of *Stevia* and steviol glycosidic production (Angelini et al. 2014; Tavarini et al. 2015). The nodal explants collected in different season, gave poor survival percentage and mostly showed fungal contamination. This could be due to predominant higher precipitation rate and moisture content in the environment in North-eastern part of India. Therefore, the nodal segment explants were collected from the mother plants to raise in vitro cultures, during the months from March to July (**Figure 4.3A** and **Figure 4.4A**). For subsequent micropropagation of *Stevia*, the nodal segments were collected from established in vitro plants only to avoid dependency of season for the availability of the explants. With this procedure, 100% contamination-free cultures were raised throughout the year.

4.3.2 Induction of axillary-bud proliferation

The sterilized nodal segments of both the plant lines, Sr1 and Sr2, were inoculated on MS and DKW₁ basal media fortified with cytokinins, 6-benzylaminopurine (BAP) and 6-

furfurylaminopurine (kinetin) at various concentrations. Bud-break response from axillary buds on MS and DKW₁ basal media was <23% in nodal segment explants. The response on percent bud-break improved upon addition of cytokinins, BAP and kinetin, to the basal media. Cytokinins are predominantly effective in triggering the process of shoot development in several plant species, such as *Azadirachta indica* (Chaturvedi et al. 2003), *Scutellaria alpine* (Grzegorzczak-Karolak et al. 2015), grapevine (Novak and Jůvová 1983), *Spilanthes acmella* (Singh and Chaturvedi 2010) and apple (Srisankarajah et al. 1990). Of the two cytokinins tested, BAP was found to be better in terms of enhanced shoot growth and higher bud-break frequency and was found prolific in long-term cultures. Similar observation was also noticed by other authors working on the micropropagation aspects of *Stevia* (Khalil et al. 2014; Pawar et al. 2015; Yang et al. 1981).

Eventually, in the present study, induction of bud-break in nodal segment cultures, bearing two axillary buds, started on 3rd day of culture initiation and it varied up to 16 days on different media (**Figure 4.2A** and **Figure 4.3A**). Development of a cluster of 3-4 shoots was noticeable, after 4 weeks of initiation of cultures, on DKW₁ medium containing 5 µM BAP, where an average of 2.4 cm long shoots developed in Sr1 and Sr2 lines. As per current study, modified DKW medium (DKW₁) was found better in terms of higher bud-break frequency and shoot proliferation, compared to the response obtained on MS medium (**Table 4.3**). Among the BAP concentrations tested, insignificant shoot proliferation was observed at 1 µM BAP, while higher concentrations of BAP at 7.5 µM and 10 µM, led to the development of hyper-hydric shoots with flappy leaves. Overall, DKW₁ + 5 µM BAP medium gave best response and 100% of cultures showed bud-break within 4 weeks in both the plant lines, Sr1 and Sr2 (**Table 4.3**). Ahmed et al. (2007) reported bud-break response in 85.3% nodal explants, within 6 weeks of culture initiation, on MS + 6.6 µM BAP + 2.3 µM kinetin medium.

Table 4.3: Influence of growth regulators on axillary bud proliferation in *S. rebaudiana*

PGR concentrations in μM		Sr1 line			Sr2 line		
BAP	Kinetin	% Bud break	Time taken for bud- break (days)	Shoot length (cm)	% Bud break	Time taken for bud- break (days)	Shoot length (cm)
0.0	0.0	12.5	15	$0.2^a \pm 0.1$	18.5	16	$0.2^A \pm 0.1$
1.0	0.0	20.8	8	$0.3^b \pm 0.6$	25.9	8	$0.2^B \pm 0.1$
5.0	0.0	58.3	4	$1.4^{ab} \pm 1.0$	55.6	4	$1.4^{AB} \pm 0.5$
7.5	0.0	50.0	5	$1.2^{bc} \pm 1.1$	37.0	5	$1.1^{ABC} \pm 0.3$
10.0	0.0	37.5	5	$1.3^{bcd} \pm 1.1$	29.6	5	$1.1^{ABC} \pm 0.8$
0.0	3.0	25.9	8	$1.2^{acd} \pm 0.4$	18.5	8	$1.5^A \pm 0.5$
0.0	5.0	29.6	8	$1.8^{ace} \pm 0.6$	29.6	8	$1.6^{A*} \pm 0.7$
PGR concentrations in μM		Sr1 line			Sr2 line		
BAP	Kinetin	% Bud break	Time taken for bud- break (days)	Shoot length (cm)	% Bud break	Time taken for bud- break (days)	Shoot length (cm)
0.0	0.0	0.0	12	$0.2^a \pm 0.1$	22.2	12	$0.2^A \pm 0.1$
1.0	0.0	74.1	6	$0.3^b \pm 0.2$	59.3	7	$0.3^B \pm 0.2$
5.0	0.0	100	3	$2.4^a \pm 1.0$	100	3	$2.4^{A*} \pm 0.8$
7.5	0.0	85.2	4	$1.2^{ab} \pm 0.8$	96.3	4	$1.3^{AC*} \pm 0.7$
10.0	0.0	100	5	$1.4^{abc} \pm 0.7$	100	5	$1.9^{A*} \pm 0.6$
0.0	3.0	33.3	6	$1.3^{ad} \pm 0.5$	22.5	6	$1.1^{ADE} \pm 0.6$
0.0	5.0	40.7	6	$1.4^{ae} \pm 0.5$	37.0	7	$1.2^{ADF} \pm 0.4$

Control: MS and DKW₁ basal media are devoid of growth regulators. Growth period is 4 weeks. PGR: Plant growth regulators.

Data are represented as mean $\pm$ standard deviation (SD), each with 24 replicates. All the values are calculated by two-way ANOVA (Tukey's multiple comparison test at $p \leq 0.05$). Different lower- and upper-case letters denote the data is not significant with each other; '*' represents $p < 0.05$, and other values represents $p \leq 0.0001$.

4.3.3 Elongation and mass multiplication of shoots

The best medium for highest percentage of shoot induction in lesser time from nodal segment explants was DKW₁ medium consisting of 5 µM BAP. The medium induced multiple shoots development where a cluster of 3-4 small shoots were observed within 4 weeks of raising the cultures (**Figure 4.2B** and **4.3B**). On transferring the cluster of shoots to the fresh medium of same composition, emergence of additional number of small shoots were seen, making a cluster of 6-7 shoots. Slight increase in the length of a few shoots was also observed but significant improvement in the length of shoots was not observed. In order to attain substantial shoot proliferation and elongation, the shoot cultures were given a pre-treatment of 0.5 µM GA₃ for one-week duration, followed by their subsequent transfer to DKW₁ + 5 µM BAP + 0.05 µM IBA medium. After the growth cycle of 6 weeks, multinodal, elongated, multiple and branched shoots were obtained, in Sr1 and Sr2 plant lines (**Figure 4.2C** and **4.3C**). Supplementation of IBA at low concentration in the medium promoted the shoot proliferation of the plant while gibberellic acid pretreatment enhanced the length of the shoots. Thereafter, the multinodal, elongated shoots were cut into individual nodal segments, bearing two axillary buds, and subcultured to the same medium composition for next cycle of shoot multiplication. The shoots grew well and attained an height of 7.1 cm with 10.0 number of shoots and 5.4 number of nodes per in vitro shoot in Sr1 plant line (**Figure 4.2D**). Similar outcome was also observed in the Sr2 line of *S. rebaudiana*, where, multinodal, multiple shoots were obtained with shoot length of 6.8 cm and 11.0 number of shoots with 4.8 number of nodes per in vitro shoot (**Figure 4.3D**). Occasionally, the shoots reached up to a maximum height of 20.0 cm in both the plant lines. By following the protocol mentioned here, and the higher rate of shoot multiplication was achieved as 54.04-fold and 52.06-fold per nodal explant, in Sr1 and Sr2 plant lines (**Table 4.4**) at every 6 weeks.

Table 4.4: Shoot proliferation using nodal explants of *S. rebaudiana*

Plant lines	Shoot length (cm)	Number of shoots	Number of nodes	Rate of multiplication
Sr1	7.1 ± 0.9	10.0 ± 2.5	5.4 ± 1.0	54.0
Sr2	6.8 ± 1.2	11.0 ± 2.0	4.8 ± 0.7	52.1

The data is represented as mean ± SD of three independent experiments, each analyzed with 24 replicates. Growth period is 6 weeks. Rate of multiplication is calculated as number of shoots × number of nodes per nodal explant containing two opposite axillary buds, at the end of the multiplication cycle (growth period) of 6 weeks.

Blend usage of BAP with auxins was experimented by various researchers in obtaining higher rate of shoot multiplication in *Stevia*. Laribi et al. (2012) obtained 92.1% response on MS + 4.4 μM BAP and 2.5 μM IBA for shoot-bud induction in *S. rebaudiana*. Similarly, in another report, the medium was fortified with 0.5 mg L^{-1} of IAA, IBA and NAA in combination with BAP (1 mg L^{-1}), where MS medium supplemented with IAA + BAP medium resulted into 92% shoot regeneration (Thiyagarajan and Venkatachalam 2012). This combination of growth regulators (BAP with auxin) was found to be favorable for enhanced shoot multiplication in *Azadirachta indica* (Srivastava and Chaturvedi 2011) and *Eucalyptus globulus* (Trindade et al. 1990). The authors described that auxin usage compensates with low cytokinins levels and, thereby, promotes shoot growth. On the other hand, pretreatment of shoots with GA_3 was found to be promotory in other plant species as well, such as *Azadirachta indica*, where 15 days of pretreatment triggered the elongation of in vitro shoots (Srivastava and Chaturvedi 2011). Role of gibberellic acid in stimulating the process of elongation in in vitro shoots is quite widespread and it has been critically emphasized in terms of physiological, molecular and biochemical ways (Jones and Kaufman 1983). According to supporting literature, Thiyagarajan and Venkatachalam (2012) obtained maximum rate of multiplication of 123 shoots/explant (49 shoots/nodal segment in 1st subculture) after subsequent three-subcultures on MS + 1 mg L^{-1} BAP in 65 days. Another work reported the large-scale in vitro production of shoots in *S. rebaudiana* by indirect organogenesis, producing 106 shoots/explant on MS + 3 mg L^{-1} BAP in 4 weeks (Khalil et al. 2014). However, it poses higher genetic variability in terms of long-term cultures. The present investigation achieved 54.0- and 52.1-fold shoot multiplication per nodal explant per cycle of 6 weeks growth phase. The results are quite favorable for large-scale shoot multiplication of both plant lines of *S. rebaudiana*. Until before, none of the reports mentioned the usage of DKW medium for large-scale propagation of *S. rebaudiana*.

4.3.4 Rooting and transplantation

To develop complete plantlets, terminal 4 cm long portion from elongated in vitro developed shoots were transferred on MS medium supplemented with an auxin, indole-3-butyric acid (IBA) at 0.5 μM and 1.0 μM concentration. MS basal medium was used at full and half strength (macronutrients reduced to half strength) concentration (Table 4.5). The highest percentage of root development was observed on MS + 0.5 μM IBA, where 88.9% of Sr1 plant lines (Figure 4.2E) and 94.4% of Sr2 plants lines were rooted (Figure 4.3E). The root initiation occurred directly from the basal end of the shoot in 9 days and it was accompanied with further growth of shoots. On the other hand, ½ MS (major salts reduced to half strength) with 0.5 μM IBA

medium was also beneficial in rooting of the shoots within 12 days. It could be noted that higher concentration of auxin at 1 μM imparted callusing at the base of the shoot in both MS and $\frac{1}{2}$ MS basal media. Rooting on these media are observed after two-weeks. Similarly, delayed rooting was also observed on the basal media, MS and $\frac{1}{2}$ MS and only <30% of the cultures responded. Sivaram and Mukundan (2003) also reported rooting of *Stevia* shoots on $\frac{1}{2}$ MS + 4.9 μM IBA medium in 100% cultures with subsequent shoot elongation. Correspondingly, several other reports emphasized upon the usage of IBA as rooting plant growth regulator (Beniwal et al. 2018; Sreedhar et al. 2008; Thilakarathne et al. 2019). Researchers also mentioned upon the usage of IAA (Kumari and Chandra 2015; Singh et al. 2017; Yücesan et al. 2016), BAP+IBA (Giridhar et al. 2010), NAA+IBA (Khalil et al. 2014; Khan et al. 2014) and NAA (Yang et al. 1981) for successful root initiation in *Stevia*.

After a period of 4 weeks of rooting cycle, the in vitro plantlets were transferred to hycotrays containing sterile soil: sand in 2:3 ratio and maintained in the greenhouse at $30\pm 2^\circ\text{C}$ temperature and 70-80% humidity. It gave rise to 70% survival rate (**Figure 4.2F and 4.3F**). After two weeks, the plants were transferred to the field conditions for acclimatization. Usage of sand was found to be productive, promote proper drainage of the potting mixture and, thereby, prevent water logging phenomenon. The reported literatures mentioned the hardening of in vitro plantlets with 70-100% survival rate in mixture of soil, sand and peat moss at 1:1:1 ratio (Ojha et al. 2010), sand, soil and vermicompost at 1:1:1 ratio (Kalpana et al. 2010) and in sandy loam soil (Yang et al. 1981).

Table 4.5: Influence of IBA concentration on rooting in *S. rebaudiana*

Medium composition	Sr1 line		Sr2 line	
	Days	% shoots rooted	Days	% shoots rooted
MS	18	27.8 ± 18.8	20	22.2 ± 9.6
$\frac{1}{2}$ MS	25	11.1 ± 3.1	22	5.5 ± 9.6
MS + 0.5 μM IBA	9	88.9 ± 9.6^a	9	94.4 ± 9.6^A
$\frac{1}{2}$ MS + 0.5 μM IBA	12	83.3 ± 16.7^b	12	88.9 ± 9.6^B
MS + 1 μM IBA [§]	18	$61.1 \pm 19.3^*$	20	55.6 ± 9.6
$\frac{1}{2}$ MS + 1 μM IBA [§]	15	$72.2 \pm 9.6^*$	14	$77.8 \pm 16.7^*$

$\frac{1}{2}$ MS (Major salts reduced to half strength). Growth period: 4 weeks. Control: MS and $\frac{1}{2}$ MS basal medium without any growth regulator.

Data are represented as mean $\pm$ SD of three independent experiments, each with 24 replicates. The values have been statistically analysed using two-way ANOVA (Tukey's multiple comparison test), wherein, '*' signifies $p > 0.05$, and other values represents $p \leq 0.0001$. Different lower and upper letters signify 'not significant' with each other.



Figure 4.2: Micropropagation of Sr1 plant line of *S. rebaudiana* through nodal segment cultures

- (A) A nodal segment inoculated on $DKW_1 + 5 \mu M$ BAP (Bar=0.25 cm),
- (B) Same as A, 4-week-old culture, showing multiple shoots from axillary buds (Bar=1 cm),
- (C) A shoot from B, after pre-treatment with $0.5 \mu M$ GA_3 , showing significant shoot growth on $DKW_1 + 5 \mu M$ BAP + $0.05 \mu M$ IBA in 6-weeks (Bar=0.6 cm),
- (D) Multinodal, elongated, multiple shoots obtained on $DKW_1 + 5 \mu M$ BAP + $0.05 \mu M$ IBA in 6-weeks (Bar=1 cm),
- (E) A shoot from D, rooted on MS + $0.5 \mu M$ IBA within two-weeks (Bar=0.7 cm),
- (F) Hardened plants in sand:soil (3:2) in greenhouse after two-weeks (Bar=2.8 cm).

