MODULATION OF ABIOTIC STRESS SIGNALS IN *IN VITRO* MORPHOGENIC RESPONSES AND SECONDARY METABOLITES PRODUCTION OF *AYAPANA TRIPLINERVIS* (VAHL) R.M.King & H.Rob.

Thesis submitted to the

University of Calicut

For the award of the Degree of

DOCTOR OF PHILOSOPHY IN

BOTANY

Under the Faculty of Science

By

APARNA BALAKRISHNAN

Under the Guidance of

Dr. DELSE P. SEBASTIAN

CENTRE FOR POST GRADUATE STUDIES AND RESEARCH IN BOTANY

ST. JOSEPH'S COLLEGE (AUTONOMOUS) DEVAGIRI

CALICUT- 673008, KERALA, INDIA

JANUARY 2024

DECLARATION

I hereby declare that the work presented in the thesis entitled **"MODULATION OF ABIOTIC STRESS SIGNALS IN *IN VITRO* MORPHOGENIC RESPONSES AND SECONDARY METABOLITES PRODUCTION OF *AYAPANA TRIPLINERVIS* (VAHL) R.M.King & H.Rob."** is based on the original work done by me under the guidance of **Dr. Delse P. Sebastian,** Assistant Professor, Department of Botany, St. Joseph's College (Autonomous) Devagiri, Calicut, Kerala and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis are undergone plagiarism check using iThenticate software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI generated contents.

Aparna Balakrishnan

Dr. Delse P. Sebastian
(Supervising Teacher)

Dr. DELSE P. SEBASTIAN
Assistant Professor
Department of Botany
St. Joseph's College (Autonomous)
Devagiri, Kozhikode - 673 008

Place: Kozhikode

Date: 26/06/2024

ST. JOSEPH'S COLLEGE (AUTONOMOUS),
DEVAGIRI, CALICUT, KERALA, INDIA
PIN-673 008
Re-accredited by NAAC with A++
Affiliated to the University of Calicut
Phone:+91 9447331351
e-mail:delsbotany@gmail.com

Dr. Delse P. Sebastian M.Sc., Ph.D.
Assistant Professor of Botany

CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"MODULATION OF ABIOTIC STRESS SIGNALS IN *IN VITRO* MORPHOGENIC RESPONSES AND SECONDARY METABOLITES PRODUCTION OF *AYAPANA TRIPLINERVIS* (VAHL) R.M.King & H.Rob."** is an authentic record of the original research work accomplished by **Ms. Aparna Balakrishnan** under my supervision and guidance at the Centre for Post Graduate Studies and Research in Botany, St. Joseph's College (Autonomous) Devagiri, Calicut, Kerala and that no part of this thesis has been published earlier for the award of any other degree or diploma. Also certified that the contents in the thesis are subjected to **Plagiarism Check** using the software **iThenticate** and that no text or data is reproduced from other's work.

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Dr. Delse P. Sebastian

Dr. DELSE P. SEBASTIAN
Assistant Professor
Department of Botany
St. Joseph's College (Autonomous)
Devagiri, Kozhikode - 673 008

UNIVERSITY OF CALICUT

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3.	Name of the Supervisor	Dr. Delse P. Sebastian	
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St. Joseph's College (Autonomous)
Devagiri, Calicut - 673 008

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to the almighty for enabling me to successfully complete my Ph.D. program. I am immensely grateful for the unwavering support and encouragement extended to me by countless individuals during the duration of my research. Their timely assistance proved to be invaluable, as I could not have concluded my thesis without them.

I am immensely thankful to ***Dr. Delse P. Sebastian****, my esteemed research mentor, for his consistent support and guidance throughout my research journey. His exceptional expertise, patience, and encouragement have been invaluable in shaping the final outcome. I express my heartfelt appreciation for his constructive feedback and insightful advice, which have significantly enhanced the quality of my work. Without his dedication and incredible patience, the successful completion of my research work would not have been possible.*

I express my profound gratitude to the ***Council of Scientific and Industrial Research-Human Resource Development Group (CSIR-HRDG)****, New Delhi, for bestowing upon me financial support in the form of a fellowship. The generous grant provided a crucial impetus in achieving the successful culmination of my research work. It would not be an overstatement to acknowledge that the fellowship played an instrumental role in facilitating my research endeavor.*

I express my heartfelt gratitude to ***Dr. Satheesh George****, Principal in-charge, St. Joseph's College (Autonomous), Devagiri, for generously providing me with the necessary resources to conduct my research. His vast knowledge and*

insightful input proved to be of immense value in shaping my research problem, and his guidance remained indispensable throughout my research project.

I am extremely grateful to Principal ***Dr. Bobby Jose*** *for providing wholehearted assistance in completing this work. I would also like to express my gratitude to former principals* ***Dr. Sabu K. Thomas****,* ***Dr. Jose John Mallikasseri****, and* ***Dr. Sibichen M. Thomas*** *for their support and help during my research.*

I would like to express my utmost gratitude to ***Dr. Sulaiman C.T.*** *for his invaluable guidance in comprehending different methodologies and interpreting results. Lastly, I extend my sincerest appreciation to* ***Mr. Deepak M.*** *and* ***Mr. Salman*** *for their invaluable support throughout the different stages of this project.*

I am deeply thankful to ***Dr. Rahul P. R.*** *for the molecular work and results. Also, express my gratitude to* ***Dr. Geetha S. Pillai*** *and* ***Dr. Sadheeshna Kumari S.*** *during the study.*

I would like to convey my heartfelt gratitude to ***Dr. Jojo Joseph,*** *Dept. of Botany, St. Joseph's College (Autonomous), Devagiri,* ***Dr. Abis V. Cherussery, and Dr. George P.S.*** *former faculty members of the Dept. of Botany, St. Joseph's College (Autonomous), Devagiri for their guidance and the immense knowledge shared, which helped me to build a strong foundation in my journey. I also express my gratitude to* ***Dr. Binu Thomas, Dr. Manudev K.M., Dr. Ron Sunny, Mr. Peter A. A,*** *and* ***Mr. Jithin J.*** *Dept. of Botany, St. Joseph's College (Autonomous), Devagiri for their timely help during the research period.*

I am deeply indebted to my fellow labmates, ***Akshaya Prakash C****.,* ***Dilsha M.V****.,* ***Aparna Prasad****,* ***Mahesh Mohanan****,* ***Neena A****.,* ***Aswindas T.P****.,* ***Bhavana R****.,* ***Rekha C****.,* ***Jameema Sidhic****,* ***Navya C.P****.,* ***Sindhu E.S****.,* ***Naseeha C.P.,*** *and* ***Prasanth M.K****. of St. Joseph's College (Autonomous), Devagiri for their valuable help, support and encouragement during the research period.*

I extend my sincere gratitude to all my former teachers who supported me to build my academic career: ***Mr. Reji T. Thomas****,* ***Dr. Dennis Thomas T.****,* ***Mr. Paul V. Karamthanam****,* ***Dr. Jomy Augustine****,* ***Dr. Bince Mani****,* ***Dr. P. O. Augusthy****,* ***Dr. Toji Thomas****, Late* ***Dr. Jhons Augustine, and Dr. Theja Joseph.***

*Finally, I dedicate this work to my Achan (****Balakrishnan P. S.****), Amma (****Sobhana****), and brother (****Abhijith****) for the concern, care and encouragement that gave me the strength and patience to complete my work successfully. I realize the depth of their sacrifices and am forever indebted to them.*

ABSTRACT

Ayapana triplinervis (Vahl) R.M.King & H.Rob, belonging to the family Asteraceae, is a medicinal plant with immense medicinal properties due to the presence of a number of secondary metabolites like ayapanin (7-methoxycoumarin), hydragetin, daphnetin etc. *In vitro* production of this plant can be a convenient method for the commercial production of these metabolites since this method prevents the exploitation of natural population of this plant. The present research work revealed how abiotic stress signals affect *in vitro* morphogenic responses and secondary metabolites production of *A. triplinervis*. The entire plant culture study was conducted using MS medium in an *in vitro* system.

The initial objective of the study was to develop protocols for direct and indirect organogenesis of *A. triplinervis* using varying concentrations of plant growth regulators. Nodal explants were selected for direct organogenesis, and leaf and internodal explants were used for indirect organogenesis and inoculated to the MS media containing different combinations and concentrations of Kin, BAP, NAA, IBA, and TDZ. 0.5 mg l^{-1} Kin was found to be best for the rapid multiplication of shoots from the single nodal explants (direct organogenesis) while TDZ (1.0 mg l^{-1} and 1.5 mg l^{-1}) alone and TDZ (1.5 mg l^{-1} and 2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) supplemented media was found to be most effective in shoot regeneration from the leaf explants (indirect organogenesis). The plants developed from the leaf explants through indirect organogenesis were subjected to genetic fidelity analysis using ISSR markers and it was found that the regenerants were genetically 100% similar to the mother plant.

The effect of different concentrations of various stress signals, NaCl, SA, Sucrose, MS medium strengths, Put, Trp, JA, and Chitosan, were also studied in *A. triplinervis in vitro* cultures. It was found that the morphogenic responses of the plant varied for each stress signals used. The secondary metabolite profile of the plant was studied using HPTLC. The HPTLC studies on the methanolic extracts of *A. triplinervis*, revealed the presence of maximum number of compounds in plants grown in SA supplemented media as well as in the various strengths of MS media. In the case of 'Ayapanin' content, the production was enhanced in the media fortified with NaCl, Sucrose, Put and Trp compared to the control plants. The study thus, validated the effects of several abiotic stress signals at different concentrations in the secondary metabolite production of *Ayapana triplinervis*.

Keywords: Direct and indirect organogenesis, genetic fidelity, ISSR, stress response, Ayapanin, HPTLC

സംഗ്രഹം

അയപാനിൻ (7-മെത്തോക്സികൗമാരിൻ), ഹൈഡ്രാജെറ്റിൻ, ഡാപ്നെറ്റിൻ തുടങ്ങിയ അനേകം ദ്വിതീയ ഉപാപചയങ്ങളുടെ സാന്നിദ്ധ്യം നിമിത്തം അപാരമായ ഔഷധഗുണങ്ങളുള്ള ഒരു ഔഷധസസ്യമാണ് ആസ്റ്ററേസി കുടുംബത്തിൽപ്പെട്ട *അയപാന ട്രിപ്ലിനർവിസ്* (വഹ്ൽ) ആർ.എം.കിംഗ് & എച്ച്.റോബ്. ഈ ചെടിയുടെ മെറ്റബോളിറ്റുകളുടെ ഇൻ വിട്രോ ഉൽപ്പാദനം വാണിജ്യ ഉൽപാദനത്തിന് സൗകര്യപ്രദമായ ഒരു മാർഗ്ഗം ആണ്, കാരണം ഈ രീതി ഈ ചെടിയുടെ സ്വാഭാവിക ജനസംഖ്യയെ ചൂഷണം ചെയ്യുന്നത് തടയുന്നു. അബയോട്ടിക് സ്ട്രെസ് സിഗ്നലുകൾ ഇൻ വിട്രോ മോർഫോജെനിക് പ്രതികരണങ്ങളിലും എ. ട്രിപ്ലിനേർവിസ്സിന്റെ ദ്വിതീയ മെറ്റബോളിറ്റുകളുടെ ഉൽപാദനത്തിലും എങ്ങനെ സ്വാധീനം ചെലുത്തുന്നുവെന്ന് നിലവിലെ ഗവേഷണ പ്രവർത്തനങ്ങൾ വെളിപ്പെടുത്തി. മുഴുവൻ പ്ലാന്റ് കൾച്ചർ പഠനവും എംഎസ് മീഡിയം ഇൻ വിട്രോ സിസ്റ്റത്തിൽ ഉപയോഗിച്ചാണ് നടത്തിയത്.

സസ്യവളർച്ച റെഗുലേറ്ററുകളുടെ വ്യത്യസ്ത സാന്ദ്രത ഉപയോഗിച്ച് എ. ട്രിപ്പിനെർവിസിന്റെ പ്രത്യക്ഷവും പരോക്ഷവുമായ ഓർഗനോജനസിസിനായി പ്രോട്ടോക്കോളുകൾ വികസിപ്പിക്കുക എന്നതായിരുന്നു പഠനത്തിന്റെ പ്രാരംഭ ലക്ഷ്യം. നേരിട്ടുള്ള ഓർഗാനോജെനനിസിനായി നോഡൽ എക്സ്പ്ലാന്റുകൾ തിരഞ്ഞെടുത്തു, പരോക്ഷമായ ഓർഗാനോജെനിസിസിനായി ഇലയും ഇന്റർനോഡൽ എക്സ്പ്ലാന്റുകളും ഉപയോഗിച്ചു, കൂടാതെ കിൻ, ബിഎപി, എൻഎഎ, ഐബിഎ, ടിഡിസെഡ് എന്നിവയുടെ വ്യത്യസ്ത കോമ്പിനേഷനുകളും കോൺസൺട്രേഷനുകളും അടങ്ങിയ എംഎസ് മീഡിയയിലേക്ക് ഇനോക്കുലേറ്റ ചെയ്തു. 0.5 mg l^{-1} കൈനെറ്റിൻ ആണ് സിംഗിൾ നോഡൽ എക്സ്പ്ലാൻറുകളിൽ നിന്ന് (ഡയറക്ട് ഓർഗാനോജെനസിസ്) ചിനപ്പുപൊട്ടലിന്റെ ദ്രുതഗതിയിലുള്ള പെരുകലിന് ഏറ്റവും നല്ലതെന്ന് കണ്ടെത്തി, അതേസമയം ടിഡിസെഡ് (1.0 mg l^{-1}, 1.5 mg l^{-1}) മാത്രം, ടിഡിസെഡ് (1.5 mg l^{-1}) കൂടാതെ 2.0 mg l^{-1}) എൻഎഎ (0.5 mg l^{-1}) സപ്ലിമെന്റഡ് മീഡിയ ഇല എക്സ്പ്ലാൻറുകളിൽ നിന്നുള്ള ഷൂട്ട് പുനരുജ്ജീവനത്തിന് (പരോക്ഷ ഓർഗാനോജെനസിസ്) ഏറ്റവും ഫലപ്രദമാണെന്ന് കണ്ടെത്തി.

പരോക്ഷമായ ഓർഗാനോജെനിസിസ് വഴി ഇല എക്സ്പ്ലാൻറുകളിൽ നിന്ന് വികസിപ്പിച്ച സസ്യങ്ങൾ ഐഎസ്എസ്ആർ മാർക്കറുകൾ ഉപയോഗിച്ച് ജനിതക വിശ്വാസ്യത വിശകലനത്തിന് വിധേയമാക്കി, പുനരുൽപ്പാദനം ജനിതകപരമായി മാതൃ സസ്യവുമായി 100% സാമ്യമുള്ളതാണെന്ന് കണ്ടെത്തി. വിവിധ സ്ട്രെസ് സിഗ്നലുകൾ, എൻഎസിഎൽ, എസ്എ, സുക്രോസ്, എംഎസ് മീഡിയം ശക്തികൾ, പ്യൂട്രെസിൻ, ട്രിപ്റ്റോഫാൻ, ജെഎ, കൈറ്റോസാൻ എന്നിവയുടെ വ്യത്യസ്ത സാന്ദ്രതകളുടെ ഫലവും എ. ട്രിപ്ലിനർവിസ് ഇൻ വിട്രോ കൾച്ചറുകളിൽ പഠിച്ചു. ഉപയോഗിച്ച ഓരോ

സ്ട്രെസ് സിഗ്നലുകൾക്കും ചെടിയുടെ മോർഫോജെനിക് പ്രതികരണങ്ങൾ വ്യത്യസ്തമാണെന്ന് കണ്ടെത്തി. ചെടിയുടെ ദ്വിതീയ മെറ്റാബോലൈറ്റ് പ്രൊഫൈൽ എച്ച്പിടിഎൽസി ഉപയോഗിച്ച് പഠിച്ചു. എ. ടിപ്ലിനർവിസിന്റെ മെത്തനോളിക് സത്തിൽ എച്ച്പിടിഎൽസി പഠനങ്ങൾ, എസ്എ അനുബന്ധ മാധ്യമങ്ങളിലും അതുപോലെ വിവിധ എംഎസ് ശക്തി മാധ്യമങ്ങളിലും വളരുന്ന സസ്യങ്ങളിൽ പരമാവധി എണ്ണം സംയുക്തങ്ങളുടെ സാന്നിധ്യം വെളിപ്പെടുത്തി. 'അയാപാനിൻ' ഉള്ളടക്കത്തിന്റെ കാര്യത്തിൽ, കൺട്രോൾ പ്ലാന്റുകളെ അപേക്ഷിച്ച് എൻഎസിഎൽ, സുക്രോസ്, പുട്രെസിൻ, ട്രിപ്റ്റോഫാൻ എന്നിവ ഉപയോഗിച്ച് സമൃഷ്ടമാക്കിയ മീഡിയയിൽ ഉത്പാദനം വർദ്ധിപ്പിച്ചു. അതിനാൽ, അയപാന ടിപ്ലിനർവിസിന്റെ ദ്വിതീയ മെറ്റാബോലൈറ്റ് ഉൽപാദനത്തിൽ വ്യത്യസ്ത സാന്ദ്രതകളിലുള്ള നിരവധി അബയോട്ടിക് സ്ട്രെസ് സിഗ്നലുകൾ പഠന ഫലങ്ങളെ സാധൂകരിക്കുന്നു.

കീവേഡുകൾ: പ്രത്യക്ഷവും പരോക്ഷവുമായ ഓർഗാനോജെനിസിസ്, ജനിതക വിശ്വസ്തത, ഐഎസ്എസ്ആർ, സമ്മർദ്ദ പ്രതികരണം, അയപാനിൻ, എച്ച്പിടിഎൽസി

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ABBREVIATIONS

µg mg^{-1}	: microgram per milligram
µl^{-1}	: milligram per litre
µM l^{-1}	: micromolar per litre
2,4-D	: 2,4-Dichlorophenoxyacetic acid
2-iP	: 2-isopentyl Adenine
ABTS	: 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid
ADC	: Arginine Decarboxylase
AFLP	: Amplified Fragment Length Polymorphism
AgNPs	: Silver Nanoparticles
ANOVA	: Analysis of Variance
ANS	: Anisaldehyde
APX	: Ascorbate peroxidase
Arg	: Arginine
BAP	: 6-Benzyl Amino Purine
Ca	: Calcium
$CaCl_2$	: Calcium chloride
CAT	: Catalase
CCl_4	: Carbon tetrachloride
Chit	: Chitosan
cm	: centimeter
cm^2	: square centimeter
CMPR	: Centre for Medicinal Plants Research
CO_2	: Carbon Dioxide
$CoCl_2.6H_2O$	: Cobalt chloride hexahydrate
$CuSO_4.5H_2O$	: Copper(II) sulphate pentahydrate
DNA	: Deoxyribonucleic acid
DPPH	: 2,2-diphenyl-2-picrylhydrazyl

$dS\ m^{-1}$	: decisiemens per metre
DW	: Dry Weight
EtOH	: Ethyl alcohol
$FeSO_4.7H_2O$	: Ferrous sulphate heptahydrate
FRAP	: Ferric Reducing Antioxidant Power
FTIR	: Fourier-transform infrared spectroscopy
FW	: Fresh Weight
$g\ l^{-1}$	: gram per litre
GA_3	: Gibberellic Acid
GABA	: Gamma-aminobutyric acid
GC/MS	: Gas Chromatography Mass Spectrometry
GPx	: Glutathione Peroxidase
GSH	: Glutathione
H_2O_2	: Hydrogen peroxide
H_3BO_3	: Boric acid
HAc	: Acetic acid
HCC	: Hepatocellular carcinoma
HCl	: Hydrochloric acid
HDF	: High Diet-Factor
$HgCl_2$	: Mercuric Chloride
HPLC	: High Performance liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
IAA	: Indole-3-acetic acid
IAOx	: Indole-3-acetaldoxime
IBA	: Indole-3-butyric acid
iP	: inorganic phosphate
ISR	: Induced Systemic Resistance
ISSR	: Inter-Simple Sequence Repeats
JA	: Jasmonic acid

K	: Potassium
KH_2PO_4	: Potassium dihydrogen phosphate
KI	: Potassium Iodide
Kin	: Kinetin
KNO_3	: Potassium nitrate
KOH	: Potassium Hydroxide
LAF	: Laminar Air Flow
LC-MS	: Liquid Chromatography- Mass Spectrometry
MDA	: Malondialdehyde
MeJA	: Methyl jasmonate
mg	: milligram
mg dm^{-3}	: milligram per cubic decimeter
MIC	: Minimum Inhibitory Concentration
mM	: Millimolar
$MnSO_4.4H_2O$	: Manganese sulfate tetrahydrate
MnSOD	: Manganese Superoxide Dismutase
MS Excel	: Microsoft Excel
MS medium	: Murashige Skoog medium
N	: Nitrogen
Na	: Sodium
Na_2EDTA	: Ethylenediaminetetraacetic acid disodium salt dihydrate
$Na_2MoO_4.2H_2O$	: Sodium molybdate dihydrate
NAA	: 1-Naphthalene acetic acid
NaCl	: Sodium chloride
$NaHCO_3$	: Sodium bicarbonate
NaOH	: Sodium Hydroxide
NH_4^+	: Ammonium cation
NH_4NO_3	: Ammonium nitrate
nm	: nanometer

NMR : Nuclear Magentic Resonance

O_2- : Superoxide radicals

OD : Optical Density

ODC : Ornithine Decarboxylase

OH- : Hydroxyl radicals

Orn : Ornithine

P : Phosphorus

PAL : Phenylalanine Ammonia Lyase

PAs : Polyamines

PCR : Polymerase Chain Reaction

PGRs : Plant Growth Regulators

pH : Potential of Hydrogen

POD : Peroxidase

PPO : Polyphenol Oxidase

PPX : Exopolyphosphate

PRPP : Phosphoribosyl diphosphate

PSM : Plant Secondary Metabolite

PUFA : Polyunsaturated fatty acids

Put : Diamine putrescine

q-rtPCR : Real-Time Quantitative Reverse Transcription PCR

R.B. flask : Round Bottom flask

RA : Rosmarinic acid

RAPD : Random-Amplified Polymorphic DNA

Rf : Retention factor

RFLP : Restriction Fragment Length Polymorphism

ROS : Reactive Oxygen Species

RP-HPLC-PDA : Reversed-phase high-pressure liquid Chromatography quipped with photodiode array detector

RuBisCO : Ribulose biphosphate Carboxylase-Oxygenase

RWC	: Relative Water Content
SA	: Salicylic acid
SAR	: Systemic Acquired Resistance
SBAP	: Sequence-Based Amplified Polymorphism
SCAR	: Sequence Characterized Amplified Regions
SE	: Standard Error
SEM	: Scanning Electron Microscopy
SM	: Signal molecules
SOD	: Suproxide dismutase
Spd	: Triamine spermidine
Spm	: Tetramine spermine
STS	: Sequence Tag Sites
TAE	: Tris-acetate-EDTA buffer
TDZ	: Thidiazuron
THQ	: Thymohydroquinone dimethyl ether
TLC	: Thin Layer Chromatography
Trp	: Tryptophan
TWC	: Tissue Water Content
UC	: Ulcerative colitis
UPLC-ESI-MS-MS	: Ultra-performance liquid chromatography coupled to electrospray ionization-tandem mass spectrometry
UV	: Ultraviolet
UV-Vis Spectroscopy	: Ultraviolet-visible spectroscopy
v/v	: volume/volume
w/v	: weight/volume
ZIKV	: Zika Virus
$ZnSO_4.4H_2O$	: Zinc sulphate

CHAPTER I

INTRODUCTION

Since ancient times, plants have been a significant source of medicine (Tripathi & Tripathi, 2003). The medicinal importance of plants is one of the most important features; every plant in nature has its medicinal properties. Plants are an excellent resource for the synthesis of novel compounds with therapeutic value in drug development. For a large percentage of the world's population, medicinal plants are the sole reservoir of life-saving medications (Hussein *et al.*, 2011). As per the estimates of World Health Organization, approximately 80% of people still rely upon herbs for medicinal requirements. Moreover, plants are the mainstay of a number of modern medicines. Plant extracts or the components that are produced from plant extracts or designed after plant compounds make up almost one fourth of all prescription medications that are available in market today (Tripathi & Tripathi, 2003).

Herbal medicines represent safety in contrast to synthetics, which are deemed hazardous to both humans and the environment. The practice of healthcare using medicinal plants started millions of years ago; Ayurveda, Siddha, Unani, and Naturopathy were also based on medicinal plants. Ayurveda, Unani, Siddha, Amchi and modern medicines use approximately 700, 700, 600, 600 and 30 species of plants respectively. More than 80,000 of the world's 2,50,000 higher plant species are therapeutically important. With approximately 45000 plant species, India is one of the global 12 biodiversity hotspots (Joy *et al.*, 2001).

Traditional systems of medicine greatly gained popularity among the people and they supported it for many valid reasons such as side effects raised by the chemical drugs, high cost of the chemical drugs and treatments,

scarcity raised when demand increased, many infection agents became resistant towards the drugs, etc. Out of ancient civilizations, India was recognized to have a great reservoir of medicinal herbs. The majority of India's medicinal and aromatic plants are found in the country's forests, where they are primarily collected as raw materials for the production of pharmaceuticals and perfumery goods (Joy *et al.*, 2001).

Plant secondary metabolites are frequently defined as metabolites that are not essential for the sustainability of plant developmental functions but are necessarily required for the plant to communicate with its environment for adaptability as well as defence (Akula & Ravishankar, 2011). In addition to being a significant source of potent therapeutics, these compounds are known to play a significant role in plants' environmental adaptation. In comparison to the primary compounds found in plants, these secondary metabolites were quickly recognized by their poor production, frequently less than 1% of total carbon, or storage in specific cells or organs (Bourgaud *et al.*, 2001). In the past 50 years, research on plant secondary metabolites has grown. A variety of phytocompounds are found in plants such as: - phenolics, terpenoids, steroids, alkaloids, coumarins, saponins, flavonoids, etc., and have various functions other than medicinal properties. Other useful hacks include perfumes, dyes, flavors, insecticides and so on.

Approximately 120 medicinal compounds with known structures have been discovered from the limited group of flowering plants that have so far been studied, which represents about 90 plant species. Atropine, aspirin, allicin, capsaicin, curcumin, camptothecin, codeine, vinblastine, taxol,

morphine, artemisinin, and ephedrine are some examples of valuable plant medications (Joy *et al.*, 2001).

Induction due to various adverse conditions like environmental stresses, pathogen attacks, or any other stress signals, can cause the accumulation of secondary metabolites. In natural ways, the production of these valuable compounds was less so, various biotechnological tools such as plant cell, tissue, or organ culture were employed nowadays to enhance their accumulation.

Plant tissue culture (PTC) methods are unaffected by environmental and geographical variables and will allow continuous, sustainable, affordable, and effective secondary metabolite production (Chandran *et al.,* 2020). Other than secondary metabolite production, the PTC can also be employed as a crop improvement technique. New varieties of plants were created by exploiting the somaclonal variations generated from the callus formed from the explants.

Plant improvement by somaclonal variation is projected to supplement traditional breeding as an alternate method. This approach might produce new superior variations with enhanced efficiency and a more interesting texture. The plant experiences somaclonal variation as an impact of cell regeneration within the *in vitro* culture period; these variations typically do not come from the axillary shoot or shoot tip (Lestari, 2006).

Plants that have been regenerated from undifferentiated callus cultures have a diverse set of genetic modifications (Phillips *et al.,* 1994). The improvement of more productive cultivation techniques for the species may depend on understanding the genetic variations within each cultivated

population and the genetic makeup of populations, which may help to guide either comparable or distinct cultivation practices (Luz *et al.*, 2020). It is required to look for significant variations at the molecular level to figure out the positions and severity of deviation from the true-to-type (Smýkal *et al.*, 2007).

Understanding the molecular basis of many biological processes in plants can be aided by the identification and evaluation of genetic variation. This was achieved by the use of phenotype based genetic markers earlier. But the creation and use of molecular markers recently have been found to be a better and reliable source compared to phenotype-based genetic markers. The stability and prominence of molecular markers in all tissues disregarding the growth, development, differentiation, or state of a cell's defence means that they have several benefits over traditional phenotype-based methods (Agarwal *et al.*, 2008).

Amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSR), Random-amplified polymorphic DNA (RAPD), Restrictions fragment length polymorphism (RFLP), sequence characterized amplified regions (SCAR), sequence-based amplified polymorphism (SBAP), and sequence tag sites (STS) are some of the molecular markers used in genetic fingerprinting (Sharma *et al.*, 2012).

ISSR markers have been presented to be an efficient, rapid, clear, reproducible, and affordable mode of analysing genetic diversity as well as identifying variations among closely related cultivars in several species of plants with limited sequence information (González *et al.*, 2000). The distribution of nucleotide repeats (inter-simple sequence repeats) all over the

genome can be discriminating, as shown in clonal plant species for the evaluation of genetic diversity (Verma *et al.*, 2017).

The limitations of conventional *in vivo* methods of secondary metabolite production can be rectified using the *in vitro* culture methods. *In vitro*, cell suspension cultures and bio-reactors were excellent sources of commercial production of desired phytocompounds.

Secondary metabolites that are utilized as medications, food additives, flavors, perfumes, colouring agents, biopesticides etc, have begun to be produced in plant cell and organ cultures, and this has raised the possibility of alternative sources for these phytocompounds. The factors that affect the growth as well as proliferation of cultured cells/organs, and the accumulation of biomass, are managed in the initial phase. The second step involves the regulation of factors that play a role in the production of metabolites (Murthy *et al.*, 2014).

The chemical composition and therapeutic properties of medicinal plants are significantly influenced by stress (Naik & Al-Khayri, 2016). Plants have been known to undergo stress due to various physical or chemical factors, such as nutrient deficiency or toxicity. As a result of these stressors, plants may display changes in their physiological, biochemical, and molecular mechanisms that are noticeable in their morphological characteristics (Pandey *et al.*, 2021). Vargas-Hernandez *et al.* (2017) found that the response from the plants depended upon the dose of growth stimulants used, and during the different stages of growth and development, the nutrient supply can act as both eustress (favourable to plant growth) or distress (unfavourable to plant growth).

An elicitation process stimulates the plant's stress response to achieve the desired chemical response. This process utilizes the correlation between plant stress and phytochemistry. An elicitor is a substance that acts as a stressor and, when applied in minute quantities to a living system, enhances the biosynthesis of particular compounds vital to a plant's adaptation to stressful conditions. The production of plant cell and organ cultures for the biotechnological generation of secondary metabolites is a viable alternative to extracting the whole plant material (Naik & Al-Khayri, 2016).

Elicitation or stress induction is an effective way to enhance the production of secondary metabolites in a short period. Elicitors or stress signals are substances derived from living (biotic) and non-living (abiotic) sources that can promote stress responses in plants, leading to augmented secondary metabolite production or the generation of novel secondary metabolites. The impact on the formation of secondary metabolites is primarily influenced by the elicitor type, dosage, and treatment period. A variety of factors, including elicitor concentrations, treatment time, cell line, nutritional content, and culture phase or stage, are also significant determinants of the efficacy of biomass production and secondary metabolite accumulation (Naik & Al-Khayri, 2016). The elicitors can initiate the signal-transduction pathway and trigger the upregulation of genes that are associated with the biosynthesis of secondary metabolites (Zhao *et al.*, 2005).

The elicitors were divided into abiotic and biotic in behalf of their nature (Naik & Al-Khayri, 2016). Both biotic and abiotic elicitors have been widely utilized to promote or trigger the *de novo* formation of secondary metabolites in plant tissue culture (Kaur *et al.*, 2021). Physical, chemical, and

hormonal components make up the categories of abiotic elicitors, which are substances with nonbiological origins. Polysaccharides derived from plant cell walls and microorganisms are examples of biological elicitors (Naik & Al-Khayri, 2016). Abiotic stress signals or elicitors induce morphological, biochemical and molecular changes in the plants.

The most commonly found abiotic stress is excessive levels of salinity (NaCl). The cultivation and growth of crops are severely hampered by soil salinity, which affects crop yields and the utilization of formerly uncultivated areas. Ions that can be transferred inside and outside of cells, primarily Na^+ and Cl^-, are what induce salt stress (Sabir *et al.,* 2012). The osmotic and toxic effects of salt, as well as the intensity and period of the stress, all influence negative plant responses to salinity stress (Hasegawa *et al.,* 2000). As a result of salinity stress oxidative damage frequently occurs and the production of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), is a secondary effect of salinity stress on plants (Halliwell *et al.,* 1986).

Sucrose is used as a carbon source in plant *in vitro* cultures since it can be conveniently transported across membranes, allowing it to be quickly metabolized by the majority of cells due to the presence of a sucrose transporter (Slone *et al.,* 1991). Exogenous carbon sources are vital for culture growth, since growth is halted when they are depleted (Kretzschmar *et al.,* 2007). The other imperative functions of sucrose include osmotic pressure maintenance and act as a rate-limiting factor in the biosynthesis of primary or secondary metabolites (Ali *et al.,* 2016).

Jasmonic acid (JA) is a family of indigenous plant growth regulators that is extensively widespread in the whole plant kingdom (Ulloa *et al.*, 2002). And JA is a plant signaling molecule that is strongly connected to plant tolerance to abiotic stress, mainly involved in the physiological (activation of antioxidant systems, regulating guard cells, accumulation of amino acids, etc.) and molecular responses of plants (Wang *et al.*, 2020).

Salicylic acid (SA) is also an important endogenous signal molecule in plants that triggers systemic acquired resistance (SAR) and induced systemic resistance (ISR) counteracting pathogen or herbivore attacks (Mendoza *et al.*, 2018); it also influences the production of ROS (Kaur *et al.*, 2020).

The culture medium contains mineral salts as the key elements and several combinations of macro- and micro-salts were found. The MS medium is one of the high salt-containing media used for *in vitro* cultures (Fadel *et al.*, 2010). Limiting mineral salts (MS medium strength) in the medium creates nutritional stress.