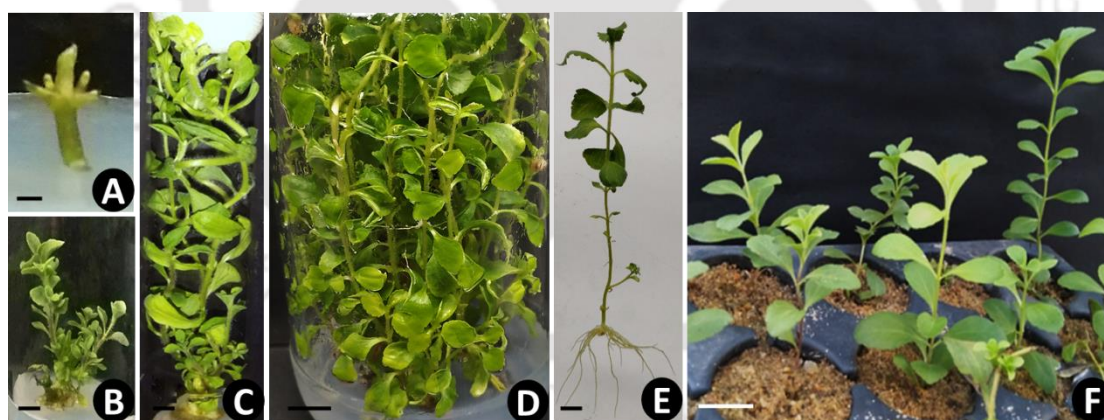


Figure 4.3: Micropropagation of Sr2 plant line of *S. rebaudiana* through nodal segment cultures

- (A) A nodal segment inoculated on $DKW_1 + 5 \mu M$ BAP (Bar=0.25 cm),
- (B) Same as A, 4-week-old culture, showing multiple shoots from axillary buds (Bar=1 cm),
- (C) A shoot from B, after pre-treatment with $0.5 \mu M$ GA_3 showing significant shoot growth on $DKW_1 + 5 \mu M$ BAP + $0.05 \mu M$ IBA in 6-weeks (Bar=0.7 cm),
- (D) Multinodal, elongated, multiple shoots obtained on $DKW_1 + 5 \mu M$ BAP + $0.05 \mu M$ IBA in 6-weeks (Bar=1 cm),
- (E) A shoot from D, rooted on MS + $0.5 \mu M$ IBA within two-weeks (Bar=0.7 cm),
- (F) Hardened plants in sand:soil (3:2) in greenhouse after two-weeks (Bar=2.3 cm).

4.3.5 Clonal fidelity assessment

The veracity of traits in the in vitro generated clones could be skilfully assessed via DNA-based molecular markers and flowcytometric analysis. Former technique offers reproducibility and accuracy in data analysis, while the latter is a rapid technique for analysing large-scale sample data. Both the techniques assist in determining the genetic diversity among the in vitro generated plantlets as the plants are exposed to various physical and chemical environments during various stages of micropropagation, that may induce the change in genetic constitution of the cells sometimes.

4.3.5.1 Clonal fidelity assessment via ISSR marker analysis

DNA extraction methodology was adopted as mentioned in **Chapter 2 section 2.2.1.7Ai**. In case of *S. rebaudiana*, DNA extraction was an intricate experiment pertaining a high content of phenolic compounds and polysaccharides, which are common contaminations of downstream processing of nucleic acids. Therefore, the current DNA extraction protocol pertaining mainly to the sorbitol pre-wash was an important step in removal of polysaccharides and phenolic compounds. The gelatinous polysaccharides, if not properly removed, causes turbidity in the final extracted DNA, and, thereby, resulting improper primer binding during polymerase chain reaction (PCR) amplification. In addition to this, PVP along with β -mercaptoethanol reduced the phenolic content of the plant sample. Reduction in protein levels was carried on with the addition of phenol-chloroform-isoamyl alcohol. A thorough wash with chloroform-isoamyl alcohol successfully removed the residual effects of phenol. Finally, the purified DNA was checked for its quality and quantity for further PCR reactions. Similarly, few researchers reported the extraction of high-quality nucleic acid from varied plant samples rich in phenolics, cambium and herbarium samples.

ISSR marker analysis: In vitro leaf samples from five randomly selected plants, each from Sr1 and Sr2 lines, were chosen for the study and the data was compared with the sample from leaves of the mother plant. Genomic DNA was used at 25 ng μL^{-1} . While the annealing temperature was set up according to the specificity of the UBC primers, in case of *Stevia*. All the ISSR fingerprinting analysis was carried out on with dinucleotide primers, which also reflected variable intensities. Similar repeats of 'AG'/'GA' and 'AC'/'CA' (**Table 2.5**) produced discrete scorable bands with least polymorphisms. Blair et al. (1999) also evaluated the genetic diversity in *Oryza sativa* using ISSR molecular markers and concluded the variable specificities of di-, tri-, tetra- and penta-nucleotide primers on DNA polymorphism.

The mother plants and its respective micropropagated plants were analysed for their clonal fidelity assessment by scoring of the amplified sequence products obtained by PCR of genomic DNA. A total of 998 bands were amplified using 8 ISSR primers, with 11 and 10.4 mean number of amplified bands per primer per experimental line (**Figure 4.4A, B, C, D**). The range of amplification varied from minimum of 500 (UBC-817) to 4500 bp (UBC-834). Percent monomorphism was observed to be 98.0% and 95.2% among the regenerated clones and mother plants of Sr1 and Sr2 lines (**Table 4.6**). The results obtained were also substantiated with other similar reports. Lata et al. (2013) also utilized UBC series ISSR primer for genetic fidelity analysis of *S. rebaudiana* and reported no variation in the in vitro clones. In another study, Ramírez-Mosqueda et al. (2016) evaluated 16.6% polymorphism in in vitro clones of *S. rebaudiana* generated via temporary immersion system.

Table 4.6: List of ISSR primers and their amplified bands per primer and monomorphic percentage of randomly selected in vitro regenerants of *S. rebaudiana*

S. No.	UBC series	Sr1 line		Sr2 line		Range of amplification (bp)
		No. of scorable bands	% monomorphism	No. of scorable bands	% monomorphism	
1	807	14	98.7	14	94.9	700-2200
2	808	9	100	9	94.4	900-3000
3	809	10	95.0	9	88.9	600-3000
4	811	14	100	15	96.7	800-4500
5	817	11	98.5	10	95.0	500-3000
6	820	8	91.7	4	95.8	1300-4000
7	826	12	100	11	95.5	700-4000
8	834	10	100	11	100	600-4500
Total		88	98.0	83	95.2	500-4500

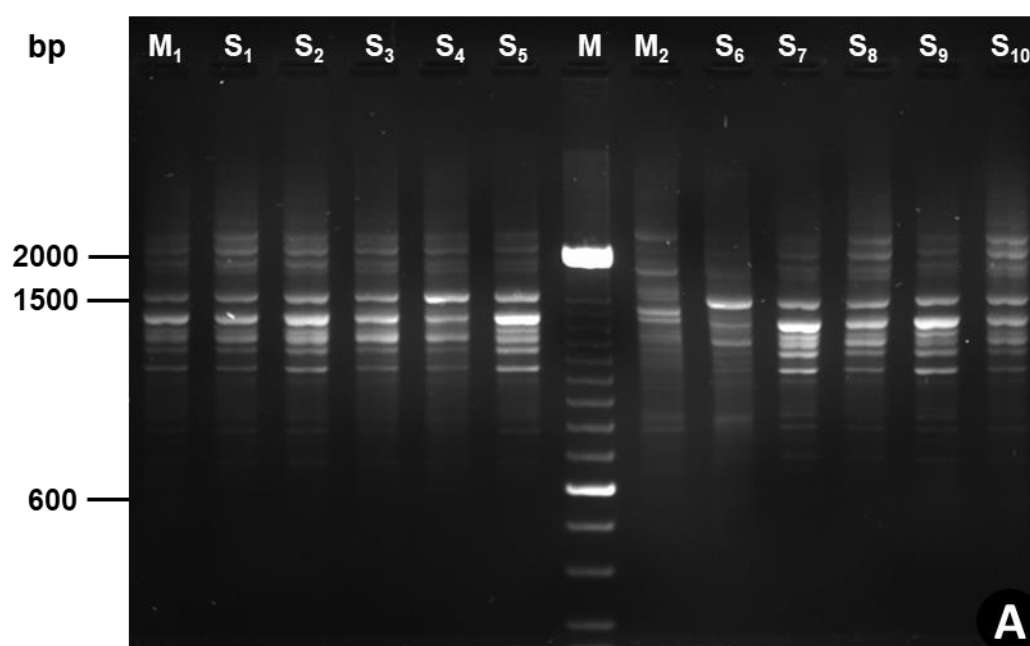


Figure 4.4 (A): ISSR marker analysis of mother plant and randomly selected in vitro clones of *S. rebaudiana* using UBC-807 primer.

Lane M: 100 bp ladder (Invitrogen); lane M1 and M2: Mother plants of Sr1 and Sr2 plant lines; lane S₁-S₅: In vitro raised clones of Sr1 plant line, and S₆-S₁₀: In vitro raised clones of Sr2 plant line.

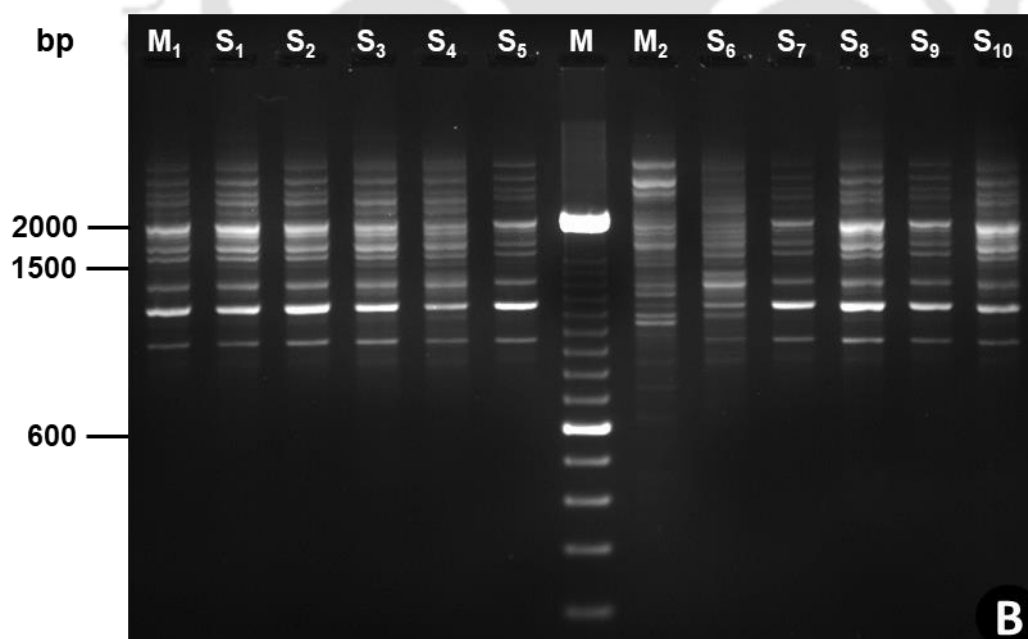


Figure 4.4 (B): ISSR marker analysis of mother plant and randomly selected in vitro clones of *S. rebaudiana* using UBC-811 primer.

Lane M: 100 bp ladder (Invitrogen); lane M1 and M2: Mother plants of Sr1 and Sr2 plant lines; lane S₁-S₅: In vitro raised clones of Sr1 plant line, and S₆-S₁₀: In vitro raised clones of Sr2 plant line.

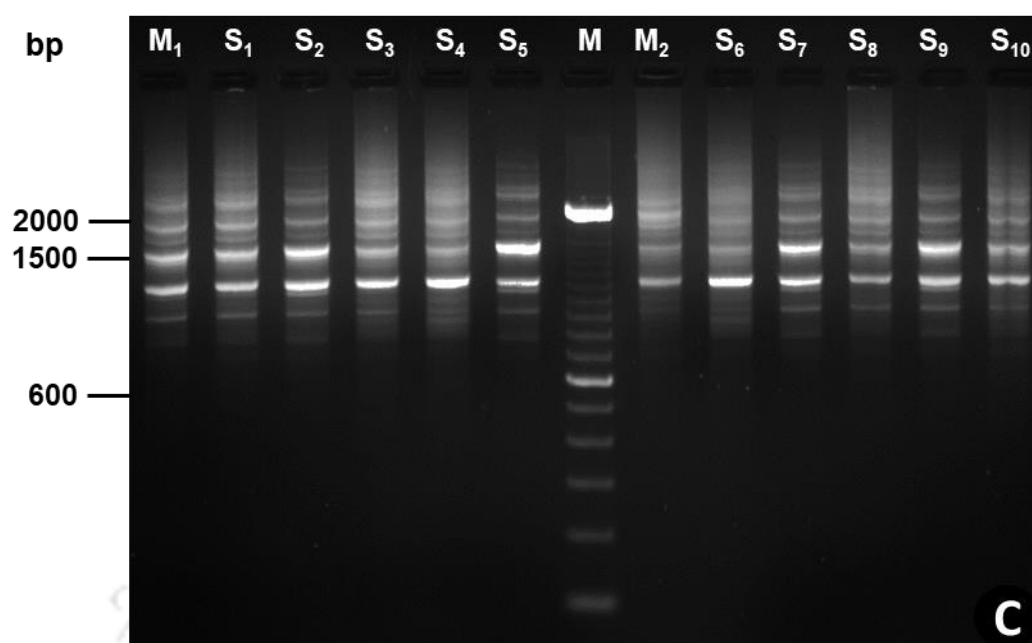


Figure 4.4 (C): ISSR marker analysis of mother plant and randomly selected in vitro clones of *S. rebaudiana* using UBC-826 primer.

Lane M: 100 bp ladder (Invitrogen); lane M1 and M2: Mother plants of Sr1 and Sr2 plant lines; lane S₁-S₅: In vitro raised clones of Sr1 plant line, and S₆-S₁₀: In vitro raised clones of Sr2 plant line.