Polyamines (PAs), which include diamine putrescine (Put), triamine spermidine (Spd), and tetramine spermine (Spm), are chemical molecules having several primary amino groups that are ubiquitous in nature and present in plant cells (Wu *et al.*, 2010). Putrescine (Put) is the most prevalent polyamine produced in higher plants. Put might be essential for both plant growth and development and plant tolerance reactions to the various stresses impacting agricultural yield (González-Hernández *et al.*, 2022). Put enhances biomass accumulation and increases the production of indigenous growth regulators.

Tryptophan (Trp) is a key precursor of plant secondary metabolites. Indole-3-acetaldoxime (IAOx) and tryptamine are the two main intermediates which leads to the production of Trp. Trp also has a role in the synthesis of the phytohormone indole-3-acetic acid (IAA).

Chitosan is a biodegradable and non-toxic biopolymer formed by the deacetylation of chitin, a natural polysaccharide found in the crustacean and insect exoskeletons as well as in the cell walls of some fungi (Sathiyabama *et al.,* 2016).

The development of analytical techniques such as chromatography in the middle of the twentieth century enabled the separation of an increasing number of these phytocompounds, providing the groundwork for the foundation of the subject of phytochemistry (Bourgaud *et al.,* 2001). HPTLC is a technique for developing chromatographic fingerprints to identify the key compounds of medicinal plants. Compared to TLC, the separation rate and precision are substantially greater, and the results are far more accurate and reproducible. The visually appealing graphical HPTLC image adds further, straightforward visible colour and/or fluorescence parameters for simultaneous evaluation on the same plate. It also showed better secondary metabolite separation (Senguttuvan & Subramaniam, 2016).

Ayapana triplinervis (Vahl) R.M.King & H.Rob. (Syn. *Eupatorium ayapana*, *Eupatorium tripliverve*) belongs to the family Asteraceae, has stomachic, diaphoretic, antiseptic, anti-ulcerous, haemostatic (Rajasekaran *et al.,* 2010), anti-tumerous, anticoagulant, hepatoprotective (Bose *et al.,* 2007), astringent and emollient properties (Gauvin-Bialecki & Marodon, 2008).

A. triplinervis consists of a number of coumarins; mainly 'ayapanin and ayapin'. These metabolites have been found to have anticoagulant properties as a result of which it is used for the production of drugs like 'Warfarin' (Bose, 1937). The active secondary compound present is 7- methoxycoumarin called 'herniarin or ayapanin' and the rest of the constituents found in leaves are 6, 7-dimethoxycoumarin (ayapin), vitamin C, carotene, and stigmasterol (Bose & Roy, 1936). In addition to these, other coumarins like hydragetin, daphnetin, daphnetin-7-methyl ether dimethyl ether and umbelliferone are present in the plant (Chaturvedi & Mulchandani, 1989).

The 7-methoxycoumarin has antitumerous activity and is toxic to multi-drug resistant cancer cells (Kawase *et al.,* 2005) and inhibitory against chemicals dispensed from leukemic cells (Watanabe *et al.,* 2005), it also has anti-nociceptive activity (Cheriyan *et al.,* 2017). Phytochemical analysis of *A. triplinervis* revealed various phytochemicals except for steroids and saponins (Mamatha & Thangavel, 2018).

Considering all these facts into account*, Ayapana triplinervis* was selected as the plant material for *in vitro* secondary metabolite production in the present investigations.

A. triplinervis is a perennial plant that needs constant watering and attention throughout the summer months to avoid drying up. It is possible to have plenty of plants throughout the year through the application of the plant tissue culture technique. Furthermore, plants grown in the field are susceptible to pathogens, pests, and abiotic stresses. Therefore, further studies of plant propagation in this research work were carried out using *in vitro* plant tissue culture methods.

Objectives of the proposed study

Despite discussing the above aspects, the present study was organized to investigate the impacts of different abiotic stress signals in the *in vitro* morphogenic responses and secondary metabolites production of *Ayapana triplinervis*. The research work was conducted as per the following objectives:

- Develop an efficient protocol for the direct and indirect organogenesis of *A. triplinervis* through *in vitro* PTC.
- Analyze the genetic fidelity between the mother plant and plant generated through indirect organogenesis through ISSR markers.
- Investigate the influence of various abiotic stress signals in the morphogenic responses of *in vitro* cultured *A. triplinervis* plants.
- Prepare and analyze HPTLC fingerprints of *in vitro* abiotic stress-induced secondary metabolites production in *A. triplinervis* methanolic plant extracts.

CHAPTER 2

REVIEW OF LITERATURE

Mankind has been depending upon plants as a means of medicine for over thousands of years. Tissue culture is a technique that can be exploited to replicate, conserve and prevent the extinction of various plant species that are hard to produce by conventional methods. In addition, this method can be utilised to produce secondary metabolites from plants of medicinal importance. The production of secondary metabolites *in vitro* can be increased by the application of various stress signals. The enhancement of biosynthetic potential of tissue culture systems can be achieved through precursor feeding, elicitation, etc. Therefore, *in vitro* culture systems have greater potential in conserving medicinal plants and commercializing herbal medicines (Thangavel *et al.*, 2014).

2.1. *In Vitro* culture studies

Modern pharmaceuticals are based on metabolites that are derived from plants, and the identification of novel products are continually occurring. Nonetheless, plant pharmaceuticals' reliable and constant supply has frequently been jeopardized. *In vitro* plant tissue culture is one option for producing essential plant active compounds because it eliminates the need to rely on wild plants (Espinosa-Leal *et al.,* 2018). Nutritious culture media and carefully controlled aseptic conditions are used in plant cell as well as tissue culture to promote the development of plant cells, tissues, and organs (Espinosa-Leal *et al.,* 2018). In many studies, including micropropagation, producing virus-free material, genetic modification, etc., plants must be grown *in vitro* (Georgieva *et al.,* 1996). Prakash and Van-Staden (2006) said that true-to-type plants can be produced in a small amount of time and area with *in vitro* propagation. In comparison to conventional field culture, tissue

culture of plants for the production of significant biomolecules has several benefits, including freedom from geographic, seasonal, and environmental variations; No need to apply pesticides or herbicides; uninterrupted production of uniform quality and yield, and relatively brief growth cycles (Rao & Ravishankar, 2002; Debnath *et al.*, 2006).

The crucial elements that are required to be set for every culture includes the kind of culture media, the salt content of the medium and the concentration and type of plant growth regulators used (Rao & Ravishankar, 2002; Monfort *et al.*, 2018). An appropriate medium is critical for establishing *in vitro* cultures (Nagella & Murthy, 2010; Monfort *et al.*, 2018). MS, B5, and WPM are three of the most commonly used culture media, and MS media contains total salts and nitrogen. For the proper growth of explants, nitrogen is essential. This is because amino acid production and nucleic acid production in plant cells requires nitrogen (Espinosa-Leal *et al.*, 2018).

The successful establishment of the *in vitro* cultures depends on the type and concentration of PGRs used. The auxins and cytokinins were mainly used in this work. The cytokinin concentration (e.g., BAP) used in the multiplication medium is one of the main factors affecting the morphology of micro cuttings and rhizogenic competence. This growth regulator promotes the creation of axillary shoots while suppressing root formation (Podwyszynska, 2002). Auxins are a group of phytohormones that regulate various plant growth and development processes (Davies, 2013). Auxins are best recognized for their ability to stimulate cell elongation. They also promote cell proliferation, vascular differentiation, and root formation (Khadr *et al.*, 2020).

Thidiazuron (TDZ) (N-phenyl-N′-1,2,3-thiadiazol-5-ylurea), a thiadiazole-substituted phenylurea that was previously authorized as a cotton defoliant (Arndt *et al.,* 1976) under the brand name "Dropp," has shown high cytokinin activity (Mok *et al.,* 1981). Murthy *et al.,* (1998) and Guo *et al.* (2011) reported that TDZ was discovered to have a plant growth regulator effect comparable to auxin and cytokinin despite having different chemical structures.

Espinosa-Leal *et al.* (2018) reported that altering the culture media by adding precursors or elicitors, or changing the surroundings, can increase the production of significant metabolites. The synthesis of natural products in plants can be stimulated by the addition of elicitors.

2.2. *Ayapana triplinervis*

Ayapana triplinervis (Vahl) R.M.King & H.Rob. (Syn. *Eupatorium ayapana*, *Eupatorium tripliverve*) belonging to the family Asteraceae is an erect perennial herb with aromatic properties. It is commonly found in India and in tropical countries as well. Nery *et al.* (2014) reported two morphotypes of *A. triplinervis* – Japana Branca and Japana roxa from Brazil. The plant has antiseptic, stomachic, antitussive, diaphoretic, anti-ulcerous, hemostatic (Rajasekaran *et al.,* 2010), antitumor, anticoagulant, hepatoprotective (Bose *et al.,* 2007), astringent and emollient properties (Gauvin-Bialecki & Marodon, 2008). *A. triplinervis* contains a variety of coumarins, mainly ‘ayapanin and ayapin,’ which have anticoagulant properties, as a result of which it is utilised as the precursor for drugs like ‘Warfarin’ (Bose, 1937). The anti-ulcer effect and radical scavenging activity of *A. triplinervis* extract were used to treat

ulcerative colitis in mice (Krishnan *et al.,* 2014). Checkouri *et al.* (2020) viewed the anti-inflammatory properties of *A. triplinervis*.

The active secondary compound present is 7- methoxy coumarin called 'herniarin or ayapanin,' and the rest of the constituents found in leaves are 6, 7- dimethoxy coumarin (ayapin), vitamin C, carotene, and stigmasterol (Bose & Roy, 1936). In addition to these, other coumarins like hydragetin, daphnetin, daphnetin-7-methyl ether dimethyl ether, and umbelliferone are present in the plant (Chaturvedi & Mulchandani, 1989). The 7-methoxy coumarin has antitumoral activity and is toxic to multi-drug resistant cancer cells (Kawase *et al.,* 2005) and inhibitory against chemicals dispensed from leukemic cells (Watanabe *et al.,* 2005); it also has anti-nociceptive activity (Cheriyan *et al.,* 2017).

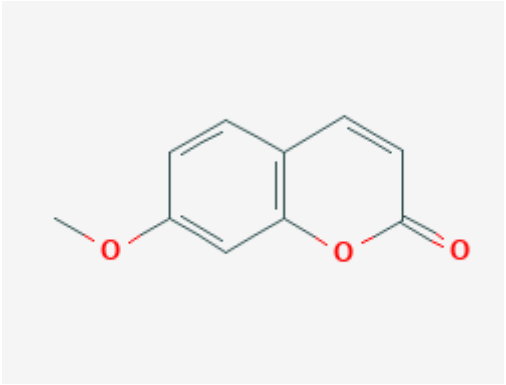

Figure 1: Structure of Ayapanin (7-methoxycoumarin)

Maximum production of multiple shoots of *Eupatorium triplinerve* was reported by Samydurai *et al*. (2012) in the MS medium treated with 0.2 mg l^{-1} BAP and 0.02 mg l^{-1} GA_3 combination. Salma *et al.* (2017) developed a

protocol for the micropropagation of *Eclipta alba* in MS medium treated with 1.0 mg l^{-1} BA and 0.25 mg l^{-1} NAA.

Rapid axillary bud multiplication of 8.1 shoots per node of *Eupatorium triplinerve* was achieved through the MS medium fortified by 2.46 µM IBA and 8.87 µM BAP (Martin, 2003). An efficient protocol for the propagation of *Eupatorium triplinerve* was achieved in the MS medium fortified with 4.44 µM BA, 32.3 ±0.3 shoots per explant (nodal segments) were produced within 30 days of growth period with a mean length of 4.6 ± 0.26 cm (Janarthanam *et al.*, 2011).

2.2.1. Phytochemical & Pharmacological aspects of *Ayapana triplinervis*

Fernezelian *et al.* (2023) conducted LC-MS/MS analysis of *A. triplinervis* plant extract and depicted 25 compounds; glucaric acid, caffeoyl glucaric acid isomers (4 times), protocatechuic acid, 3-caffeoylquinic acid (chlorogenic acid), 5-caffeoylquinic acid, caffeic acid, dicaffeoyl glucaric acid isomers (4 times), dihydroxycoumarin, feruloylquinic acid, ferulic acid hexoside, quercetin 3-O-rutinoside, quercetin 3-rahmnoside, quercetin 3-O-(6-malonyl-glucoside), 3,4-dihydro-3,8-dihydroxy-3-methylisocoumarin, Ayapin, ferulic acid, 7-hydroxy-8-methoxycoumarin (hydrangetin) and 7-methoxycoumarin (Ayapanin).

Biswas and Mukherjee (2019) conducted standardization of Ayapanin through HPTLC, and the ayapanin was densitometrically analyzed in absorption mode at 254 nm. The technique produced a band at RF 0.56, which corresponded to ayapanin.

Fernezelian *et al.* (2023) conducted LC-MS/MS analysis on an aqueous extract of *A. triplinervis*. They depicted various flavonoids and phenolic acids such as caffeoyl glucaric acid, ferulic acid, caffeoylquinic acid (chlorogenic acid), and quercetin derivatives. In addition to this, they also identified coumarins, including ayapanin and ayapin. According to their study, the aqueous extract could generate the caudal fins from the amputated model of Zebrafish by recruiting more inflammatory cells. Also, in HDF (high diet-factor) larvae, the extract can lower the accumulation of lipids.

Gupta *et al.* (2004) identified 20 compounds from essential oil taken from *A. triplinervis* leaves by GC/MS analysis, and among them, selina-4(15),7(11)-dien-8-one (1), β-caryophyllene and δ-elemene were prominent ones. Other compounds included - α-pinene, sabinene, β-pinene, myrcene, α-terpinene, p-cymene, limonene, β-phellandrene, γ-terpinene, α-terpinol, α-cubebene, β-farnesene, δ-cadinene, β-sesquiphellandrene, *trans*-calamenene, and caryophyllene oxide.

A similar study was conducted by Begum *et al.* (2010) and Unnikrishnan *et al.* (2014) and identified 30 compounds from the aerial parts of *Eupatorium triplinerve* Vahl. through GC/MS analysis. The reported compounds are: - α-thujene, α-pinene, sabinene, β-pinene, β-myrcene, α-phellandrene, o-cymene, D-limonene, Terpinolene, Sabinenehydrate, Methyl chavicol, Thymol methyl ether, Terpinyl acetate, Anethole, (+) Myrtenyl acetate, Isolongifolene, 9,10-dehydro, Camphene, 3-oxabicyclo [4,2,0] oct-5-ene,endo-8-methyl -exo-8-(2-propenyl), *cis* -myrtanal acetate, β-elemene, α-gurjunene, 2-tert-butyl-1,4-methoxybenzene, α-caryophyllene, 3,4-dimethoxy-b-nitrostyrene, α-selinene, β-selinene, Patchoulene, Caryophyllene

oxide, 6-isopropenyl-4, 8a-dimethyl -1,2,3,5,6,7,8, 8a octahydronaphthalene-2-ol, and Ayapanin. Among these compounds, 2-tert-butyl-1,4-methoxybenzene (74.27%) and β-selinene (8.59%) were reported to be major and exhibit potent antimicrobial activity.

Lobato-Rodrigues *et al.* (2021) conducted a study that delved into the effects of a nano-emulsion containing essential oil from two morphotypes of *A. triplinervis*. Using GC/MS analysis, they discovered that morphotype A contains β-caryophyllene (45.93%) and thymohydroquinone dimethyl ether (32.93%), while morphotype B contains 84.53% thymohydroquinone dimethyl ether. To test the acute oral toxicity of the nano-emulsions, the researchers treated Swiss albino mice with the essential oil nano-emulsion. They found that both morphotypes (A & B) essential oil nano-emulsions caused the animals' livers to show signs of inflammatory cells. Furthermore, the nano-emulsions exhibited larvicidal activity against *Aedes aegypti*, causing malformation in the anal papillae and peeling of the cuticle of the larvae. The essential oil targeted neuromuscular action by inhibiting the octopaminergic system of the larval nervous system.

Viet-Huong *et al.* (2020) isolated ten compounds from the methanolic leaf extracts of *Eupatorium triplinerve* Vahl in the study of anti-diabetic compounds (Asteraceae). The compounds were identified as -sitosterol (1), stigmasterol (2), -sitosterol 3-O--Dglucopyranoside (3), ayapanin (4), ayapin (5), thymoquinone 5-O--D-glucopyranoside (6), thyrsifloside (8), (E)-4-methoxymelilotoside (9), and kaempferol 3.

UPLC-ESI-MS-MS analysis of the *A. triplinervis* plant extract revealed the presence of the following polyphenols: protocatechuic, isoferulic,

and caffeic acids. In addition to this, two coumarins were found: ayapin and ayapanin (Taïlé *et al.*, 2020).

Rani *et al.* (2022) conducted a study that found the aqueous leaf extract of *A. triplinervis* to be more effective in inhibiting corrosion than the alcoholic extract. Thymohydroquinone dimethyl ether (THQ) was attributed to this result, as it facilitated the adsorption of corrosion enhancers on the surface of THQ rather than the metal surface. Additionally, the study found that THQ has an increased surface coverage. According to Haddad *et al.* (2019), the THQ and essential oil of *A. triplinervis* can inhibit ZIKV (Zika Virus) infection in human cells. They studied human epithelial A549 cells and found that THQ can inhibit virus entry.

The gastroprotective effect of *A. triplinervis* leaf extracts was reported by Kathirvelu *et al.* (2019) and Maity *et al.* (2021) studied indomethacin-induced gastric ulcers in male albino rats. Ethanolic and water extracts of *E. ayapana* leaves showed antimitotic, cytotoxic, and apoptosis properties in Ehrlich's ascites carcinoma. Among them, the ethanolic extract was more active; it arrests G_0/G_1 of the cell cycle (Maity *et al.*, 2015). Bose *et al.* (2007) revealed the hepatoprotective and antioxidant effects of methanolic extract of *E. ayapana* against CCl_4-induced hepatotoxicity in Wistar Albino rats. The extract and Silymarin were administrated to the rats. The results showed that the extract showed hepatoprotective activity by reducing the serum enzyme activity, uric acid, bilirubin, and lipid peroxidation as well as inducing the production of SOD, GSH, and catalase.

An *in vivo* study was conducted on Swiss Albino Mice with Ehrlich's ascites carcinoma injected with water and ethanol extracts of *E. ayapana.* The

results showed that both the extracts have potential antitumor and hepatoprotective activity on the hemopoietic system. Among them, ethanolic leaf extract was comparatively more potent than water (Bepari *et al.*, 2015).

The antimicrobial activity of petroleum ether leaf extract and methanolic leaf extracts of *E. ayapana* was reported by Gupta *et al.* (2002). They observed increased antibacterial activity in the petroleum ether extract. An ethno–gynecological study was conducted on medicinal plants conventionally used in West Bengal. From the available information, they concluded that parts of *A. triplinervis* have the activity to cure various female diseases like infertility, menstrual problems, etc. (Pattanayak *et al.*, 2016).

Rahman and Junaid (2008) studied various extracts of *E. triplinerve* leaves, such as in petroleum ether, carbon tetra chloride, chloroform, and ethyl acetate. The antimicrobial activity of the extracts was tested against 11 and 6 pathogenic bacteria and fungi respectively. Among the crude extracts, chloroform extract showed the largest inhibition zone (22mm in diameter with 1000 µg/disc extract) against *Vibrio* and the highest inhibition against fungal radial mycelial growth of *Colletotrichum cochori* (73.5% with 100 µg extract/ml medium). The lowest MIC value was also exhibited by chloroform extract against *Vibrio* (250µg ml^{-1}) and *C. corchori* (62.5µg ml^{-1}).

A. triplinervis extracts were potent biopesticides because the growth and development of *Myzus persicae* nymphs were interrupted (Facknath & Lalljee, 2008). Essential oil from *E. triplinerve* leaves was used to test antimicrobial efficacy. The results showed the highest activity against *Escherichia coli* and *Proteus vulgaris* (Yadava & Saini, 1990).

An ethnobotanical review of *E. triplinerve* revealed that a more significant part of people in many regions use it for many diseases. It is widely used in Unani and Ayurvedic medicines. Also, the secondary metabolite plays a significant role in the following activities of this plant: anti-inflammatory, anti-fungal, antibacterial, and anti-ulcer (Cheriyan *et al.*, 2019).

Ag nanoparticles were synthesized using the leaf extracts of *A. triplinervis*. These AgNPs were analyzed using UV-Vis spectrophotometer, FTIR, and SEM, revealing the presence of functional groups that may be involved in the AgNP synthesis. The biosynthesized silver nanoparticles exhibited prime antibacterial activity against *Salmonella, Bacillus*, and *Pseudomonas*. Nanoparticles have achieved greater importance in research due to their smaller size and eco-friendly synthesis (Dev *et al.*, 2018).

The methanolic extract of the leaves of *E. triplinerve* in a melanin biosynthesis assay showed anti-melanogenesis activity. The active compound 7- methoxycoumarin inhibited the melanin formation and tyrosinase enzyme activity in B16 melanoma cells. Dayak tribe, a native of East Kalimantan, traditionally uses A. *triplinerve* leaves (Arung *et al.*, 2012).

A study was conducted to evaluate the phytochemicals present in the *A. triplinervis* and obtained the following compounds such as alkaloids, flavonoids, terpenoids, glycosides, phenols, reducing sugars, carbohydrates from the preliminary phytochemical studies using acetone extracts of leaves (Mamatha & Thangavel, 2018).

Sugumar *et al.* (2014) conducted preliminary phytochemical studies using leaf extracts with various solvents. They identified compounds like

alkaloids, flavonoids, saponins, tannins, quinones, steroids, triterpenoids, coumarins, volatile oils, carbohydrates, proteins, amino acids, glycosides, and phenols. *A. triplinervis* was used to cure dysentery and enteritis in some rural areas of three districts in West Bengal (Pattanayak *et al.*, 2016).

Different extracts of *E. triplinerve* leaf and callus were used for the phytochemical and antimicrobial studies, revealing many compounds. The ethanol extract yielded an optimum production of tannins; this was used for antimicrobial activities and showed the highest inhibition against *Bacillus cereus* (Usha & Karpagam, 2017).

Ten compounds were identified by the GC-MS analysis of the methanolic extract of *E. triplinerve*. The prominent compounds in the extract were hexadecanoic acid, tetradecanoic, and octadecanoic acid. These compounds exhibited hypocholesterolemic activity, antioxidant, and lubricating activity. Anticancer and antiproliferative activities were demonstrated by tetradecanoic acid and 2,6,10,-trimethyl,14-ethylen-14-pentadecne, while 1-hexyl-1-nitrocyclohexane and 1,14- tetradecanediol compounds showed antimicrobial and anti-inflammatory activities (Selvamangai & Bhaskar, 2012).

An antihelminthic study was conducted with *Eupatorium triplinerve* leaves against *Pheritima posthuma* and *Ascardia galli*. The ethanolic extract was used for the analysis in a dose-dependent manner, and the results revealed that it shows a broad-spectrum action against these worms (Subash *et al.*, 2012).

The GC-MS analysis of essential oil from aerial parts of *Eupatorium triplinerve* indicated the presence of thirty compounds. The major

components were 2-tert-butyl-1,4-methoxybenzene (74.3%) and b-selinene (8.6%) (Begum & Bhuiyan, 2010).

Quercetin content of *E. triplinerve* leaf extracts were estimated with ethanol and water. The HPTLC was done with the mobile phase containing toluene: ethyl acetate: formic acid: methanol (5.5:4:1:0.5). Quercetin was found at the Rf value of 0.54 in the densitometric scanning image under 254 nm and 9.29 mg g^{-1} quercetin was obtained from this plant (Nithya & Kamalam, 2019).

Krishnan *et al.* (2014) reported that the acetic-mediated ulcerative colitis (UC) could be reduced with the antiulcerative effect of *E. triplinerve* methanolic fractions. *E. triplinerve* (200 mg kg^{-1}) was observed to be more efficient in treating acetic acid molds by producing a cuticle on top of the mucosal membrane and by reducing the destruction caused by lipid peroxidation. Hence, this plant could be considered as a promising means for treating UC.

The study of Chen *et al.* (2018) revealed that essential oil from *Eupatorium adenophorum* contained sesquiterpenes, and the predominant sesquiterpenes are torreyol, aristolone, and a-bisabolol, a-curcumene, b-bisabolene, and b-sesquiphellandrene.

Paes *et al.* (2014) conducted a study on the endophytic mycobiota of *Ayapana triplinervis*, and the results showed that 12 genera of fungi were isolated from the vegetative parts of this important medicinal plant. Noyes (2007) reported apomixis in *Ayapana triplinervis*.

2.3. Direct Organogenesis Studies

Martin (2003) reported that rapid shoot multiplication of *A. triplinervis* was achieved in the MS medium fortified with 8.87 µM BAP and 2.46 µM IBA and produced a mean of 8.1 shoots per node. The basal end of the shoots was dipped in 2.46 µM IBA solution and kept for ten days to initiate roots and then transferred to small pots, which aided in the viability and persistence of all rooted shoots. *Ex vivo* rooting by directly transferring seedlings from a multiplication medium resulted in a 92% survival rate.

Samydurai *et al.* (2012) worked on *in vitro* studies on *Eupatorium triplinerve*. They worked on the MS medium treated with 0.2 mg l^{-1} BAP + 0.02 mg l^{-1} GA_3; after 30 days, the highest number of shoots (5.0) was formed. The length of the longest shoot was 2.85 cm. Half-strength MS liquid medium fortified with 0.2 mg l^{-1} of IBA and 0.2 mg l^{-1} of IAA produced the best root induction results. Plantlets were transferred to sterile vermiculite soil under controlled circumstances. Plantlets that were well established were later moved to plastic pots containing garden soil and sand (3:1). Following that, the plants were transferred to field conditions for acclimatization.

Salma *et al.* (2017) developed a rapid axillary bud multiplication protocol for *Eclipta alba* on MS medium treated with 1.0 mg l^{-1} BA and 0.25 mg l^{-1} NAA. In the same growing medium, a high shoot multiplication rate (22 shoots per axillary bud) was achieved in 21 days. The most well-developed roots per stalk were observed in MS medium containing 1.5 mg l^{-1} IBA. The plantlets grown *in vitro* were well acclimatized in coco pith and soil (3:1), with a survival rate of 91%. Within 60 days, 88 acclimatized seedlings were obtained from each nodal explant.

Hesar *et al.*, (2011) evaluated the MS medium provided with 0.5-2 mg l-1 kinetin, producing multiple shoots with roots. The shoot length of plants grown in the 2 mg l^{-1} kinetin media, was the highest (11.72 mm), and the shoots were mainly formed in clusters (4.64). The most significant number (3.40) and length (54.0 mm) of roots were obtained with 1 mg l^{-1} kinetin. Deshmukh *et al.,* (2017) carried out *in vitro* propagation studies on *Stevia rebaudiana*. A combination of 2 mg l^{-1} BAP and 0.5 mg l^{-1} kinetin produced the best results for the induction of shoots.

Sarowar *et al.* (2003) developed an effective protocol for direct shoot growth of interspecific *Cucurbita* hybrid varieties using shoot tips of 5-day-old explants. The MS medium provided with 3 mg l^{-1} of BA provided the optimal environment for shoot growth. After 30 days of culture, five seedlings were harvested from each explant, with an 84% shooting frequency. In MS medium provided with 1 mg l^{-1} IBA, shoots (11.5 cm long) were best rooted. All rooted shoots were transplanted into soil, with the most significant root formation rate being 93%.

Young shoot buds of *Chlorophytum borivilianum* were cultured on MS medium containing BAP and Kinetin separately or in combination. BAP alone was considerably effective on shoot proliferation and Kn alone was effective in shoot elongation (Ashraf *et al.,* 2014). Shekhawat *et al.* (2015) carried out *in vitro* multiplication studies, and the growth of nodal explants on MS + 2.0 mg l^{-1} BAP led to the induction of multiple shoots. On MS medium with 0.5 mg l^{-1} of each BAP and kinetin, *Passiflora foetida* shoots were produced.

Kharde *et al.* (2014) studied *in vitro* propagation of *Rosa hybrida*, and the nodal segment was grown on MS medium provided with 0.5-3.5 mg l^{-1} BAP and 0.5 mg l^{-1} kinetin fixed for shoot formation. The optimal medium for *in vitro* shoot formation from the nodal segment was 2.0 m gl^{-1} BAP and 0.5 mg l^{-1} Kinetin. The rate of shoot formation at this quantity was 86% in 8 days. On medium containing 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin, a maximum of 12 shoots per culture were produced. The *in vitro*-produced stems were rooted in a half-strength MS medium containing 2.5 mg l^{-1} IBA in 67% of the shoots. On treating the cut portions of the shoots with 300 mg l^{-1} IBA for 5 minutes, 97% of the shoots were rooted *ex-vitro* (8.33 0.29 roots per shoot). These plants were hardened and acclimatized in the greenhouse before transplanting to the field.

Vitex agnus-castus explants were grown on MS medium fortified with BAP (2.0 mg l^{-1}) and Kin (0.1 mg l^{-1}), regeneration frequency was maximum for both apical meristem and nodal explants. As many as 7.7±0.4 and 6.7±0.2 branches were formed per explant during the culture of apical meristem and nodal explants, respectively. After 30-35 days of culture, *in vitro* produced shoots were transferred to half-strength MS medium provided with 0.1 mg l^{-1} IBA, resulting in the production of roots. Almost 80% of these shoots were effectively established in the soil (Balaraju *et al.*, 2008).

Deepak *et al.* (2019) reported *Spermacoce hispida* propagation through direct organogenesis, nodal explants produced on MS medium provided with 2.0 mg dm^{-3} BAP induced the greatest shoot regeneration frequency (72%) and a large number of shoots (10.5±0.3). MS medium provided with 1.0 mg dm^{-3} of IBA resulted in highest root formation frequency (71.66%), number of

roots (7.5±0.2), and root length (3.0±0.1 cm). 70% of these plants survived in the greenhouse and 68% in the field.

Nodal explants of *Baliospermum montanum* grown on MS medium treated with 2.0 mg l^{-1} BAP resulted in the production of plants with the highest number of shoots (22.2±0.84), as well as the highest frequency of shooting reaction (82%), were obtained. The maximum shoot height was 15.8 cm. A combination of IBA and IAA resulted in the production of a large number of healthy rootlets (14.8±2.07 cm) with a 90% rooting response. With 86.2% survival, the regenerated plantlets were moved to their native habitat (Sasikumar *et al.*, 2009).

Anand *et al.* (2012) conducted direct organogenesis studies on *Passiflora foetida*. They found the development of multiple shoots on MS medium treated with 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} Kn, with an 85% survival rate. The maximum range of shoot elongation was found on MS medium treated with 1.5 mg l^{-1} BAP + 0.5 mgl^{-1} NAA. 0.5 mg l^{-1} NAA + 0.5 mg l^{-1} IBA was determined to be the optimal dosage for root induction. The hardened seedlings had an 85% survival rate.

2.4. Indirect Organogenesis

Corredoira *et al.* (2008) reported that leaf explants of *Paulownia tomentosa* cultured on an induction medium containing TDZ (22.7 or 27.3 µM) with 2.9 µM IAA for two weeks showed the best shoot regeneration. A 7-day treatment with 0.5 µM IBA increased rooting frequency by up to 90%.

Liberman *et al.* (2010) reported that shoot regeneration was highest for young leaf explants grown in 4.44 µM BA and 2.85 µM IAA and highest for mature leaf explants grown in 8.88 µM BA and 2.85 µM IAA for mature leaf

explants of *Brunfelsia calycina*. When branches were moved to MS media enriched with 1.23- 2.46 µM IBA, they generated roots. Li *et al.* (2013) reported the best results for shoot proliferation from leaf explants using MS media containing 0.5 mg l^{-1} BA alone or in combination with 0.1 mg l^{-1} NAA. High BA concentration (1.0 mg l^{-1}) in the media resulted in the generation of a high percentage of vitrified shoots. Regenerated shoots rooted effectively on half-strength MS medium containing 0.5 mgl^{-1} IBA and IAA, and the plantlets successfully acclimatized and developed aggressively in the greenhouse with a survival rate of 94.2% and 92.1%, respectively.

Regeneration of shoots from *Exacum* species leaf explants was achieved in the MS medium containing 4.44 µM BA plus 0.54 µM NAA (Unda *et al.*, 2007). When leaf explants of *Aronia mitschurinii* were wounded with two transverse cuts along the midrib and placed on an MS basal medium containing 5 µM IBA and 10 µM TDZ, the best regeneration of the leaves occurred (Mahoney *et al.*, 2018). Gupta *et al.* (2017) found that MS media enriched with 1.25 mg l^{-1} TDZ and 1.0 mg l^{-1} NAA resulted in potent adventitious shoot proliferation of 17.21±0.24 per leaf explant of *Lysimachia laxa*. By using elongation media fortified with 1.0 mgl^{-1} BAP, the average number of shoots was 31.1±0.80, and the length was 5.96±0.13 cm. 0.50 mg l^{-1} IAA induced 100% rooting, with an average root number of 11.70 and length of 7.35 cm.

Leaf segments from 20-day-old *in vitro* plantlets of *Aerva lanata* cultured on MS medium supplemented 1.0 mg l^{-1} TDZ, the highest number of shoot organogenesis (23.6±0.16) was obtained after 21 days of culture incubation. After being transferred to half-strength MS media containing 1.0

mg l^{-1} IBA, approximately 86% of the regenerated shoots developed roots and plantlets (Varutharaju *et al.*, 2013). MS with 0.272 µM TDZ was shown to be the most effective medium for shoot induction from leaf tissues of *Embelia ribes*. Without any callus phase, numerous shoot primordia appeared on the leaf margin and midrib region within 2-3 weeks. 2 to 3-cm-long shoots rooted on half-strength MS basal medium provided with 4.90 µM IBA and 3% (w/v) sucrose (Raghu *et al.*, 2006).