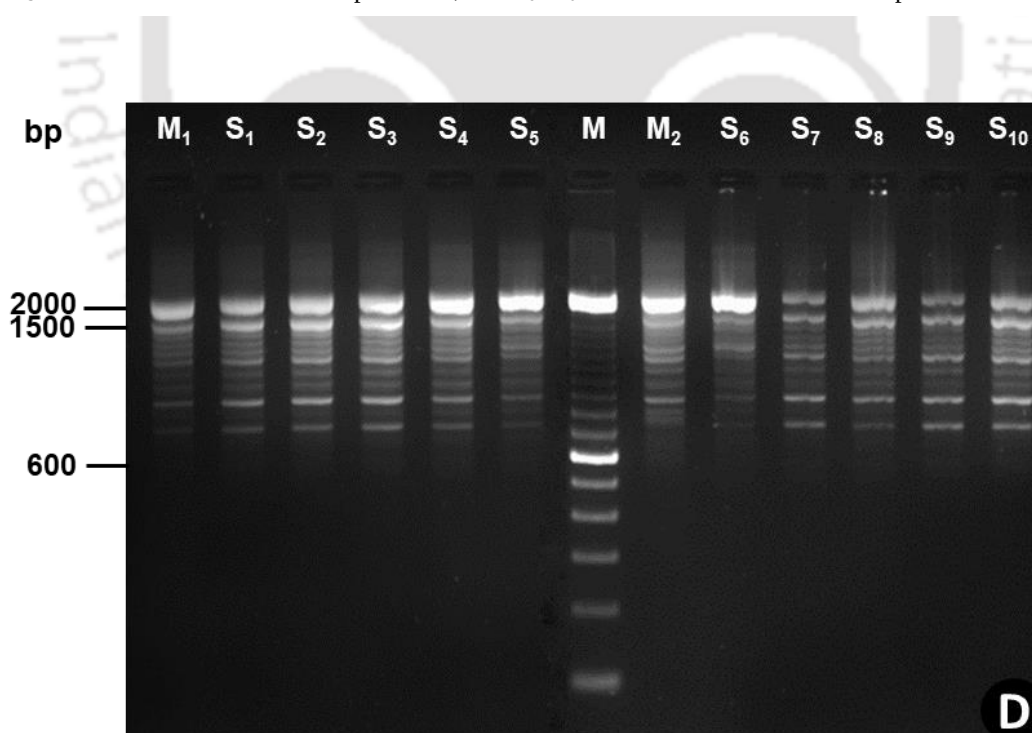


Figure 4.4 (D): ISSR marker analysis of mother plant and randomly selected in vitro clones of *S. rebaudiana* using UBC-834 primer.

Lane M: 100 bp ladder (Invitrogen); lane M1 and M2: Mother plants of Sr1 and Sr2 plant lines; lane S₁-S₅: In vitro raised clones of Sr1 plant line, and S₆-S₁₀: In vitro raised clones Sr2 plant line.

4.3.5.2 Genome size estimation using flow cytometry

Application of flow cytometry have recently been utilized in clonal fidelity assessment. The technique assist by analysing the in vitro regenerants for their nuclear DNA content and ploidy status (Bošnjak Mihovilović et al. 2019). Generally, tissue culture system tends to induce somaclonal variations especially during cell culture studies or indirect organogenesis. In the present pioneering study, clonal fidelity as well as genome size of *S. rebaudiana* have been evaluated using flow cytometry, taking *Vigna radiata* (1.20 pg genome size; Marie and Brown (1993)), as reference standard. Leaves from in vitro developed Sr1 and Sr2 plant lines of *Stevia* were analysed. Sample preparation was a crucial step to reduce the contamination of phenolic compounds, which otherwise interfere during nuclear staining. Phenolic contaminants were removed using PVP and β -mercaptoethanol as the most suitable buffer constituents.

The analysis showed synchronization in the ploidy status of in vitro regenerants and mother plant (**Figure 4.5A, B, C, D**). Mean G₁ channel positions, coefficient of variation (CV%) and relative DNA content was found to be correlated as reflected in the molecular marker analysis (**Table 4.7**). Genome size of the plant was calculated to be 1.9-2.03 bp (2.03-2.1 pg/2C). Yadav et al. (2013) initially examined the genome size of *S. rebaudiana* using flow cytometry to be 2.7 pg/2C, while generating the tetraploidy in field grown mother plants. In an another study, ploidy status of diploid and tetraploid *Stevia* were analysed using flow cytometry (Zhang et al. 2018).

Table 4.7: Relative DNA content and genome size estimation of mother plant and in vitro micropropagated clones of *S. rebaudiana*

S. No.	Sample type	Mean G ₁ channel peak position*	Relative nuclear DNA content (pg)	CV%*	2C genome size (pg $\times$ 0.978 $\times$ 10 ⁹ bp)
Sr1	Mother plant	145059.4 $\pm$ 2347.6	2.0	4.0 $\pm$ 0.4	1.95
	In vitro plant	146219.2 $\pm$ 1109.9	2.0	3.0 $\pm$ 0.2	2.01
Sr2	Mother plant	146656.1 $\pm$ 246.5	2.1	4.0 $\pm$ 0.1	2.01
	In vitro plant	147778.3 $\pm$ 1849.5	2.1	4.4 $\pm$ 0.1	2.03

* The values represented as mean $\pm$ SD (n=3).

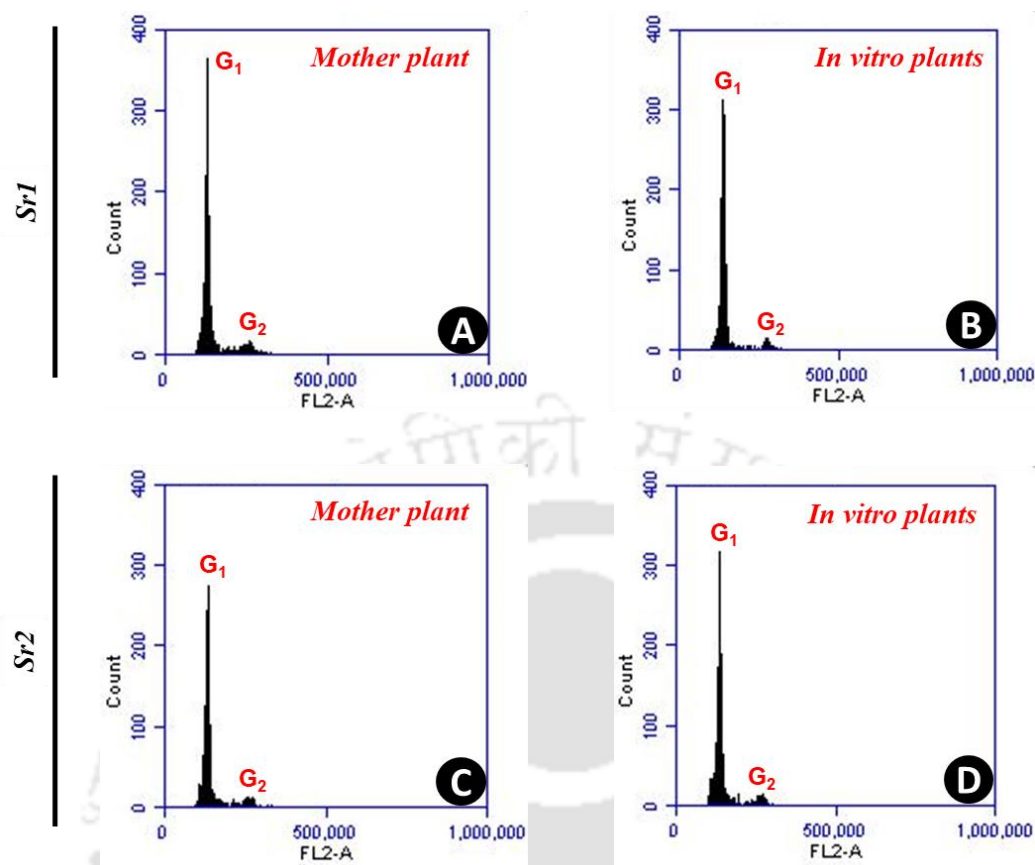


Figure 4.5: Clonal fidelity assessment via flow cytometry

(A, B) Flow cytometry of mother plant and in vitro regenerated clones of Sr1 plant line,

(C, D) Flow cytometry of mother plant and in vitro regenerated clones of Sr2 plant line.

Note: The histograms containing y-axis represents number of events (counts) and x-axis signifies relative nuclear DNA content.

4.3.6 Chemical analysis of steviol glycosides

Steviol glycosides are one of the main constituents of the plant apart from flavonoids, phenolics, alkaloids and other classes of secondary metabolites (Wölwer-Rieck 2012). In the current study, quantitative estimation of the key steviol glycosides was carried out in both, mother plant and micropropagated plants, utilizing High Performance Liquid Chromatography (HPLC). Though steviol glycosides are soluble in water, however, methanol was chosen for the study since better extraction of rebaudioside A and stevioside was observed in methanol, similar to few other reports (Ahmed and Dobberstein 1982; Gardana et al. 2010; Makapugay et al. 1984). Fresh samples were preferred for extraction over dried ones, to reduce the hindrance caused by the phenolic compounds during the analysis. Likewise, Periche et al. (2015) reported the maximum phenolic content in dried samples and highest stevioside content in fresh samples of *S. rebaudiana*. Production of phenolic compounds occurs due to oxidation

caused by the key enzymes, involved during their biosynthesis. Moreover, these enzymes are thermo-stable and their stability varies between 50°C to 90°C or more, depending upon the plants species and cultivar (Yemenicioğlu et al. 1997). Apart from this, researchers have also evaluated other extraction methodologies for higher rebaudioside A and stevioside yield. Gasmalla et al. (2015) utilized sonication to achieve shorter extraction time at 68°C and enhanced rebaudioside A productivity by 1.5-times. Similarly, Jaitak et al. (2009) evaluated microwave-assisted extraction and yielded 2.3% and 8.6% of rebaudioside A and stevioside, respectively. Use of C-18 solid-phase extraction (SPE) cartridges was also found feasible for elution and purification of analytes with simultaneous removal of impurities in the stationary phase (Martono et al. 2018).

4.3.6.1 HPLC method optimization

Another key objective of the present study was to optimize HPLC parameters for obtaining proper resolution of rebaudioside A (R_A) and stevioside (S). Mobile phase comprised of solvent A= MilliQ with 0.05% formic acid and solvent B= Acetonitrile. During the process, solvent A and B were tested in variable ratios at different flow rates but the maximum resolution was obtained in 20% milliQ and 80% acetonitrile (i.e. at 20:80 ratio of solvent A: solvent B) at temperature of 25°C and a flow rate of 0.6 mL min⁻¹. Formic acid (0.05%) was found as effective solvent modifier in improvement of splitting or tailing of peaks. The HPLC chromatogram showing elution of peak at retention time of 4.3 min corresponds to rebaudioside A standard (**Figure 4.6A**), while stevioside standard eluted at 5.01 min (**Figure 4.6B**). Linear calibration graph for both standards were generated. Similarly, all the samples were run and analyzed, showing identification of rebaudioside A and stevioside at 4.3 and 5.01 min, respectively (**Figure 4.7**). Cross-verification for their presence in the leaf samples, obtained from mother plants and in vitro regenerated plants, were done by individually spiking the isolated compounds from HPLC with their respective known standards, (**Figure 4.8**). Leaf extracts when spiked with stevioside standard also spikes up rebaudioside A due to low purity (80%) of the stevioside standard. The target peaks were collected for further confirmation through mass spectrometry studies. Mostly, quantification of key ingredients of *Stevia* leaves has been performed using HPLC or UHPLC (Ultrahigh-pressure liquid chromatography) using carbohydrate (Richman et al. 2005), C-18 columns (Vaněk et al. 2001), amino (Woelwer-Rieck et al. 2010) and HILIC (Hydrophilic interaction chromatography) (Zimmermann et al. 2012), followed by generally UV or mass spectrometry detection mode. In case of UV detection mode, the optimum wavelength according to the available literature ranged from 200-210 nm, since

the analytes are associated with alkene and carboxylic acid moieties, which decreases UV absorbing capacity. Martono et al. (2018), while using SPE cartridge system for purification of steviol glycosides, also proposed acetonitrile and water (Milli-Q) in the ratio of 80:20 for better elution with least impurities.

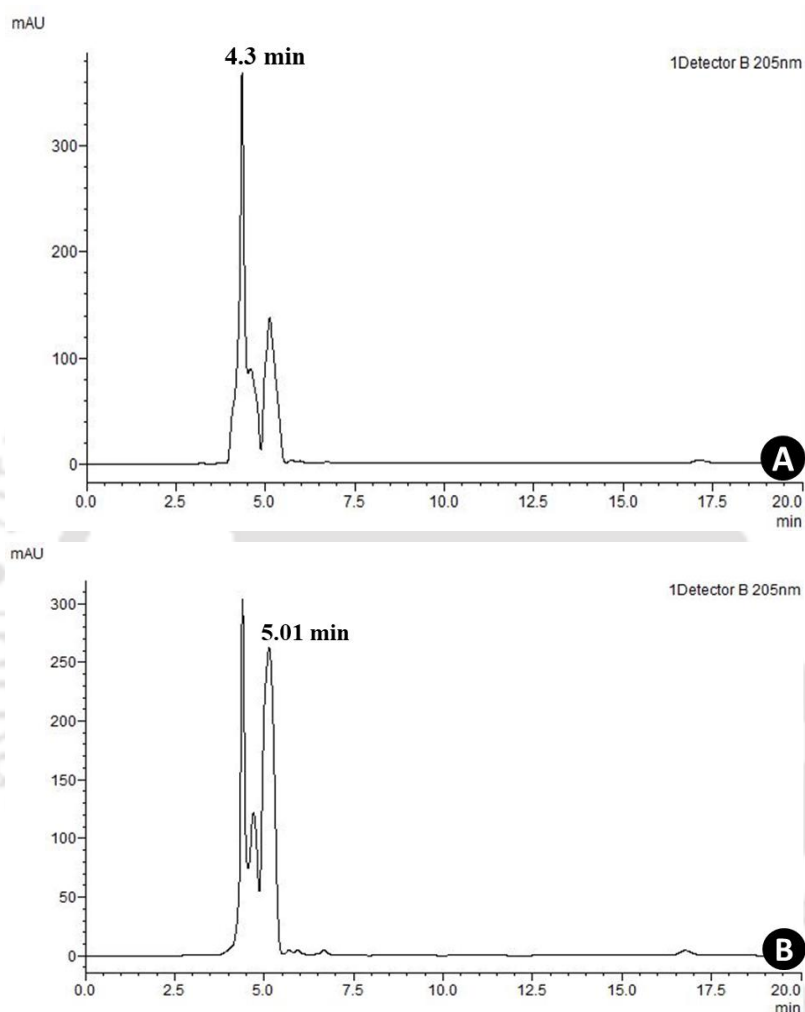


Figure 4.6: HPLC chromatograms of steviol glycosides' standards

(A) Rebaudioside A (R_A) standard (retention time: 4.3 min),

(B) Stevioside (S) standard (retention time: 5.01 min)

4.3.6.2 Linearity and method precision

Calibration curve was generated assessing the linearity of the method. Standards of rebaudioside A and stevioside were run at six different, serially diluted concentrations and a linear regression equation along with regression coefficient (R^2) was calculated (**Table 4.8**). The HPLC instrumentation and the method was precisely validated. The accuracy levels for rebaudioside A ranged from 1.1 to 2.9 % (intraday) and 0.6 to 2.0 % CV (interday). While the CV range for stevioside was observed to be 1.4 to 4.2 (intraday) and 0.9 to 6.0 %, in all the respective samples (**Table 4.9**).

Table 4.8: Retention time and linearity check of key steviol glycosides via HPLC

Type of steviol glycoside	Retention time (min)	Calibration curve ^a		
		Y-intercept	Slope	R^2
Rebaudioside A	4.3	72671	4411497	0.99
Stevioside	5.0	71057	4260000	0.99

^a Values are represented as mean $\pm$ SD ($n=3$)

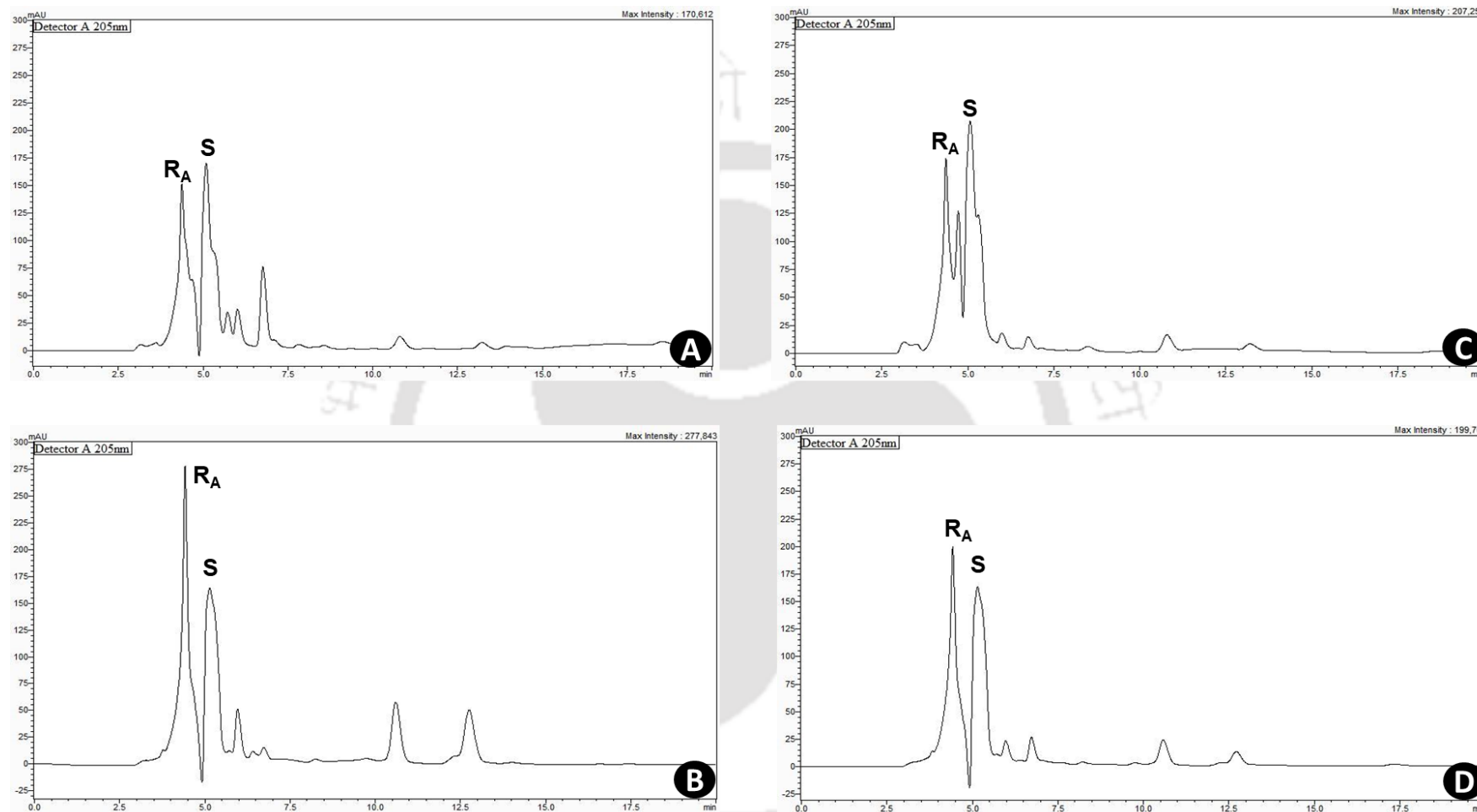


Figure 4.7: HPLC chromatograms, **A, B** showing the presence of rebaudioside A (R_A) and stevioside (S) in leaf extracts of field grown mother plants and in vitro grown plants, respectively of Sr1 line, and **C, D** showing the presence of field grown mother plants and in vitro grown plants, respectively of Sr2 line of *S. rebaudiana*

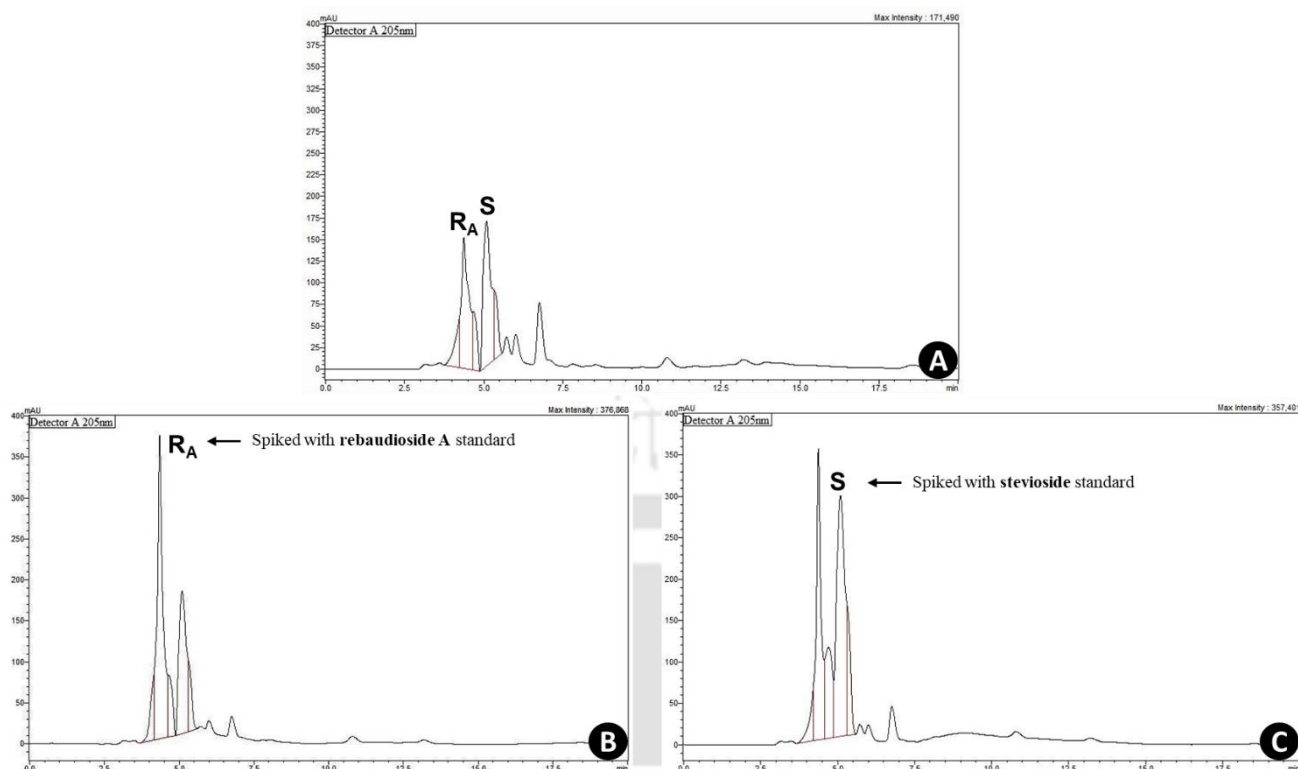


Figure 4.8: HPLC chromatograms showing confirmation of rebaudioside A and stevioside via spiking analysis, where,

- (A) Leaf extract from mother plant (Sr1),
- (B) Extract from A, spiked with known amount of rebaudioside A (R_A) standard,
- (C) Extract from A, spiked with known amount of stevioside (S) standard.

Table 4.9: Method precision studies of steviol glycosides

Sample type	Rebaudioside A ^a			Stevioside ^b			R _A /S ratio
	Amount (%)	Coefficient of Variation (%)		Amount (%)	Coefficient of Variation (%)		
		Intraday	Interday		Intraday	Interday	
Ex-1	5.8 ± 0.1	1.1	1.7	3.8 ± 0.1	1.4	6.0	1.5
In-1	10.7 ± 0.1	1.2	1.7	5.2 ± 0.1	1.5	1.8	2.1
Ex-2	5.8 ± 0.1	1.0	0.6	5.0 ± 0.2	4.2	0.9	1.2
In-2	8.7 ± 0.2	2.9	2.0	5.1 ± 0.1	2.8	1.3	1.7

Field grown mother plants Sr1 line (Ex-1); Field grown mother plant Sr2 line (Ex-2); In vitro Sr1 plant line (In-1); In vitro Sr2 plant line (In-2).

a, b

Values represented as mean ± SD (n=3).

R_A/S ratio is determined by percent amount of rebaudioside A/ percent amount of stevioside.

4.3.6.3 Mass spectrometric studies

Presence of steviol glycosides was assessed via mass spectrometry in the purified samples and crude extracts as compared to their respective standards. The detection was notified in negative ion mode. Similar confirmation studies were carried out by several researchers (Choi et al. 2002; Jackson et al. 2009; Rajasekaran et al. 2007; Zimmermann et al. 2012). The respective mass spectra showed majority of steviol glycosides in the form of adducts as the most abundant product. Presence of rebaudioside A is shown as 1001.47 ($M+Cl^-$), 965.50 ($M-H^+$), 917.43 ($M-glc+TFA-H^+$), 839.41 ($M-glc+Cl^-$), 803.44 ($M-glc$) and 641.37 ($M-2glc-H^+$) m/z values in the purified sample as compared to 1001.50 ($M+Cl^-$), 965.52 ($M-H^+$) and 803.44 ($M-glc$) m/z values in the rebaudioside A standard (**Figure 4.9A, B**). Besides this, stevioside was also successfully characterized as 917.42 ($M+TFA-H^+$), 839.43 ($M+Cl^-$) and 803.45 ($M-H^+$) m/z values in the sample as compared to 839.43 ($M+Cl^-$), 803.41 ($M-H^+$) and 641.36 ($M-glc-H^+$) m/z values in its standard (**Figure 4.10A, B**). The majority of the peaks signifies the loss of one or two units of glucose residues (162 Da), while other peaks indicated deprotonation and adduct formation or both, during the process of ionization. Loss of glucose residues resulted in the fragmentation showing peak at 641 m/z value indicating the deprotonated form of steviolbioside/rubusoside (642.74 m/z), another steviol glycoside.

Mass spectrometry represents one of the sensitive high-end techniques for sample characterization. Hence, crude methanolic samples were also assessed for analyzing the presence of other types of steviol glycosides, where certain peaks with weak intensities were observed except presence of prominent peak of steviolbioside as 641.46 m/z value. In **Figure 4.11**, peak value at 787.56 m/z corresponds to dulcoside A in the deprotonated form. Similarly, in the expanded version of the same figure, peak value at 1127.72 m/z signifies the deprotonated form of rebaudioside D. Two other fragments of rebaudioside D were also observed as 1109.70 ($R_D-H_2O-H^+$) and 1149.66 ($R_D-Na^+-2H^+$) m/z values. Presence of Rebaudioside M was verified in the sample, indicated as 1271.78 ($R_M-H_2O-2H^+$) m/z value, representing the loss of water molecule from the deprotonated form of the compound.

Confirmation of Rebaudioside A (M)

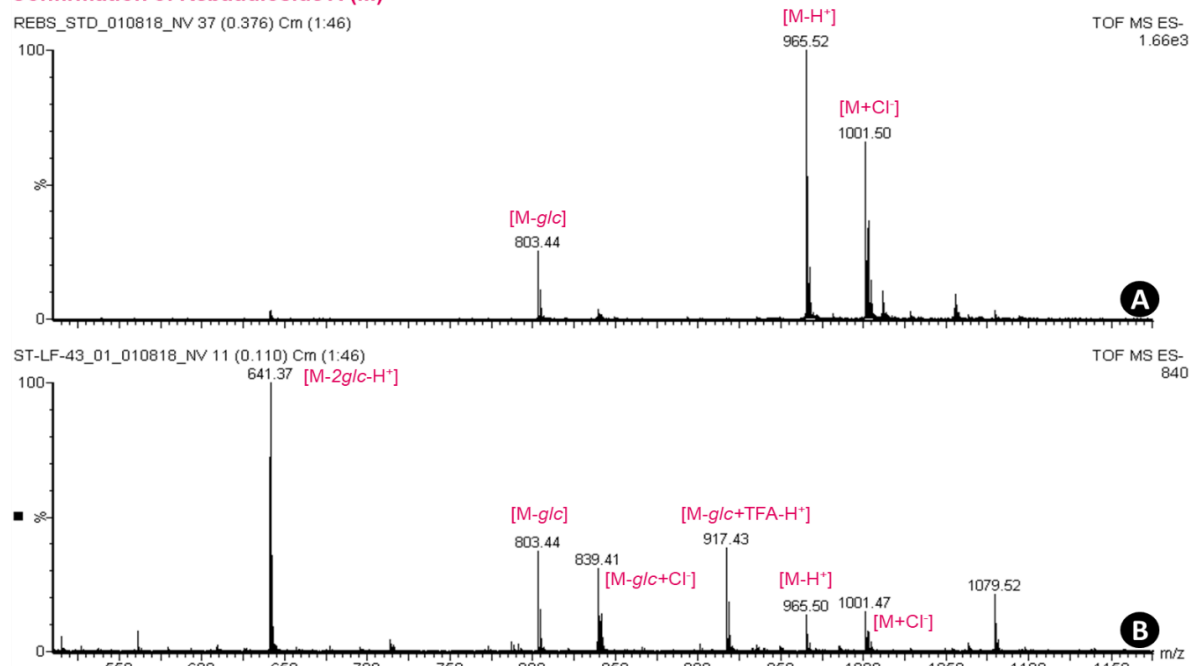


Figure 4.9: Mass spectra of rebaudioside A standard and sample

Mass spectra of (A) Rebaudioside A standard, (B) Eluted HPLC sample at 4.3 min from crude extract of in vitro *Stevia* plants, showing rebaudioside A at 965.50 m/z value with their adducts and fragments, analyzed in the negative ion mode.

Confirmation of Stevioside (M)

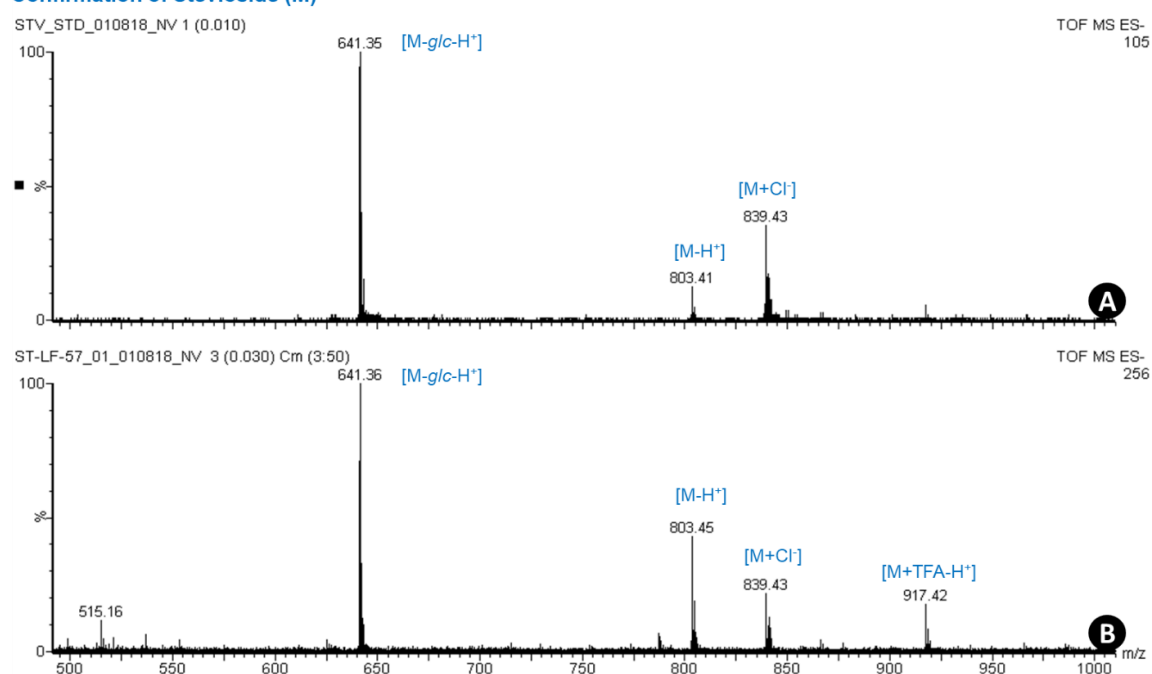


Figure 4.10: Mass spectra of stevioside standard and sample

Mass spectra of (A) Stevioside standard, (B) Eluted HPLC sample at 5.0 min from crude extract of in vitro *Stevia* plant, showing stevioside at 803.45 m/z value with their adducts and fragments, analyzed in the negative ion mode.