The combination of BAP (3.0 m gl^{-1}) and IAA (0.5 mg l^{-1}) produced the most significant number of shoots (38.0) from the *in vitro*-derived leaf explants of *Solanum nigrum*. The best rooting response was seen with 0.5 mg l^{-1} IBA (Sridhar & Naidu, 2011).

Ahmad *et al.* (2010) conducted a study on shoot multiplication of *Ruta graveolens* leaf explants, and maximum response (92.3%) was obtained on MS medium supplemented with 7.5 µM BA and 1.0 µM NAA. The shoots thus produced were rooted on MS with 0.5 µM IBA.

Lee and Pijut (2017) conducted studies on *in vitro*-grown leaf explants of *Fraxinus nigra*. They obtained the frequency of callus production ranged from 77.8 to 94.4% for single leaflets and 88.9-100% for entire compound leaves, respectively. However, regardless of the initial leaf explant type, combination of 22.2 µM BAP and 31.8 µM TDZ was the best treatment, resulting in highest shoot bud induction. The regenerated shoot buds were grown lengthier on MS medium containing 2 mg l^{-1} glycine (MSB5G), 13.3 µM BA, 1 µM IBA, and 0.29 µM gibberellic acid. In woody plant medium containing 5.7 µM IAA with 4.9 µM IBA, 85.2% of the micro shoots were effectively rooted.

Rathore *et al.* (2015) studied *Withania coagulans* and MS medium containing 4.44 µM BAP resulted in the production of 11.4±0.9 shoot buds per explant. On MS medium, regenerated shoot buds were extended (6.7±0.22 cm) with 1.11 µM BAP and 0.57 µM IAA. Both *in vitro* and *ex-vitro* germination were used to root the long shoots.

MS medium fortified with 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} TDZ increased the frequency of shoot production from explants of leaves of *Lycium chinense*. In comparison, IBA was more effective in root regeneration and growth. The rooting medium with half-strength MS salts mixed with 3% sucrose resulted in the lengthiest root development (6.67±1.25). The plantlets that survived were gradually moved to the greenhouse and natural soil. Within 85 days, more than 90% of the plantlets had survived and developed (Jung *et al.,* 2021).

2.5. Genetic Fidelity Analysis

Plant tissue culture is identified as a vital area of biotechnology due to its utility in regenerating plants while protecting valuable plant genetic resources (Saha *et al.,* 2014). Any micropropagation technology that grows up has the danger of producing genetic variability, such as somaclonal variation among the sub-clones of a single parental line (Larkin & Scowcroft, 1981). Saha *et al.* (2014) viewed that studying the somaclonal variation in regenerates is extremely important for commercializing and exploiting it. Cytological studies, molecular analysis, and phenotypic identification are among the methods for spotting genetic variation. Genetic similarity or dissimilarity of tissue culture produced plants can be effectively determined by the use of molecular markers.

Using molecular markers to measure genetic integrity has various advantages, including that it is not affected by environmental influences and is a rapid, robust, efficient, and cost-effective procedure (Cui *et al.*, 2017). Jung *et al.* (2021) assessed the genetic stability of regenerated shoots using several molecular markers, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphisms (RFLP), simple sequence repeats (SSR), and inter simple sequence repeat (ISSR). The necessity to investigate microsatellite repeats without DNA sequencing led to the development of ISSR markers (Lagercrantz *et al.*, 1993).

ISSR is a simple, rapid, accurate approach that generates amplified products ranging in length from 200 to 2000 bp (Dos-Santos *et al.*, 2011). ISSR technique avoids the need for flanking sequence information and has therefore found widespread application in diverse plants (Srivastava & Gupta, 2008). PCR analysis with ISSR primers proved a beneficial technique in investigations of genetic diversity, either intra- or inter-specific, phylogenies, and evolutionary biology (Wang *et al.*, 2009).

The genetic fidelity analysis using ISSR helps in identifying that *in vitro* regenerants were genetically similar to the mother plant and was found in the following reports on *Syzygium travancorium* (Anand, 2003) *Musa acuminata* var. Nanjanagudu (Lakshmanan *et al.*, 2007), *Cineraria maritima* (Srivastava *et al.*, 2009), *Ochreinauclea missionis* (Chandrika & Rai, 2009), *Hydrangea* 'Hyd1' (Liu *et al.*, 2011), *Zingiber rubens* (Mohanty *et al.*, 2011), *Rauvolfia serpentina* (Faisal *et al.*, 2012), *Withania somnifera* (Fatima *et al.*, 2013), *Artemisia absinthium* (Kour *et al.*, 2014), *Dendrocalamus strictus*

(Goyal *et al.*, 2015), *Cornus alba* (Ilczuk & Jacygrad, 2016), *Nothapodytes nimmoniana* (Prakash *et al.*, 2016), *Morus alba* (Saha *et al.*, 2016), Finger millet (Babu *et al.*, 2017), *Paederia foetida* (Behera *et al.*, 2018); *Tecoma stans* (Hussain *et al.*, 2018); *Lycium chinense* (Jung *et al.*, 2021).

2.6. Stress studies

The biotechnological production of plant secondary metabolites can be improved by elicitation. An elicitor is a compound that is provided in minute concentrations to living systems to enhance the production of a target metabolite. The cost-effective and sustainable commercial production of plant secondary metabolites will depend on a more in-depth knowledge of the metabolic response to elicitation in plant cells (Ramirez-Estrada *et al.*, 2016).

The commercial importance of secondary metabolites has been increasing in recent years, resulting in a greater interest in the study of secondary metabolite production, particularly in the possibility of altering the production of bioactive plant metabolites through tissue culture technology. Various researchers have extensively studied different strategies using an *in vitro* system to enhance the generation or biosynthesis of plant active principles (Aijaz *et al.*, 2011).

Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavours, and industrially essential biochemicals. Accumulation of such metabolites often occurs in plants subjected to stresses, including various elicitors or signal molecules. Secondary metabolites play a significant role in adapting plants to the environment and overcoming stress conditions. Abiotic stress factors influence growth and secondary metabolite

production in higher plants. Environmental stresses like pathogen attack, wounding, temperature, humidity, light intensity, UV-irradiation, the availability of water and nutrients, mineral deficiencies, heavy metals, methyl jasmonate, salicylic acid, plant growth regulators and CO2 influence the growth of plants and secondary metabolite production. Drought, high salinity, and freezing temperatures are environmental conditions that cause adverse effects on the growth and development of plants and the productivity of crops. *In vitro,* culturing plant tissues and cells is an excellent technique for producing and studying secondary metabolites (Akula & Ravishankar, 2011).

Plants are the primary source of pharmaceuticals in all medical systems. The whole plant is sometimes destroyed to obtain secondary metabolites, which may perish rare and endangered plant species and imbalance biodiversity. The *in vitro* culture technique is a promising tool for overcoming this condition. Various *in vitro* culture methods increase secondary metabolite production via. elicitation, media manipulation, precursor feeding, plant hormone regulation, cell immobilization, biotransformation and bioconversion, hairy root cultures, and genetically modified cells. Among them, elicitation has particular importance, and two classes of elicitors were used: abiotic and biotic. Biotic elicitors include microbial attack, protein kinases, glycoproteins, and polysaccharides from cell walls of plants and microorganisms, and the abiotic elicitors include chemicals, availability of water and nutrients, salinity, wounds or pathogen attack, climate change, light intensity or UV irradiation and temperature. A significant exposure to UV irradiation activates genes of the phenylpropanoid and flavonoid pathways, producing flavonoids, alkaloids, anthocyanins, and

polyamines. It also induces the ROS signaling pathway and elicits the stress response. Jasmonic acid regulates the genome level and induces the synthesis of enzymes. More than one pathway was cross-linked during elicitation, and due to this complexity, the actual mechanism still needed to be discovered. To unlock those secrets, it is necessary to check all aspects of stress response related to phytochemistry, biochemistry, pharmacognosy, and molecular biology.

Plants produce diverse secondary metabolites that play a vital role in environmental adaptation. Flavonoids are a class of polyphenols with many biological activities, including anti-inflammatory, anti-carcinogenic, antioxidant, and anti-mutagenic activities. Biotechnology provides an excellent opportunity to exploit these secondary metabolites through different methods. Callus culture, suspension cultures, transformation, and other techniques have been used to enhance the production of flavonoids. Such potential new biotechnological strategies result in the production of flavonoids continuosly under controlled environmental conditions (Bharati & Bansal, 2014).

Light, temperature, soil water, soil quality, and salinity all play a role in the production of SMs. The uses of elicitors in culture systems and their positive effects on secondary metabolite build-up were studied in different plant species. The aromatic amino acids, precursors to various secondary metabolites like terpenoids, alkaloids, and sulfur- and nitrogen-containing substances, are produced through the shikimate pathway. Most plants have preserved the core biosynthesis pathways of metabolites, with the bulk of primary metabolites found in all tissue types. The preservation of this

metabolic base has resulted in the emergence of a few numbers of essential metabolic frameworks. A wide range of changes in basic structures are caused by frequent glycosylation, methylation, hydroxylation, acylation, oxidation, phosphorylation, and prenylation (Jan *et al.*, 2021).

Vascular plants commonly contain coumarins (2H-benzopyran-2-one), a group of simple phenolic compounds made up of a sizeable phenolic substance created by the union of a benzene ring and a -pyrone ring (Jan *et al.*, 2021). Luca *et al.* (2006) and Glowniak and Widelski (2009) reported that they are found in natural and synthetic sources and are particularly common in the plant families of Umbelliferae, Rutaceae, Leguminosae, Compositae, and others. Coumarins are found in plants as free molecules and glycosides; they are derived from phenylpropanoid precursors such as cinnamic acid and p-coumaric acid, which undergo o-hydroxylation to form o-coumaric acid and 2, 4-dihydroxycinammic acid, respectively (Verushkin *et al.*, 2021).

Coumarins are classified as simple, furano, dihydrofurano, linear-type, phenyl, and bicoumarins, depending on their chemical structure (Venugopala *et al.*, 2013). Mueller (2004) reported that some natural coumarins had been utilized as human medicines, whereas 4-hydroxycoumarins are prominent instances of microbial alteration that gave rise to the first-generation compounds produced as anticoagulants together with aspirin and heparin. Bourgaud *et al.* (2006) found that umbelliferone, herniarin (7-methoxycoumarin or Ayapanin), and scoparone (2 methoxylated umbelliferone derivatives), esculetin, fraxetin, isofraxidin, isoscopoletin, daphnetin, and their respective glucosides are the most common hydroxylated coumarins.

Elicitors are chemical substances that can promote plant stress responses, resulting in increased production and accumulation of secondary metabolites. The elicitor type, dose, and treatment schedule heavily influence the effects on secondary metabolite production. Numerous other variables, including cell line, nutritional content, age or stage of the culture, elicitor concentrations, exposure time, and others, significantly impact biomass synthesis and accumulation of secondary metabolites (Naik & Al-Khayri, 2016).

Naik and Al-Khayri (2016) classified elicitors as abiotic or biotic based on their nature. Abiotic elicitors are nonbiological substances classified as physical (UV radiation, osmotic stress, salinity, drought, thermal stress), chemical (heavy metals, mineral salts, gaseous toxins), or hormonal variables (e.g., Salicylic acid, and Jasmonates). Biological elicitors include polysaccharides derived from plant cell walls (e.g., chitin, pectin, and cellulose) and microorganisms.

2.6.1. Effects of Stress Signals on Morphogenic Responses

2.6.1.1. NaCl (Salt stress)

The *in vitro* shoot cultures of *Actinidia deliciosa* were established with 40 and 80 mM NaCl. They showed varying degrees of mild to severe chlorosis, and the higher NaCl concentration caused shoot dieback (Sotiropoulos & Dimassi, 2004). Sabir *et al.* (2012) reported that, with increasing salt concentration relative to water content, photosynthetic pigments such as chlorophyll *a*, *b*, total chlorophyll (*a* + *b*), and carotenoid content were decreased in shoot cultures of *Withania somnifera*. El-Shennawy

et al. (2017) conducted work on *Mentha longifolia;* with various concentrations of NaCl. After 60 days of incubation plants experienced poor growth, cell yellowing, and occasionally degeneration at high salt chloride concentrations.

Muchate *et al.* (2019) investigated the effects of salt stress in *in vitro* shoot cultures of *Spinacia oleracea.* When compared to high salinity levels (300 mM), the effect of minimal to average salinity stress (100-200 mM) on shoot culture development and tissue water content (TWC) was less significant. Shoot cultures treated with salt (NaCl) showed decreased shoot quantity, leaves per culture, FW, and TWC. As NaCl concentration increased, the number of shoots and leaves per culture decreased significantly. After 21 days of incubation, it was found that the number of shoots and leaves were highest in control cultures. The salt-treated cultures resulted in a gradual decrease in fresh weight, fresh weight/dry weight and total water content per shoot culture as salinity increased.

At higher salt stress, a reduction in chlorophyll content and the growth of the plants was observed in several reports (Günes *et al.*, 1996; Khavari-Nejad & Mostofi, 1998; Marcelis and Van-Hooijdonk, 1999 and Yang *et al.*, 2009). In the *in vitro* culture of Strawberry, in comparison to salt stress (100 mM NaCl), morphological measures such as branch number, root and shoot dry weight, relative water content, chlorophyll and iron content were highest in control plants (Mozafari *et al.*, 2019).

Singh *et al.* (2000) reported a gradual decrease in the number of leaves per shoot and internodal length with increased salt stress and fresh and dry weight at 50 mM NaCl. With further raising of NaCl concentrations, these

parameters were reduced. *Vitis vinifera* showed signs of leaf damage such as reduced leaf size and marginal necrosis from 75 – 100 mM of NaCl.

Shake culture media induced salinity by introducing 0 (control), 50, 100, 150, or 200 mM NaCl. An increased salinity treatment promoted ethylene production in the headspace and leaf epinasty in *Lycopersicon esculentum*. Higher salinity treatments reduced growth, viability and osmolarity of leaf and soluble protein content of shoots while increasing electrolyte leakage, membrane damage, raffinose, and total sugars (Shibli *et al.,* 2007).

According to Coste *et al.* (2011), enhanced production of hypericin and hyperforin from the shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum* in the MS media provided with BA (0.4 mg l^{-1}) or Kin (0.4 mg l^{-1}). There was an approximately 2-fold increase in the production of hypericin from the cultures of *H. maculatum* in the MS medium modified with 3% sucrose, growth regulators such as BA (0.2 mg l^{-1}), Kin (0.1 mg l^{-1}), 2iP (0.4 mg l^{-1}) and NAA (0.05 mg l^{-1}) along with 10 mM NH^{+}_{4} (MSM medium) and of *H. hirsutum* 6.16-fold increase of hyperforin in this same medium. At 50 µM, SA enhanced the production of hypericin and pseudohypericin in *H. hirsutum*, and, at 200 µM, enhanced the production of hypericin and pseudohypericin in *H. maculatum*.

Abed-Alrahman *et al.* (2005) conducted studies on the influence of salinity on *in vitro*-grown *Cucumis sativus,* and salinity was created by adding 0, 50, 75, or 100 mM NaCl to the growth media. Microshoots were subjected to both immediate and gradual salt shock. Gradual salt stress was implemented by gradually shifting micro shoots weekly to different NaCl

concentrations ranging from 0 to 50 to 75 to 100 mM (beginning with the control and ending with 100 mM NaCl). Various growth parameters (shoot length, fresh shoot weight, dried shoot weight, root length, and root number) were generally reduced with increased salt level in both the direct and progressive salt shocks. Both salt stress treatments increased root length at 50 mM NaCl. With increased salinity, leaf osmotic potential was dramatically lowered (more negative). Although Na^+ concentration increased in salinized microshoots, K^+ and Ca^+ concentrations decreased with increased salinity, with the decrease being less prominent in the gradual salt shock. The K /Na ratio dropped in salinized microshoots corresponding to control, and progressively salinized micro shoots had a more excellent K/Na ratio than directly salinized microshoots.

According to Kiełkowska (2017), root meristem cell cross-section area and nuclear volume decreased in *in vitro* cultures of *Allium cepa* under salt stress. After 20 days, 300 mM NaCl in the culture media inhibited mitotic activity in onion root tip cells. Analysis of 200 mM NaCl cultures showed that salt stress has more potent mitodepressive effects.

2.6.1.2. Sucrose

The influence of different concentrations of sucrose on somatic embryogenesis of three genotypes of soybeans (Iroquois, Macon, and Savoy) was studied, and the cultures established with lower sucrose concentrations (1 and 2%) produced more friable calli than higher sucrose concentrations (4.5 and 6%) (Hofmann *et al.*, 2004).

Abou-Dahab *et al.* (2004) conducted a study on different concentrations of sucrose in *in vitro* cultures of *Ruscus hypoglossum*. MS media treated with sucrose at 30 g l^{-1} or 40 g l^{-1} generated the most significant number of shoots per explant (13.00 and 13.58, respectively), and the utilisation of MS medium supplemented with sucrose at 10 g l^{-1} resulted in the fewest number of shoots (5.50). The explants produced the lengthiest shoots (7.00 cm) when cultivated on MS media supplemented with sucrose at the maximum concentration (50 g l^{-1}). In contrast, the shortest shoots (3.25 cm) were formed using the minimal dosage (10 g l^{-1} sucrose). When the explants were cultivated on MS media enriched with 40 g l^{-1} of sucrose, the highest number of leaves (10.67) was formed. The average number of leaves increased significantly when the sucrose content in the media increased from 10 to 30 g l^{-1}; however, sucrose at 50 g l^{-1} dramatically decreased the number of leaves compared to 30 and 40 g l^{-1} sucrose concentrations.

Sari *et al.* (2018) used different concentrations of sucrose in cultures of *Myrmecodia tuberosa*. They observed reduced callus growth and weight, and the calli colour changed from green to yellow with the increasing sucrose concentration in the medium. The accumulation of sucrose in cells can also hinder the process of photosynthesis by causing the demand for sugar in the cell to be met. As a result, the cells block photosynthesis and chlorophyll development.

The effects of sucrose on the cell suspension cultures of *Salvia leriifolia* caused an increase in the dry weight of cells cultured on media containing 40 g l^{-1} sucrose (Modarres *et al.,* 2018). *Artemisia absinthium* cell suspension cultures were treated with sucrose (1, 3, 5, 7, and 9 %), and 3%

sucrose showed an enhancing effect on the dry biomass of the cells (Ali *et al.*, 2016). In *in vitro* root cultures of *Musa* species, sucrose (35, 40, 45, and 50 g l^{-1}) was supplemented for elicitation studies, and the average number of leaves increased noticeably when 40 and 45 g l^{-1} of sucrose were added. At 50g l^{-1} sucrose, the number of leaves was reduced (Ayoola-Oresanya *et al.*, 2021). Shohael *et al.* (2006) studied the effects of initial sucrose levels on *Eleutherococcus sessiliflorus* embryonic cell cultures. The results showed that with an increase in sucrose concentration, the ultimate dry cell weight increased from 6.16 to 23.33 g l^{-1}, but higher sucrose concentration appeared to inhibit cell growth.

Suspension cultures of *Panax notoginseng* were treated with different concentrations of sucrose (20, 30, 40, and 60 g l^{-l}). As the concentration of sucrose was increased, the overall dry cell weight increased. But at high sucrose concentration, cell growth was inhibited (Zhang *et al.*, 1996). El-Tahchy *et al.* (2011) studied the effects of sucrose on the shoot cultures of three Amaryllidaceae species (*Narcissus pseudonarcissus*, *Galanthus elwesii*, and *Leucojum aestivum*) and the sucrose-enhanced explant survival, with higher sucrose concentrations causing enhanced organogenesis and reduced callusing.

Root suspension cultures of *Hypericum perforatum* were treated with different concentrations of sucrose. It was found that the root weight and growth ratio were optimal at 3% (w/v) sucrose, which yielded the highest fresh weight, dry weight, and growth ratio but higher sucrose concentrations significantly decreased the dry weight (Cui *et al.*, 2010). The effects of carbohydrate availability and light on rhizosecretion in potato plantlets

(*Solanum tuberosum* L., cv. 'Iwa') in liquid MS media were studied by Ling and Leung (2010). It was found that the seedlings developed in light and on a sucrose-containing medium were the most robust ones.

2% sucrose with pH 4.5 fortified medium was optimum for increased biomass production in the *in vitro* cultures of *Bacopa monnieri* (Naik *et al.*, 2010). *Alocasia amazonica* plants were grown in the MS medium supplemented with sucrose (0-9%), and the sucrose at 6% or 9% increased proliferation but inhibited photoautotrophic development (leaf formation). Increasing sucrose treatment increased sugars, starch content, and the number of stomata while decreasing water potential and stomatal size during the *in vitro* growth stage (Jo *et al.,* 2009). Saeiahagh *et al.* (2019) conducted work on the effects of cytokinins and sucrose on the *in vitro* cultures of red-fleshed *Actinidia chinensis* var. *chinensis* 'Zes006' and 2% sucrose with *m*T 6 (3-hydroxy benzyl adenine or *meta*-Topolin) gives plantlets with higher leaf numbers and chlorophyll levels.

The influence of sucrose on the micropropagation and multiplication of *Vaccinium corymbosum* was studied, and it was found that as the sucrose concentration was increased from 15 mM to 29, 44, or 58 mM, shoot multiplication was increased by 1.2 to 2.2-fold for all blueberry varieties (Cao *et al.*, 2003).

García-Ramirez *et al.* (2019) investigated the influence of sucrose on biochemical and physiological modifications in *Bambusa vulgaris* Schrad Ex Wendl shoots cultured *in vitro* and in a rooting culture medium, two sucrose concentrations (20 and 30 g l^{-1}) were utilized, as well as a control treatment (sucrose-free medium). It was found that eliminating sugar from the culture

media increased the shoots' morphophysiological, biochemical, and anatomical growth and development. High sugar concentrations reduced overall chlorophyll content and leaf area in shoots. SEM revealed that leaves of shoots grown on medium containing 30 g l^{-1} sucrose had anatomical modifications in the stomata of plants, but those grown on sucrose-free media had normal structural development.

Tasheva and Kosturkova (2014) conducted a study that aided in determining the optimal sucrose concentrations in culture media for the growth of callus cultures of Bulgarian Golden Root. The 2% sucrose concentration was identified as ideal for efficient induction and maintenance of callus with grain structure suited for long-term cultivation. Hmood and Salim (2017) reported the effects of different concentrations of sucrose in the *in vitro* cultures of *Withania somnifera*. They reported that the shoots burned and became brown in MS media that contained 120 g l^{-1} sucrose.

2.6.1.3. Putrescine (Put)

Rakesh *et al.* (2021) reported that polyamines (PA) are water-soluble, low molecular weight polycationic, aliphatic nitrogenous molecules with more than two amino groups observed in all living species. They can exist alone or combined with other molecules such as phenolic acid, macromolecules (nucleic acid, proteins), etc. In living organisms, the occurring PAs are Putrescine (Put), Spermidine (Spd), and Spermine (Spm). Put [butane-1,4-diamine] and Cadaverine [pentane-1,5-diamine] are two naturally formed aromatic, volatile polyamines identified from the decaying flesh of cadavers. The cell wall contains Spd and Spm, which regulate

lignification, cell wall pH, and other processes, and they are connected to pectic polysaccharides.

González-Hernández *et al.* (2022) reported that Put is the most prevalent PA in nature and the key product of the PA biosynthetic pathway, being primarily developed by two pathways that originate from ornithine (Orn) or arginine (Arg) as a result of the activity of ornithine decarboxylase (ODC, EC 4.1.1.17) or arginine decarboxylase (ADC, EC 4.1.1.19), respectively.

Park *et al.* (2012) conducted *in vitro* shoot induction studies on *Sinningia speciose* and reported that 50 mg l^{-1} Put enhanced the shoot growth and number. Kim *et al.* (2016) reported that Put (0, 10. 30, 50, 100, 200 mg l^{-1}) mediated enhancement in shoot organogenesis of *Polygonum tinctorium*, and the maximum number of shoots and longest shoots were seen in cultures supplemented with 50 mg l^{-1} Put, and increasing the dosage inhibited shoot regeneration and elongation. Sivanandhan *et al.* (2011) studied the effects of PAs (5, 10, 15, 20, and 25 mg l^{-1}) on *in vitro* multiplication of *Withania somnifera* and up to 20 mg l^{-1} Put, there was a progressive increase in the number of roots per shoot as well as the length of roots, and after that, there was a decrease in the number of roots per shoot as well as the length of roots.

The influence of PAs on the micropropagation of *Pisum sativum* was investigated, and the highest rooting response (88%) was found with 30 mg l^{-1} Put and 0.6 mg l^{-1} NAA among the several PAs examined. Applying PAs (Put) increased rooting by twice compared to plants treated with PGR treatments. Increasing or decreasing the concentration of Put from 30 mg l^{-1} has gradually lowered root induction (Ajithan *et al.*, 2019).

Abd-Elbar *et al.* (2019) studied the effects of foliar application of Put on morpho-anatomical, biochemical, essential oil content, and growth parameters of *Thymus vulgaris.* The fresh and dry weights of shoots and roots of thyme plants under watering regimes were improved by the foliar application of Put at 0.1 or 0.2mM. Put (0.2 mM) produced a significant ($P \leq .05$) increase in growth parameters when compared to the control plants.

Bais *et al.* (1999) investigated the impact of Put on the development and generation of two coumarins, esculin and esculetin, in hairy roots of chicory (*Cichorium intybus* L. cv. Lucknow local) and the highest length of primary hairy roots in samples treated with 1.5 mM Put was 18.3±1.4 cm, which was 1.6 cm longer than in controls (11± 0.9 cm).

Amri *et al.* (2011) assessed the responses made by Put and Spd on *Punica granatum* grown under salinity. Compared to Spd, Put exhibited longitudinal root growth that could be considerably boosted. Fresh weight and root dry weight dropped dramatically as salt levels increased. Polyamine levels may raise the dry and fresh weight of the root at various salinity levels. Weight of the plant and root dry weight increased considerably when Put was used in comparison to Spd. Put significantly increased the number and length of shoots and leaf area.

Santa-Catarina *et al.* (2007) reported that Put increased the production of somatic embryos of *Ocotea catharinensis*. Bais *et al.* (2001) reported that *Cichorium intybus* L. plants that had not transformed experienced increased shoot multiplication and shoot growth when 40 mM Put was added to the MS medium. Put at a dose of 40 mM also caused flowering on day 28, with

increased levels of endogenous Put and Spm in both untransformed and transformed plants.

Application of PAs and the addition of putrescine and spermidine increased the growth and number of roots of *Beta vulgaris* and *Tagetes patula* (Bais *et al.,* 2000). Yazdanian *et al.* (2022) reported that 0.5 mM Put significantly increased the callus growth rate of *Allium jesdianum*.

The impact of Put in the *Cedrela fissilis*'s organogenesis was investigated. Adding Put at all tested doses significantly enhanced shoot induction in cotyledonary nodal segments compared to the control. The highest shoot length (1.71 cm), was attained with 2.5 mM Put treatment (Aragão *et al.,* 2017).

Put at 50 mg l^{-1} concentration was optimum for the enhanced biomass production in the callus culture of *Glycyrrhiza glabra* (Jaiswal *et al.,* 2021). *In vitro,* multiplication of *Aristolochia indica* was studied by Dey *et al.* (2021), and SH media fortified with 2 mg l^{-1} BAP + 0.5 mM Put was best for the shoot proliferation from explants.

Redha and Suleman (2011) studied the external application of PAs in the androgenesis of Wheat. Low quantities of put, alone or in combination with spd or spm, could enhance the androgenesis of some recalcitrant wheat genotypes to increase ELS (Embryo-like structures) induction and green plant regeneration. Parimalan *et al.* (2011) reported that Put enhanced the *in vitro* shoot production of *Bixa orellana*. In 4 weeks, medium supplemented with 1,000 µM Put yielded the most significant response (12.8±0.52 shoots), whereas 800 µM Put yielded the highest shoot elongation (7.3±0.31 cm), and all the Put (200–1,500 µM) concentrations triggered callus formation.

Reis *et al.* (2015) studied the effects of Put on somatic embryogenesis in callus cultures of Sugarcane. 500 µM Put was found to be the best among the treatments tested, which induced the production of 55 embryos per callus culture, compared to 19 somatic embryos per callus culture in the control treatment. The impact of various Put concentrations on the embryogenic callus and subsequent embryogenesis in *Phoenix dactylifera* cultivar Bream was investigated by Ibrahim *et al.* (2014). The calli formed in the nutrient medium were transferred to culture media with 0.0, 0.5, 1.0, 2.0, or 3.0 mM of Put. At 2.0 mM Put, embryonic callus fresh and dry weights increased significantly, reaching 2.3 and 0.3 g, respectively. The number of embryos was increased after the calli were inoculated to the fresh media with the same concentration.

The highest percentage of embryonic tissue of *Pinus gerardiana* was produced from the mature zygotic embryos in the half MSG medium provided with 9.0 µM 2, 4-D, and 2.0 mg l^{-1} Put (Malabadi & Nataraja, 2007). The study aimed to see how varying amounts of Put affected the regeneration of the date palm cultivar Barhee at all stages of regeneration (callogenesis, somatic embryo multiplication, germination, and rooting). The results showed that Put significantly affects callus growth, somatic embryogenesis, and rooting (Hussein *et al.*, 2021).

In the study conducted by Amin *et al.* (2011), different concentrations of Put and glutamine were applied topically to onions (*Allium cepa* L. cv. "Giza 20") to see how they affected their growth, yield, quality, and several metabolic aspects. Put and Glut, either separately or together applied as foliar, greatly enhanced plant height, number of leaves, fresh weight of leaves per

plant, fresh and dried weight per plant, leaf area, leaf area per plant, bulb length, bulb diameter, and weight, as well as yield of onion and quality of bulbs.

Kandil *et al.* (2011) studied the impacts of foliar spray of Put on the flower characteristics of *Chrysanthemum*. Spritzing of Put at 200 ppm considerably enhanced flower yield, floral head diameter, pedicle length of flowers, length of the flowering stalk, vase life, and fresh and dry weight of inflorescence of *Chrysanthemum* when compared to the control plants. Mustafavi *et al.* (2014) examined the influence of polyamine priming on the germination behaviour of fennel at low temperatures; the highest germination percentage was attained at 10 ppm. A lower germination percentage was found in the control.

Xu *et al.* (2011) conducted experiments to promote chilling tolerance in tobacco by seed priming with Put by utilizing seeds from two tobacco varieties, MSk326 and Honghuadajinyuan (HHDJY). Under chilling stress at 11^0C, seed priming with Put resulted in considerably higher germination percentages, germination indexes, seedling lengths, and dry weights when compared to controls that did not get the Put treatment.

A significant enhancement was observed in the plant height and fresh and dry weights of *Pelargonium graveolens* due to the foliar application of Put (Ayad *et al.,* 2010). Baniasadi *et al.* (2014) experimented with a completely randomized design in a greenhouse setting using NaCl (1, 3, 6, and 9 dS m^{-1}) and Put (0, 1, and 2 mM) to determine the effects of salt stress and Put on certain morphological and biochemical traits and the pigment content of pot marigold plants. The soil was treated with NaCl, and the leaves

were sprayed with Put solution. Spritzing of 2 mM Put, under salt stress level of 9 dS m^{-1}, enhanced fresh flower weight and shoots dry weight.

Amin *et al.* (2013) conducted a study to analyse the effects of Put and IBA applied alone or in combination in chickpea (*Cicer arientinum* L. cv. Giza 3). The plant height, quantity, dry weight of branches, leaves and pods/plant, and leaf area/plant during the two growth stages were considerably raised by spraying Put and IBA separately or in combination. The highest number of pods was generated when Put and IBA were applied at 100 mg l^{-1}, which led to a much higher seed output.

The effects of Put in the micropropagation of *Decalepis hamiltonii* were examined by Matam and Parvatam (2017). Put was added at a concentration of 50 µM, resulting in the induction of roots with greater root length. The root fresh weight was also found to be increased. The optimum response for *in vitro* rooting in terms of quality and quantity was supported when Put was used alone in a rooting medium without auxins.

The influence of Put on the vegetative growth and flowering characteristics of *Antirrhinum majus* was investigated by Badawy *et al.* (2015). At 75 days after transplanting, foliar treatment of Putrescine at a dosage of 200 ppm resulted in the maximum increase in vegetative growth and flowering characteristics.

Wu *et al.* (2010) reported a study on the effects of Put on plant growth and root mycorrhizal development in *Glomus versiforme*. Put substantially enhanced the number of entry sites, arbuscules, and vesicles, as well as the mycorrhizal colonization of the area. The Put treatment considerably enhanced total root length, projected area, surface area, and root volume.

2.6.1.4. Tryptophan

Trp was used as an inductive signal in the root culture of *Hypericum perforatum.* Trp exposure improved *de novo* shoot organogenesis in liquid pulse exposure trials, and prolonged incubations of tissues in tryptophan solutions reduced regeneration. After 24 hours of exposure, tryptophan significantly enhanced the shoot generation from root explants, although micro shoot (shoots < 1 mm in length) production increased more modestly (Erland & Saxena, 2019).

Taha *et al.* (2009) studied the effects of precursor feeding of amino acids and L-Trp at 300 mg l^{-1} and enhanced cell growth parameters such as cell number, fresh weight, dry weight, and packed cell volume in Egyptian *Catharanthus roseus* suspension cultures. Klimek-Szczykutowicz *et al.* (2021) reported a study on the effects of precursor feeding on *Nasturtium officinale* microshoots, and the application of 3.0 mM Trp enhanced the growth index and biomass.

2.6.1.5. Medium Strength (MS)

Wan-Nurul-Hidayah *et al.* (2012) studied the effect of full and half MS media strengths on *Pogostemon cablin* and found that the number of shoots formed in full MS medium was relatively higher than in half MS medium. The rooting response of *Stevia rebaudiana* plants regenerated through callus culture was more effective in quarter strength MS medium treated with 0.1 mg l^{-1} IBA (Patel & Shah, 2009).

Rezali *et al.* (2017) studied the effect of different liquid and semi-solid MS media strengths in the *in vitro* cultures of *Typhonium flagelliforme*. When

MS strength was reduced in semi-solid media, the leaves and roots number were significantly increased. In contrast, when MS strength was decreased in liquid media, the number of leaves and roots was reduced. The full-strength MS liquid medium has the highest number of leaves, and the lowest was in full-strength MS semi-solid media. Quarter-strength MS media had the greatest FW and DW values in solid media, followed by half- and full-strength MS. The FW was higher in liquid media and became lower in semi-solid media. Full-strength MS media had the highest FW in liquid media, followed by half and quarter-strength MS media. The highest total chlorophyll, chlorophyll a, and b levels were found in quarter-strength MS liquid media. Chlorophyll a and total chlorophyll were reduced to their lowest levels by half-strength MS in solid and liquid media.

Altered MS media strength was applied in *in vitro* organogenesis of *Mentha spicata*, and the results showed that the highest shoot and root number was produced per explant in half-strength MS medium. However, the most significant leaf number and average root length were related to regeneration on a full-strength medium (Fadel *et al.,* 2010).

In vitro, propagation of *Boswellia serrata* was tested with varying agar concentrations. Shoot cultures produced on conventional shoot multiplication media solidified using 0.8% agar multiplied 2.2-folds, after 42 days, each cluster had an average of 11.3 shoots and 76.3 leaves. In this medium, shoots grown were 2.51±0.49 cm long with 3.08±1.49 g fresh weight and 0.31±0.10 g dry weight. However, more than 20 shoots were formed in liquid medium and in weakly gelled medium (0.2% agar) (almost 2.0 times more than the

control), and the most significant shoot length (2.830.15 cm) was measured in this medium (Suthar *et al.*, 2010).

Abou-Dahab *et al.* (2005) worked on *Ruscus hypoglossum* to study the effects of different media strengths. In comparison to 1/2 MS strength (9.50) and 1/4 MS strength (6.16), the number of shootlets produced per explant was highest in the case of full MS strength (12.50). The 1/2 strength of MS salts had a lengthy shoot (5.29 cm), proceeded by the full strength (4.50 cm), and the 1/4 strength of MS salts generated the shortest shoot (3.66 cm). The explants cultured on full-strength MS medium, resulted in the most significant number of leaves/shoot (11.5), and on 1/4 strength MS medium, which resulted in the least number of leaves (6.41).

The effect of media strength on *Manihot esculenta* somatic embryogenesis was studied by Groll *et al.* (2002), and the results showed that in full- and half-strength MS media, more embryos were differentiated and germinated. Embryos with reduced size and pale-yellow colour were observed in the quarter-strength MS medium. Cotyledonary abnormalities, such as excessive enlargement of cotyledons, were observed in the medium with double-strength MS.

Tylophora indica tissues produced in the full-strength MS medium were proliferating and highly chlorophyllous. In contrast, those grown in 1/2 and 1/4 strength MS medium exhibited pigmentation loss accompanied by a significant decrease in growth rate (Benjamin *et al.*, 1978).

2.6.1.6. Jasmonic Acid (JA)

Jasmonic acid (JA), as well as its derivatives and precursors, collectively known as jasmonates (JAs), are crucial molecules in the control

of numerous physiological processes of plants, particularly in mediating plant responses to various types of stressors (Ruan *et al.*, 2019).

Kamińska (2021) viewed that JAs increase the multiplication rate of shoots, roots, and calli, as well as the production of micro tubers and bulblets. Adverse effects of JAs on plant tissue have also been described, such as leaf senescence, decreased growth, and inhibition of somatic embryogenesis. Numerous studies demonstrated that Jasmonic acid and Methyl jasmonate promote the breakdown of chlorophyll, which contributes to leaf senescence. JA stress damages chloroplasts, reduces photosynthetic performance by stimulating RuBisCO breakdown, encourages loss of the structure of plasma membrane during the process of lipid peroxidation, and promotes the expression of genes involved in senescence.

The effects of JA elicitation on the cell suspension cultures of *Hypericum performers* were investigated by Gadzovska *et al.* (2007). Exogenously administered JA induced specific stress responses in *Hypericum* cells, resulting in negative control of growth and development. Cell viability was decreased in JA-treated cells and depended on the post-elicitation period. The fresh weight of elicited cells reduced gradually throughout the culture, and when compared to control levels, JA-elicited cells produced less biomass. Compared to cells treated with 50 µM JA, cell suspensions treated with 100–250 µM JA produced less biomass.

Krzyzanowska *et al.* (2012) conducted work on suspension cultures of *Mentha* x *piperita* to study the effects of Jasmonic acid and JA marginally suppressed biomass production at higher concentrations (100 and 200 µM). The suspension cultures' color changed from greenish to green-brown or even

brown at the final growth stage. The Browning of the cell cultures is an evident indication of increasing phenolic compound deposition. This style of behaviour was also observed in many elicited cultures.

Al-Huqail *et al.,* (2021) reported a study on the effects of JA in the micropropagation of *Catharanthus roseus* treated with salinity stress. The root dry weight of saline irrigated plants increased by the application of JA and 1.5 mM was found as the optimum concentration of JA for enhancing the plant height.

The application of exogenous JA reduced the biomass by 25% in the cell cultures of *Jatropha curcas* (Zaragoza-Martınez *et al.,* 2016). Walker *et al.* (2002) reported JA-induced effects in the cell suspension cultures of *Hypericum perforatum*. Under light conditions, 250 µM JA treated on the suspension cultures of *H. perforatum* induced higher biomass production than light-grown control, and the cultures triggered the cell growth under dark conditions than the dark-grown control and light-grown JA elicited cultures.

Jasmonic acid increased dry weight in the cell suspension cultures of *Artemisia absinthium* (Ali *et al.,* 2014). *Narcissus triandrus* shoot cultures were induced with JA alone, JA+2-iP, and JA+NAA; among them, the plantlets produced in the medium with JA alone showed the highest number and diameter of the bulb (Santos & Salema, 2000).

The media fortified with JA (10 µM) enhanced the shoot multiplication and tuber formation in *Dioscorea cayenensis–D. rotundata* complex (Ovono *et al.,* 2007). Blázquez *et al.* (2003) reported that the application of 0.5 mgl^{-1} JA in the *in vitro* medium significantly enhanced the somatic embryogenesis of *Crocus sativus*. To develop an efficient protocol for the micropropagation

of *Pistacia lentiscus,* Koç *et al.* (2014) added JA as an elicitor, and the following results were obtained. The number of shoots that proliferated per explant was much smaller after adding JA, which decreased the production of numerous shoots. JA also did not affect the quality of the shoots since proliferating shoots were unhealthy and had poor leaf development.

Kamińska *et al.* (2018) reported the physiological effects of JA treatments on *Taraxacum pieninicum* during the micropropagation of shoots and cold storage at 4 °C in the dark or with reduced light. The maximum proliferation rate was seen in shoots cultivated on media containing JA at the lowest concentration (24 µM), and the shoot growth was limited as JA concentration was raised. Leaves fell off quickly from vulnerable plants on medium with the greatest concentration of JA.

The effects of JA in the *in vitro* callus production of Blackgram were studied by Lingakumar *et al.* (2014). At 1 ppm of JA, the frequency of root production was 50%, and at 1.5 ppm of JA, a significant response was seen. Despite going through callus development, JA directly induced roots at 0.5 ppm. Long, well-branched roots with a 100% root development frequency were seen at 1.5 ppm of JA and moderate root growth at 2 ppm of JA. JA concentrations ranging from 0.5 to 2.5 ppm encouraged root initiation and subsequent root development with the formation of lateral branches.

Ružić *et al.* (2013) examined JA's ability to enhance the *in vitro* propagation phase in micro-propagated shoots of Pyrodwarf and Gisela, low vigorous pear and cherry rootstocks. The following were the main features of shoots grown in JA alone media: extensive plants/shoots, huge, dark green

leaves, small firm callus, and the emergence of rhizogenesis with small firm radially oriented roots up to 3 mm in length were observed.

Ravnikar *et al.* (1993) reported that the length of potato plants was significantly increased by concentrations of JA ranging from 0.1-1 µM, and the treatment of JA at these concentrations resulted in a significantly varied root system with numerous lateral branches. The primary roots became shorter and thicker as the JA concentration increased.

Zhang *et al.* (2006) studied the effects of JA in the *in vitro* growth of *Solanum tuberosum.* The JA reduced the development of potato explants at higher concentrations (20 - 50 mg dm^{-3}), and the amount of chlorophyll in explant leaves reduced as the concentration of JA increased.

Kumlay *et al.* (2015) reported the response of JA treatment on the *in vitro* cultures of *Solanum tuberosum*. The elongation of stems and roots was stimulated in MS medium supplemented with JA.

2.6.1.7. Salicylic Acid (SA)

Zare-Hassani *et al.* (2019) reported that elicitation with SA in the shoot cultures of *Ziziphora persica* significantly reduced the fresh weight, number, and length of roots and shoots. Khan *et al.* (2019) studied the effects of chitosan treatment in the callus cultures of *Fagonia indica* and found inhibition of callus proliferation at greater SA concentrations. Chaichana and Dheeranupattana (2012) worked on *in vitro* cultures of *Stemona* species, and various concentrations of SA (0.1, 0.3, 0.5, and 1.0 mM) were applied. SA partially inhibited root and overall growth in various treatments, notably at 1.0 mM. Açikgöz *et al.* (2019) found that the cell suspension culture of *Achillea gypsicola* was treated with various concentrations of SA (0, 10, 50,

and 100 µM), and in comparison, to the control, the suspension culture treated with 100 µM SA had a significantly higher cell number and dry weight.

Elicitation studies on the root cultures of *Datura metel* were conducted by Ajungla *et al.* (2009), and varying concentrations of SA (25, 250, and 500 µM) were added to the culture medium; the growth index was slightly reduced as a result of the SA addition. Miclea *et al.* (2020) viewed that in a nutrient medium supplemented with 1.5 mg l^{-1} of SA, the shoots produced per explant were significantly higher in number than the control in the *in vitro* shoot cultures of *Lavandula angustifolia*. However, the lowest tissue weight per explant was observed at 15 mg l^{-1} SA.

The effects of SA on different explants (cells, calli, and shoots) of *Hypericum perforatum* were studied by Gadzovska *et al.* (2013); the viability of cell suspension cultures was reduced approximately by 20 and 35%, respectively, at the lowest (50 µM) and maximum (250 µM) SA concentrations selected. During the last week of callus culture, the green callus turned red and brown in the presence of 100 µM SA. A spontaneous adventitious root production also occurred towards the culture's end. Jirakiattikul *et al.* (2021) worked on SA-mediated elicitation studies on *Musa acuminata* cv. 'Gros Michel' shoots and found that SA significantly affects growth, and the fresh and dry weight of the plants was reduced when the concentration of SA increased.

2.6.1.8. Chitosan

Shah *et al.* (2021) conducted studies on the effect of chitosan on cell suspension cultures of *Silybum marianum*. The cell suspensions treated with

5.0 mg l^{-1} chitosan produced the highest biomass levels. Fooladi-Vanda *et al.* (2019) found that application of chitosan improved the shoot fresh weight of *Melissa officinalis*.

Sathiyabama *et al.* (2016) reported that the exogenous foliar application of chitosan promoted the shoot and rhizome development in *Curcuma longa*. Mastuti *et al.* (2021) developed an elicitation medium consisting of MS basal medium fortified with BAP + IAA and chitosan elicitor. The results showed that *in vitro* application of chitosan as an elicitor on *Physalis*' shoot tip and nodal explants produces multiple shoots and enhanced growth from both explants.

In cell suspension cultures of *Vitis vinifera*, applying 50 µg ml^{-1} chitosan reduced the cell viability along with the preceding growth periods (Ferri *et al.*, 2009). Brasili *et al.* (2014) viewed that the root biomass was increased in *in vitro* root cultures of *Hypericum perforatum* due to chitosan elicitation. Sivanandhan *et al.* (2012) reported a 1.02-fold decrease in adventitious root growth in the *in vitro* root cultures of *Withania somnifera* in all the chitosan concentrations for 8 hours of exposure.

Baque *et al.* (2012) conducted studies by chitosan elicitation on *Morinda citrifolia* adventitious root suspension culture and its effects on growth. A significant reduction in the growth and development of adventitious roots with the chitosan treatment was observed. Brasili *et al.* (2016) found that the growth rate was reduced in the liquid root cultures of *Hypericum perforatum* due to chitosan induction. Apart from the control culture, the chitosan-treated roots with an increased biomass intensity and swelling at the root apex were also noticed.

Shah *et al.* (2021) observed that applying chitosan increases biomass production in cell suspension cultures of *Silybum marianum*. The maximum production was found in the medium containing 5.0 mg l^{-1} chitosan .

2.6.2. Effect of stress signals in *in vitro* production of secondary metabolites

2.6.2.1. HPTLC STUDY

HPTLC is an important phytochemical estimation method (Attimarad *et al.,* 2011; Misra *et al.,* 2014) and is more efficient for identifying plants using secondary metabolites in the field of plant taxonomy (Khan *et al.,* 2011). Plant identification via HPTLC fingerprinting has been demonstrated to be a linear, precise, and accurate method (Cortés *et al.,* 2014). The testing of quality and adulterants of mark available herbal drugs can be greatly achieved through this type of advanced phytochemical estimation (Gandhi *et al.,* 2012; Meena & Sandhya, 2013; Teo *et al.,* 2013).

The plant extract comprises a variety of secondary metabolites such as volatile oils, saponins, coumarins, phenolics, flavonoids, terpenoids, terpenes, alkaloids, etc. The presence of these medicinally important metabolites was also detected in the detailed HPTLC profiles, lending support to the species' traditional therapeutic applications and the examination of the crude extract is an essential aspect of determining proper identification (Senguttuvan & Subramaniam, 2016).

2.6.2.2. NaCl (Salt stress)

Hussein and Aqlan (2011) reported that salt stress caused by sodium chloride concentration lowered growth, and increased total phenolics,

flavonoids, and tannins. However, higher salt concentration dramatically inhibited growth and secondary metabolite accumulation.

Sabir *et al.* (2012) reported that, under salt treatments, the activity of peroxidises increased significantly activity, while catalase was the least sensitive. El-Shennawy *et al.* (2017) reported that in the shoot culture of *Mentha longifolia*, 2000 mgl^{-1} NaCl was influential in rosmaric acid and phenolic acid production.

Muchate *et al.* (2019) reported that salt stress induced the accumulation of 20-hydroxyecdysone in *in vitro* shoot cultures of *Spinacia oleracea*. Mozafari *et al.* (2019) reported that in *in vitro* cultures of Strawberry, salt stress of 100 mM NaCl increased the peroxidation of lipids.

Singh *et al.* (2000) studied that Na, K, Cl, Ca, Mg, total sugar, and proline contents were increased at 100 mM NaCl, and a reduction of leaf chlorophyll *a* + *b* was noticed in the *in vitro* shoot cultures of *Vitis vinifera*.

Shibli *et al.,* (2007) reported decline in the levels of P, K, Mg, Ca, N, and S with increasing salinity in *Lycopersicon esculentum* Mill. Abed-Alrahman *et al.* (2005) reported that higher salt stress increased the ash percent and reduced soluble protein percent, fat, and fibre in microshoots of *Cucumis sativus*. Reduced the moisture percentage in microshoots and carbohydrate content at 50 and 75 mM NaCl; an elevation occurred at 100 mM NaCl. In contrast to increased proline and reducing sugars, elevated salinity dramatically decreased the amount of crude protein in microshoots. When microshoots were gradually salinized instead of exposure to salt stress, they gathered higher moisture, reducing sugars, soluble protein, crude protein, proline, and less fat and carbohydrates.

Pandey *et al.* (2016) reported that a higher amount of artemisinin was produced under salt stress from the gamma-irradiated soma clonal variant (ASV12) from callus-generated plants of *Artemisia annua*.

2.6.2.3. Sucrose

In the study conducted by Abou-Dahab *et al.* (2004) on *Ruscus hypoglossum*, different concentrations of sucrose induced the following results: sucrose concentrations substantially affected chlorophyll content. The maximum quantity of chlorophyll-*a* was produced by sucrose at 30 g l^{-1}, whereas the least amount of chlorophyll-*a* (133.8 mg $100g^{-1}$ FW) was produced by sucrose at 20 g l^{-1}. The maximum indole content was obtained when sucrose was used in a concentration of 50 g l^{-1}.

Treatement with 88 mM and 176 mM sucrose, enhanced the contents of glucosinolates in sprouts of Broccoli; increased the FRAP (Ferric reducing antioxidant power) value and the activity of PAL enzyme (Guo *et al.*, 2011). Taxol production in *Taxus cuspidata* nodule cultures was increased to around 12 g taxol g^{-1} nodule dry weight (dw) with an increased sucrose content (0.5% or 1.0%) (Ellis *et al.*, 1996).

Sari *et al.* (2018) used reported highest secondary metabolite (alkaloids, phenols, flavonoids, saponins, and steroids) production in callus cultures of *Myrmecodia tuberosa* in nutrient medium containing 30 g l^{-1} of sucrose.

Melissa officinalis leaves were treated with different concentrations of sucrose and at higher concentrations of sucrose (150 and 300 mM) the levels of several flavonoids were found to be increased (Kim *et al.*, 2020). In *Salvia*

leriifolia cell suspension cultures, the levels of caffeic acid and salvianolic acid B were increased in the medium containing 40 g l^{-1} sucrose and in the medium containing 50 g l^{-1} sucrose, enhanced production of rosmarinic acid was observed (Modarres *et al.*, 2018). In cell suspension cultures of *Artemisia absinthium*, total phenolics and flavonoids showed maximum accumulation in the media containing 5 and 7% sucrose, respectively (Ali *et al.*, 2016).

Elicitation studies on *in vitro* root cultures of *Musa* species, high accumulation of total phenolic content was reported in the nutrient media containing 40 and 45 g l^{-1} of sucrose (Ayoola-Oresanya *et al.*, 2021). The study of taxane diterpene—taxuyunnanine C (Tc) production in the *Taxus chinensis* with a combined process of elicitation and sucrose feed in suspension cultures was carried out by Dong and Zhong (2001), and it was found that in the cells elicited with MeJ and fed by sucrose and the content of Tc was rapidly elevated.

Shohael *et al.* (2006) studied the effects of initial sucrose concentration on *Eleutherococcus sessiliflorus* embryonic cell cultures. They found that the contents of eleutherosides, total phenols, and flavonoids increased with the sucrose concentration. However, the polysaccharide content was unaffected by the high sugar treatment. SOD, CAT, APX, GR, GST, MDHAR, and DHAR activities were significantly increased with the higher sucrose concentrations.

The effects of initial sucrose concentrations (20, 30, 40, and 60 $g1^{-l}$) on *Panax notoginseng* suspension cultures were studied, and the initial sucrose levels did not appear to impact the content of ginseng polysaccharides. The

optimum amount of ginseng polysaccharide and saponin was produced at a sucrose concentration of 40 gl^{-1} (Zhang *et al.,* 1996).

The impact of sucrose and auxin on the alkaloid profiles of three Amaryllidaceae species was investigated by El-Tahchy *et al.* (2011). GC-MS was used to analyze culture extracts of *Narcissus pseudonarcissus*, *Galanthus elwesii*, and *Leucojum aestivum* grown on MSA or MSB medium with varying sucrose. *Narcissus* tissues cultured on MSA and 30 g l^{-1} sucrose produced the highest amount of alkaloids (which included five known alkaloids). In extracts of *G. elwesii* tissues cultivated in nutrient medium containing 60 g l^{-1} sucrose, gal, trispheridine, crinine, and demethylmaritidine were found. The tissues of *L. aestivumgrown* cultured on MSA and 30 g l^{-1} sucrose exhibited the production of four identified alkaloids. Galanthamine (Gal) accumulation in *G. elwesii* was 20-fold (0.02% DW) enhanced in nutrient medium containing 60–90 g l^{-1} of sucrose. In the shoot cultures of *N. pseudonarcissus* highest Gal content (0.1% DW) was observed in medium containing 30 g l^{-1} sucrose, in contrast, in medium containing 120 g l^{-1} of sucrose, the gal content enhanced to 0.08% DW. Shoots cultured in medium containing 30-60 g l^{-1} sucrose had the highest Gal content (0.032-0.045% DW). As a result, shoots cultured with 60 g l^{-1} sucrose and 10 µM NAA produced the most effective outcomes in terms of Gal accumulation (0.074% DW).

Cui *et al.* (2010) reported a study on the sucrose-induced (0, 1, 3, 5, 7, or 9% in w/v) osmotic stress on the root suspension cultures of *Hypericum perforatum.* Increased total phenolic, flavonoid, chlorogenic acid, and total hypericin accumulation was facilitated by increased sucrose content (3, 5, and

7% in w/v). The H_2O_2, MDA, and proline levels increased in the higher sucrose-treated adventitious roots.

The influence of sucrose in *in vitro* cultures of *Bacopa monnieri* and bacoside A content was studied with varying sucrose concentrations (0, 1, 2, 3, 4, 5, and 6%), and the HPLC analysis showed that 2% sucrose at pH 4.5 was optimum for the bacoside A production; also, increasing sucrose concentration reduces the phytocompound accumulation in the regenerants (Naik *et al.*,2010). Park *et al.* (2006) reported that addition of 5–15 g l^{-1} of sucrose to the cell cultures of *Eschscholtzia californica,* increased the accumulation of alkaloids such as sanguinarine, chelerythrine, and chelirubin. With the further addition of sucrose (20-30 g l^{-1}), Dihydroform benzophenanthridine alkaloid (DBPA) production was increased.

The effects of exogenous sucrose on the *Bambusa vulgaris in vitro* cultures were studied, and the concentration of hydrogen peroxide and malondialdehyde increased in shoots cultured with 30 g l^{-1} sucrose (García-Ramirez *et al.,* 2019). Hmood and Salim (2017) studied the effects of sucrose in the *in vitro* production of alkaloids and steroids from *Withania somnifera*. The addition of variouslevels of sucrose (30, 60, 90, and 120 g l^{-1}) in the MS medium resulted in variations in the production of ten compounds. Withanone, 27-hydroxy withanolide D, and compound 17-hydroxy 27 deoxy withaferin all produced at their highest and most significant rates when exposed to 30, 60, and 90 g l^{-1} sucrose.

2.6.2.4. Putrescine (Put)

PAs (Polyamines) are involved in various growth and development activities of plants (Gill & Tuteja, 2010). González-Hernández *et al.* (2022) viewed that PAs can be observed in higher plants not only as freely accessible but also in conjugates linked to phenolic acids (ferulic, caffeic, coumaric or hydroxycinnamic acid) or biomacromolecules. Put is the most prevalent PA in nature and the key product of the PA biosynthetic pathway. Put interacts with many compounds, including gas molecules, phytohormones, and signal molecules.

Diwan and Malpathak (2012) studied the effects of PAs in *Ruta graveolens in vitro* cultures; adding Put increased the accumulation of furanocoumarins, psoralen, xanthotoxin, and bergapten. Abd-Elbar *et al.* (2019) researched the effects of foliar application of Put on *Thymus vulgaris* morpho-anatomical, biochemical, essential oil content, and growth characteristics. Put at 0.1 or 0.2 mM was applied as a foliar spray, and it significantly increased ($P \leq .05$) the activities of PAL and polyphenol oxidase (PPO) in the leaf tissues of thyme plants. The Put (0.2mM) application increased the essential oil percentage by reducing the adverse effects of drought. That was anticipated since putrescine inhibits photosynthesis and chlorophyll concentrations, accumulates phenolic chemicals to guard against reactive oxygen species (ROS), and promotes growth, which leads to thyme plants producing more herbage biomass. In hairy roots of chicory, the production of esculetin and esculin was enhanced by the treatment of 1.5 mM Put (Bais *et al.*, 1999).

Punica granatum grown under salinity were treated by Put and Spd, and the responses include: the application of PAs (Put and Spd) decreases the Na^+ and Cl^- concentrations and simultaneously increased the K^+ concentrations and proline content (Amri *et al.*, 2011). Aragão *et al.* (2017) reported the Put-induced changes in the *Cedrela fissilis,* and the explants grown with 2.5 mM Put had considerably higher indigenous total free-PA levels.

Jaiswal *et al.* (2021) studied the callus culture of *Glycyrrhiza glabra* on enhancing phytocompounds. Put was the most effective in increasing primary metabolite content and increased peroxidase activity. The embryonic callus culture of Sugarcane was treated with Put by Reis *et al.* (2015), and the biochemical changes were monitored. 500 µM Put was effective in the enhancement of PAs in callus cultures.

A study was conducted to assess the response of onion (*Allium cepa* L. cv. 'Giza 20') in terms of growth, yield quality, and several metabolic elements to foliar application of glutamine (Glut; 50, 100, and 200 mg l^{-1}) and putrescine (Put; 25, 50, and 100 mg l^{-1}). Increased Put concentrations up to 100 and increased Glut concentrations up to 200 mg l^{-1} enhanced photosynthetic pigment content in leaves, sulphur compounds, free amino acids, total soluble sugars and soluble phenols (Amin *et al.*, 2011).

Kandil *et al.* (2011) studied the effects of Put in *Chrysanthemum* flowers and the foliar application of Put to enhance the contents of Chl *a* and *b*, carbohydrates, and carotenoids in the plants. Xu *et al.* (2011) reported that seed priming with 0.1 mM Put enhanced the activities of antioxidant enzymes in tobacco.

Ayad *et al.* (2010) reported the effects of Put foliar spray on the essential oil content, photosynthetic pigments, and lipid peroxidation on *Pelargonium graveolens.* The treatment with and 20 mg l^{-1} Put increased the oil percent and yields, citronellal, geraniol, and linalool, and decreased lipid peroxidation. Shen *et al.* (2019) experimented in a greenhouse to better understand how exogenous putrescine (Put) reduced the amount of starch accumulated in cucumber leaves during salt stress.

Esfandiari-Ghalati *et al.* (2020) reported that foliar spray of NaCl increased the content of Catalase, polyphenol oxidase, carotenoids, and proline in guava seedlings. However, the foliar spray of Put reduced Catalase and peroxidase activity.

Baniasadi *et al.* (2015) experimented with a completely randomized design in a greenhouse to study the impacts of Put and salt stress on pot marigold plants. The contents of chlorophyll *a*, total chlorophyll, leaf carotenoids, and reduced sugars were enhanced after Put foliar spray.

El–Bassiouny *et al.* (2008) reported the effects of foliar spray of Put on wheat plants to study various aspects. The exogenous application of Put increased the percentage of total carbohydrates, proteins and endogenous phytohormones.

Amin *et al.* (2013) investigated the effects of Put and IBA application alone or in combination on the chickpea (*Cicer arientinum* L. cv. Giza 3). The levels of total free amino acids, soluble sugars, N, P and K were significantly higher after adding IBA to the treatment.

2.6.2.5. Tryptophan

Trp belongs to the group of aromatic amino acids produced by the shikimate pathway, which begins with the condensation of erythrose-4-phosphate with phosphoenol pyruvate to produce Phe and Tyr in addition to Trp. After the production of chorismate from shikimate, the pathway splits into two paths: one leads to Phe and Tyr, whereas the other gives Trp via a critical intermediary, anthranilate (Ishihara *et al.*, 2007). Tryptophan (Trp) is a precursor of secondary metabolites in plants (Ishihara *et al.*, 2011).

Quittenden *et al.* (2009) studied the characterization of the tryptamine pathway in *Pisum sativum* and found that the tryptamine pathway leads to bioactive auxin, indole-3-acetic acid. Glawischnig *et al.* (2004) reported that in *Arabidopsis thaliana* indole phytoalexin; camalexin is synthesized from tryptophan via indole-3-acetaldoxime (IAOx) in a process catalyzed by CYP79B2 and CYP79B3A.

Erland and Saxena (2019) hypothesized that tryptophan is metabolized to auxin, melatonin, or serotonin, which causes organogenesis in *Hypericum perforatum* L. Taha *et al.* (2009) reported the effects of adding precursor amino acids in Egyptian *Catharanthus roseus* suspension cultures. Different concentrations of amino acids were used (0, 100, 300, or 500 mg l^{-1}), including the L-Trp. The L-Trp at 300 mg l^{-1} was highly effective in producing total alkaloids, vinblastine, and vincristine.

Whitmer *et al.* (2002) reported that the effect of precursor feeding of Loganin/tryptamine and loganin/tryptophan combinations boosted terpenoid indole alkaloid accumulation in cell cultures of *Catharanthus roseus*.

Klimek-Szczykutowicz *et al.* (2021) conducted a precursor-feeding study on *Nasturtium officinale* microshoots to produce metabolites in bioreactors. Precursor feeding of Trp boosted the synthesis of polyphenols .0.5 mM Trp exhibited maximum antioxidant activity in the microshoots

2.6.2.6. Medium Strength (MS)

Fadel *et al.* (2010) studied *in vitro* organogenesis of *Mentha spicata* with different strengths of MS media and the extracts from shoots and roots cultivated on quarter- and half-strength medium, had the highest average phenolic content.

The effect of different media strengths on *Ruscus hypoglossum in vitro* cultures was worked by Abou-Dahab *et al.* (2005), and the following results were obtained. The highest concentrations of chlorophyll-*a* (127.2 and 126.7 mg 100 g^{-1} FW, respectively) were obtained using MS salts at 1/2 and 1/4 strength, whereas the lowest concentration (110.8 mg 100 g^{-1} FW) was obtained using full-strength MS. From the results it was evident that the MS salt strength had no discernible impact on the chlorophyll-*b* levels. The maximum concentrations of carotenoids (42.65 and 42.11 mg 100 g^{-1} FW) were obtained when MS at 1/2 and 1/4 strength was used, whereas the lowest concentration (26.13 mg 100 g^{-1} FW) was observed when full salt strength MS medium was used. The maximum indole concentration (6.293 mg 100g^{-1} FW) was obtained by employing MS salts at 1/4 strength, whereas the least amount (2.687 and 2.101 mg 100 g^{-1} FW) was obtained using MS salts at full and 1/2 strength.

2.6.2.7. Jasmonic Acid (JA)

Jasmonates (JAs), including jasmonic acid are lipid-derived substances which influence plant growth, development, and stress responses. JAs were utilized to elicit and stimulate secondary metabolite synthesis in various *in vitro* culture systems (Kamińska, 2021).

Gadzovska *et al.* (2007) used JA elicitation on *Hypericum perforatum* suspension cultures, and cells showed an increase in the production of phenolic substances, flavonols, and flavonols. On the other hand, the production of Anthocyanins were found to be decreased. JA elicitation increased the production of hypericin and pseudohypericin in *H. perforatum*. In JA-induced cells, PAL and CHI activity increased 6-8-fold, demonstrating a substantial activation of the phenylpropanoid pathway.

The effects of JA in the rosmarinic acid (RA) production on the suspension cultures of *Mentha* x *piperita* were studied by Krzyzanowska *et al.* (2012), and JA at a concentration of 200µM was most effective in the production of RA. Miclea *et al.* (2020) studied the effects of JA in the *in vitro* cultures of *Lavandula angustifolia*. Explants cultured in media supplemented with 0.5 mg l^{-1} JA total phenolics, Chl *b*, carotenoid, lutein, β-carotene, *cis*-β-carotene, and violaxanthin levels were found to be high.

Micropropagation of *Hypericum avicullariifolium* and *H. pruinatum* from Turkey and the effects of JA elicitation on the secondary metabolites production were studied by Cirak *et al.* (2020). The production of various secondary metabolites was found to be increased in plants of both species as a result of the administration of JA. The most significant impact of JA elicitation was reported for chlorogenic and neochlorogenic acids.

Anusha *et al.* (2016) conducted elicitation studies using JA in cell suspension cultures of *Celastrus paniculatus*. They found that 250 µM JA produced the maximum total phenolics content after 24 hours, which was higher than the control. However, at 50 µM JA, the accumulation of total phenolic content was higher.

The effects of JA on *in vitro* cultures of *Catharanthus roseus* under salinity stress were studied. Application of JA enhanced the concentration of nutrients such as N, P, K, and Ca in salinity-stressed plants. The osmo-metabolic and antioxidant compounds studied in this experiment were dramatically enhanced due to water salinity and JA. The exogenous supply of JA to the plants increased the production of soluble carbohydrates, proline and total phenols, and increased the activity of APX, PPX, and PPO (Al-Huqail *et al.,* 2021).

Zaragoza-Martınez *et al.* (2016) conducted a work on the *Jatropha curcas* suspension cultures to study the effects of JA elicitation, and 200 and 400 µM JA were applied to the culture. As a result of JA treatment, maximum accumulation of triterpenes, betulin, betulinic acid, and lupeol was observed. H_2O_2 and MDA levels and APX, CAT, and POD activities were increased; catalase showed high activity against APX and CAT activities.

Walker *et al.* (2002) reported that 250 µM JA generated an increase in hypericin accumulation in cultured cells maintained under dark (0.318±0.02 mg g^{-1} DW basis). According to Ali *et al.* (2016), the production of total phenolics and flavonoids were increased in the cell suspension cultures of *Artemisia absinthium,* and the highest radical scavenging activity was also noticed due to JA elicitation.

Kamińska *et al.* (2018) investigated the physiological impacts of JA treatments on *Taraxacum pieninicum* during shoot micropropagation and cold preservation at 4 °C in low light or darkness. JA (24-72 M) reduced the impact of cold stress during cold storage, which was seen in a decreased accumulation of proline and TBARS (Thiobarbituric acid reactive substances).

The effects of JA on the *Solanum tuberosum in vitro* growth were studied by Zhang *et al.* (2006). When leaves were treated with 0.2 mg dm-3 JA, acid peroxidase activity increased; however, when leaves were treated with more than 2 mg dm-3 JA, it decreased.

A work was conducted by Chavan *et al.* (2021) on *Salacia chinensis* to study the effects of elicitors. The total phenolics, flavonoids, and mangiferin contents of the callus grown on MS medium enriched with 2, 4-D, BAP, and JA were found to be increased. Additionally, the same treatment induced the calli's antioxidant characteristics.