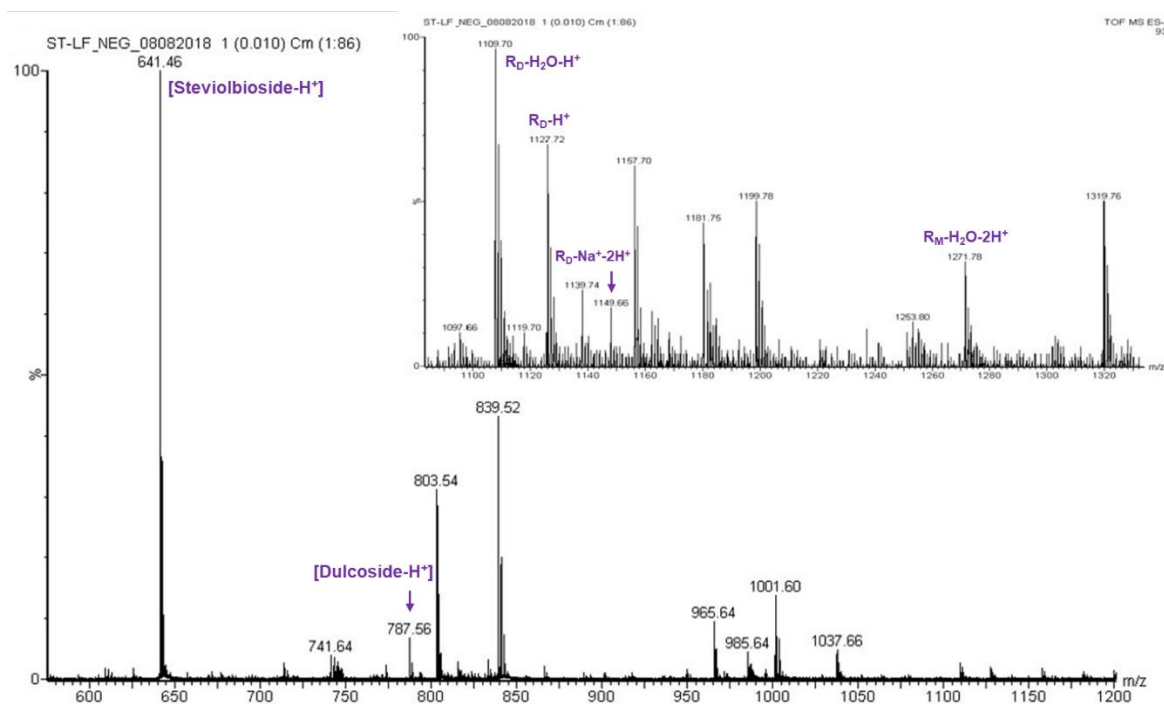


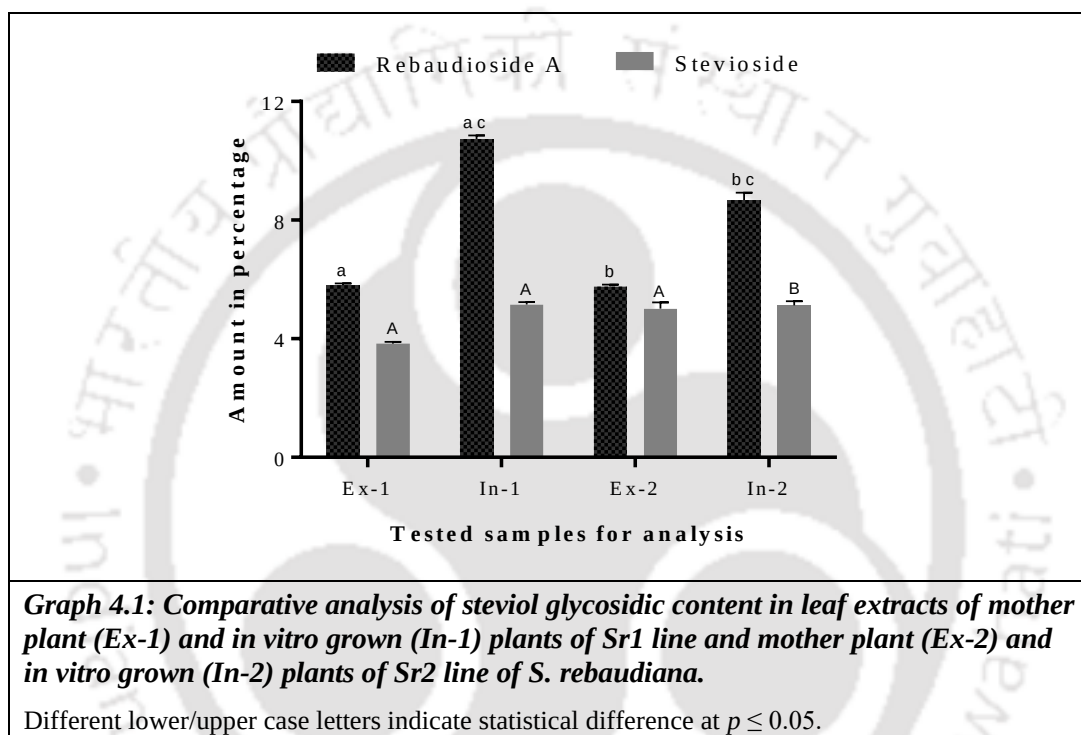
Figure 4.11: MS analysis showing steviolbioside (641.46 m/z), dulcoside (787.56 m/z), rebaudioside D (R_D :1127.72 m/z) and rebaudioside M (R_M :1271.78 m/z) in their adducts forms, in the leaf extracts of *S. rebaudiana*, apart from rebaudioside A and stevioside.

4.3.6.4 Comparative assessment of key steviol glycosides content

With respect to *S. rebaudiana*, most species contain a high stevioside to rebaudioside A ratio (Jaitak et al. 2009; Lorenzo et al. 2014; Makapugay et al. 1984; Woelwer-Rieck et al. 2010; Yamazaki et al. 1991), although the market demand favors the cultivars that are rich in rebaudioside A content. There are a few reports, which verified in vitro grown regenerants of *S. rebaudiana* to be rich in rebaudioside A content (Gupta et al. 2015; Javed et al. 2017a; Javed et al. 2017b; Mejía-Espejel et al. 2018; Yücesan et al. 2016). Rajasekaran et al. (2007) also mentioned a high rebaudioside A to stevioside ratio, 1.6 and 0.9 in the ex vitro and in vitro plant leaves, respectively.

Our findings report a higher production of rebaudioside A than stevioside in mother plant of both the plant lines, Sr1 and Sr2, as well as their in vitro propagated plants. However, yield percent of rebaudioside A was greater in the in vitro propagated plants (**Table 4.9; Graph 4.1**). Highest rebaudioside A content was found in in vitro line of Sr1 (10.7%), followed by Sr2 (8.7%). Stevioside was accounted to be 5.2% and 5.1% in in vitro lines of Sr1 and Sr2, respectively. With respect to the ratio of rebaudioside A to stevioside, as a significant factor for development of sweeter plant lines, in vitro propagated plants generate a vividly better ratio

of rebaudioside A and stevioside as 2.1 and 1.7, while in the field grown mother plants, the ratio was 1.5 and 1.2 (**Table 4.9**). Thus, contributing successfully in decreasing the bitter taste of the leaves, which is primarily due to higher content of stevioside. The environmental conditions affect rebaudioside A content in the plant as confirmed by the previous reports that high steviol glycosides content have been mostly reported during summers as favored by long-day conditions (Angelini et al. 2018; Metivier and Viana 1979; Tavarini et al. 2015).



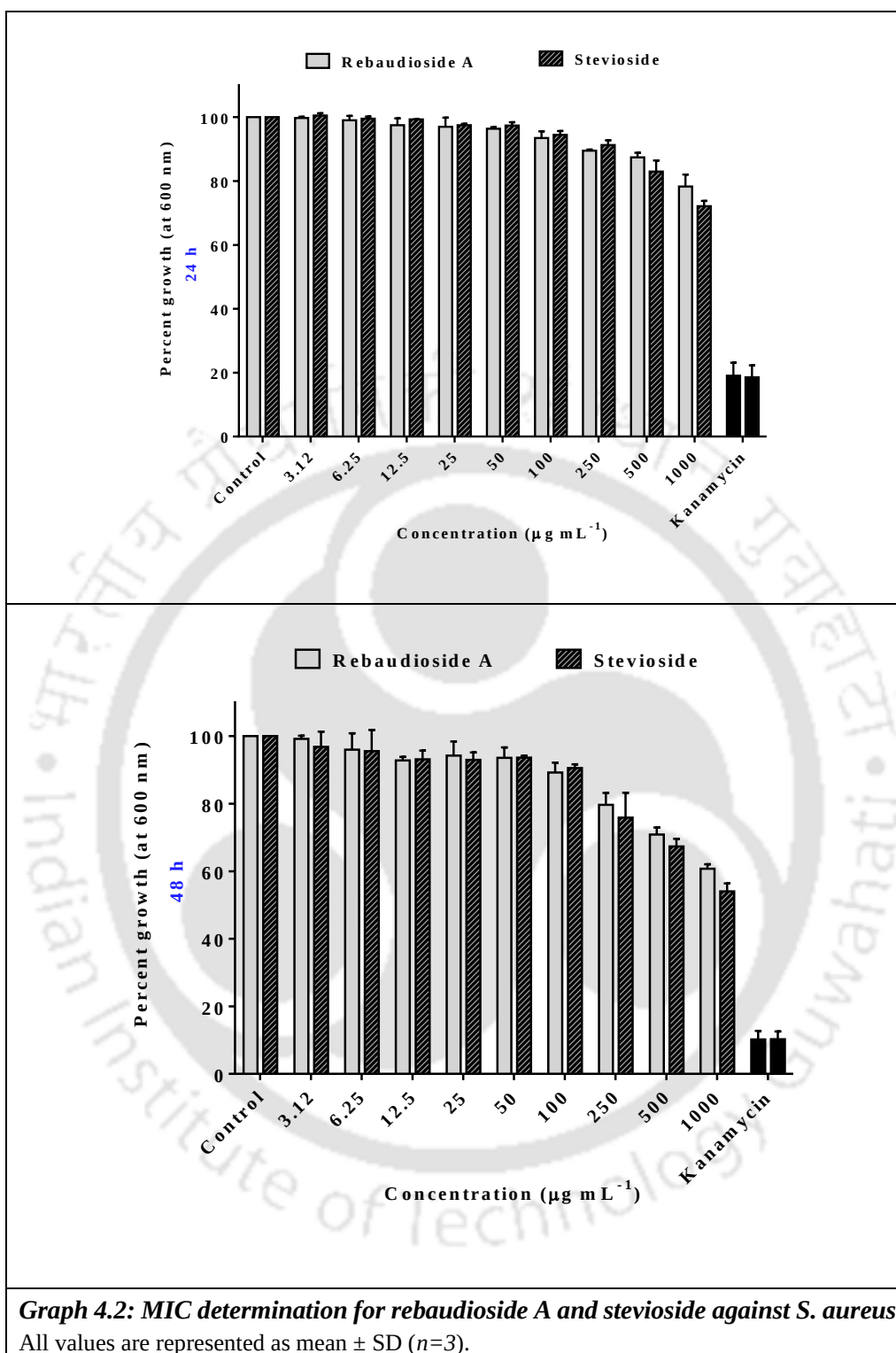
Researches have proved that a cross talk exists between the medium components and the production of secondary metabolites. According to available literature, (de Lima Filho et al. 1997) have mentioned that calcium deficiency in the plant can lead to decrease in the content of glycosides (Yadav et al. 2011). In accordance to this, dramatic increase of rebaudioside A in the in vitro cultures, would be the influential role of the supplemented medium containing high calcium content (3X) in the basal DKW medium as compared to MS medium. Bramble et al. (1991) also found that calcium supplementation to callus culture of *Coffea arabica* inhibited the shoot growth but enhanced caffeine production. They concluded that calcium precipitated phosphate, thereby, increasing the rate of alkaloid production. Moreover, in *Agave amanuensis*, high amount of magnesium, copper and cobalt ions in the medium inhibited the production of sapogenin steroid formation, but, absence of calcium in the medium supported steroid production in the in vitro callus cultures (Andrijany et al. 1998). In another literature on *S.*

rebaudiana, impact of the medium components on the vitro shoots have been recently reported, where two important macronutrients (nitrogen and phosphorus) at different concentrations were supplemented, but, the authors found a negative correlation between the macro salts and rebaudioside A and stevioside content (Magangana et al. 2018). In conclusion, exogenous supply of medium components is crucial in the biosynthesis and accumulation of secondary metabolites and vary depending upon the plant species.

4.3.7 Biofilm inhibition studies

4.3.7.1 MIC determination

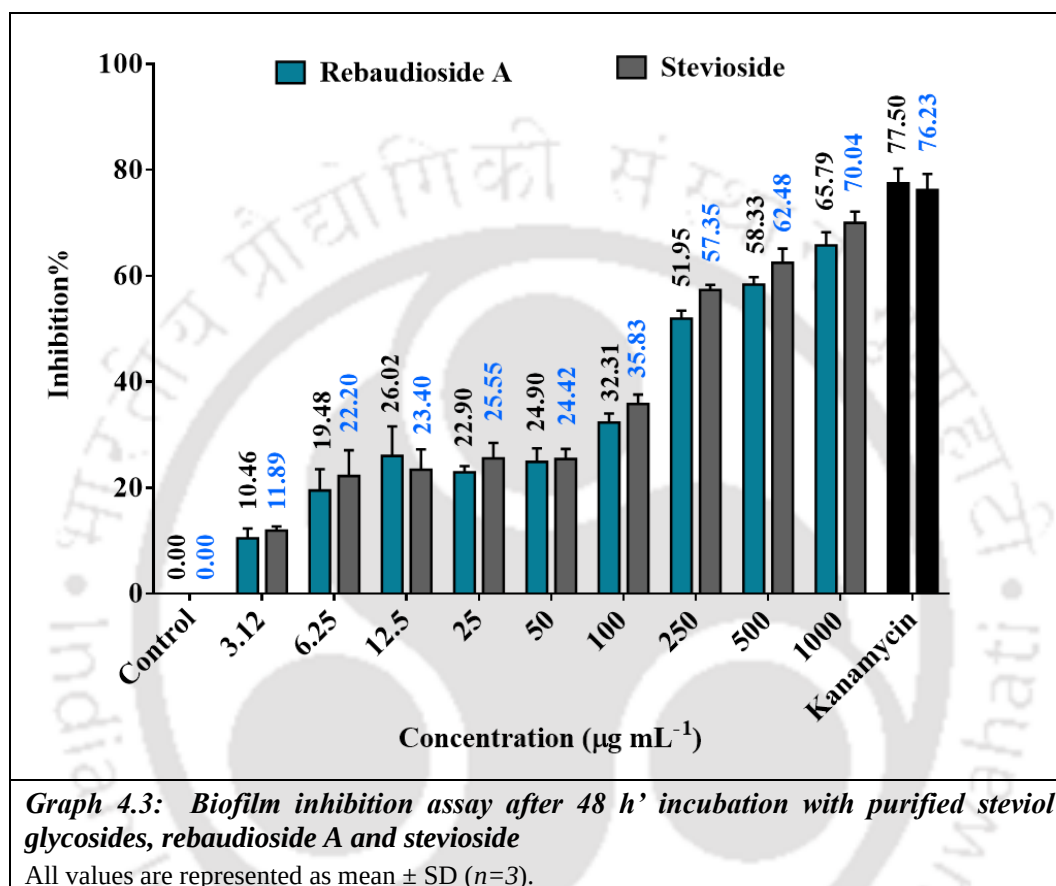
Assessing the inhibiting effect of plant-based anti-microbial agents had become crucial nowadays in the era of increasing antibiotic resistance. Therefore, the present study puts forth the initial set of experiments analysing the biofilm inhibition capability of steviol glycosides, rebaudioside A and stevioside. According to the broth-dilution assay for MIC determination, varied concentrations from $3.12 \mu\text{g mL}^{-1}$ to $1000 \mu\text{g mL}^{-1}$ was used for the analysis. After 24 h incubation, only 21.7% and 27.9% reduction in the bacterial population was observed at a concentration of $1000 \mu\text{g mL}^{-1}$ of rebaudioside A and stevioside, respectively. A higher reduction of 39.3% and 46.0% in the planktonic bacterial cells was observed at same concentration of rebaudioside A and stevioside, respectively, upon 48 h of incubation (**Graph 4.2**). This is perhaps due to presence of both alycone and glucose moieties. It has also been stated that glycosylation improves the hydrophilicity, bioavailability and pharmacokinetic properties of the compounds (Křen 2008). According to the updated literature, the purified rebaudioside A have not been assessed against *S. aureus* infection, while, Wang et al. (2014) reported the inhibitory effect of stevioside (33, 100 and $300 \mu\text{g mL}^{-1}$) on TLR-2, apoptotic, NF- κ B and MAPK proteins of *S. aureus* infected mouse mammary epithelial cells. There were reports about the anti-microbial effect of different solvent extracts of *S. rebaudiana* leaves (Debnath 2008; Jayaraman et al. 2008). As mentioned in **section 1.5.1.1 of Chapter 1**, stevioside and steviolbioside have been experimented to have anti-tuberculosis effect (Kataev et al. 2011), while rebaudioside A and stevioside possessed anti-cariogenic effect against *S. mutans* (Das et al. 1992).



4.3.7.2 Biofilm inhibition assay and MBIC determination

Addressing the biofilm forming capacity of *Staphylococcus aureus* with an intake of purified steviol glycosides for a diabetic patient is extremely necessary. Therefore, after achieving ~40% inhibition in planktonic cells, the accessibility of target steviol glycosides on the biofilm inhibition was a crucial phase. According to the **Graph 4.3**, percent inhibition of biofilm

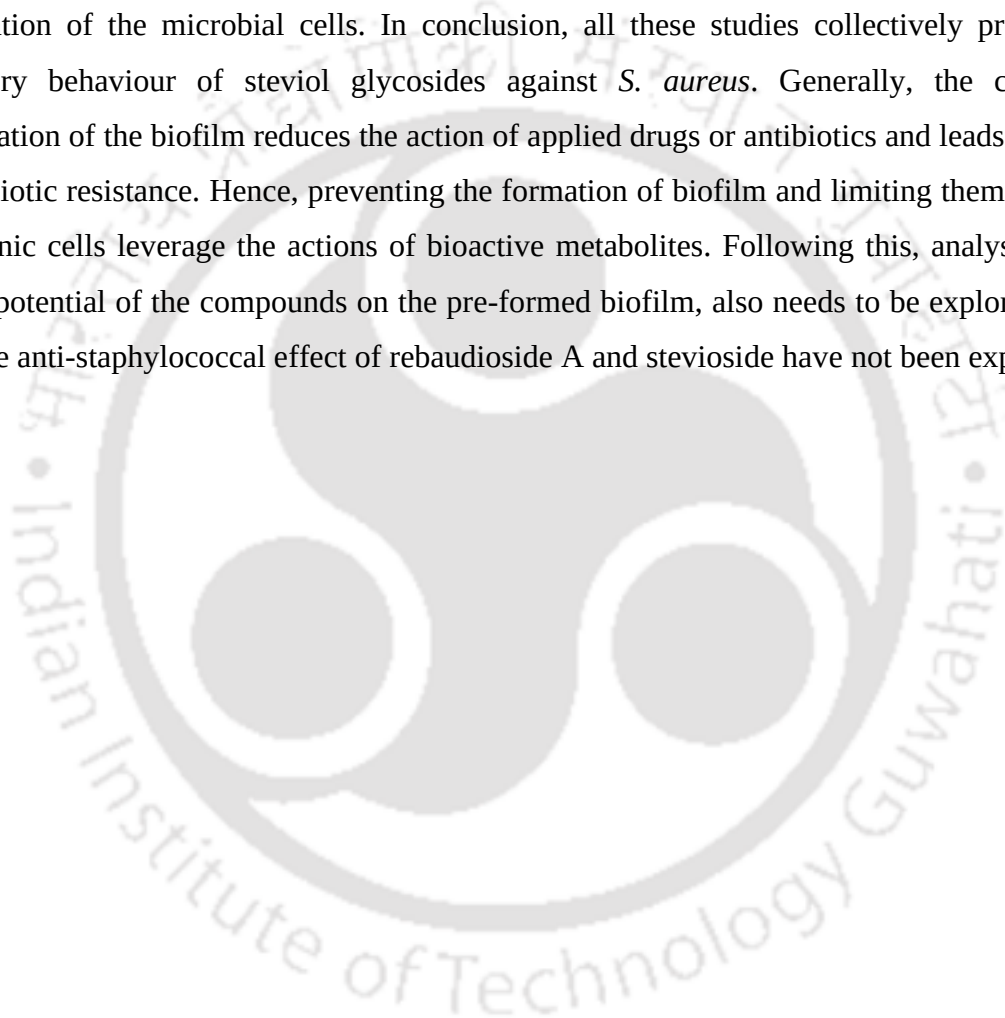
formation was observed to be 65.8% and 70.0% in case of rebaudioside A and stevioside, respectively, at a MBIC of 1000 $\mu\text{g mL}^{-1}$, upon 48 h of incubation. As per the assay, $\leq 100 \mu\text{g mL}^{-1}$ was found to have minor inhibitory effect, however, as the concentration increased in dose-dependent manner, the biovolume of *S. aureus* declined.



4.3.7.3 Qualitative analysis of biofilm inhibition and surface morphological analysis of steviol glycosides via FESEM

Biofilm inhibition was reanalysed qualitatively via fluorescence microscopy. Acridine orange is an efficient fluorescent indicator, staining the live and dead bacterial cells. The non-treated (control) bacterial cells forming biofilm as visible under bright field and fluorescence (**Figure 4.12A, D**). The increased biovolume of *S. aureus* could be seen within 48 h, almost covering the entire coverslip, whereas, there was a reduction in biofilm in steviol glycoside treated population of *S. aureus*. Upon treatment at their MBIC, presence of discrete population of planktonic cells was visualized in comparison to the dense clustered population (**Figure 4.12B-C, E-F**). With respect to the type of steviol glycosides, stevioside was found to be better in response as compared to rebaudioside A, as corroborated with MIC and biofilm inhibition

analysis. Chu et al. (2016) also utilized acridine orange for qualitative analysis of *S. aureus* biofilm. To further confirm our results, inhibitory role of the steviol glycosides on the bacterial population could be visualized at an ultra-magnification via FESEM. Although, the non-treated bacterial cells (control) exhibited spherical morphology with smooth surface (**Figure 4.12G**), the treated bacterial cells showed distorted/wrinkled surface, pore formations, more mucus secretions, thus, indicating membrane disruption and cell lysis as possible mode of action of key metabolites (**Figure 4.12H, I**). Cytoplasmic leakage may have contributed to the aggregation of the microbial cells. In conclusion, all these studies collectively prove the inhibitory behaviour of steviol glycosides against *S. aureus*. Generally, the complex organization of the biofilm reduces the action of applied drugs or antibiotics and leads to drug or antibiotic resistance. Hence, preventing the formation of biofilm and limiting them to only planktonic cells leverage the actions of bioactive metabolites. Following this, analysing the killing potential of the compounds on the pre-formed biofilm, also needs to be explored. Till date, the anti-staphylococcal effect of rebaudioside A and stevioside have not been explored.



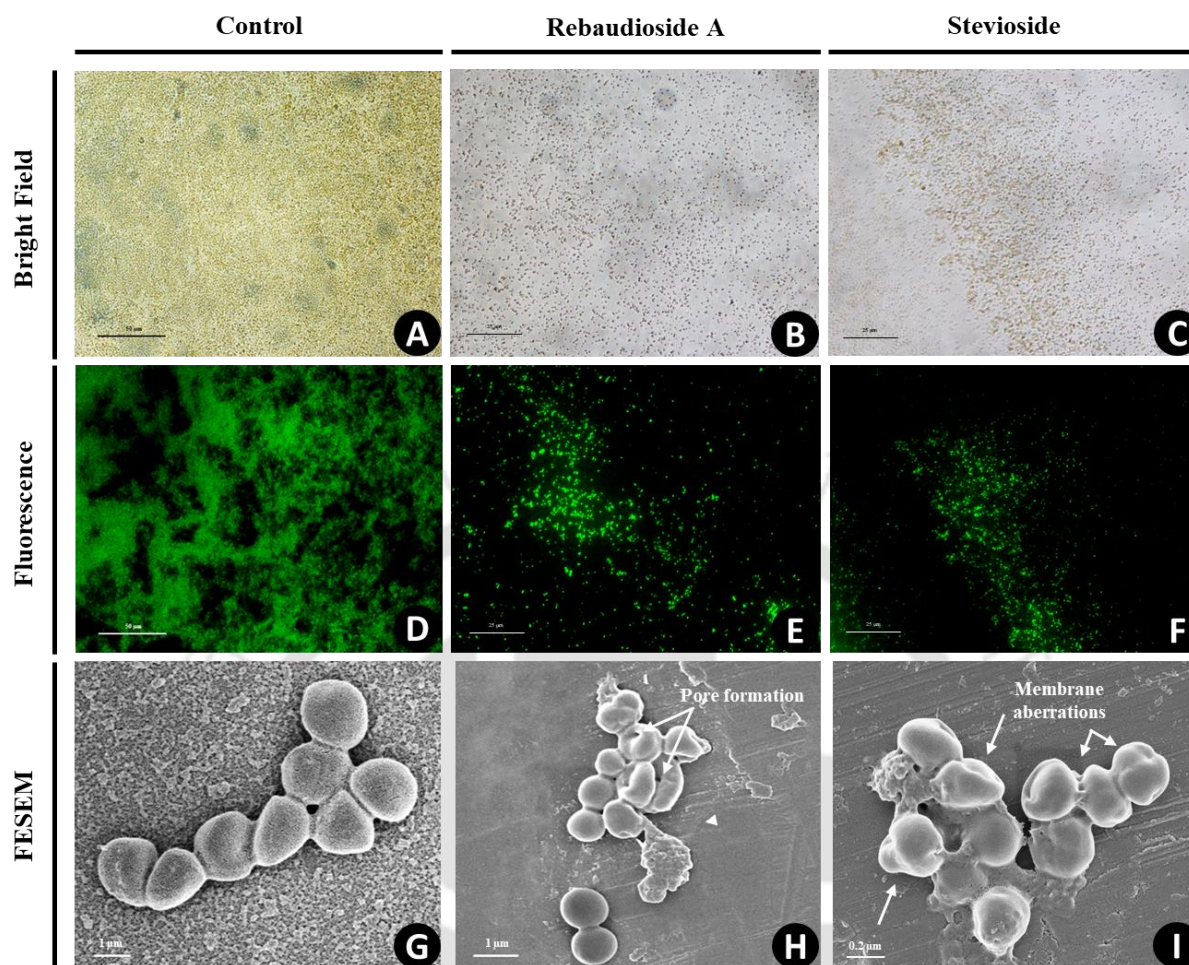


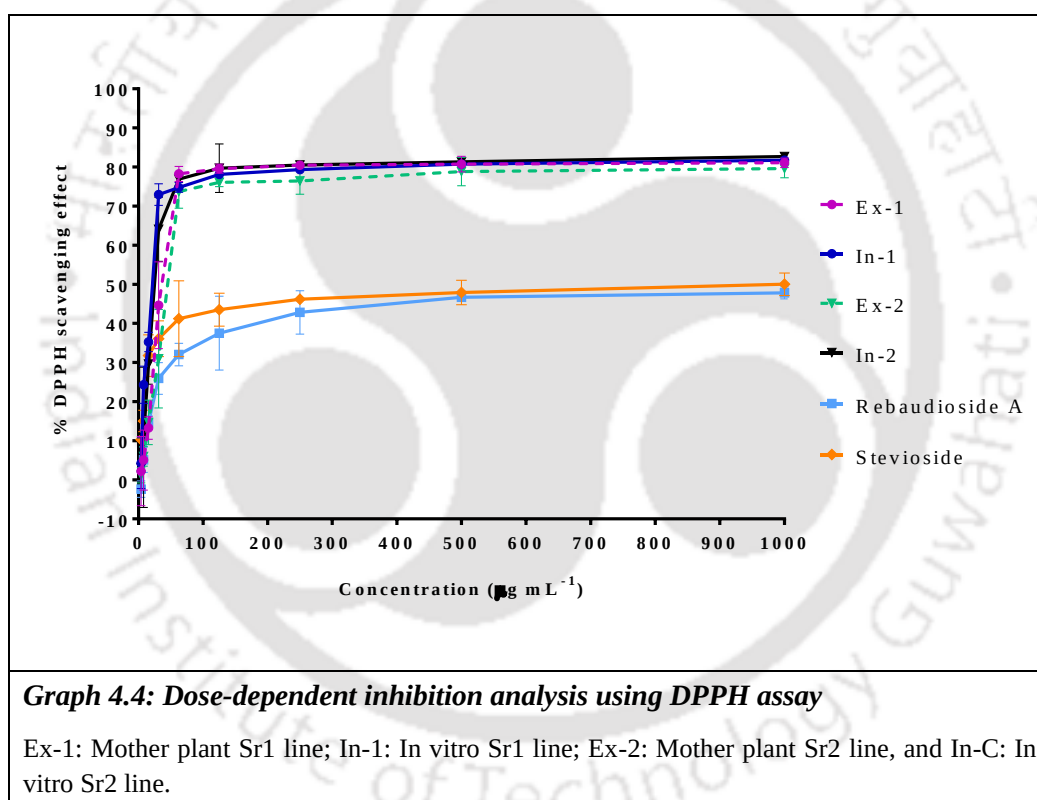
Figure 4.12: Qualitative analysis of steviol glycoside treatment on *S. aureus*

- (A, D) Non-treated bacterial biofilm (Bar=50 μm),
 (B, E) Rebaudioside A (1000 $\mu\text{g mL}^{-1}$) treated *S. aureus* population (Bar=25 μm),
 (C, F) Stevioside (1000 $\mu\text{g mL}^{-1}$) treated *S. aureus* population (Bar=25 μm);
 (G) Ultrastructural view of non-treated bacterial cells (Bar=1 μm),
 (H) Ultrastructural view of purified rebaudioside A (1000 $\mu\text{g mL}^{-1}$) treated bacterial cells, showing membrane distortions and pore formation (Bar=1 μm)
 (I) Ultrastructural view of purified stevioside (1000 $\mu\text{g mL}^{-1}$) treated bacterial cells showing irregularities of bacterial membrane and pore formations (Bar=0.2 μm)

4.3.8 Assessment of antioxidant potential of crude extracts and purified steviol glycosides using DPPH method

Gaging through the antioxidant prospective of plant seems quite common but is a crucial aspect. In addition to this, consumption of natural sweeteners possessing antioxidant potential seems an added-advantage to the consumers. Since the free radicals are highly unstable and are generated unlikely, whenever oxygen is burnt out. Reduction in their population is successfully

performed by plants. In the present study, *S. rebaudiana* has been evaluated for its potential to curb these free radicals. Fresh leaf methanolic crude extracts of two selected parent plant lines of *Stevia*, Sr1 and Sr2, possessed 81.1% and 79.6% effect on reduction of DPPH free radical scavenging activity in dose-dependent manner. Their respective in vitro developed plant lines contributed 81.7% and 82.7% activity on reduction of DPPH free radical scavenging activity (**Graph 4.4**). Among all plant lines, the crude extracts from in vitro plant lines of Sr1 at IC_{50} of $27.4 \mu\text{g mL}^{-1}$ gave significant influence on scavenging effect which is next higher to the positive control (Ascorbic acid) (**Table 4.10**). Similarly purified steviol glycosides, rebaudioside A and stevioside, were also measured for scavenging effect, where they showed 47.8% and 50.0% reduction in DPPH free radicals.