2.6.2.8. Salicylic Acid (SA)

Zare-Hassani *et al.* (2019) worked on SA elicitation studies on *Ziziphora persica*. They observed that in 50 µM SA treatment, six volatile compounds were identified, with alkane hydrocarbons showing the highest concentration of these compounds and the α-Tetradecene compound accounting for the highest percentage of the total number of compounds. Nine compounds were found for 100 µM of SA treatment, with alkane hydrocarbons showing an enormous quantity and containing durene monoterpene and ethyl palmitate (a fatty acid). The most significant percentage of chemicals in this treatment belongs to Tridecane.

Reports from the SA-induced studies on the callus cultures of *Fagonia indica* by Khan *et al.* (2019) showed that the total phenolics and flavonoid contents were enhanced than the control culture due to SA induction. Chaichana and Dheeranupattana (2012) reported that in the *in vitro* cultures of *Stemona* species salicylic acid treatment increased the production of 1′, 2′-didehydrostemo-foline and stemofoline. According to Açikgöz *et al.* (2019), cell suspension culture of *Achillea gypsicola* treated with 10 and 50 µM SA resulted in a reduction in total flavonol content when compared to the initial culture; meanwhile, treatment with 100 µM SA enhanced total flavonol content to a level more significant than that of the control culture. When juxtaposed with the original culture, the total anthocyanin content increased considerably in the SA-treated suspensions, with a 33.9% hike observed with 50 M SA treatment.

Exogenous SA was applied to the adventitious root cultures of *Aloe vera*. The SA treatment significantly boosted the production of aloe-emodin and chrysophanol. A total of 37 SA-induced compounds were found using ultra-performance liquid chromatography-electrospray ionization mass spectrometry (Lee *et al.*, 2013). SA-mediated elicitation in the cell suspension cultures of *Taxus chinensis* var. *mairei* was conducted by Wang *et al.* (2004), and they reported that 72.5 – 290 µM concentrations of SA enhanced taxol production than controls. The maximum taxol production was recorded at 145 µM SA, and SA effectively increased the production of cephalomannine.

Jatropha curcas cell cultures were treated with 200, 300, 400, and 500 µM SA in MS medium. The addition of salicylic acid modified the chemical composition of all treatments, raised the percentage of chemicals present, and

produced more alkanes and fatty acids (Mahalakshmi *et al.,* 2013). SA effectively enhanced swertiamarin and mangiferin in the *in vitro* shoot cultures of *Swertia paniculata* (Kaur *et al.,* 2020).

Mendoza *et al.* (2018) reported that 300 µM SA effectively enhanced the antioxidant activity flavonoid content and phenolic compound content, in the *in vitro* cultures of *Thevetia peruviana*. A report from work conducted on callus cultures of *Rumex vesicarius* by Sayed *et al.* (2017) showed that SA was the most efficient elicitor in the presence of fructose, dramatically increasing the accumulation of flavonoids and scoring the highest scavenging activity and dramatically increased phenylalanine ammonia-lyase activity. Also, according to HPLC analysis, orientin, isoorientin, vitexin, and isovitexin could not be found in the control sample. However, they were found in the SA-elicited callus.

The highest hyoscyamine and scopolamine accumulation was observed in the root cultures of *Datura metel* at 500 µM SA; they were 3.5- and 4-fold higher than the control culture. Moreover, the SA addition was highly favourable for the accumulation of tropane alkaloids in root culture (Ajungla *et al.,* 2009). Miclea *et al.* (2020) studied the effects of SA on *in vitro* cultures of *Lavandula angustifolia*. They observed that Chl *b*'s content was higher in explants treated with SA, and shoots raised on a medium containing 5 mg l^{-1} SA had the highest neoxanthin content.

In vitro cultures of *Rauvolfia serpentine* were elicited with SA, and the production of reserpine and ajmalicine was enhanced due to the elicitation. Also, SOD, CAT, and DPPH activities were relatively higher in SA-treated samples (Dey *et al.,* 2020). The effects of SA on different explants (cells,

calli, and shoots) of *Hypericum perforatum* were studied by Gadzovska *et al.* (2013). In comparison, to control cell suspension cultures, the amounts of both hypericin and pseudohypericin doubled in the presence of SA, regardless of its concentration. In the presence of SA, flavonols increased by 2 to 5 times after 7-21 days, while flavanols increased in 1-4 days.

100 mgl^{-1} of SA was sprayed on fruits of *Vitis vinifera,* and the overall phenol content and free radical scavenging activity were elevated. The total phenol content increased, whereas the IC50 values reduced from 45.2 to 13.2 mg ml^{-1}. The content of various phenolics enhanced significantly with 100 mg l^{-1} salicylic acid, except for anthocyanins (Blanch *et al.,* 2020). Nair *et al.* (2013) studied the effects of salicylic acid, methyl salicylate, and acetylsalicylic acid on *Rauvolfia serpentina* by spraying it on whole plants. The content of rutin, caftaric, cichoric, caffeic acid, and chlorogenic acids, was significantly enhanced by using salicylic acid and its derivatives in both the shoots and roots of *R. serpentine*.

Jirakiattikul *et al.* (2021) worked on SA-mediated elicitation studies, and SA effectively increased secondary metabolite synthesis in *Musa acuminata*. The highest concentrations of total phenolics, total flavonoids, and total saponins were found in shoots treated with 100 µM SA.

Teixeira *et al.* (2019) conducted a study to assess the influence of 2-iP, 2,4-D, and Salicylic acid on the synthesis of total phenolics, flavonoids, fukugetin and 7-epiclusianone in *Garcinia brasiliensis* zygotic embryo callus. The maximum production of total phenolic compounds was observed in callus subcultures treated with 100 µM SA, and irrespective of the concentration, the

interaction between the elicitor SA and the 2,4-D and 2-iP reduced flavonoid synthesis.

The root cultures of *Stemona curtisii* were induced with SA, and *the* stimulation of the roots with 500 mgl^{-1} salicylic acid resulted in the maximum synthesis of oxyprotostemonine, stemocurtisine, and stemocurtisinol (Chotikadachanarong *et al.*, 2011). Varying concentrations of SA (75 μM, 150 μM, 300 μM) were applied to the shoot cultures of *Ajuga integrifolia*, and as a result, the rosmaric acid and caffeic acid levels were increased. The SA (150 μM) treatment significantly improved antioxidant and anti-inflammatory activity *in vitro* (Haider-Abbasi *et al.*, 2020).

2.6.2.9. Chitosan

Shah *et al.* (2021) reported that applying all chitosan treatments augmented the production of total flavonoids, phenolics, and silymarin in *in vitro* cultures of *Silybum marianum*. Among the chitosan concentrations, 5.0 mg l^{-1} chitosan showed a slight increase in the total flavonoid content to others. The extracts from the chitosan treatments showed improved antioxidant and anti-inflammatory properties.

Qiu *et al.* (2021) reported that saponin production in the hairy root cultures of Psammosilene tunicoides was augmented by chitosan by about 4.55-fold than the control. The result from the HPLC showed a remarkable increase in the production of gallic acid, gypsogenin, and gypsogenin- 3-*O*-*β*-D-glucuronopyranoside concerning the chitosan treatments. Moreover, ROS and nitric oxide (NO) activity was also activated in the cultures. Putalun *et al.* (2007) reported that the application of chitosan elevated the production of

artemisinin after six days in hairy root cultures of *Artemisia annua*, which was six-fold higher than the control cultures.

Lertphadungkit *et al.* (2020) conducted elicitation studies on *Trichosanthes cucumerina,* and the cell suspension cultures were treated with 1 mg ml^{-1} chitosan for eight days and had greater bryonolic acid concentration (23.56±1.68 mg g^{-1} dry weight). Results from the studies of Fooladi-Vanda *et al.* (2019) found that different concentrations of exogenous chitosan significantly affect the secondary metabolite production in shoot cultures of *Melissa officinalis*. The highest PAL and guaiacol peroxidase (GPX) activities were observed at 50 mgl-1 chitosan. The GPX activity rate was four-fold higher than the control, and the maximum catalase activity occurred at 100 mg l^{-1} chitosan. At all the chitosan treatments, phenolic compounds and rosmarinic acid contents were increased than the control culture (both were higher at 100 mg l^{-1} chitosan concentration).

Results from the studies of foliar application of chitosan on *Curcuma longa* found that the curcumin production in the rhizomes was increased by 56%, and the total production was augmented (Sathiyabama *et al.,* 2016). Ferri *et al.* (2009) viewed induction with 50 µg ml^{-1} chitosan in *Vitis vinifera* cell suspensions down stimulated the accumulation of *trans*-resveratrol and some flavonoids and hydroxycinnamic acids.

Chitosan elicitation studies by Brasili *et al.* (2014) reported an increase of isoleucine, valine, glutamine, GABA, and alanine and a reduction of histidine in *in vitro* root cultures of *Hypericum perforatum*. An increase of dimethylallyl-pyrophosphate (DMAPP) was noticed in chitosan-elicited cultures, and DMAPP is the substrate for the synthesis of isoprenylated

xanthones. The effects of chitosan elicitation include (a) stimulation of the Hexose Mono Phosphate shunt pathway; as a result, there was a large production of erythrose-4-phosphate, an intermediary of the shikimate pathway and hence of phenylpropanoid synthesis, (b) decreased utilization for energy production, and protein synthesis, carbohydrates and amino acids (mostly glutamine and GABA) were stored; this was associated with a slowing or halting of growth, (c) change in the use of acetyl-CoA from mitochondrion energy production to cytoplasmic lipid synthesis, such as PUFA and sterols.

Kasem (2018) discovered that the cell suspension cultures of *Impatiens balsamina,* when induced with 50 mg l^{-1} chitosan, elevated the total flavonoid contents. Skorupinska-Tudek *et al.* (2007) reported that due to chitosan induction, the higher polyprenol contents were slightly augmented in *Taxus baccata*. Sivanandhan *et al.* (2012) studied the effect of chitosan application in the *in vitro* adventitious root cultures of *Withania somnifera* and found that the application increased the production of several metabolites. Udomsuk *et al.* (2011) reported that chitosan at 150 mg l^{-1} increased isoflavonoid synthesis in *Pueraria candolllei* hairy root cultures.

Elicitation of *Hypericum perforatum* subsp. *angustifolium* cell cultures with 200 mg l^{-1} chitosan increased xanthone production while the flavonoid contents were decreased. The production of paxanthone, cadensin G, 1,3,6,7- and 1,3,5,6-tetrahydroxyxanthone was also noticed due to elicitation by Tocci *et al.* (2010). Cell suspension cultures of *Phyllanthus debilis* when treated with different concentrations of chitosan, at 150 mg l^{-1} chitosan, hydrolyzable tannins' content was accumulated compared to the control and other

concentrations of chitosan. Also, a reduction of hydrolyzable tannin production was noticed when the elicitor concentration was increased (200 mg l^{-1}) (Malayaman *et al.*, 2017).

Gai *et al.* (2019) reported that in the hairy root cultures of *Astragalus membranaceus* elicited by chitosan, 100 and 150 mg l^{-1} showed a significant augmentation in the levels of the isoflavones, formononetin, and calycosin. The 100 mg l^{-1} chitosan was the highly effective concentration of the elicitor, and when compared with the control, both compounds have 12.45- and 6.17-fold enhancement. H_2O_2 content and CAT activity were also increased concerning the elicitation. Pliankong *et al.*, (2018) applied chitosan (50, 100, 250 and 500 mg l^{-1}) on cell suspension cultures of *Catharanthus roseus*. Vinblastine and vincristine accumulation were increased by chitosan treatment and 100 mg l^{-1} was the most efficacious concentration.

The elicitation with chitosan on *Morinda citrifolia* adventitious root cultures and phytocompounds production was studied by Baque *et al.* (2012b). The time exposure of elicitation increased the enhancement of anthraquinones (AQ), phenolics, and flavonoids in the system. Lei *et al.* (2011) reported that foliar application of 100 mgl-1 chitosan increased the dihydroartemisinic acid and artemisinin content by 72% and 53% compared to the control. Enhancement of phenolic compound production by 8-fold was observed in callus cultures of *Pseudarthria viscida* by using 1.5 mg l^{-1} chitosan as elicitor (Sangeetha *et al.*,2016).

Brasili *et al.* (2016), found that chitosan application increased the production of phenolic compounds and brasilixanthone B (one of the xanthones which were first reported in the chitosan induced roots) in the

liquid root cultures of *Hypericum perforatum*, The withaferin-A and withanolide production was enhanced after the chitosan treatment in the hairy root cultures of *Withania somnifera* (Shajahan, 2013).

Kaur *et al.* (2020) conducted chitosan induction studies in the *in vitro* cultures of *Swertia paniculate* to produce mangiferin, swertiamarin and amarogentin. The results showed that chitosan was very effective in the maximum accumulation of mangiferin and swertiamarin and less effective in producing amarogentin. Shah *et al.* (2021) worked on chitosan elicitation studies on cell suspension cultures of *Silybum marianum*. The results from the HPLC analysis showed that chitosan treatment increased the accumulation of silymarin, phenolic compounds and flavonoids and elevated levels of silybin A, silybin B, and silydianin. The related extracts demonstrated intriguing antioxidant and anti-inflammatory properties. Significant ABTS antioxidant activity was found in extracts from cultures obtained from medium containing 0.5 mg l^{-1} chitosan.

The cell suspension cultures of *Andrographis paniculata* were treated with different concentrations of chitosan (5 mg, 10 mg, and 20 mg per 50 ml). The chitosan treatment (20 mg for 48 hours) elicited the most andrographolide (119.0 g g^{-1}, 59.5-fold) corresponding to the relevant control and other treatments (Vakil & Medhulkar, 2013).

Chapter 3

MATERIALS AND METHODS

3.1. Plant Selected for the Study

Ayapana triplinervis (Vahl) R.M.King & H.Rob. (**Plate 1**), an important medicinal plant, was chosen for the present study. The twigs of the plant were collected from Kumily, Idukki (Dist), Kerala, and they were planted and maintained well in the Botanical garden of St. Joseph's College (Autonomous), Devagiri, Kozhikode.

A. triplinervis is an ascending, slender perennial shrub belonging to the family Asteraceae. Stem: stem cylindrical, Leaf: phyllotaxy opposite; blade oblong/lanceolate, 3-nerved; margin entire; indumentum glabrate; petiole subsessile. Inflorescence: phyllary, sub imbricate/lanceolate; involucre campanulate; type paniculiform/lax. Flower: anther rounded; appendix ovate; necklace oblong; colour white: style filiform; stylopodium present/glabrous; type infundibular. Fruit: carpopodium conspicuous; indumentum present; pappus bristle scabridous; type obpyramidal (Gamble, 1921).

3.2. *In vitro* culture Studies

3.2.1. Plant material selected for the study

Various plant parts of *A. triplinervis,* such as nodes, leaves, and internodes, were excised and used as the explants for the present *in vitro* culture studies.

3.3. Glasswares

The glass wares of the brand Borosil were used for the *in vitro* culture studies and other experiments in the present study.

3.4. Preparation of MS medium stock solution

Table 1: Constituents of MS medium

Constituents	Amount required for 1 L medium
Macrosalts	NH_4NO_3 – 16.5 g KNO_3 – 19 g $CaCl_2$ – 3.7 g KH_2PO_4 – 1.7 g
Microsalts	$MnSO_4.4H_2O$ – 2.230 g $ZnSO_4.4H_2O$ – 0.860 g H_3BO_3 – 0.620 g KI – 0.083 g $Na_2MoO_4.2H_2O$ – 0.025 g $CuSO_4.5H_2O$ – 0.0025 $CoCl_2.6H_2O$ – 0.0025
Iron	Na_2EDTA – 0.3725 g $FeSO_4.7H_2O$ – 0.2785 g
Vitamins	Glycine – 0.04 g Niacin – 0.01 g Pyridoxine HCl – 0.01 g Thiamine HCl – 0.002 g Meso – inositol – 2 g
Sucrose	30 g
Agar	8 g

Murashige and Skoog's (MS) medium (1962) was selected for the culture studies. It is known as an excellent basal medium for culture initiation. Macronutrients, micronutrients, vitamins, iron chelators, 3% sucrose, and 0.8% Agar **(Table 1) a**re the components of the medium. Each chemical was

Plate 1: *Ayapana triplinervis*; A) Habit, B) Single twig of the plant

carefully weighed and dissolved separately during the stock solution preparation. Later, all of them required to make a stock were mixed and made up to the volume. The stock solutions were carefully stored inside the refrigerator in suitable containers.

3.5. Preparation of the culture medium

Appropriate quantities of macronutrients, micronutrients, vitamins, and iron stock solutions were measured using measuring cylinders and pipettes and poured into a 1 L beaker. 3% sucrose was weighed and added to this, and then the medium was made up to 1000 ml with distilled water. Before the pH adjustment, the plant growth regulators or the stress factors were added to the media. Prior to adding Agar, the pH of the medium was adjusted to 5.7-5.8 using 0.1 N NaOH or 0.1N HCl. Media were boiled after adding agar, then poured into cleaned culture tubes or glass bottles of about 10-15 ml each; caps or cotton plugs were used to close the culture vessels, and they were autoclaved at 121ºC temperature and 15 lbs pressure for 20 min, and after the autoclaving, they were kept in the culture room until inoculation.

3.5.1. Preparation of Plant Growth Regulators (PGRs)

For direct and indirect protocol development studies PGRs such as NAA, IBA, BAP, Kinetin, and TDZ were used.

3.5.1.1. Preparation of stock solutions for Auxin PGRs

0.1 mg ml^{-1} of NAA/IBA containing stock solution was prepared by weighing 25 mg of NAA/IBA and placed into a 500 ml beaker. Several drops of 1M NaOH or KOH were added with a dropper with constant stirring until the powder was dissolved. 250 ml of distilled water was quickly added to it

and mixed properly. The prepared stock solutions were transferred to containers, sealed tightly, labelled properly, and placed inside the refrigerator.

3.5.1.2. Preparation of stock solutions for Cytokinin PGRs

0.1 mg ml^{-1} of BAP/Kinetin/TDZ containing stock solution was prepared by weighing 25 mg of NAA/IBA and placed into a 500 ml beaker. Several drops of 1M HCl (for BAP/Kinetin) and 1M NaOH or KOH (for TDZ) were added with a dropper with constant stirring until the powder was dissolved. 250 ml of distilled water was quickly added to it and mixed properly. The prepared stock solutions were transferred to containers, sealed tightly, labelled properly, and placed inside the refrigerator.

3.5.2. Preparation of Sterilant

$HgCl_2$ was used as the sterilant for the explant's surface sterilization procedure. 0.1 % stock solution was prepared by dissolving 0.5 g $HgCl_2$ in 500 ml distilled water. The stock solution was carefully dispensed to a container and stored inside the Laminar Air Flow chamber.

3.6. Preparation of the Explants – Direct organogenesis

The nodal segments collected from the mother plant were used as explants. The excised explants were thoroughly washed in running tap water for twenty minutes to remove the surface dirt. Then, explants were soaked in liquid detergent (Extran) for ten minutes, shaken with the solution, and rinsed multiple times with sterile distilled water. Later, the explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution for about 8-9 min and rinsed with sterile distilled water three to five times to remove all traces of the sterilant.

3.7. Inoculation to the Medium

A pair of forceps, a scalpel, 8-10 Petri plates, one or two bottles of distilled water, and other glassware were autoclaved and kept inside the Laminar Air Flow (LAF) chamber to perform the inoculation procedure. Before inoculation, the sterile media were brought from the culture room to the LAF chamber in the inoculation room. The stock of ethyl alcohol, cotton, surgical blades, scissors, spirit lamp, and Paraffin film were checked and restocked inside the chamber. UV light was switched on for 20 minutes, and after switching off, the UV light blower was switched on. After 15 minutes, the inoculation procedure was started.

Table 2: Concentrations and combinations of PGRs used for direct organogenesis.

Plant growth regulators (PGRs)			
Kinetin (mg l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	IBA (mg l^{-1})
0.5	-	-	-
-	0.5	-	-
-	0.5	0.5	-
-	1.0	1.0	-
-	1.0	0.5	-
-	1.5	2.0	
0.1	1.0	-	-
1.0	0.1	-	-
0.5	1.0	-	-
1.0	0.5	-	-
0.5	-	0.5	-
1.0	-	0.5	-
-	1.0	-	0.5
-	-	-	0.5
-	-	-	1.0

Hands were finely sanitized with ethyl alcohol, and the floor of the LAF chamber was wiped with cotton soaked with ethyl alcohol. Following

the lighting of the spirit lamp and insertion of the surgical blade into the scalpel, inoculation was started. The explants were washed with sterile water multiple times. Then, the explants were finely trimmed and inoculated to the medium, and the culture vessels were sealed with the Paraffin tape. The culture vessels were then transferred to the culture room under the conditions of 25± 2ºC and relative humidity of 60-70 percent under 16/8 hours light/dark of photoperiod with approximately 1000-2000 lux provided by cool white fluorescent tubes. Cultures were well maintained, and contamination was checked frequently.

3.8. Preparation of Explants – Indirect organogenesis

Table 3: Concentrations and combinations of PGRs used for indirect organogenesis.

Plant Growth Regulators (PGRs)			
Kinetin (mg l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	TDZ (mg l^{-1})
0.5	-	-	-
-	0.5	0.5	-
-	1.0	1.0	-
-	-	-	1.0
-	-	-	1.5
-	-	-	2.0
-	-	0.5	1.0
-	-	0.5	1.5
-	-	0.5	2.0

The leaf and internodal explants of *A. triplinervis* were collected from the *in vitro* grown plants. Explants were selected from the contamination-free plants; because of this mercuric chloride treatment procedure was not used.

They were dipped in 70% ethyl alcohol and quickly removed. Then, they were finely washed with sterile water for multiple times.

3.9. Preparation of 70% Ethyl alcohol solution

70 ml of absolute EtOH was measured and poured into a 100 ml beaker and 30 ml of distilled water was added to the beaker and made up to the volume.

3.10. Hardening of *in vitro* grown plantlets

Soil from the field was taken and kept in an autoclavable polypropylene bag and then the soil was autoclaved and distributed to plastic cups. The plantlets were taken out from the culture medium and the roots were thoroughly washed in running tap water to remove the remnants of agar. Subsequently, the plants were fixed to the soil in plastic cups, watered and bagged with polythene bags. After 2 weeks the plantlets were transferred to the pots containing potting mixture placed inside the greenhouse. The plants were watered frequently.

3.11. Genetic Fidelity Analysis – ISSR (Inter Simple Sequence Repeat) Profiling

The analytical study was performed at CMPR (Centre for Medicinal Plant Research) Arya Vaidyasala, Kottakkal, Malappuram, Kerala.

3.11.1. Plant Material for genetic fidelity analysis

Fresh leaves of tissue culture (indirect organogenesis) raised and the mother plants of *A. triplinervis* (from 5 plants each) were collected for genetic fidelity analysis.

3.11.2. Genomic DNA extraction

The genomic DNA extraction was done by the column-based kit method using the DNeasy plant mini kit (Qiagen, Germany). 100 mg of the leaf tissue/tissue cultured leaf was ground to a fine powder using liquid nitrogen in microfuge tubes with the help of micropestle. 400 µl AP1 buffer (lysis buffer) and 4 µl RNase A were added and mixed by vortex and micropestle. The tubes were incubated at 65°C for 10 min in a water bath with intermittent mixing 2-3 times by inverting the tubes. Then 130 µl buffer P3 (neutralization buffer) was added to the tube, mixed and incubated for 5 min on ice. The lysate was centrifuged for 5 min at 14,000 rpm. The samples were then loaded onto the QIA shredder spin columns and centrifuged at 14,000 rpm for 2 min. The flow-through was transferred to a new tube without disturbing the pellet. After measuring the flow-through its 1.5^{th} volume of AW1 buffer was added (wash buffer 1) and mixed by pipetting. The contents were then loaded in 650 µl fractions onto the DNeasy mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through was discarded. The spin column was placed into a new 2 ml collection tube and 500 µl buffer AW2 (wash buffer 2) was added, followed by centrifugation for 1 min at 8000 rpm. This last step with buffer AW2 step was repeated, and centrifuged at 14,000 rpm for 2 min. The spin columns were placed in fresh microfuge tubes and 100 µl AE buffer (Tris-EDTA buffer or DNA elution buffer) was added onto the membranes and incubated at room temperature for 5 min. The tubes were then centrifuged at 8000 rpm for 1 min. This step was repeated with another 50 µl of AE buffer. The eluted samples were stored at -20^{o}C.

3.11.3. Quantification of DNA & Quality check

The quality of the DNA was checked in 0.8 % agarose gel by visualizing the intactness of DNA bands and the absence of any contaminating protein or RNA using the gel-documentation system MultiImage II Alpha Imager-HP (Alpha Innotech, USA). DNA was quantified by the spectrophotometric method using the Biophotometer plus (Eppendorf, Germany) **(Table 4)**. The pre-set program of the Biophotometer was used for DNA quantification.

The blank was set using an AE buffer. 50µl of DNA sample was taken in a cuvette and the OD was measured automatically at 260nm, 280nm and 340nm. The amount of DNA present in 1µl of the sample was calculated using the formula:

If OD at 260nm= A260

If OD at 280nm= A280

DNA concentration (ng/µl) = 50 × (A260-A340)

DNA purity (A260/A280) = (A260 reading – A340 reading) ÷ (A280 reading – A340 reading)

Table 4: Estimated DNA concentration of *Ayapana triplinervis* samples using UV-spectrophotometer method (BioPhotometer plus, Eppendorf, Germany).

S.No.	Sample Name	A260	A280	A340	A260/A280	Concentration (ng μl^{-1})
1	Mother plant	0.634	0.365	0.03	1.80	30.2
2	TC1	0.106	0.067	0.019	1.81	4.4
3	TC2	0.122	0.074	0.016	1.83	5.3
4	TC3	0.137	0.083	0.016	1.81	6.1
5	TC4	0.239	0.132	0.009	1.87	11.5
6	TC5	0.156	0.091	0.008	1.78	7.4

3.11.4. Inter simple sequence repeat – Polymerase chain reaction (ISSR-PCR)

PCR amplification was performed in a 20 µl reaction mixture consisting of 15 ng genomic DNA, 2 µl of 10x buffer, $MgCl_2$ (2.5 mM), dNTP (200µM), ISSR primer (0.5µM) and Taq polymerase (1U). The annealing temperature of 10 ISSR primers **(Table 5)** was tested individually using the DNA of the mother plant at temperatures ranging from 46-60°C. Further PCR amplification was done for all 6 DNA samples using optimum annealing temperature. DNA amplification was performed in a Veriti^{TM} (ThermoFisher Scientific, USA) thermal cycler. The cycling program was (i) 1 cycle of 95° C for 2 min; (ii) 35 cycles of 95° C for 30 seconds for denaturation, (46-60° C) for 1 min 10 seconds for annealing of primer, and 72°C for 2 min for extension; and (iii) a final extension at 72°C for 8 min. The PCR products were stored at 4°C till electrophoresis.

3.11.5. Agarose Gel Electrophoresis

1.8 % agarose gel was prepared by adding 2.34 g of agarose in 130 ml 1X TAE (Tris-acetate-EDTA) buffer. The agarose was dispersed in a buffer and heated near to boiling point in a microwave oven for several minutes. The melted agarose was cooled sufficiently and 6.5 µl of ethidium bromide (10mg/ml) was added to the gel before the gel sets. After mixing the gel, it was poured immediately onto a sealed gel casting tray. A comb was placed in the slot provided in the casting tray to create wells for loading the samples. It was made sure that no air bubbles were trapped. A 0.8 % agarose gel was also cast in the same manner as above, for running the DNA samples.

Table 5: List of ISSR Primer, Sequences, Tm (°C) and optimum annealing temperature

Primer	Sequence (5´-3´)	Tm (°C)	Optimum Annealing temperature (°C)
UBC 807	AGAGAGAGAGAGAGAGT(17)	50.4	52
UBC 808	AGAGAGAGAGAGAGAGC(17)	52.8	-
UBC 810	GAGAGAGAGAGAGAGAT(17)	50.4	48
UBC 825	ACACACACACACACACT(17)	50.4	54
UBC 840	GAGAGAGAGAGAGAGACTT(19)	54.5	-
UBC 841	GAGAGAGAGAGAGAGAYC(18)	54.8	-
UBC 842	GAGAGAGAGAGAGAGAYG(18)	54.8	-
UBC 847	CACACACACACACACARC(18)	54.8	56
UBC 851	GTGTGTGTGTGTGTGTYG(18)	54.8	-
UBC 857	ACACACACACACACACYG(18)	54.8	56

Once the gel was set, the comb was removed, leaving wells where the samples could be loaded. The gel was placed in the electrophoresis unit (Wide Mini-Subcell GT, Bio-rad, USA). Electrophoresis buffer (1X TAE) was added into the electrophoresis tank, whereby the slab gel was completely submerged in the buffer during electrophoresis. The reaction products were mixed with 5 µl of 6x gel-loading dye. The Samples were then loaded in the wells with a micropipette. Care was taken to prevent the mixing of the sample between wells. A standard 100bp-3kb DNA ladder was also loaded in one of the wells. The lid of the electrophoresis unit was carefully closed. The black lead was connected to the negative terminal, and the red lead was connected to the positive terminal of the power supply (PowerPac Basic, Bio-rad, USA).

Electrophoresis was carried out at 70 volts. The distance the DNA migrated in the gel was assessed visually by monitoring the migration of the tracking dyes. The power supply was turned off when the bromophenol blue dye migrated a distance estimated to be sufficient for separating the DNA fragments.

3.11.6. Visualization of PCR Products

The gels were documented under UV illumination using the MultiImage II Alpha Imager-HP gel documentation system (Alpha Innotech, USA).

3.12. Stress Study

Eight stress signals in different concentrations were selected for the study **(Table 6)**. They were weighed and applied to the MS media and the nodal segments from the *in vitro* grown basal medium cultures were inoculated to these media.

3.12.1. Preparation of Stock solutions for stress study

3.12.1.1. Salicylic acid

1.3812 g of salicylic acid was dissolved in the 1% solution of $NaHCO_3$ by continuous stirring for 1 hour.

3.12.1.2. Jasmonic acid

1 mg of Jasmonic acid liquid was diluted with a few drops of 5 mM EtOH and then the solution was made up to 100 ml using distilled water. The stock solution was stored in the refrigerator for further usage.

Table 6: Concentrations of stress signals used in the present study

Abiotic Stress	Concentration of each stress used per litre of MS media				
NaCl	10 mM	25 mM	50 mM	100 mM	-
Sucrose	10 g	20 g	40 g	50 g	-
Putrescine	20 mg	40 mg	60 mg	80 mg	100 mg
Tryptophan	20 mg	40 mg	60 mg	80 mg	100 mg
MS Medium strength	1/6	¼	½	3/4	-
Jasmonic Acid	1.25 µM	2.5 µM	5.0 µM	10 µM	15 µM
Salicylic acid (SA)	10 µM	25 µM	50 µM	75 µM	100 µM
NaHCO3 Soln. (SA Control)	1 ml	2.5 ml	5.0 ml	7.5 ml	10 ml
Chitosan	50 mg	100 mg	150 mg	200 mg	250 mg
1% Acetic ā soln. (Chit. control)	10 ml	20 ml	30 ml	40 ml	50 ml

3.12.1.3. Chitosan

1 % Chitosan solution was made by dissolving 0.5 g deacetylated chitosan in 100 ml of 1% Acetic acid solution. After continuous stirring for 2-3 hours, a colloidal solution was obtained. Later, the solution was filtered through a cheese cloth and stored in a glass bottle in the refrigerator.

3.12.1.4. Preparation of Medium with Jasmonic acid through Filter sterilization

Appropriate volumes of macronutrients, micronutrients, vitamins, and iron stock solutions were measured using measuring cylinders and pipettes.

3% sucrose was weighed and added to this, and then the medium was made up to 1000 ml with distilled water. Before the pH adjustment, the plant growth regulators and or stress signals were applied to the media. Then, the pH of the medium was adjusted to 5.7-5.8 by using 0.1 N NaOH or 0.1N HCl before adding Agar. After that, the media were boiled with Agar, and the whole medium was transferred to a 1 litre conical flask, closed with a cotton plug, and autoclaving was done.

The empty bottles, test tubes, and other glass wares required for the medium preparation and media were autoclaved and transferred to a pre-sterilized Laminar airflow (LAF) chamber. Pre-sterilized 0.22 µ sterile syringe filter (Sartorius) and syringes were also transferred to the pre-sterilized Laminar airflow (LAF) chamber. The required volume of Jasmonic acid for the media preparation was measured with a pipette or a measuring cylinder and transferred to a sterile beaker. The Jasmonic acid solution was sucked into the syringe. The needles from the syringes were removed, and then the sterile filter was fixed to the tip of the syringe. The required volume of JA solution was passed through the sterile filter. The sterile filtrate infiltrated through the membrane to the cooled sterilized media. After the whole solution was filtered, they were mixed with the help of a glass rod, and then the media was poured into the sterilized bottles or test tubes; the media were transferred to the culture room for further use.

3.13. Statistical Analysis

One Way ANOVA for significance and Duncan's multiple range test for significance differences of means at $P \leq 0.05$ level were done using IBM SPSS (Ver. 25).

3.14. Graph Preparation

All the graphs used in the present study were prepared with MS Excel.

3.15. Phytochemical Analysis

3.15.1. Preparation of the Extracts

Plant materials were harvested after attaining 60 days of growth. They were washed thoroughly in distilled water to remove the remnants of the agar from the roots. After that, the plants were allowed to dry under shade at room temperature. The dried plant materials were powdered using a mixer and safely stored in suitable containers. 1 g was weighed and transferred from the powdered sample to a round bottom (R.B.) flask. 50 ml of Methanol was poured into the RB flask, and the flask was placed on a heating mantle. Hot extraction with Methanol in the Reflux condenser was conducted for three hours. The extracts were filtered with filter paper and then reduced to 5 ml in a water bath. The final aliquots were kept in the refrigerator in appropriate vials.

3.15.2. HPTLC (High-Performance Thin Layer Chromatography)

The HPTLC studies were carried out at the CMPR (Centre for Medicinal Plants Research), Arya Vaidya Sala, Kottakkal, Kerala, on CAMAG Automatic TLC Sampler 4 (ATS4) with a syringe size of 25µl. Each sample was injected in a volume of 3.0 µl and the 7-methoxy coumarin

(marker – Alfa Aesar) in a 5.0 µl volume. The mobile phase consisted of toluene: ethylacetate: methanol (7:3:1) in a Twin Trough Chamber 20×10cm. The TLC plate was detected with CAMAG TLC Scanner 3 and visualized by CAMAG Visualizer at 254 nm, 366 nm, and 550 nm wavelengths of light. The derivatization was done by spraying with the ANS (anisaldehyde) reagent, and after this, the plate was visualized at 550 nm. All the steps of the HPTLC process and instruments were controlled by the winCATS Planar-Chromatography Manager software (CAMAG, Switzerland).

Chapter 4

RESULTS AND DISCUSSION

4.1. Protocol Development for Direct Organogenesis

The plant tissue culture technique is widely employed for micropropagation of medicinal plants. It offers a reliable means of producing sufficient amounts of drugs and secondary metabolites to meet the growing needs of humanity. This technology makes it possible to supply natural products all year round, regardless of the season, while allowing for precise control of the conditions for optimal plant growth (Sidhu, 2010). In many applications, such as producing virus-free material, genetic modification, and micropropagation, *in vitro* growth of plants is necessary (Georgieva *et al.,* 1996). As per Prakash and Van-Staden (2007), *in vitro* propagation is highly effective for producing true-to-type plants quickly and with minimal space requirements.

Utilizing nodal explants for direct organogenesis is a highly efficient method for plant propagation. Rapid and positive results can be achieved using MS media supplemented with varying concentrations of PGRs. This method is particularly effective due to axillary buds in the explants, which enhances plant propagation. Shoot tips and nodal explants display the most evident *in vitro* morphogenic responses.

In this study, nodal segments of *A. triplinervis* were utilized as explants and cultured on MS media with varying PGR concentrations and combinations. The experiment was evaluated over a 60-day growth period, with regeneration typically beginning 5-6 days after inoculation. Rooting was observed after one week of inoculation, though the timing may differ depending on the PGRs used.

The nodal explants did not exhibit rapid proliferation when cultured in the PGR-free MS basal medium. The number of shoots formed in this medium was less; however, the second-highest shoot length (4.8 cm) was observed (**Table 7**). For further studies, it was crucial to produce multiple shoots. For this reason, several PGRs were utilized during the study.

In the medium supplemented with 0.5 mgl^{-1} of Kinetin, a substantial number of multiple shoots (13.9±7.86) was formed (**Plate 2B**). However, in this medium plant height (4.4 cm) was slightly reduced. The leaves of the plants formed in this medium were small and yellowish-green. The shoots were short, and both pale red and whitish-green coloured stems were present. Several roots were also formed in this medium, possibly due to adequate amounts of indigenous auxins within the plant.

In the 0.5 mgl^{-1} BAP supplemented medium, a smaller number of shoots were formed from the explants. However, the leaves were dark green and large, and the stem was thick. Even though fewer shoots were formed, the plants formed in this medium had long shoots (4.7 cm), the stems were highly branched and green-coloured, and dark green roots were also formed in this medium (**Plate 2C**). This outcome was consistent with the findings of Krishnamurthy *et al.* (2001) in *Polianthes tuberosa* L., where 0.5 mg l^{-1} BAP produced the long shoots.

In the MS medium supplemented with 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA, the plants formed were shorter, with an average length of 3.04 cm, and mostly with single shoots. The leaves had a yellowish hue, but the veins were green (**Plate 2D**). The root system was heavily branched, and some

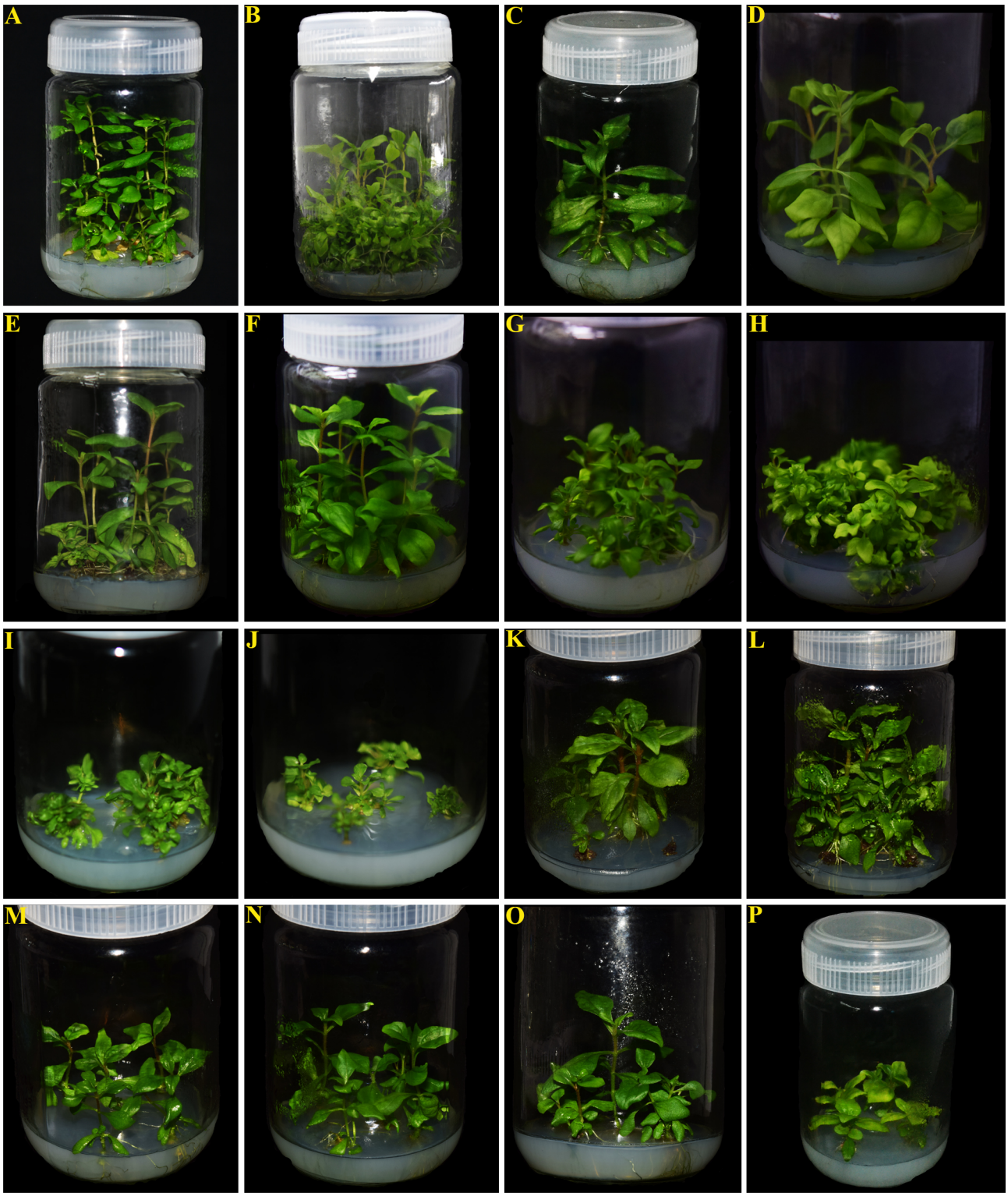

Plate 2: *In vitro* cultures of *A. triplinervis* in MS media containing various concentrations of plant growth regulators; **A)** MS basal, **B)** 0.5 mgl^{-1} Kinetin, **C)** 0.5 mgl^{-1} BAP, **D)** 0.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, **E)** 1.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA, **F)** 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, **G)** 1.0 mgl^{-1} BAP + 0.1 mgl^{-1} Kinetin, **H)** 0.1 mgl^{-1} BAP + 1.0 mgl^{-1} Kinetin, **I)** 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} Kinetin, **J)** 0.5 mgl^{-1} BAP + 1.0 mgl^{-1} Kinetin, **K)** 0.5 mgl^{-1} Kinetin + 0.5 mgl^{-1} NAA, **L)** 1.0 mgl^{-1} Kinetin + 0.5 mgl^{-1} NAA, **M)** 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} IBA, **N)** 0.5 mgl^{-1} IBA, **O)** 1.0 mgl^{-1} IBA, **P)** 1.5 mgl^{-1} BAP + 2.0 mgl^{-1} NAA.

phyllotaxy abnormalities, such as an alternate arrangement of leaves, were observed.

The shoot length was highest (5.08 cm) for the plants in the MS medium supplemented with 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, possibly due to the synergistic effect of cytokinin and auxin in promoting shoot elongation. However, the number of shoots was reduced, and the stems appeared pale red with large green leaves. Further, the medium supported the production of abundant light green roots, and root formation was noticed from the stem above the media surface (**Plate 2E**).

Table 7: Effect of PGRs on shoot multiplication from nodal segments of *A. triplinervis* in MS medium

KIN (mg l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	IBA (mg l^{-1})	No. of Shoot	Shoot length (cm)	No. of Root
-	-	-	-	1.7±0.64c	4.8±1.16a	4±0.7fg
0.5	-	-	-	13.9±7.86b	4.4±1.04ab	10.4±0.5bc
-	0.5	-	-	0.9±0.63c	4.7±2.05a	4.2±0.58fg
-	0.5	0.5	-	0.8±0.46c	3.04±1.66b	8±0.7e
-	1.0	1.0	-	1.1±0.63c	5.08±1.94a	12.2±1.46b
-	1.0	0.5	-	1.7±0.93c	3.5±1.51b	10.6±0.67bc
0.1	1.0	-	-	3.2±2.09c	1.7±0.97c	2.2±0.66gh
1.0	0.1	-	-	18.1±11.85a	0.6±0.35c	1.4±0.24h
0.5	1.0	-	-	3.08±2.18c	2.3±0.71bc	0
1.0	0.5	-	-	2.4±1.32c	1.5±0.58c	2.2±0.37gh
0.5	-	0.5	-	1.9±1.11c	3.2±1.39b	5.4±0.5f
1.0	-	0.5	-	0.9±0.57c	4.2±2.3ab	3.8±0.37fg
-	1.0	-	0.5	1.5±0.57c	2.9±1.05b	9.6±0.67cd
-	-	-	0.5	1.4±0.78c	4.2±1.54ab	10.8±0.86bc
-	-	-	1.0	1.4±0.64c	3.6±1.37b	14.8±1.06a

Data in each column represents mean ± standard deviation. According to Duncan's multiple-range test, significant differences are denoted as $p< 0.05$ (Duncan, 1955).

In the MS medium supplemented with 1.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA, increased number of shoots were formed in the plants in comparison to the

previous medium (**Plate 2F**). The shoots had large green leaves. Many green coloured roots were also formed in this medium. Anand *et al.* (2012) observed the best shoot elongation in *Passiflora foetida* was observed in the medium supplemented with a higher concentration of BAP and a lower concentration of NAA. Similarly, previous studies have documented the synergistic effect of BAP and NAA in promoting shoot and root multiplication rate, root length, and leaf production in *Sphenostylis stenocarpa* (Ogunsola *et al.,* 2016) and *Eclipta alba* (Salma *et al.* 2017). Similar effects were also found in legumes, BAP + NAA, causing shoot regeneration and proliferation (Yadav *et al.,* 2010).

In medium supplemented with 0.1 mgl^{-1} Kin + 1.0 mg l^{-1} BAP, a less number of shoots were formed when compared to the medium supplemented with 0.5 mg l^{-1} Kin. (**Plate 2G**). These plants exhibited stunted growth, with small yellowish-green leaves and deficient root production. Some roots were also formed, they were predominantly cream or white-colored and lacked a highly branched root system.

Conversely, reversing the concentration of the growth regulators used in the previous medium (1.0 mg l^{-1} Kin + 0.1 mg l^{-1} BAP) resulted in the formation of highest number of shoots (18.1±11.85) (**Plate 2H**), but the shoots were very short in length (0.6 cm) with tiny yellowish-colored leaves. Some white-colored roots were also formed in this medium.

In the medium supplemented with 0.5 mg l^{-1} Kin + 1.0 mg l^{-1} BAP, 3.08±2.18 shoots were formed from the explant with a shoot length of 2.3 cm, and closely arranged green leaves. Root formation was not observed in this medium (**Plate 2I**). The shortest plants (1.5 cm) were formed in the MS

medium supplemented with 1.0 mg l^{-1} Kin + 0.5 mg l^{-1} BAP, and the number of shoots (2.4±1.32) was also decreased. The leaves were tiny, with yellowish-green or pale-green coloration. Plant growth decreased, and only tiny shoots were produced (**Plate 2J**). Roots were not formed in this medium.

Less number of shoots (1.9 ±1.11) was formed when the explants were cultured in a nutrient medium supplemented with equal concentrations of Kin and NAA (0.5 mg l^{-1} Kin + 0.5 mg l^{-1} NAA), roots and a small amount of reddish-brown callus was formed from the bottom cut ends of the shoots. The root system was not highly branched. However, the stems were thick and had a reddish hue, while most of the leaves were large with broad lamina and green in color, but some were small (**Plate 2K**). The regeneration percentage of the explants was found to be low.

In the medium supplemented with 1.0 mg l^{-1} Kin + 0.5 mg l^{-1} NAA lowest number of shoots was formed (0.9±0.57). The leaves were primarily large with broad lamina and green in color, and the stems were thick. However, it promoted shoot elongation (4.2 cm); they had white-colored roots, and the formation of the reddish-brown callus was observed (**Plate 2L**).

The explants of *A. triplinervis* grown in a medium containing a combination of BAP and IBA (1.0 mg l^{-1} BAP + 0.5 mg l^{-1} IBA) produced a smaller number of shoots (1.5±0.57). The leaves of the plants were moderately sized and green-colored, with a red hue on the top portion of the shoot (**Plate 2M**). The shoots were 2.9 cm in length, and the secondary shoot was observed as dormant. Highly branched root systems with green roots were present.

To promote *in vitro* root induction, the rooting media was prepared with different concentrations of IBA (0.5 mg l^{-1} IBA and 1.0 mg l^{-1} IBA). When the explants were cultured in rooting media green-colored, highly-branched root system with lots of roots was formed. Plants formed in the medium supplemented with 0.5 mg l^{-1} IBA medium showed better elongation of shoots (4.2 cm), mostly single shoots were formed in both IBA media (**Plate 2N**). But in the 1.0 mg l^{-1} IBA supplemented medium, length of shoots was found to be slightly reduced (3.6 cm) (**Plate 2O**), In both media, the leaves were large and green in the upper portion of the shoots and small in the lower part, and most of the stems were greenish.

The plants formed in the media containing 1.5 mg l^{-1} BAP + 2.0 mg l^{-1} NAA showed yellowish-green leaves with broad lamina (**Plate 2P**). Mostly single shoots were formed from the explants in this medium. The plants were short, and many explants failed to regenerate in this medium. The roots were small, but numerous ones were produced from the basal cut ends of the shoot, and slight callus formation was also noticed.

In the present studies on *A. triplinervis*, the highest number of shoots were formed in the medium supplemented with 1.0 mg l^{-1} Kin + 0.1 mg l^{-1} BAP. The synergistic effect of Kinetin and BAP combination in multiple shoot induction was reported earlier in several plants. Anand *et al.* (2012) and Shekhawat *et al.* (2015) reported that the combined effects of BAP and Kin could increase the shoot multiplication rate in the nodal explants of *Passiflora foetida* through direct organogenesis. Similar reports of the synergistic effect of BAP and Kin was observed in *Vitex agnus-castus* (Balaraju *et al.*, 2008), *Manihot esculenta* (Demeke *et al.*, 2014), *Rosa hybrida* (Kharde *et al.*, 2014),

Plate 3: Acclimatization of *in vitro* cultured *A. triplinervis*; **Stage I)** *In vitro* cultured plants transferred to autoclaved soil, **Stage II)** Stage I plants transferred to the external environment - pot with potting mixture, **Stage III)** Fully grownup plants in the green house.

Vetiveria zizanioides (Sompornpailin & Khunchuay, 2016); *Chlorophytum borivilianum* (Ashraf *et al.* 2014) and *Stevia rebaudiana* (Deshmukh *et al.,* 2017).

In the present studies on *A. triplinervis*, shoot length was highest in the MS medium supplemented with 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA. The synergistic effect of cytokinin and auxin in promoting shoot elongation and axillary bud multiplication was reported earlier in plants like *A. triplinervis* (Martin, 2003); *Arnebia hispidissima* (Shekhawat & Shekhawat, 2011) and *Leptadenia reticulata* (Rathore *et al.,* 2013).

Balaraju *et al.,* (2008) reported that Kin proved to be effective for bud break and multiple shoot formation in *Vitex agnus-castus.*, Hesar *et al.,* (2011) also reported that Kinetin was found to be effective for shoot multiplication in *Matthiola incana*. These reports were corroborative with the results observed in the present studies on *A. triplinervis*. In the present study, Kinetin was found to be the most effective cytokinin in multiple shoot induction in *A. triplinervis.* However, in several reports BAP was found to be highly effective in shoot multiplication in several plants like *Withania somnifera* (Saritha & Naidu, 2007), *Baliospermum montanum* (Sasikumar *et al.,* 2009), *Thymus vulgaris* (Ozudogru *et al.*, 2011), *Indigofera zollingeriana* (Royani *et al.,* 2021) and *Scutellaria alpine* (Grzegorczyk-Karolak *et al.,* 2016).

In the present studies, MS medium supplemented with IBA was highly effective in root induction in the cultures of *A. triplinervis*. This was corroborative with results reported earlier in plants like *Caralluma edulis* (Patel *et al.,* 2014) and *Passiflora foetida* (Shekhawat *et al.,* 2015).

4.2. Hardening

The plantlets of *A. triplinervis* raised *in vitro* were successfully transferred to *ex-vitro* conditions, where they thrived and remained healthy (**Plate 3**). Under optimal care and watering, their shoots and leaves flourished, resulting in larger and greener foliage in field conditions. It should be noted that the plants generated via micropropagation were similar to the mother plants in both morphology and physiology.

4.3. Indirect organogenesis – Protocol Development

The present study was conducted to develop a suitable protocol for indirect organogenesis from the leaves and internodes of *A. triplinervis.* Explants for the study were collected from the sterile stock cultures raised on MS (Murashige and Skoog, 1962) basal medium. Wounds were made along the lamina of the leaf explants using a surgical blade and then the whole leaf or the pieces of the leaves and trimmed internodes were inoculated into the culture media. The leaves were placed both abaxially and adaxially in the media. MS media fortified with various concentrations and combinations of PGRs such as TDZ, NAA, BAP and Kinetin were used during the study. The effects of different PGRs on indirect organogenesis were studied earlier by Nalousi *et al.,* (2019), Scotton *et al.,* (2013) and Ntui *et al.,* (2010).

The MS basal and ½ MS media were not suitable for the indirect shoot organogenesis of *A. triplinervis*. The explants inoculated into the MS basal medium (without PGRs) did not show any growth and some of the explants dried completely. In the ½ MS medium, leaf explants (full leaf) placed abaxially produced highly branched green-coloured roots from the ends of the petiole and little amount of light green callus formation was also observed

(**Plate 4**). Small amounts of light green calli were formed from both the cut ends of the internodal explants.

In the MS medium supplemented with 0.5 mg l^{-1} Kn, the adaxially placed leaf explants (full leaves) responded, and roots (1.4±0.24) were formed from the tip of the petiole and no callus formation was noticed, however, internodal explants did not show any response. The combination of 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA in MS medium induced the production of a large number of roots (16±1.7b) (**Table 8**), but shoots were not formed. Callus formation was also observed in this medium from the lamina and the petiole portion irrespective of the side of the explant touching the medium (adaxial or abaxial). The callus formed was initially pale green coloured, which later turned to dark brown. Later roots were formed from the calli. Callus formation was observed from the internodal explants, but no further development was observed.

In MS medium supplemented with 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} NAA, plants with few shoots (1.8±0.37) and the highest number of roots (28.4±1.36) was formed. Although the number of shoots was less, they were grown into plants with 8 – 10 leaves; some of the leaves showed hyperhydricity. The efficacy of the BAP and NAA combination in morphogenesis was reported earlier in *Nicotiana tabacum* by Gill and Saxena (1993). Das and Rout, (2002) also reported shoot regeneration in a medium supplemented with BAP.

In the present study, TDZ either alone or in combination with other growth regulators produced both shoots and roots from the explants. In the medium supplemented with 1.0 mg l^{-1} TDZ, initially, a light green callus was formed from the leaf explants, especially from the basal portion of the petiole. Then numerous pale green tiny shootlets (20.2±2.53) were formed from the

calli and the colour of the calli later turned to dark brown. Less number (1.2±0.2) of roots was formed in this media and there was no response from the internodal explants. In the 1.5 mg l^{-1} TDZ, supplemented medium, a good

Table 8: Effect of different PGRs on indirect organogenesis from leaf explants of *Ayapana triplinervis*

Kinetin (mg l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	TDZ (mg l^{-1})	Mean no. of Shoots	Mean no. of Roots
0.5	-	-	-	-	1.4±0.24c
-	0.5	0.5	-	-	16±1.7b
-	1.0	1.0	-	1.8±0.37d	28.4±1.36a
-	-	-	1.0	20.2±2.53c	1.2±0.2c
-	-	-	1.5	28.6±2.4c	3.6±0.5c
-	-	-	2.0	49.4±3.57a	1.8±0.37c
-	-	0.5	1.0	42±4ab	2.4±0.24c
-	-	0.5	1.5	32.2±8.54bc	3.2±0.37c
-	-	0.5	2.0	42.2±3.21ab	1.4±0.24c

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range tests.

number of shoots and roots were formed from leaf explants, but internodal explants did not show any response. The highest number of shoots (49.4±3.57) was observed in the 2.0 mg l^{-1} TDZ supplemented medium but, only a lesser number of roots (1.8±0.37) were formed in this medium. The leaf explants were completely turned to green calli in this medium and clumps of small shootlets were produced and many of them showed hyperhydricity.

The efficacy of TDZ in shoot proliferation was reported earlier in sweet cherry (Bhagwat and Lane, 2004), *Malus* (Fasolo *et al.,* 1989) *Pyrus* (Chevreau and Skirvin, 1992), Nemaguard peach (Zhou et al., 2010) and

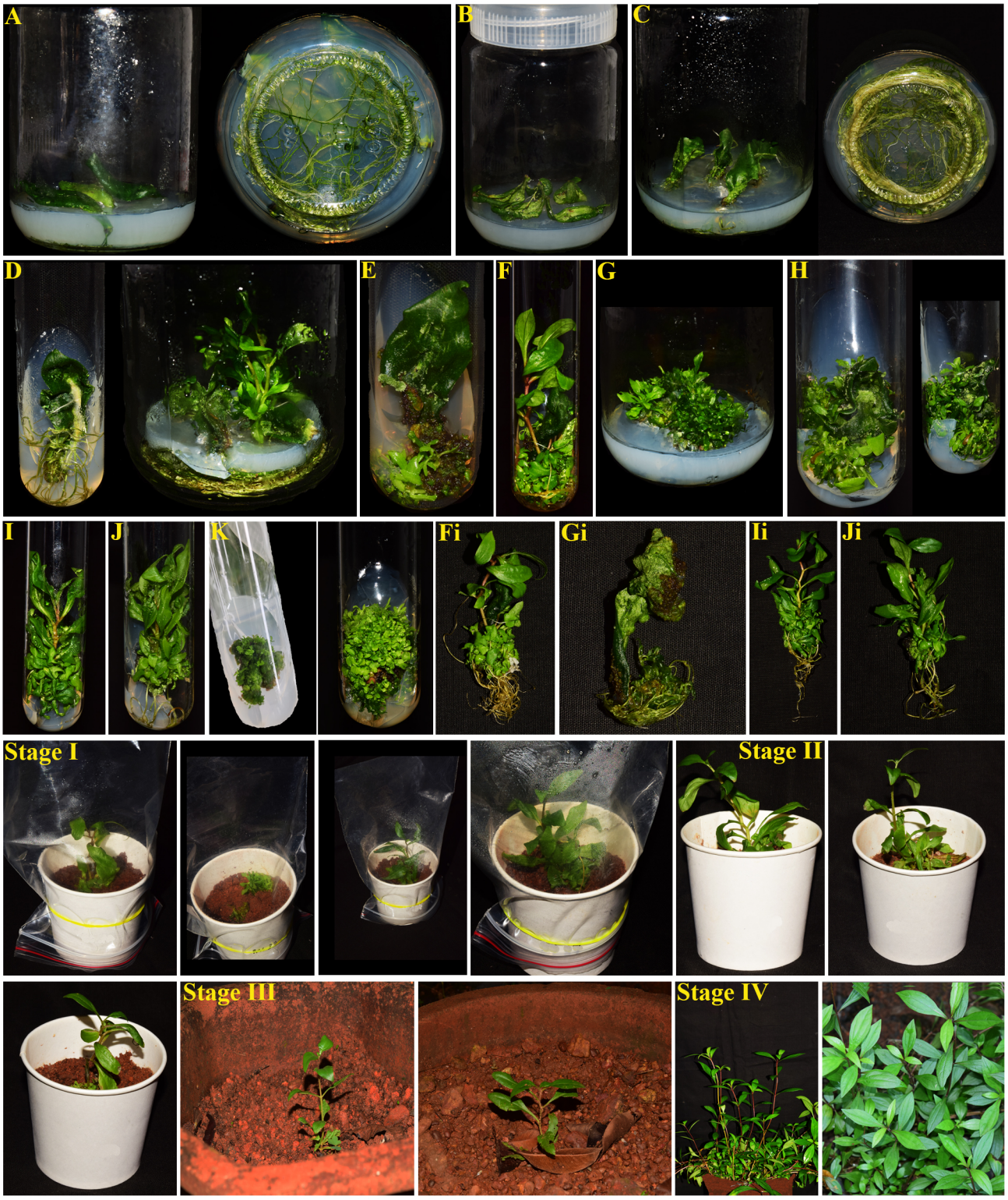

Plate 4: Indirect organogenesis of *A. triplinervis* from different *in vitro* cultures of MS media containing various concentrations of plant growth regulators; **A)** ½ MS, **B)** 0.5 mgl^{-1} Kinetin, **C)** 0.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, **D)** 1.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA, **E)** 1.0 mgl^{-1} TDZ, **F)** 1.5 mgl^{-1} TDZ, **G)** 2.0 mgl^{-1} TDZ, **H)** 1.0 mgl^{-1} TDZ + 0.5 mgl^{-1} NAA, **I)** 1.5 mgl^{-1} TDZ + 0.5 mgl^{-1} NAA, **J)** 2.0 mgl^{-1} TDZ + 0.5 mgl^{-1} NAA, **K)** Indirect organogenesis from internode in the medium 2.0 mgl^{-1} TDZ + 0.5 mgl^{-1} NAA. Individual plants harvested and cleaned for hardening; **Fi, Gi, Ii** and **Ji**. Different stages of acclimatization of *in vitro* generated plantlets through indirect organogenesis; **Stage I)** Plantlets transferred to autoclaved soil and covered with polythene bag, **Stage II)** Bags were removed, **Stage III)** Transferred to green house, **Stage IV)** Fully grown plants in the green house.

Prunus serotina (Hammatt and Grant, 1998). Lee and Pijut (2017), Ramakrishnan *et al.,* (2014) and Jung *et al.,* (2021) reported the effectiveness of BAP and TDZ combination in shoot proliferation. Lee and Pijut (2017) reported that adventitious shoot buds were primarily formed from the abaxial side and some of the buds were formed from the adaxial and petiole portion of the leaf explants of *Fraximus nigra*. Raghu *et al.,* (2006) studied shoot organogenesis from leaf explants of *Embelia ribes* and reported that adventitious shoot formation was increased with increasing TDZ concentrations.

The medium containing a combination of 1.0 mg l^{-1} TDZ + 0.5 mg l^{-1} NAA induced the production of a large number of adventitious shoots in clusters and a moderate number of roots than the individual 1.0 mgl^{-1} TDZ containing medium. In this medium also the internodal explants did not show any response and from the leaf explants light green calli were formed followed by the formation of numerous small green shootlets. Among the various TDZ and NAA combinations tested, the highest number of shoots was formed in medium supplemented with 2.0 mg l^{-1} TDZ + 0.5 mg l^{-1} NAA. A combination of TDZ and NAA produced good results in the *in vitro* cultures of *Aerva lanata* (Varutharaju *et al.,* 2014). The effectiveness of TDZ or its combination with NAA in the medium for shoot regeneration was reported earlier in many plants (Bhagwat & Lane, 2004; Escalettes & Dosba, 1993; Mandal & Laxminarayana, 2014; Gupta *et al.,* 2017).

Of the various combinations tested the internodal explants produced shoots only in the 2.0 mg l^{-1} TDZ + 0.5 mg l^{-1} NAA fortified MS medium; in

this medium, the internodal explants produced green calli and a large number of tiny pale green shootlets, but roots were not formed.

The *in vitro* raised plantlets with roots were taken out from the culture vessels, washed thoroughly to remove the remnants of Agar and transferred to the paper cups containing autoclaved soil. They were watered, covered with polythene bags and kept in a greenhouse for acclimatization. After two weeks the plants were relocated to the pots with potting mixture and kept in the field. The plants did not show any variation in morphology from the mother plant.

4.4. Genetic Fidelity Analysis

ISSR (inter-simple sequence repeat) profiling (Zietkiewicz *et al.,* 1994) of *in vitro* grown plantlets through indirect organogenesis and the mother plant of *Ayapana triplinervis* were carried out for testing the genetic fidelity. ISSR technique avoids the need for flanking sequence information and has therefore found widespread application in diverse plants (Srivastava and Gupta, 2008). The primary concern for maintaining the quality of germplasm is the homogeneity of the plantlets developed through tissue culture (Fatima *et al.,* 2013). The regenerants and their further progenies developed through tissue culture were affected as a result of phenotypic mutations (Phillips *et al.,* 1994). *In vitro*-raised plants, which exhibit a variety of genetic and epigenetic variants, are expected to frequently exhibit somaclonal variation (Peredo *et al.,* 2006). Diaz *et al.,* (2003) suggested that to produce exact copies of the parental plants through *in vitro* cultures genetic purity testing was necessary. Soniya et al., (2001) reported that callus-based genetic variations were common in *in vitro* cultures.

Various methodologies, such as morphological evaluations, physiological supervisions, cytological studies, isoenzymes, and so on, have been established to evaluate the genetic validity of tissue-culture grown plants (Gupta *et al.,* 1999). PCR analysis with ISSR primers proved to be a beneficial technique in investigations of genetic diversity, either intra- or inter-specific, phylogenies, and evolutionary biology (Wang *et al.,* 2009). Several plant species benefit greatly from the use of PCR-based methods for determining the genetic stability of *in vitro*-regenerated plantlets, such as random amplified polymorphic DNA (RAPD) and ISSR (Faisal *et al.,* 2012). RAPD and ISSR markers are relatively simple, rapid, cost-effective, highly discrete, and dependable; they need only a small amount of DNA sample and no prior sequencing data to construct the primer and the most important necessities in the *in vitro* cultures of plant species is the genetic stability and conservation of the germplasm (Fatima *et al.,* 2013).

The application of molecular techniques was one of the best methods to test the genetic fidelity of *in vitro* regenerants (Chandrika and Rai, 2009). The existence of subtle genetic flaws in regenerants mediated through somaclonal variation might significantly impair the *in vitro* culture system (Salvi *et al.,* 2001). In plants, the molecular basis of various biological phenomena was identified and studied with the help of genetic variation studies using molecular markers such as RFLP, RAPD, AFLP and SSR (Agarwal *et al.,* 2008).

The genomic DNA from the leaf tissues of *in vitro* grown plants and mother plant were successfully extracted to obtain sufficient quantity of DNA for ISSR analysis (**Plate 6**) by the kit method using the DNeasy plant mini kit.

The DNA concentration was estimated using UV-Spectrophotometer and was found to be in the range between 4 - 31 ng μl^{-1} for different samples and the ratio of OD260/OD280 ranged between 1.78 - 1.87, which was good for further processes. Ten ISSR primers were used to monitor the samples and out of them five (UBC807, UBC810, UBC825, UBC847 and UBC857) gave good reproducible amplification (**Plate 7**) which was used in screening all the six DNA samples in the present study.

Table 9: Primers details and number of scorable bands used in the genetic fidelity analysis of *A. triplinervis*

Primer	5′-3′ motif	Optimum annealing temp. (°C)	No. of scorable bands per primer	Size range (bp)
UBC807	$(AG)_8T$	52	6	280 - 2,500
UBC810	$(GA)_8T$	48	7	800 - 2,500
UBC825	$(AC)_8T$	54	13	450 - 2,800
UBC847	$(CA)_8RC$	56	14	380 - 2,250
UBC857	$(AC)_8YG$	56	12	400 - 3,500

Forty-three monomorphic bands were obtained from all five primers after PCR amplification and the number of bands varied from 6 – 14 per ISSR primer (**Table 9**). The amplified products were separated using 1.8% Agarose gel electrophoresis and the five primers showed monomorphic bands for all six samples. The results of the fidelity analysis based on the ISSR profiling of the samples using five primers showed that the *in vitro* grown plants of *A. triplinervis* were 100% similar to the mother plant.