According to other reports on antioxidant activity, Shukla et al. (2009) found the DPPH effect between 36.9% to 68.8% in dose-dependent manner. Kim et al. (2011) found leaf aqueous extract to have better antioxidant activity than leaf-induced callus aqueous extract. In another report by Ghanta et al. (2007), out of different solvent extracts, crude ethylacetate extract possessed the highest scavenging effect. According to the literature, there have been other reports regarding the antioxidant potential of *S. rebaudiana* leaf extracts assessed via DPPH radical scavenging assay (Ahmad et al. 2016; Barba et al. 2015; Barroso et al. 2016; Criado et

al. 2014; Kaushik et al. 2010; Periche et al. 2015; Raut and Aruna 2017; Ruiz et al. 2015; Shivanna et al. 2013; Tavarini et al. 2015; Zayova et al. 2013; Zeng et al. 2013). In addition to this, in vitro cultures of other plant species have also been evaluated for their anti-oxidative effect such as *Camellia sinensis* (L.) O. Kuntz (Hazarika and Chaturvedi 2013), *Spilanthes acmella* (Singh et al. 2014) and several other plant species as well.

Table 4.10: DPPH scavenging activity of crude extracts and purified steviol glycosides displaying maximum DPPH scavenging effect and IC_{50} and IC_{99} values

Sample	Maximum effect (%) *	IC_{50} ($\mu\text{g mL}^{-1}$)	IC_{99} ($\mu\text{g mL}^{-1}$)
Ascorbic acid	93.6 ± 0.6	16.9	787.4
Ex-1	81.1 ± 1.2	194.5	989.9
In-1	81.7 ± 0.5	27.4	1085.7
Ex-2	79.6 ± 2.4	243.9	1016.8
In-2	82.7 ± 0.9	100.6	1009.7
Purified rebaudioside A	47.8 ± 1.5	802.0	2581.2
Purified stevioside	50.0 ± 2.9	752.8	2098.3

Values represented as mean $\pm$ SD ($n=3$).

*The values are 'non-significant (*ns*)' with each other, according to one-way ANOVA (Tukey's multiple comparison test). 'Ex-' and 'In-' represents crude leaf extracts of mother plant and in vitro grown plants of Sr1 and Sr2 lines of *S. rebaudiana*.

With respect to the in vitro cultures of *S. rebaudiana*, Tadhani et al. (2007) induced leaf callus and evaluated the highest effect in the range of 33.2 to 56.8% in methanolic callus extract. On the other hand, Ahmad et al. (2016) speculated that red spectral light provided to the leaf-induced callus, caused 80% reduction DPPH induced free radicals. In relation to the drying process, oven-dried leaves at 40°C proved to possess better antioxidant activity assessed via Oxygen Radical Absorbance Capacity (ORAC) assay, however, DPPH assay was found to be insignificant in differentiating on the basis of fresh or dried leaves (Lemus-Mondaca et al. 2016). Different plant parts of *Stevia* were evaluated for their phenolic content and DPPH radical scavenging activity of which root methanolic extract possessed highest total phenolic content, anti-oxidative enzymatic levels (catalase, peroxidase and SOD) and 97% DPPH radical scavenging activity (Singh et al. 2012).

Chapter 5

Conclusions and Future Prospects

5.1 CONCLUSIONS

Plant tissue culture technique is one of the prominent approaches in establishment of plant species for their large-scale propagation and conservation. It also provides renewable source of bioactive secondary metabolites for development of therapeutics. The same was the rationale of the present study encircled around two plant species, *T. cordifolia* and *S. rebaudiana*.

- ➔ To generate, true-to-type elite plants of *T. cordifolia*, nodal segment cultures were utilized. The cultures were successfully established on Murashige and Skoog (MS; 1962) medium fortified with 10 μM 6-benzylaminopurine (BAP), producing multinodal and long shoots, which grew up to a height of 10.3 cm with a number of nodes per shoot as 10.1 in 6 weeks of growth cycle. The rate of shoot multiplication was found to be 10-fold at every 6 weeks. However, after subsequent two cycles of multiplications by nodes, taken from in vitro shoots at the end of the growth cycle, on the fresh medium of same composition, the medium did not support axillary shoot growth beyond 2 cm. Therefore, these small shoots were transferred to elongation medium. Elongation medium, consisted of MS₁ (ammonium nitrate and potassium nitrate concentrations reduced to half strength) + 5 μM BAP + 3 μM IAA + 100 mg L⁻¹ ammonium sulfate, proved to be effective in producing an average of 7.8 cm long shoot with an average of 8 nodes per shoot at the end of the 6 weeks. Hence after, regularly, the shoots were multiplied via single nodal segment cultures on MS + 10 μM BAP medium and elongation was achieved on MS₁ + 5 μM BAP + 3 μM IAA + 100 mg L⁻¹ AS medium. The rooting of the shoots was achieved on MS + 1 μM IBA with 90% success. The plantlets were hardened in the field conditions with 80% survival rate.
- ➔ The in vitro generated elite lines were randomly selected for clonal fidelity assessment using Inter Simple Sequence Repeats (ISSR) molecular marker. Total of 800 bands were amplified in range between 300 to 3000 bp, while percent monomorphism was analyzed to be 96.3% between mother plant and in vitro lines, displaying the true-to-type behavior of the in vitro plants. Similar study was verified using flow cytometry, where, mother plant and in vitro plants shared the similar G₁ peak channel position (~110000). The genome size was also explored to be 1.5×10^9 bp, which remained untouched in the published reports.

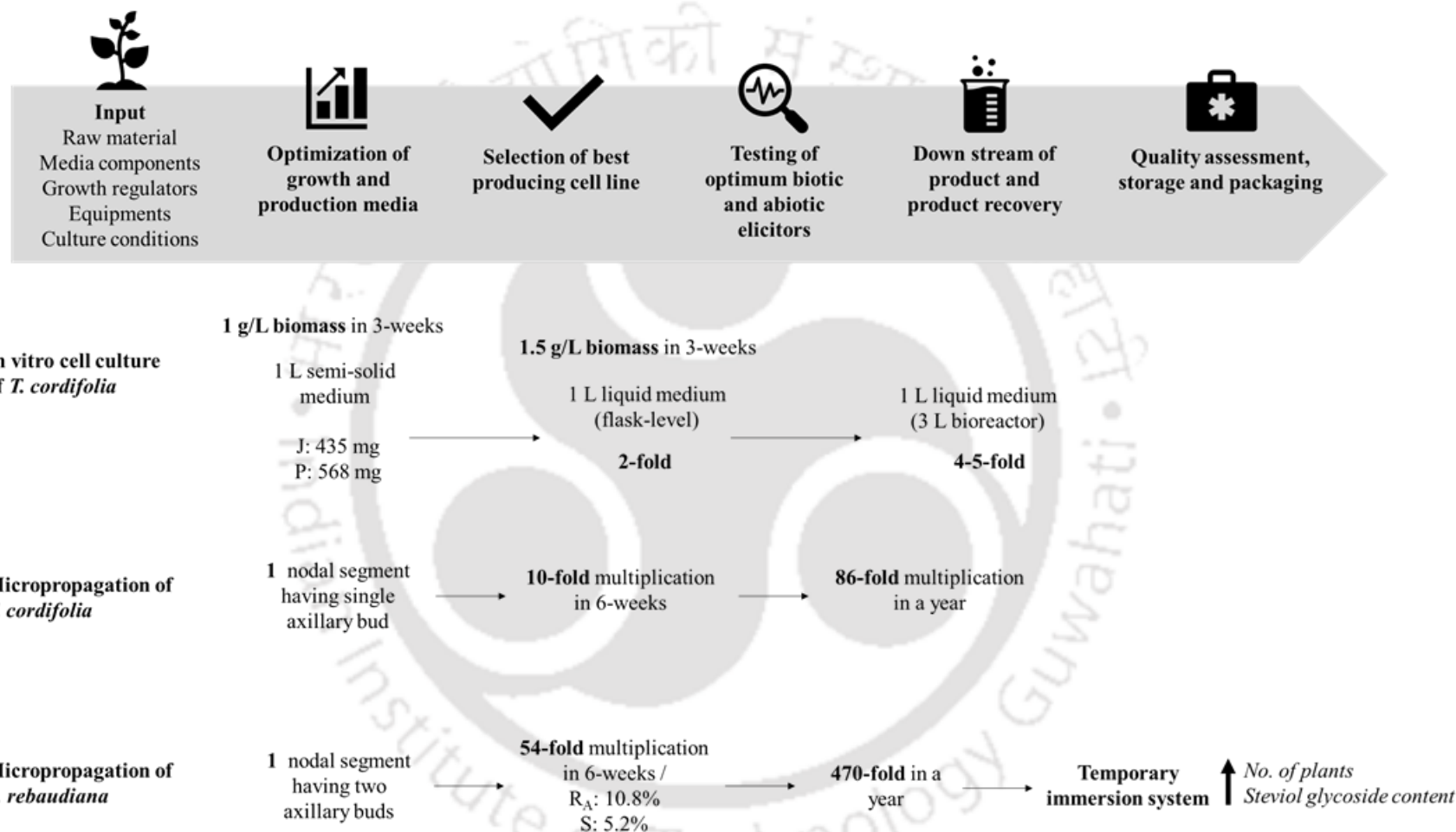
- ➔ In the present study, *in vitro* cultures of *T. cordifolia* was also initiated using leaf explants. Out of several tested media compositions, MS + 3 μ M BAP + 10 μ M NAA was found to be most competent in inducing callus in 100% leaf-disc explants. The beginning of the subculturing process produced proliferated, dark brown and hard callus and with subsequent passages at regular intervals, browning of *in vitro* calluses drastically reduced with an increase in their friability. Biomass growth curve analysis revealed the lag phase of callus in semi-solid medium till 6th day, following which the exponential phase began and cell biomass increased until 27th day.
- ➔ The proliferated light brown friable calluses were utilized for the extraction of target protoberberine alkaloids, jatrorrhizine and palmatine using acid-base extraction approach. HPLC method optimization was performed and the alkaloids were clearly identified at 6.8 min and 7.4 min retention time for jatrorrhizine and palmatine, respectively. This extraction was quite feasible in enhancing the productivity of alkaloids in both leaf extract and *in vitro* callus extracts. However, it was notable in later where jatrorrhizine was 435.9 μ g mg⁻¹ and palmatine was 567.5 μ g mg⁻¹. The purified alkaloids were verified via mass spectrometry and distinctly observed at 338.11 and 352.14 *m/z* value as compared to their respective standards. The present study lay an evident observation of the presence of protoberberine alkaloids in the leaf as well, although, they were reported in stem portion of the plant in other published reports. Besides, leaf-induced *in vitro* callus cultures have been proved to be the foremost alternative for isolation of protoberberine alkaloids.
- ➔ The purified protoberberine alkaloids, jatrorrhizine and palmatine, were found to demonstrate a competent activity against planktonic cells of *S. aureus* and inhibited biofilm formation as envisioned through fluorescence microscopy. Jatrorrhizine unveiled 78.3% reduction while palmatine exhibited 87.0% reduction in 48 h, at their respective MBIC (minimum biofilm inhibition concentration). The ultrastructural analysis distinctively showed rough surface, membrane distortion, cytoplasmic leakage and pore formations in bacterial cell after alkaloidal treatment.

- ➔ Analyzing the antioxidant feature is prerequisite in development of any therapeutics. Eventually, among the analysis of protoberberine alkaloids, purified palmatine was observed to depict 75.6% DPPH radical scavenging effect. However, scavenging effect of crude extracts were also analyzed, of which, callus extract was found to be 87.1% potent while leaf extract showed 76.7% scavenging potential.
- ➔ Corresponding research on *Stevia rebaudiana* have enabled us to develop an improved, productive and reproducible micropropagation methodology for establishment of nodal segment cultures of Sr1 and Sr2 plant lines. They were successfully initiated on modified Driver Kuniyuki Walnut (DKW₁; 1984; with Murashige and Skoog (MS; 1962) iron and vitamin supplements) medium supplemented with 5 μ M benzylaminopurine (BAP). An average of 3-4 shoots per node were obtained, which grew up to a height of 2.4 cm in Sr1 and Sr2 plant lines, at the end of 4 weeks. Further, multiplication and elongation of shoots were obtained by pre-treating the cluster of shoots with 0.5 μ M GA₃ for 7 days, before transferring them on modified DKW₁ medium + 5 μ M BAP + 0.05 μ M IBA. At the end of 6 weeks of growth cycle, multiple, multinodal, branched and elongated shoots were obtained in both the experimental plant lines, Sr1 and Sr2. The shoots attained a height of 7.1 cm with 10.0 number of shoots and 5.4 number of nodes per in vitro shoot in Sr1 plant line. Similar outcome was also observed in the Sr2 plant line, where 10.8 number of shoots of 6.8 cm shoot length with 4.8 number of nodes per in vitro shoot were obtained. By following the protocol mentioned here, the higher rate of shoot multiplication was achieved as 54.0-fold and 52.1-fold per nodal explant, in Sr1 and Sr2 plant lines, at every 6 weeks. The following protocol represents a reproducible, economic and productive method for procuring higher plant yield.
- ➔ The in vitro developed lines were evaluated for their genetic integrity tests via ISSR molecular marker analysis and flow cytometry. Using ISSR molecular marker analysis, 998 bands were amplified in an amplification range of 500 bp to 4500 bp. The analysis displayed 98.0% and 95.2% monomorphism among the randomly selected in vitro and mother plants of Sr1 and Sr2 plant lines of *Stevia*, undoubtedly exhibiting the exact parental nature of in vitro plants. Similar study was corroborated using flow cytometry

analysis depicting similar G₁ peak channel profile (~140000) with respect to the in vitro and mother plants. Genome size was estimated to be $1.9\text{-}2.03 \times 10^9$ bp.