The present study was in corroboration with the reports of genetic fidelity analysis using ISSR of *in vitro* generated plantlets of *Syzygium*

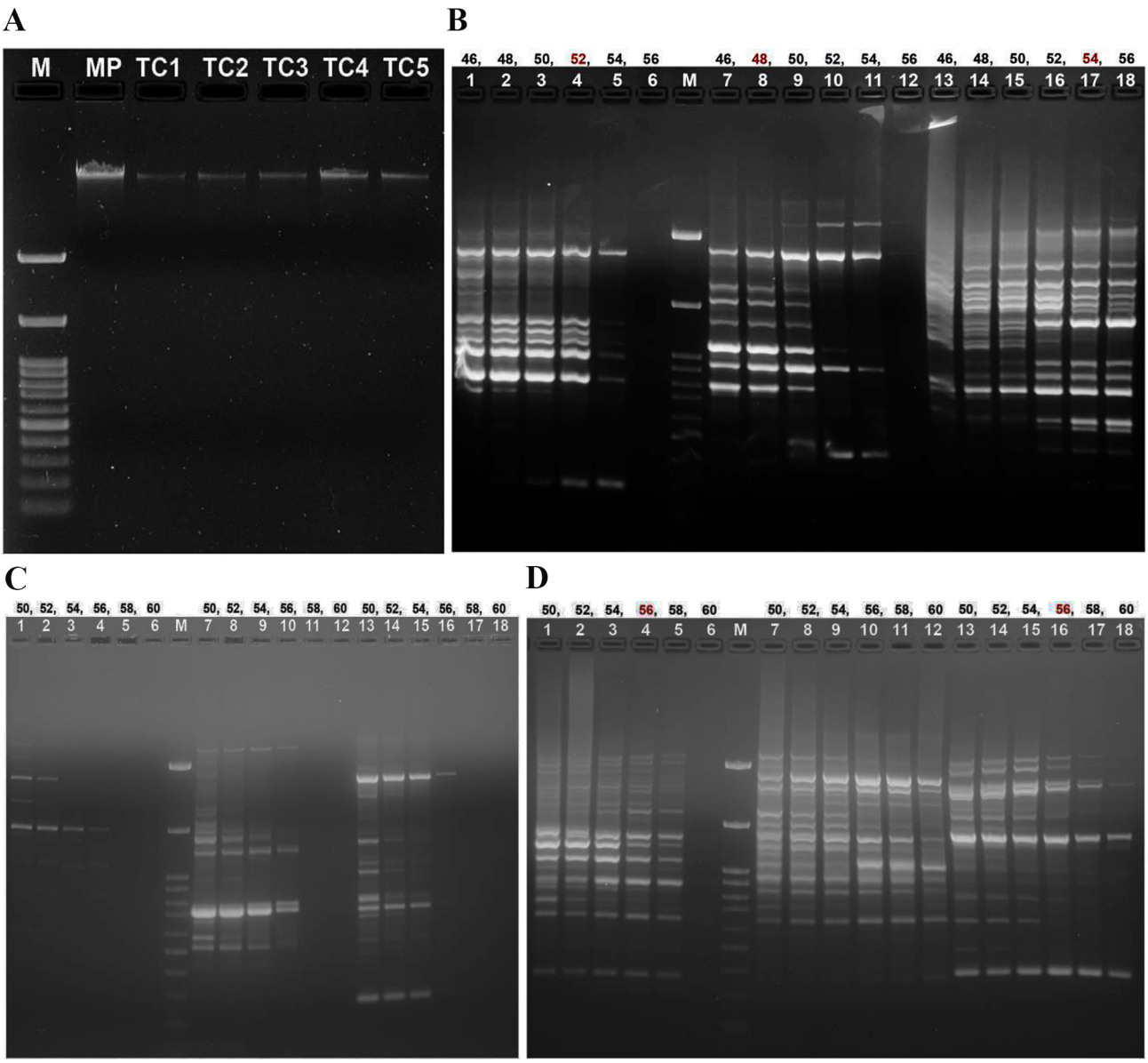

Plate 5: A) Agarose gel (0.8%) showing seperation of DNA isolated from 6 leaf samples of *A. triplinervis*. (Lanes: M - 100bp - 3kb DNA ladder, MP - Mother Plant, TC - Tissue culture raised plant).

Agarose gel (1.8%) image showing optimaisation of annealing temperatures of ISSR primers using primers (and DNA tempelate of Mother plant).
B) UBC807 (Lane 1 - 6), UBC810 (Lane 7 - 12) and UBC825 (Lane 13 - 18).
C) UBC840 (Lane 1 - 6), UBC841 (Lane 7 - 12) and UBC842 (Lane 13 - 18).
D) UBC847 (Lane 1 - 6), UBC851 (Lane 7 - 12) and UBC857 (Lane 13 - 18).

(The numbers above each lane indicates the temperatures (°C) used in gradient PCR reaction and red colour indicates selected temperature)

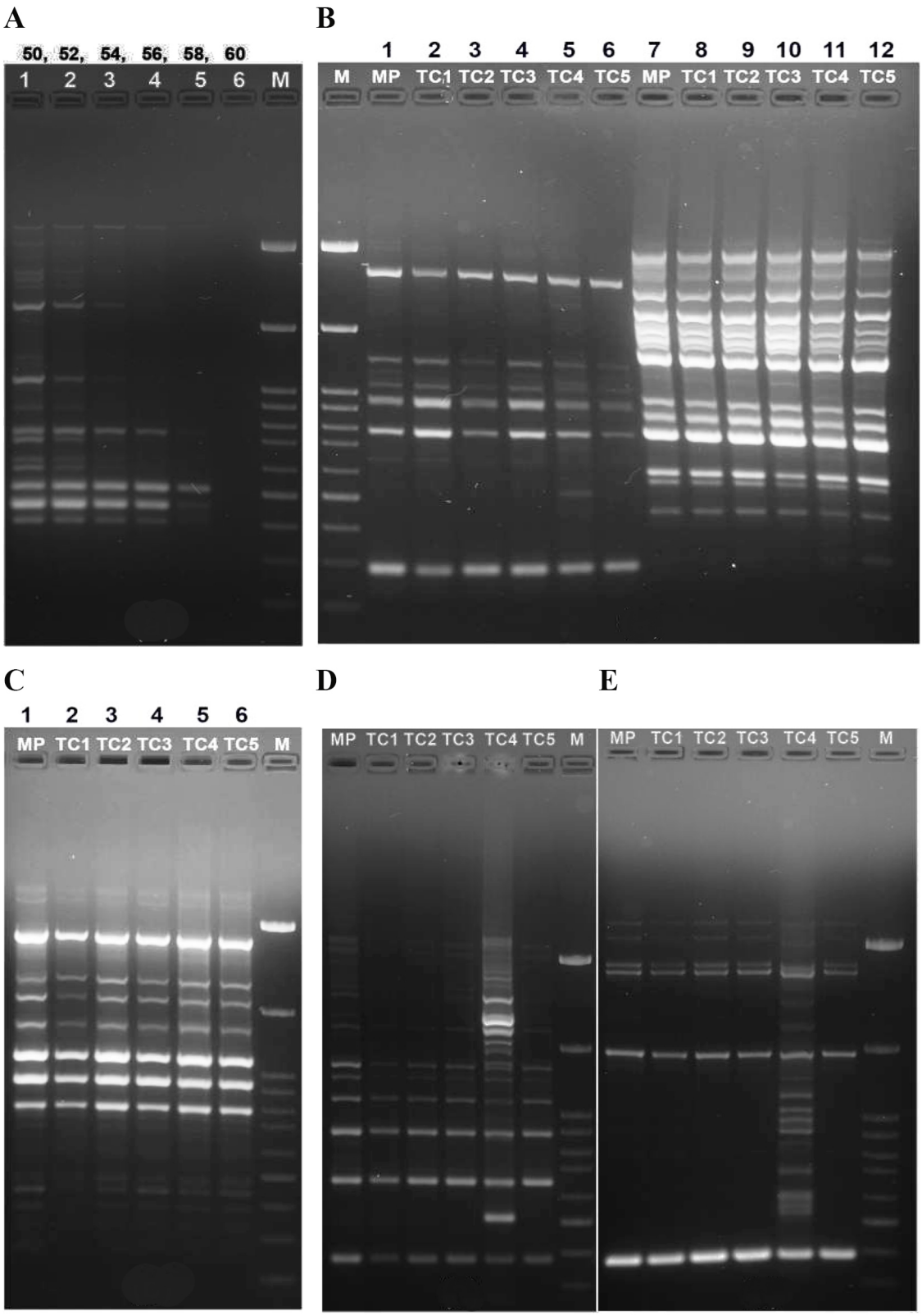

Plate 6: A) Agarose gel (1.8%) image showing optimaisation of annealing temperatures of ISSR primers using primers (and DNA tempelate of Mother plant):- UBC808 (Lane 1 - 6). Agarose gel (1.8%) image showing PCR amplification of all the 5 TC plants and the mother plant DNA samples using ISSR primers:- **B)** UBC807 (Lane 1 -6), UBC825 (Lane 7 - 12): **C)** UBC810 (Lane 1 - 6): **D)** UBC847: **E)** UBC857.

travancorium (Anand, 2003), *Musa acuminata* var. Nanjanagudu (Lakshmanan *et al.,* 2007), *Cineraria maritima* (Srivastava *et al.,* 2009), *Ochreinauclea missionis* (Chandrika and Rai, 2009), *Hydrangea* 'Hyd1' (Liu *et al.,* 2011), *Zingiber rubens* (Mohanty *et al.*, 2011), *Rauvolfia serpentina* (Faisal *et al.,* 2012), *Withania somnifera* (Fatima *et al.,* 2013), *Artemisia absinthium* (Kour *et al.,* 2014), *Dendrocalamus strictus* (Goyal *et al.,* 2015), *Cornus alba* (Ilczuk and Jacygrad, 2016), *Nothapodytes nimmoniana* (Prakash *et al.,* 2016), *Morus alba* (Saha *et al.,* 2016), Finger millet (Babu *et al.,* 2018), *Paederia foetida* (Behera *et al.,* 2018); *Tecoma stans* (Hussain *et al.,* 2018) and *Lycium chinense* (Jung *et al.,* 2021). ISSR-based genetic fidelity analysis was reported earlier in *Robina ambigua* (Guo *et al.,* 2006b), *Swertia chirayita* (Joshi and Dhawan, 2007), *Glycyrrhiza uralensis* (Yao *et al.,* 2008) and *Bambusa balcooa* (Negi and Saxena, 2010). Guo *et al.,* (2006a) reported polymorphism in ISSR analysis of *Codonopsis lanceolate*. Somaclonal variations were detected in the *in vitro* raised plantlets of *Dendrobium nobile* Lindl (Bhattacharyya et al. 2014); *Clerodendrum thomsoniae* Balf. F. (Kar *et al.* 2019).

4.5. *In vitro* stress study

4.5.1. Morphogenic responses

4.5.1.1. NaCl

The transfer of specific ions, mainly Na^+ and Cl^- inside and outside cells, can lead to salt stress (Sabir *et al.,* 2012). This type of stress, caused by salinity or NaCl induction, is quite common in plants and can create harsh conditions. One significant impact of salinity stress is the production of free radicals.

During the present study, nodal segments from *A. triplinervis* were cultured in MS media containing 10, 25, 50, and 100 mM l^{-1} NaCl concentrations. After a 60-day growth period, the plants were harvested, and various growth parameters were analyzed to assess the morphogenic response in the *in vitro* cultures of *A. triplinervis* (**Plate 7** and **Table 10**).

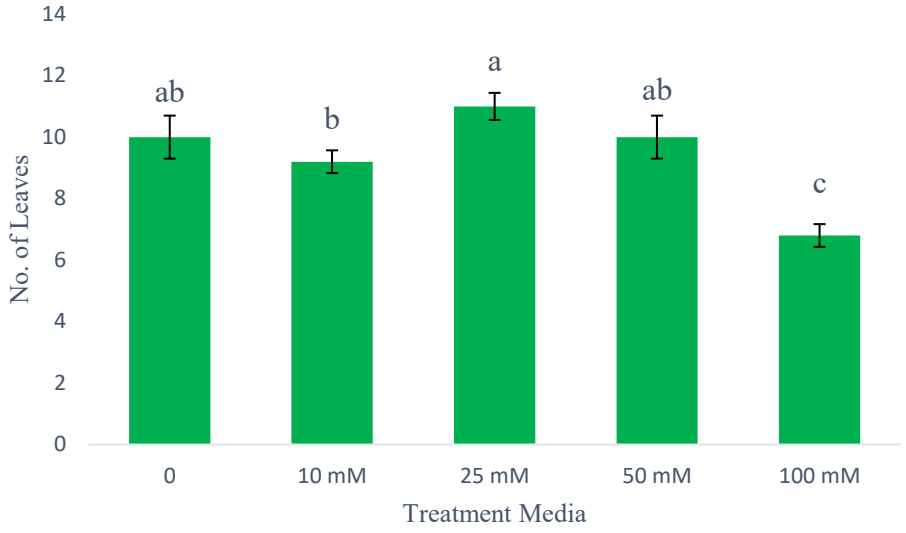

Figure 2: Effects of NaCl on the no. of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

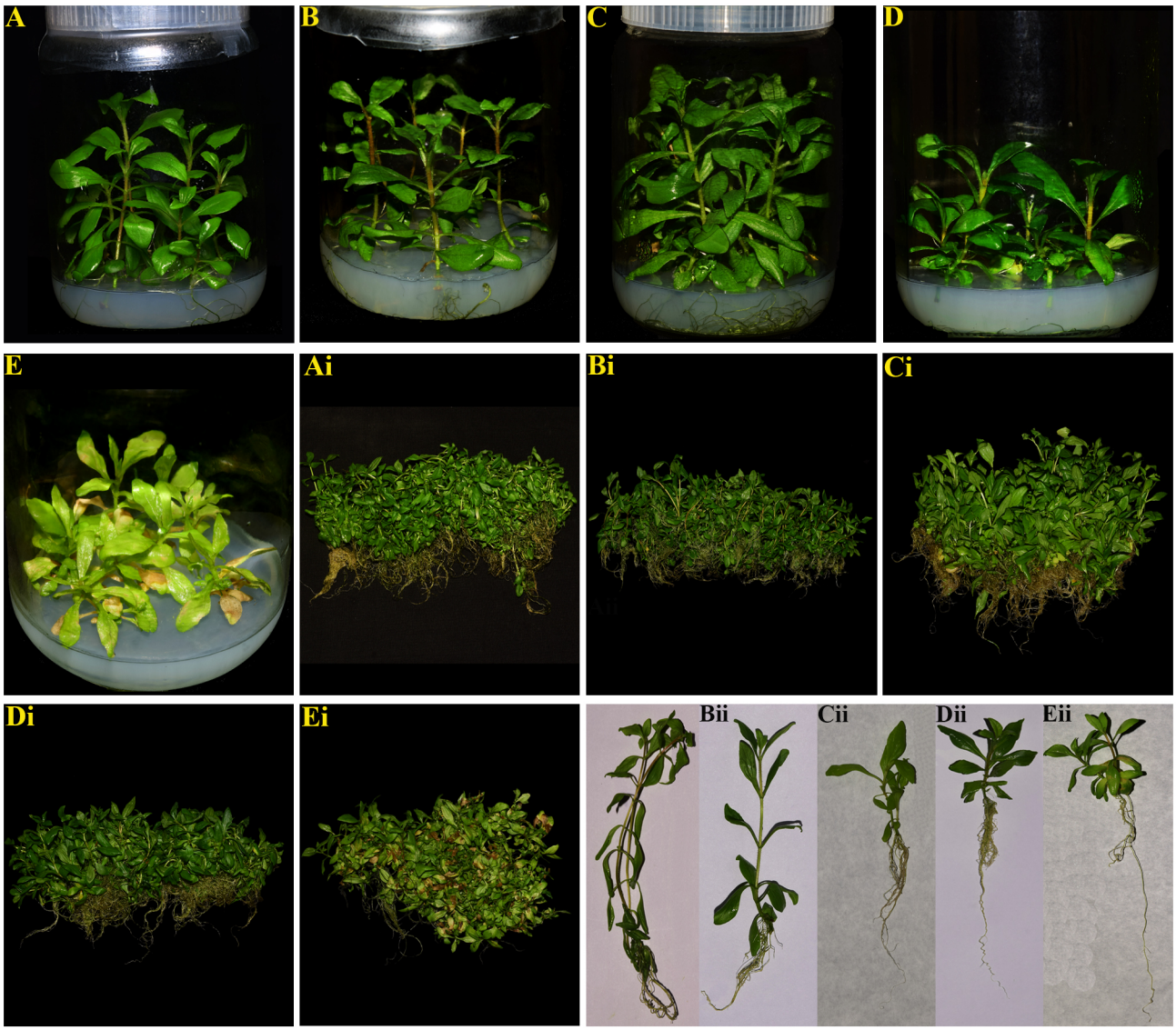

Plate 7: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 10 mM l^{-1} NaCl, **C)** 25 mM l^{-1} NaCl, **D)** 50 mM l^{-1} NaCl, **E)** 100 mM l^{-1} NaCl. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Ei**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Eii**.

Table 10: Effects of NaCl in morphogenic responses of *in vitro* cultures of *A. triplinervis*

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	3.289±0.23ab	10±0.7ab	2.1±0.18a	3.44±0.09b	6.1±0.23a	6.46±0.10b	35.489	2.5417
10 mM	1.527± 0.09b	9.2±0.37b	1.8±0.20a	3.99±0.13a	3.4±0.27b	4.52±0.11d	38.0332	2.3741
25 mM	5.155±2.43a	11±0.44a	1.6±0.16a	4.22±0.12a	5.6±0.16a	5.67±0.14c	33.3797	2.3103
50 mM	2.827±0.26ab	10±0.7ab	1.6±0.16a	1.82±0.21c	6±0.26a	7.39±0.12a	44.0683	3.8956
100 mM	1.105±0.08b	6.8±0.37c	1.9±0.18a	0.96±0.10d	1.6±0.16c	4.98±0.43d	29.066	1.5681

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test

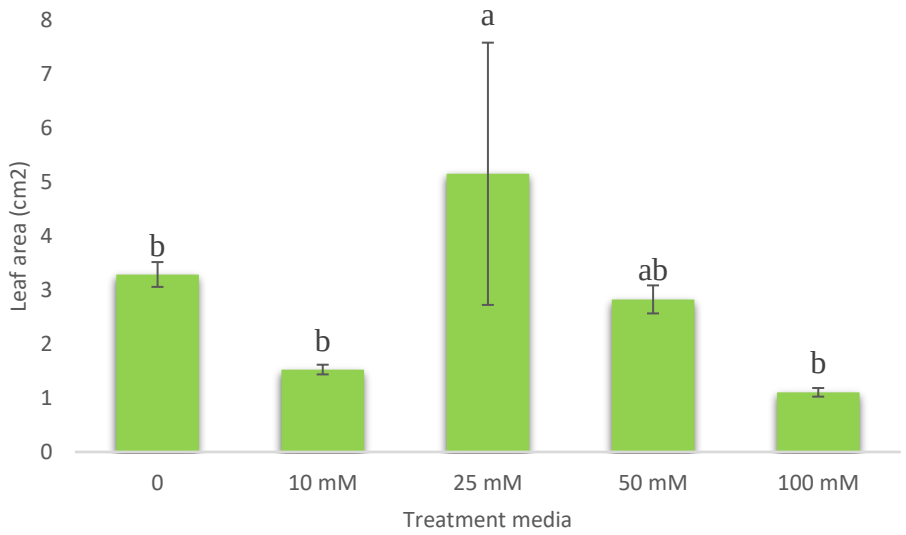

Figure 3: Effects of NaCl on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

4.5.1.1.1. Leaves

The plants formed in the control (MS basal) medium, 10±0.7 green leaves per shoot were formed (**Figure 2**), with a large leaf area of 3.289±0.23 cm^2 (**Figure 3**). On the other hand, plants formed in 10 mM l^{-1} NaCl-induced medium had 9.2±0.37 pale green leaves per shoot with a reduced leaf area of 1.527±0.09 cm^2 compared to the control plants. The plants formed in 25 mM l^{-1} NaCl-induced medium had 11±0.44 green leaves per shoot, with the highest leaf area of 5.155±2.43 cm^2. In 50 mM l^{-1} NaCl-induced plant cultures, 10±0.7 green leaves per shoot were formed, with a leaf area of 2.827±0.26 cm^2. In 100 mM l^{-1} NaCl containing medium, the plants showed the lowest leaf area of 1.105±0.08 cm^2, and only 6.8±0.37 yellowish pale green leaves were formed per shoot. The leaves were chlorotic, and many

were dried due to excess salt in the medium. As internodes were shorter, the arrangement of leaves provided a rosette-like appearance.

4.5.1.1.2. Shoots

The shoot number values of the control and NaCl-induced cultures showed a slight difference, but they still fell within the same mean group, as depicted in **Figure 4**. The control plants had a slightly higher shoot number (2.1±0.18) when compared to the other stress-induced plants. The plants treated with 10mM l^{-1} NaCl had an increased shoot length of 3.99±0.13cm, surpassing the control plants, which had a shoot length of 3.44±0.09cm (**Figure 5)**. The plants cultured in 25mM l^{-1} NaCl medium exhibited the highest shoot length of 4.22±0.12cm and grew faster than the control plants. However, in the 50mM l^{-1} NaCl-induced medium, the length of shoots decreased significantly to 1.82±0.21cm, leading to decreased growth

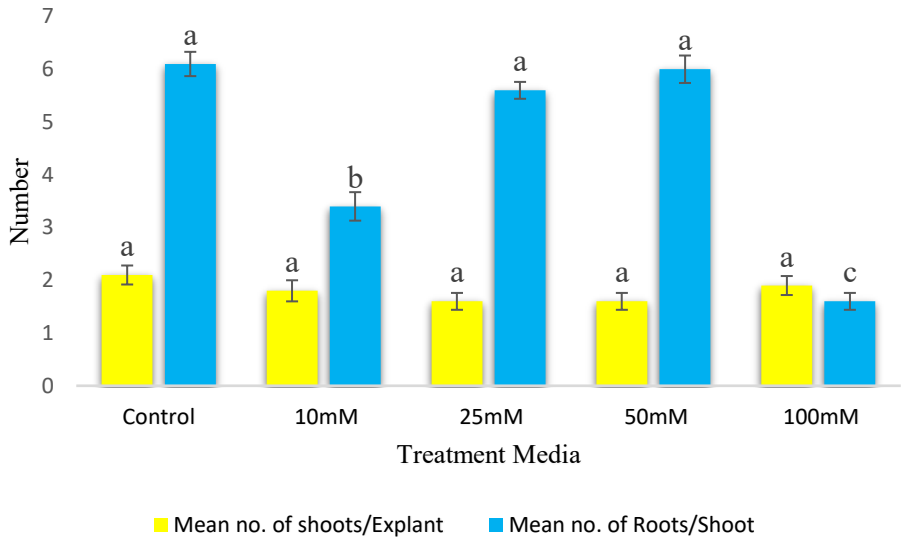

Figure 4: Effects of NaCl on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

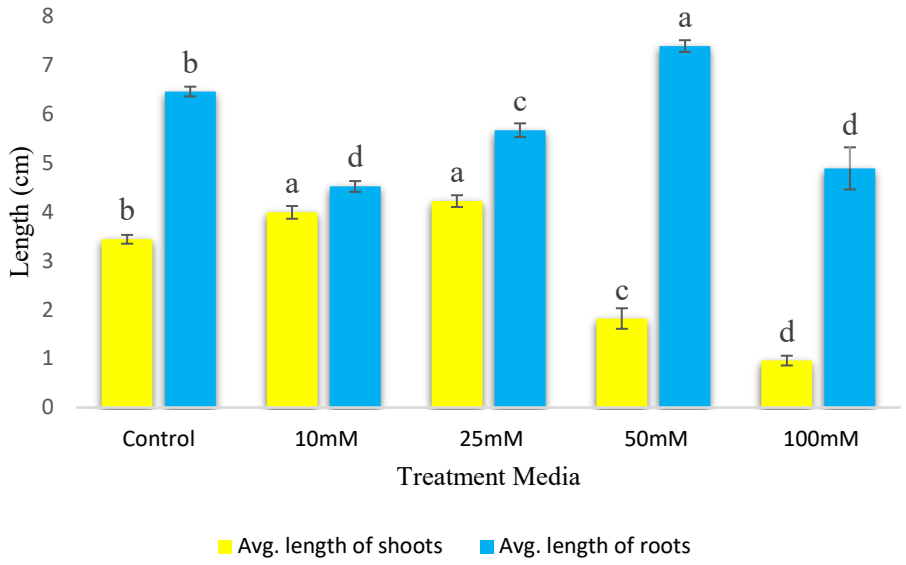

Figure 5: Effects of NaCl on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

and multiplication of shoots. Shoots with necrotic patches were observed in the 100mM l^{-1} NaCl-containing medium, and they were very short (0.96±0.10cm) with shorter internodes.

4.5.1.1.3. Roots

In the control medium substantial number of roots (6.1±0.23) (**Figure 4**) with a length of 6.46±0.10cm (**Figure 5**) was formed. In the nutrient medium containing 10mM l^{-1} NaCl medium, less number of roots was formed (3.4±0.27) with a root length of 4.52±0.11cm. In the nutrient medium containing 25 mM l^{-1} NaCl, the root number and length increased to 5.6±0.16 and 5.67±0.14cm respectively. The most favourable growth was observed in the nutrient medium containing 50 mM l^{-1} NaCl, where roots reached their maximum length (7.39±0.12cm). However, the number of roots produced

(1.60.16) decreased in the nutrient medium with a greater salt content of 100mM l^{-1} NaCl.

4.5.1.1.4. Biomass Production

The biomass production (fresh and dry weight) of harvested plants increased slightly when grown in the nutrient medium containing 10 mM l^{-1} NaCl compared to the control medium (**Figure 6**). However, the plants harvested from the 25 mM l^{-1} NaCl-containing nutrient medium showed a decrease in biomass production. The maximum biomass production (fresh and dry weight) was observed in the harvested plants grown in a 50 mM l^{-1} NaCl-containing nutrient medium. On the other hand, it was also observed that the biomass production of plants grown in a 100 mM l^{-1} NaCl-containing nutrient medium was the lowest.

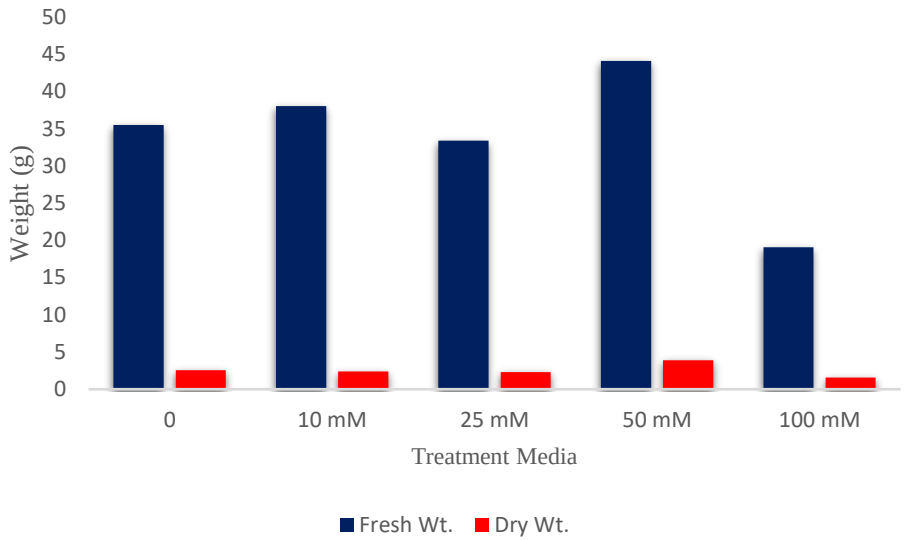

Figure 6: Effects of NaCl on the total biomass production in *in vitro* cultures of *A. triplinervis*

Weimberg (1975) in *Pisum sativum*, Güens *et al.,* 1996 in pepper, Khavari-Nejad & Mostofi, 1998 in tomato cultivars, Marcelis and Van-Hooijdonk (1999) in *Raphanus sativus*, and Yang *et al.* (2009) in *Populus cathayana* also reported a reduction in the growth of the plants with an increase in salt concentration. Zhao *et al.* (2009) reported that the growth rate of the calli of *Thellungiella holophila* was maximum at 100 mM NaCl, and the accumulation of glycine betaine, trehalose, and total flavonoids was increased.

In the present study, different NaCl concentrations caused multiple impacts on the plant cultures. Various growth parameters were gradually reduced with increased NaCl concentration in the medium. This was corroborated by the earlier reports on *Aloe vera*; *Arabidopsis*; *Spinacia oleracea*; *Cucumis sativus*; and Tomato (Asghari & Ahmadvand, 2018; Zhao *et al.,* 2009; Muchate *et al.,* 2019; Alrahman *et al.,* 2005; Sam *et al.,* 2003; Srinieng *et al.,* 2015). The reports showed that ionic stress or imbalance affects plant growth and metabolism. As a result, the growth was retarded.

In the present study, higher salt concentration in the medium resulted in necrosis of tissues. Nawaz *et al.* (2013) earlier reported necrosis of tissues in wheat cultures in the presence of high concentrations of NaCl in the medium.

Allakhverdiev *et al.* (2000) reported that stress induced by NaCl in *Synechococcus* sp. has both osmotic and ionic effects; cytosolic water content was reduced, and simultaneously, intercellular salt concentration was elevated in the osmotic effect and the ionic effects resulting in the increase in the influx of Na^+ ions to the cytosol and inactivated the PSI and PSII irreversibly.

Burssens *et al.* (2000) reviewed alterations in cell division and growth in response to salt stress with the transcriptional level control of the cell cycle. Salt stress decreases the photosynthesis rate by limiting CO_2 diffusion to the chloroplast through stomatal closure and structural change in the mesophyll (Delfine *et al.,* 1998). The study of Gong *et al.* (2001) pointed out that the SOS (salt overly sensitive) pathway downregulates the expression of the following salt-regulated genes negatively: *SAMT*, *C0R 6.6/KIN2*, *STZ*, and *CCR1* in NaCl-induced treatments. This might have caused a decrease in various growth parameters observed in the present study.

4.5.1.2. Sucrose

Sucrose is the essential and most dependable carbon source in the culture media for plants. In the present study, different concentrations of sucrose (10, 20, 30 (control), 40 and 50 g l^{-1}) were applied to the *in vitro* cultures of *A. triplinervis* to investigate its effects on various *in vitro* morphogenic responses (**Plate 8** and **Table 11**).