- ➔ Moreover, another crucial aspect of the thesis work on *S. rebaudiana* includes quantification of steviol glycosides, rebaudioside A and stevioside. HPLC studies identified rebaudioside A at 4.3 min and stevioside elution at 5.0 min of retention time. Quantification studies revealed in vitro lines of Sr1 plant line possess highest rebaudioside A content (10.7%), followed by in vitro Sr2 plant line (8.7%). Although, both mother plant and in vitro lines are observed to have higher rebaudioside A (R_A) content than stevioside (S), in vitro lines were quite profitable in endowment of better R_A/S ratio (2.1 and 1.7 for in vitro Sr1 and Sr2 lines, respectively), illustrating superior sweetened lines of *S. rebaudiana*. Moreover, stevioside was found to be 5.2% and 5.1% in Sr1 and Sr2 in vitro plant lines, respectively.
- ➔ Further, the purified steviol glycosides were examined for their anti-staphylococcal effect, in which, rebaudioside A and stevioside depicted 65.8% and 70.0% inhibition in biofilm formation.
- ➔ In analysis of antioxidant potential of purified steviol glycosides, rebaudioside A and stevioside exhibited 47.8% and 50.0%, scavenging effects, respectively. In comparison to this, crude extracts of *S. rebaudiana* showed 79.6% to 82.7% reduction in the DPPH free radicals.
- ➔ The outcome of the thesis can form a basis of commercial plan for propagation of plants and production of potential secondary metabolites, as described in the sketch below.

Proposed outline for commercial production of secondary metabolites of *Tinospora cordifolia* and *Stevia rebaudiana*



5.2 FUTURE PROSPECTS



Establishment of in vitro callus cultures using stem explants of *T. cordifolia* could be pursued for chemical profiling of protoberberine alkaloids.



The cytotoxic effects of palmatine, jatrorrhizine, rebaudioside A and stevioside, need to be evaluated using viability assays on animal cell lines.



Combitorial benefits of protoberberine alkaloids or with known anti-bacterial antibiotic could be examined for enhanced anti-bacterial effects.



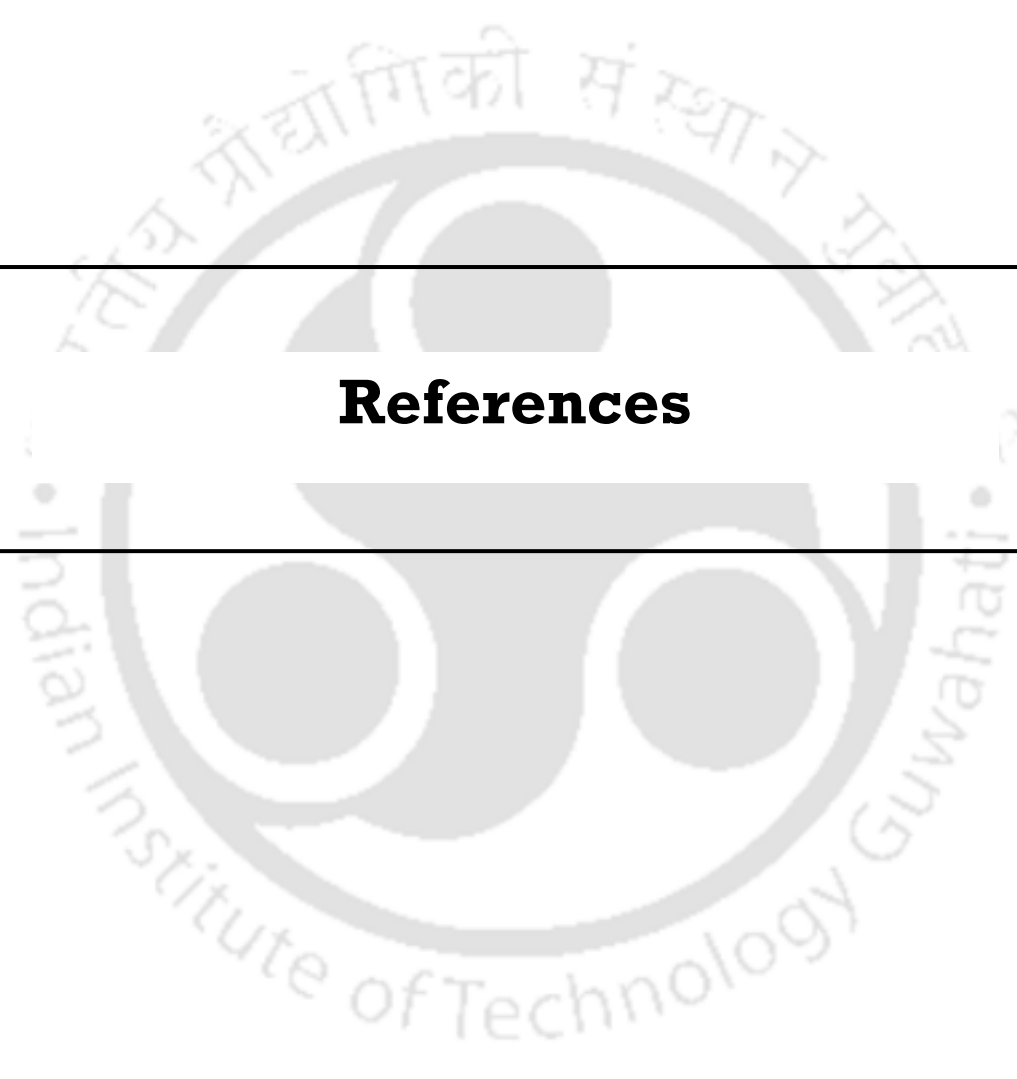
Similarly, combitorial effects of rebaudioside A and stevioside need to be examined at the tested as well as at higher concentrations, for improved therapeutic effects.



Elicitor mediated studies could be an interesting area in case for enhanced productivity of protoberberine alkaloids and steviol glycosides.



Jatrorrhizine and palmatine undoubtedly reduced the formation of *Staphylococcus aureus* biofilm, however, they could also be tested at other stages of biofilm forming nature of *S. aureus* and other microorganisms, such as, time-kill assay post-formation of biofilm as well as against persistent microbial strains.



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Appendix

***Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms**

Kingdom - **Plantae**

Sub-kingdom - **Tracheobionta**

Division - **Magnoliophyta**

Class - **Magnoliopsida**

Sub-class - **Ranunculidae**

Order - **Ranunculales**

Family - **Menispermaceae**

Genus - ***Tinospora***

Species - ***cordifolia***

***Stevia rebaudiana* (Bertoni)**

Kingdom - **Plantae**

Sub-kingdom - **Tracheobionta**

Division - **Magnoliophyta**

Class - **Magnoliopsida**

Sub-class - **Asteridae**

Order - **Asterales**

Family - **Asteraceae**

Genus - ***Stevia***

Species - ***rebaudiana***

LIST OF PUBLICATIONS

International Peer reviewed Journals

1. Kumar P., **Srivastava Vartika**, Chaturvedi Rakhi, Durai S. and Bisaria V. S.*. Elicitor enhanced production of protoberberine alkaloids from in vitro cell suspension cultures of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. Plant Cell Tissue Organ Culture. **2017** 130: 417- 426.
2. **Srivastava Vartika** and Chaturvedi Rakhi. Micropropagation and clonal fidelity assessment of in vitro elite plants of an essential medicinal woody liana, *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. (*Communicated*)
3. **Srivastava Vartika** and Chaturvedi Rakhi. Improved production of protoberberine alkaloids using in vitro callus cultures of *Tinospora cordifolia* and their anti-staphylococcal and antioxidant studies. (*Communicated*)
4. **Srivastava Vartika** and Chaturvedi Rakhi. Development of elite plant lines of *Stevia rebaudiana* Bertoni, their steviol glycosidic analysis and assessment of their anti-staphylococcal and antioxidant effects. (*Communicated*)
5. **Srivastava Vartika** and Chaturvedi Rakhi. A critical updated review on present and future prospects of *Stevia rebaudiana*. (*Communicated*)

Book chapters

6. **Srivastava Vartika**. and Chaturvedi Rakhi*. Somatic Embryogenesis in Neem. In: Mohan Jain and Pramod Gupta (Eds.) Step Wise Protocols for Somatic Embryogenesis of Important Woody Plants. Springer Publications, New York, USA, pp. 369-386, **2018**.
7. **Srivastava Vartika**. and Chaturvedi Rakhi*. Micropropagation of *Tinospora cordifolia* Miers ex Hook. F. & Thoms: A high yielding platform for sustainable production of metabolites. In: S. K Borthakur, A Bawri and N. Nath (Eds.) Recent Advances in Botanical Research in North East India. EBH Publishers, Assam, India, **2018**.

Awards

8. Recipient of **Hope E. Hopps Award** at the In vitro Biology Annual Meeting from June 5-9, organized by the Society for In Vitro Biology (SIVB), USA, **2021**

International and National Conferences

9. **Srivastava Vartika** and Chaturvedi Rakhi*. Exploring the totipotency of plants using plant tissue culture technology for sustainable production of potent natural compounds. In: Research and Industrial Conclave, January 20-23, Indian Institute of Technology Guwahati, Guwahati, India, **2022 (Best Oral)**
10. **Srivastava Vartika** and Chaturvedi Rakhi*. Enhanced Production of Bioactive Protoberberine Alkaloids from In vitro cultures of *Tinospora cordifolia* (Willd.) Miers Ex Hook F. & Thoms. In: In Vitro Biology Meeting 2021, USA on June 5-9, Presentation No: P-1012, **2021 (Oral)**
11. **Srivastava Vartika** and Chaturvedi Rakhi*. Isolation of steviol glycosides from in vitro generated elite cell lines of *Stevia rebaudiana* and its antioxidant analysis. In: 42nd Annual Meeting of Plant Tissue Culture Association – India (PTCA-I) & International Symposium on “Advances in Plant Biotechnology and Genome Editing” (APBGE-2021), April 8-10, ICAR-Indian Institute of Agricultural Biotechnology, Ranchi, Jharkhand, INDIA, **2021 (Poster)**
12. Bajpai R., **Srivastava Vartika**, Das T., Pachauri K. K., Adak R., Imnanaro, Debbarma A., Balla N. and Chaturvedi Rakhi*. Plant tissue culture: A promising approach to quality plant production and sustainable utilization of bioactive metabolites. In: First Departmental Retreat “Biotech Express” December 21, Indian Institute of Technology Guwahati, India **2019 (Poster)**
13. **Srivastava Vartika** and Chaturvedi Rakhi*. Exploring the anti-bacterial potential of palmatine, an alkaloid, identified from in-vitro culture of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. and Thoms. In: Research Conclave, March 14-17, Indian Institute of Technology Guwahati, Guwahati, India, **2019 (Poster)**
14. Organizing committee member and participated in International Conference on Next Generation Plant Production and Bioresources Utilization Technologies (NGPPBUT-2019) from February 11-13, organized by International Plant Propagators' Society (IPPS), USA and Indian Institute of Technology Guwahati, Guwahati, India, **2019**
15. Organizing committee member and participated in 40th Annual Meeting of Plant Tissue Culture Association - India (PTCA-I) and International Conference on Trends in Plant Sciences and Agrobiotechnology (ICTPA-2019) from February 14-16, organized by Plant Tissue Culture Association of India (PTCA-I) and Indian Institute of Technology Guwahati, Guwahati, India, **2019**

16. **Srivastava Vartika** and Chaturvedi Rakhi*. Optimized micropropagation protocol to establish high-yielding true-to-type plantations of elite genotypes of *Tinospora cordifolia* for consistent production of therapeutic compounds. In: International Plant Propagators Society (IPPS), October 17-20, Wilsonville, Oregon, USA, Page No: 5, **2017 (Poster)**
17. Participated in International conference on ‘Genomics Analysis and Technology Conference (GATC-2018) from January 7-9, at Gauhati University, Guwahati, Assam, India, **2018**
18. Participated in International Symposium of ‘Plant Biotechnology for Crop Improvement’ from January 20-21, organized by Indian Institute of Technology Guwahati, Guwahati, Assam, **2017**
19. **Srivastava Vartika** and Chaturvedi Rakhi*. Development of reproducible micropropagation method and screening of bioactive metabolites from *Tinospora cordifolia* (Willd.) Miers ex Hook. F. Thoms. In: World Congress on In Vitro Biology, June 11-15, San Diego, California, USA, Page No: 563, **2016 (Poster)**
20. **Srivastava Vartika** and Chaturvedi Rakhi*. Establishment of in vitro callus cultures for the analysis of essential phytochemical constituents from *Tinospora cordifolia* (Willd.) Miers ex Hook. F. Thoms against challenging diseases. In: International Seminar on Prevention, Promotion, and Pacification: Ayurvedic Landscape, February 9-11, Science City Auditorium, Kolkata, India, Page No: 185, **2016 (Best poster)**
21. **Srivastava Vartika** and Chaturvedi Rakhi*. Screening of two important alkaloids from cell suspension cultures of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms using high-throughput screening methods. In: 22nd ISCB International Conference - Recent trends in Affordable and Sustainable Drug Discovery and Developments, February 6-8, Uka Tarsadia University, Surat, India, Page No: 87, **2016 (Oral)**
22. **Srivastava Vartika** and Chaturvedi Rakhi*. Impact of plant tissue culture in sustainable drug development. In: Research Wave, held on October 11, 2015, organized by Association of Civil Engineering (ACE) and Student Alumni Linkage cell (SAIL), Indian Institute of Technology Guwahati, Guwahati, Assam, India, **2015 (Oral)**
23. **Srivastava Vartika**, Jannat W, Chaturvedi Rakhi*. Establishment of in vitro culture and micropropagation protocol to raise high yielding true-to-type plantations of *Tinospora cordifolia* (Willd.) Miers ex Hook. F. & Thoms. In: Asian Plant Science Conference (APSC), November 1-3, Bhairahawa, Nepal, Page No: 29, **2014 (Oral)**

24. Dutta R., **Srivastava Vartika**, Chaturvedi Rakhi*. Extraction and Identification of Biologically Active Compounds from In vivo and In vitro Cultured *Tinospora cordifolia* (Willd.) Miers ex Hook. F. Thomas. In: Asian Plant Science Conference (APSC), November 1-3, Bhairahawa, Nepal, Page No: 59, **2014**

Workshops

25. 'Targeted Approach using Nutraceuticals for Prevention & Treatment of Cancer' from July 25-26, organized by Rapture Biotech, India, **2020**
26. 'Next Generation Sequencing' from January 10-13, at Gauhati University, Guwahati, India, **2018**
27. 'Biomedical Device Technology' as part of 5th International Conference on Advanced Nanomaterials and Technology (ICANN) on December 18, at Indian Institute of Technology Guwahati, Guwahati, Assam, India, **2017**
28. 'Flow Cytometry Data Analysis' held from January 23-24, at Indian Institute of Technology Guwahati, Guwahati, Assam, India, **2015**