4.5.1.2.1. Leaves

The plants formed in MS medium containing 30 g l^{-1} of sucrose produced 10±0.7 green leaves per shoot (**Figure 7**). The highest leaf area value of 1.491±0.12cm² was also observed in this medium (**Figure 8**). Plants formed in the medium containing a sucrose concentration of 10 g l^{-1} produced 12±0.7 yellowish-green leaves per shoot. The leaf area of these plants (1.359±0.10cm²) showed a slightly significant difference compared to the

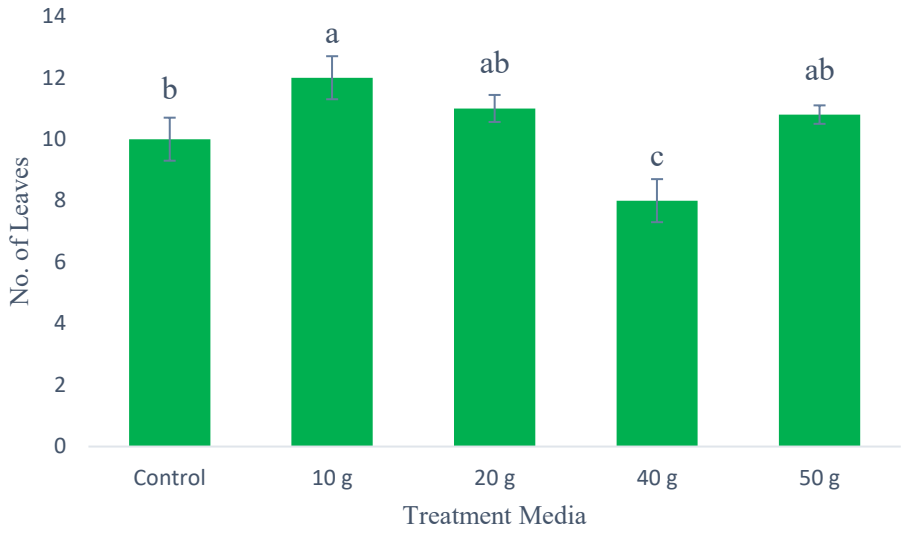

Figure 7: Effects of Sucrose on the numbers of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

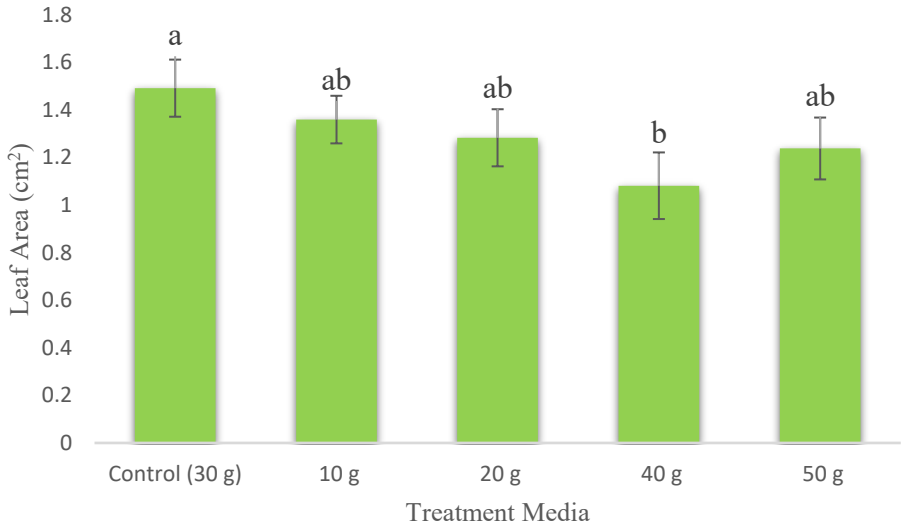

Figure 8: Effects of Sucrose on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

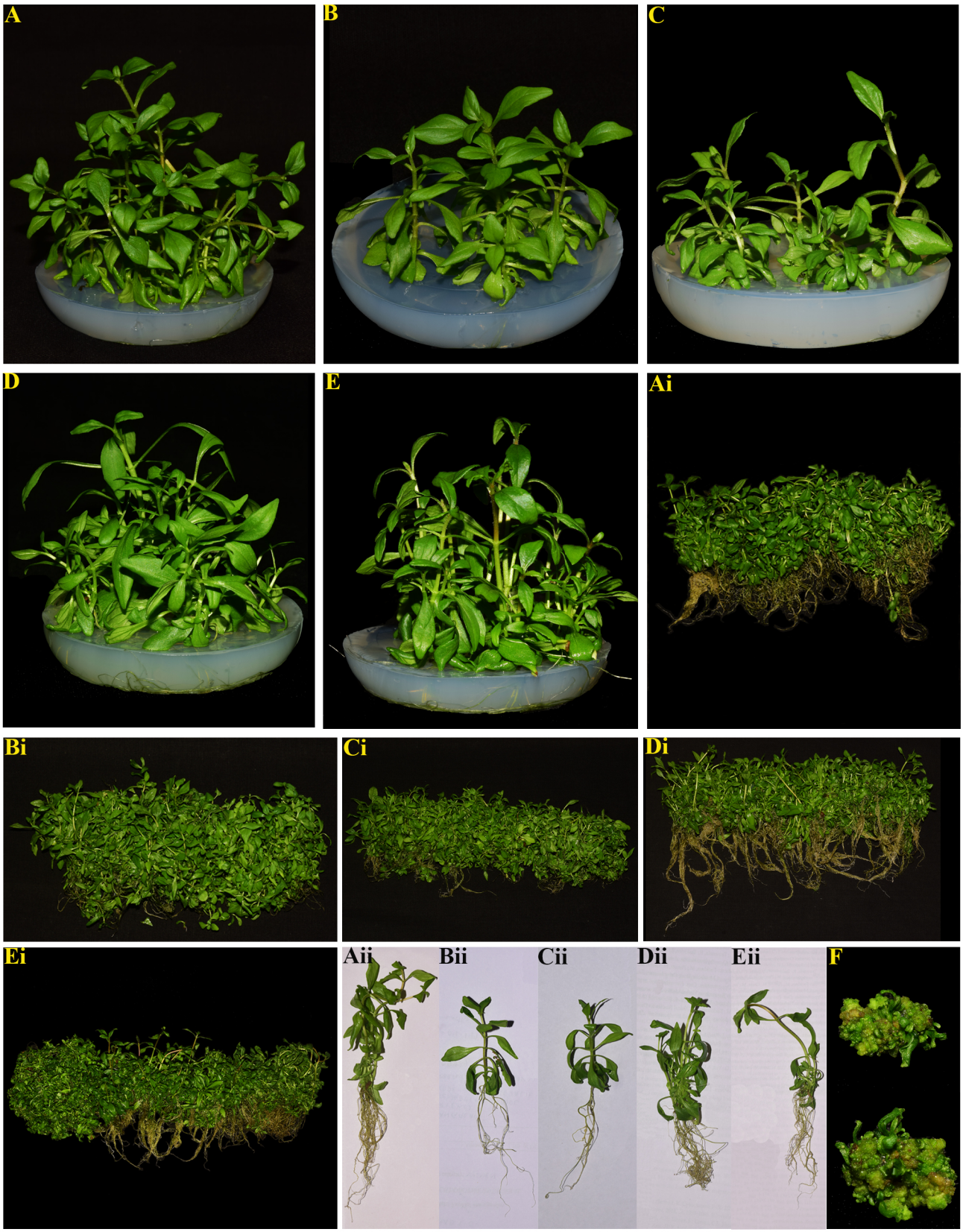

Plate 8: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal - 30 g l^{-1} Sucrose (control), **B)** 10 g l^{-1} Sucrose, **C)** 20 g l^{-1} Sucrose, **D)** 40 g l^{-1} Sucrose, **E)** 50 g l^{-1} Sucrose. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Ei**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Eii**. **F)** Callus with tiny plantlets produced from the 50 gl-1 Sucrose induced *in vitro* culture medium.

Table 11: Effects of Sucrose in morphogenic responses of *in vitro* cultures of *A. triplinervis*.

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
30 g (control)	1.491±0.12a	10±0.7b	2.2±0.20ab	3.24±0.15a	3.1±0.28c	7.83±0.17a	72.4612	6.3824
10 g	1.359±0.10ab	12±0.7a	1.7±0.15b	1.72±0.11b	2.4±0.16c	4.67±0.26a	42.4051	2.5866
20 g	1.2825±0.12ab	11±0.44ab	1.7±0.15b	1.52±0.12b	2.5±0.17c	5.82±0.22a	29.5239	2.5804
40 g	1.081±0.14b	8±0.7c	2.5±0.17a	1.76±0.13b	4±0.21b	7.65±0.34a	57.3543	5.3926
50 g	1.2375±0.13ab	10.8±0.37ab	2.3±0.21a	1.94±0.79b	5.2±0.33a	6.46±2.73a	46.152	5.4205

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.

control medium. In the nutrient medium containing 20 g l^{-1} sucrose, the leaf area (1.2825±0.12cm²) was found to be reduced. The plants grown in this medium produced 11±0.44 yellowish-pale green leaves per shoot. The lowest leaf area value of 1.081±0.14cm² was observed in plants formed in the 40 g l^{-1}sucrose containing medium. The plants produced 8±0.7 yellowish-pale green leaves per shoot. The leaves of plants formed in 50 g l^{-1} sucrose containing medium had a leaf area of 1.2375±0.13cm² and 10.8±0.37 dark green leaves were formed per shoot.

4.5.1.2.2. Shoots

In the present study, the longest shoots, measuring 3.24±0.15cm (**Figure 10**) were found in plants formed in the nutrient medium containing 30 g l^{-1} sucrose (control). The lowest shoot numbers (1.7±0.15) were observed

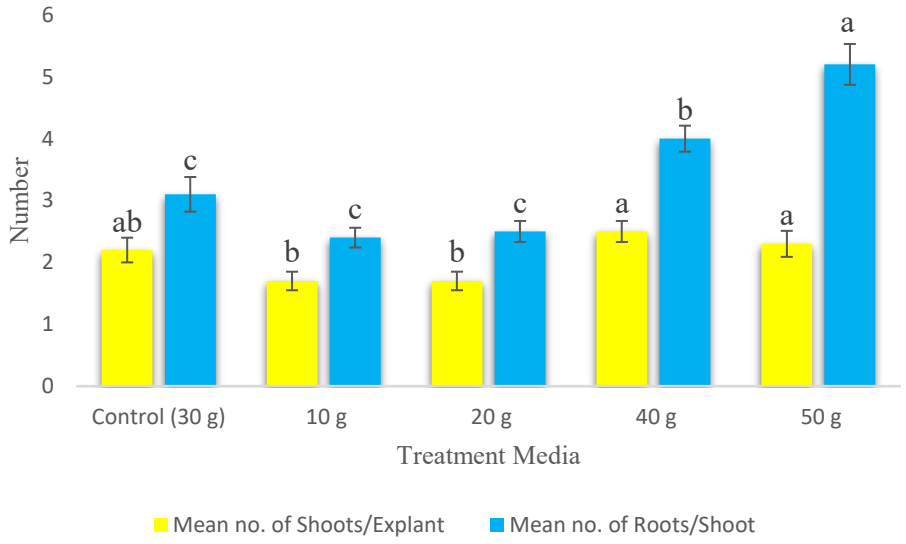

Figure 9: Effects of Sucrose on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

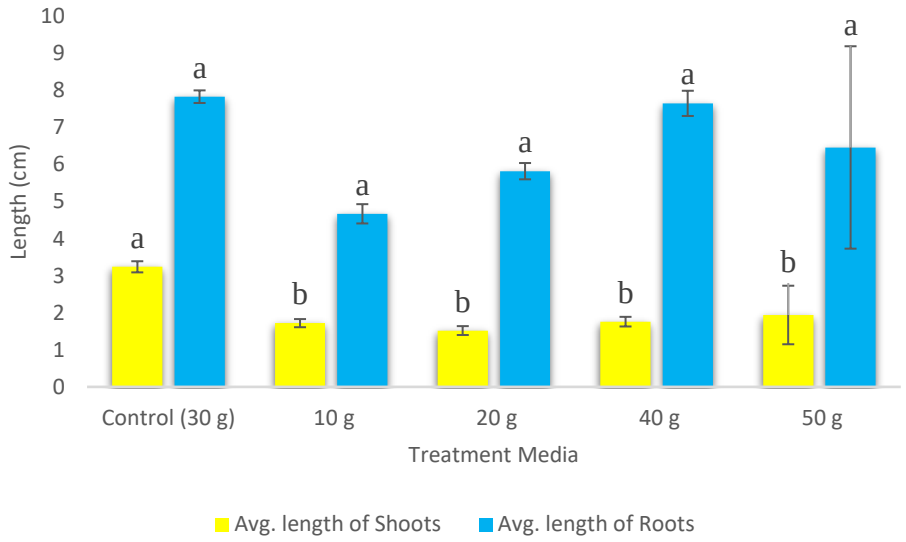

Figure 10: Effects of Sucrose on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

in the nutrient medium containing 10 g l^{-1} and 20 g l^{-1} sucrose. The shoots were whitish-green with reduced shoot length (1.72±0.11cm). The stem had a whitish-pale green colour on the upper portion and a green colour on the lower part. Above the control sucrose concentration, the growth of axillary buds was observed. The 40 g l^{-1} sucrose-induced medium shows the maximum multiplication of shoots, with highest number of shoots (2.5±0.17), and have a whitish-green stem.

The plants grown in the medium with 50 g l^{-1} sucrose showed a slight decrease in shoot number (2.3±0.21) (**Figure 9**) and shoot length (1.94±0.79cm) compared to the control plants. The stems of the plants were whitish-green and pale reddish coloured. The of axillary bud growth was also observed in these plants.

4.5.1.2.3. Roots

In the present study, 3.1±0.28 (**Figure 8**) roots were formed in the plants of the control medium. The average length of roots in this medium was 7.83±0.17cm ((**Figure 9**). On the other hand, plants formed in 10 g l^{-1} sucrose containing medium had the lowest number of roots (2.4±0.16) and an average root length of 4.67±0.26cm. The roots were thin and green-coloured. Plants formed in the 20 g l^{-1} sucrose-containing medium showed a root number of 2.5±0.17 and a root length of 5.82±0.22cm. These roots were slender and green with white rootlets.

A higher number of roots (4±0.21), were formed in the nutrient medium containing 40 g l^{-1} sucrose. The roots were long, slender, and green-coloured with thin white rootlets. The roots had a length measuring 7.65±0.34cm. The highest number of roots (5.2±0.33) was formed in the nutrient medium containing 50 g l^{-1} sucrose. The roots were long, slender, and green-coloured with thin, white rootlets. Callus formation was also observed in this medium.

4.5.1.2.4. Biomass Production

The plants formed in 10 g l^{-1} and 20 g l^{-1} sucrose containing medium had low biomass production. The plants formed in the medium containing 40 g l^{-1} sucrose had a slightly higher biomass production. However, in plants formed in the nutrient medium containing 50 g l^{-1} of sucrose, the biomass production was found to be decreased. The harvested plants formed in the nutrient medium containing very high or very low sucrose concentrations had

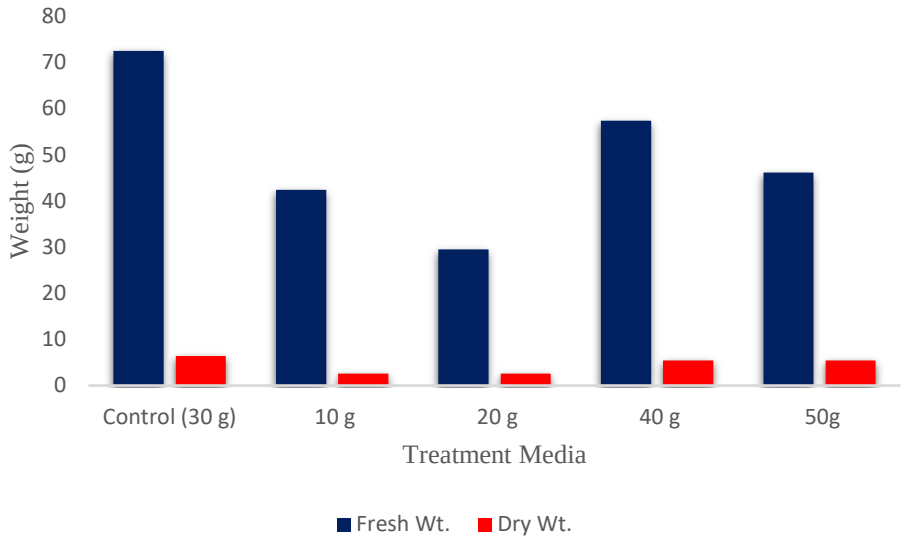

Figure 11: Effects of Sucrose on the total biomass production in *in vitro* cultures of *A. triplinervis*

reduced biomass production. This shows the importance of using optimum sucrose concentration in the medium.

The only medium that exhibited callus production and the highest number of roots was the 50 g l^{-1} sucrose-containing medium.

The 30 g l^{-1} sucrose in the medium was found to be optimal for morphogenesis in the *in vitro* cultures of *Nephrolepis exaltata (*Pasqual *et al*., 1994); *Syngonium podophyllum* (Nower, 1998); *Hypericum perforatum* L. (Cui *et al.*, 2010). and *Rucus hypoglossum* L. (Abou-Dahab *et al.*, 2005). These reports were in consensus with the results observed in the present studies on *A. triplinervis.* However, Naik *et al.*, (2010) reported that the medium containing 2% sucrose was suitable for the shoot regeneration from leaf explants of *Bacopa monnieri*. The callus induction of Bulgarian Golden root was optimum at 2% sucrose in the culture medium (Tasheva & Kosturkova, 2014). Ayoola-Oresanya *et al.* (2021) reported that the 40 g l^{-1} sucrose-fortified medium was optimum for growth and leaf initiation in Musa

species. In the suspension cultures of *Salvia leriifolia,* the highest dry weight was observed in the medium supplemented with 40 g l^{-1} sucrose (Modarres *et al.,* 2018).

4.5.1.3. Putrescine

Polyamines are low molecular weight, aliphatic nitrogenous compounds in the cells influencing plants' growth and stress-related functions (Redha & Suleman, 2011; Chen *et al.,* 2019). The three main forms of polyamines are putrescine (Put), spermidine (Spd), and spermine (Spm).

In the present study, different concentrations of Put were used: 20, 40, 60, 80, and 100 mg l^{-1}. Different growth parameters were selected to study the effects of various Put concentrations in the *in vitro* morphogenic responses of *A. triplinervis* (**Table 12** and **Plate 9**).

4.5.1.3.1. Leaves

Plants formed in the control (MS basal) medium, had a high leaf area (1.687±0.16cm^2) (**Figure 13**), and each shoot had 10.2±1.35 light green leaves (**Figure 12**). The plants produced in the MS medium induced with 20 mg l^{-1} Put had 13±0.44 pale green leaves per shoot, but there was a significant reduction in the leaf area (1.1415±0.13cm^2) compared to the control plants. In the 40 mg l^{-1} Put-induced medium, the number of leaves increased compared to the control plants, and each shoot had 12±0.7 slightly dark green leaves. However, the leaves of these plants had the lowest leaf area (0.9005±0.08cm^2) among the Put induced cultures.

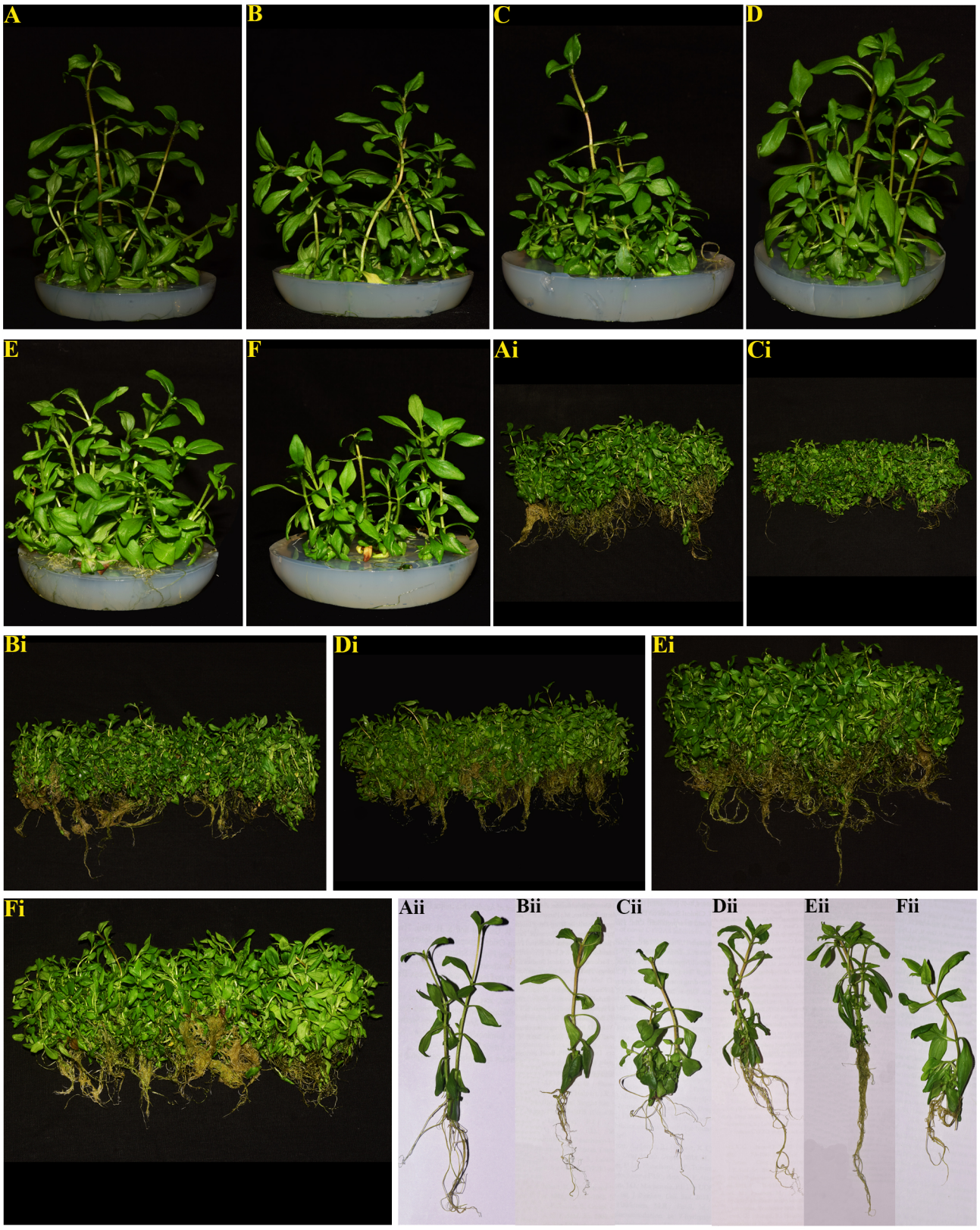

Plate 9: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 20 mg l^{-1} Putrescine, **C)** 40 mg l^{-1} Putrescine, **D)** 60 mg l^{-1} Putrescine, **E)** 80 mg l^{-1} Putrescine, **F)** 100 mg l^{-1} Putrescine. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Fi**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Fii**.

Table 12: Effects of Putrescine in morphogenic responses of *in vitro* cultures of *A. triplinervis*.

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	1.687±0.16a	10.2±1.35b	2.1±0.23a	3.77±0.20a	4.7±0.54a	7.25±0.17ab	67.9331	6.0145
20 mg	1.1415±0.13bc	13±0.44ab	1.9±0.23a	2.42±0.12b	2.2±0.13c	5.96±0.18c	37.9967	4.0199
40 mg	0.9005±0.08c	12±0.7ab	2±0.21a	1.63±0.14c	2.4±0.16c	4.1±0.15d	21.3394	2.8838
60 mg	1.847±0.18a	13.4±1.07ab	1.6±0.16a	3.85±0.16a	2.6±0.16c	7.77±0.12a	59.1328	4.7831
80 mg	1.3995±0.16ab	15.2±1.39a	1.9±0.23a	2.2±0.12b	3.5±0.17b	6.87±0.21b	64.4304	4.9639
100 mg	1.78±0.16a	11.8±0.8b	1.7±0.21a	2.36±0.16b	2.5±0.17c	7.68±0.23a	52.1039	3.4735

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.

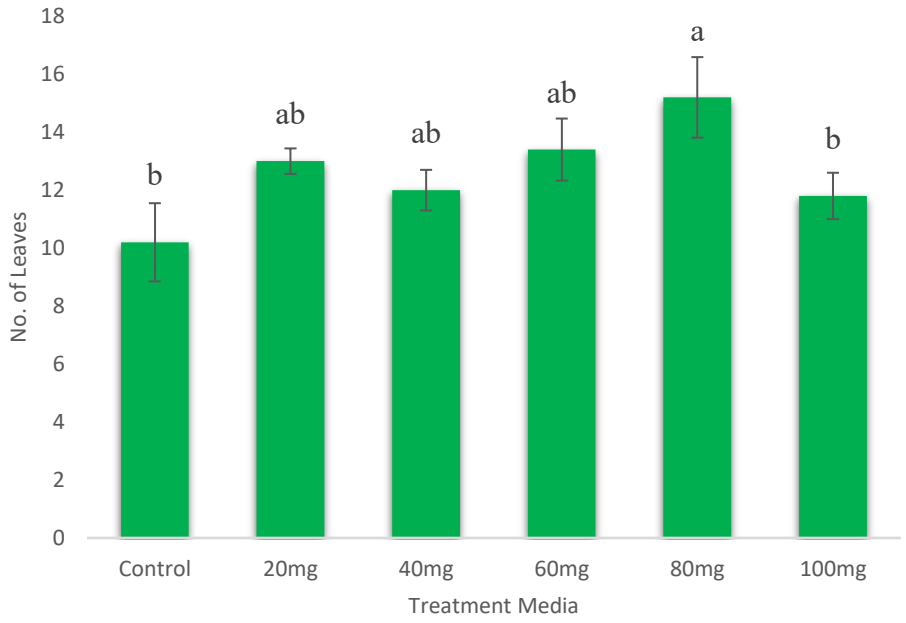

Figure 12: Effects of Putrescine on the no. of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

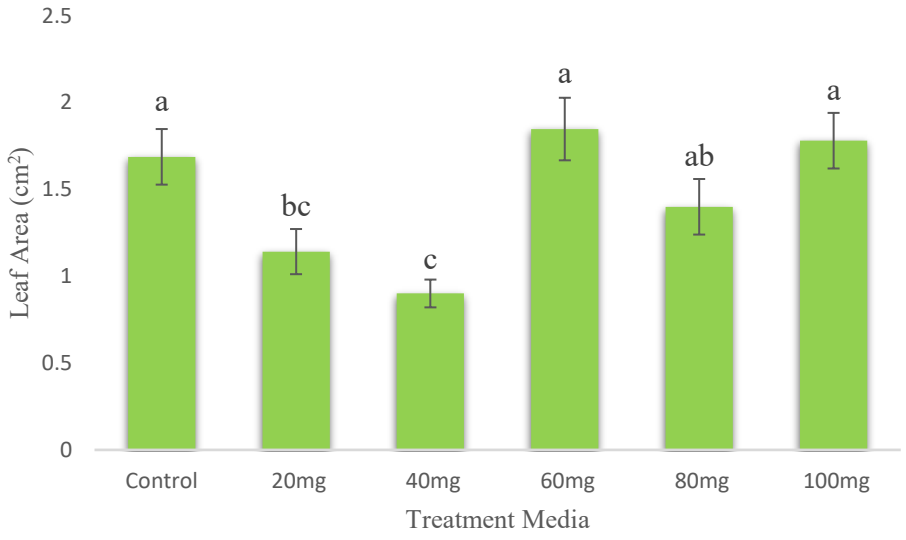

Figure 13: Effects of Putrescine on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

The plants formed in the 60 mg l^{-1} Put-containing culture medium had 13.4±1.07 light green leaves per shoot, and the highest leaf area (1.847±0.18cm^2) was also observed in these plants. The plants formed in the 80 mg l^{-1} Put induced medium had 15.2±1.39 green leaves per shoot, and this medium also promoted the production of leaves. The leaf area observed in plants of this medium was 1.3995±0.16cm^2. In the 100 mg l^{-1} Put medium, the plants produced 11.8±0.8 leaves per shoot, and the leaf colour ranged from green to yellowish green. The leaf area (1.78±0.16cm^2) showed an increase compared to the plants of the previous media.

Shoots

The plants in the control medium showed the most significant number of shoots (2.1±0.23) **(Figure 14**) and the longest shoot length (3.77±0.20cm) (**(Figure 15**) and the shoots were whitish-green. In the 20 mg l^{-1} Put-induced

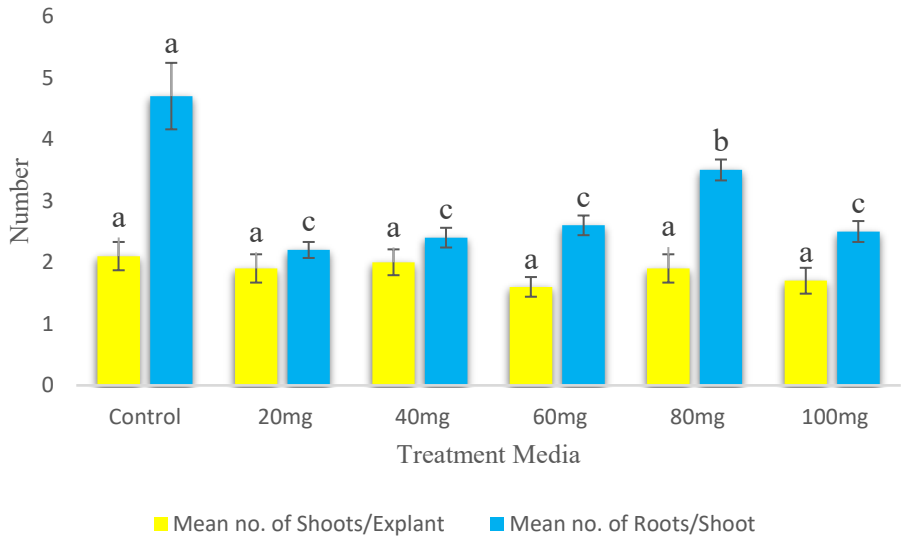

Figure 14: Effects of Putrescine on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

medium, shoot length significantly decreased (2.42±0.12cm), and stems often displayed a pale reddish-green hue, but the number of shoots (1.9±0.23) remained almost the same as that formed in the control medium.

Plants grown in the 40 mg l^{-1} Put-induced medium also showed axillary bud growth, with the maximum number of shoots (2±0.21) observed among the Put-induced cultures. The number of shoots did not differ significantly from the control group, but the shoots were stunted, and shortest (1.63±0.14cm). The stems were mostly whitish-green, but some displayed intermittent pale reddish coloring.

In the 60 mg l^{-1} Put-induced medium, there was a slight decrease in shoot production compared to other cultures. However, the plants grown in this medium had the longest shoots (3.85±0.16cm) and pale brownish stems. Plants grown in both 80 mg l^{-1} and 20 mgl^{-1} Put-induced medium had similar shoot numbers and lengths, with stems mostly whitish-green in color and occasionally pale red. The highest concentration of Put, 100 mg l^{-1}, reduced the number (1.7±0.21) and length (2.36±0.16cm) of shoots. The stems of these plants were green and occasionally pale red.

4.5.1.3.3. Roots

The plants formed in the control medium, had the highest root count (4.7±0.54) (**Figure 14**) and longest roots(7.25±0.17cm). These roots were slender, long, and pale green with white rootlets. In contrast, the plants grown in the 20 mg l^{-1} Put cultures displayed limited growth and root multiplication compared to those in the control medium. These plants produced the lowest number of roots (2.2±0.13), which were thin, green, and had green rootlets.

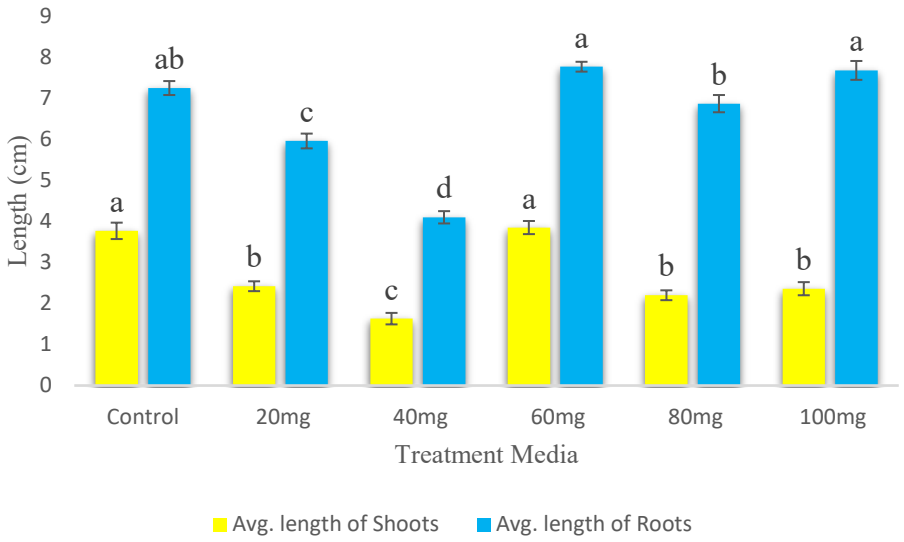

Figure 15: Effects of Putrescine on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

Compared to the control and other Put-induced cultures, the plants grown in the 40 mg l^{-1} Put-induced medium had fewer roots (2.4±0.16), and shorter roots (4.1±0.15cm). These roots were thin, green, and had white rootlets. However, the plants grown in the 60 mg l^{-1} Put-induced medium demonstrated the longest roots (7.77±0.12cm), which were green. In contrast, the highest number of roots (3.5±0.17) was observed in plants cultured in the 80 mg l^{-1} Put-induced medium compared to other Put-induced media. There was only a minor difference in the length of roots (6.87±0.21cm). In the medium with the highest concentration of Put (100 mg l^{-1}), the number of roots (2.5±0.17) decreased, but the length of the roots (7.68±0.23cm) increased.

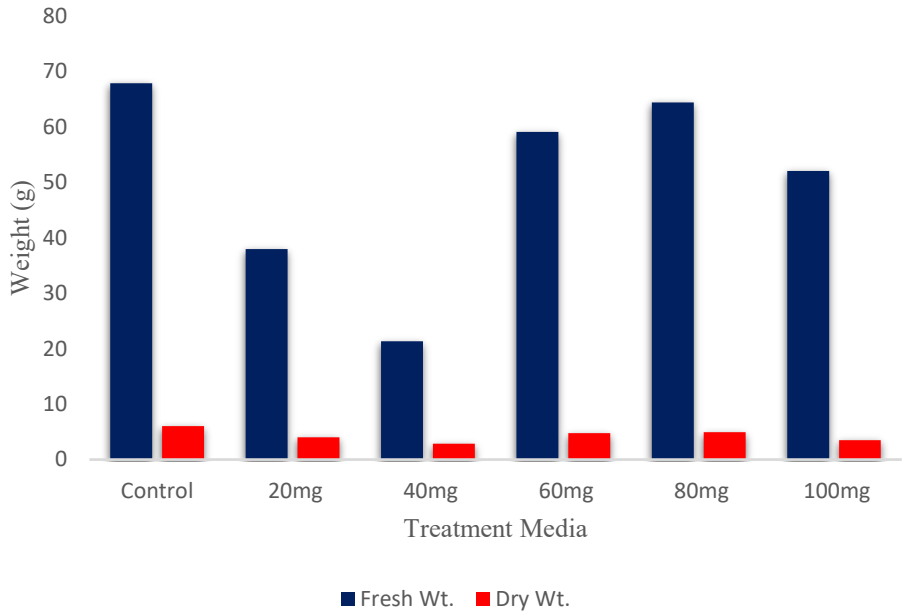

Figure 16: Effects of Putrescine on the total biomass production in *in vitro* cultures of *A. triplinervis*

4.5.1.3.4. Biomass Production

The plants harvested from the control medium showed the highest biomass production (**Figure 16**). These plants' fresh and dry weights were higher than those from the 20 and 40 mg l^{-1} Put-induced media. Reduced biomass production was observed in plants formed in 20 mg l^{-1} Put-induced medium. Plants formed in 40 mg l^{-1} Put-induced medium showed decreased fresh and dry weights than those formed in control medium and other Put-induced media. However, 60 mg l^{-1} Put-induced medium showed a sudden surge in biomass production compared to medium containing lower concentrations of Put. The highest biomass production among Put-induced media was found in the 80 mg l^{-1} Put-induced medium. At 100 mg l^{-1} Put-induced medium, the harvested plants showed a decrease in biomass production.

In the present studies on *A. triplinervis* the application of Put in the MS medium increased the plant's leaf area and shoot length. However, the highest

biomass production, and the production of the highest number of shoots and roots were found in the control medium. The effect Put in *in vitro* morphogeneic responses was reported earlier in several plants; the application of Put increased the biomass production of *Beta vulgaris* and *Tagetes patula* (Bais *et al.,* 2000); enhanced the biomass production of *Thymus vulgaris*(Abd-Elbar *et al.,* 2019); enhanced callus growth of *Allium jesdianum* (Yazdanian *et al.,* 2022); enhanced root growth of *Cichorium intybus* (Bais *et al.,* 1999); enhanced shoot number of *Cichorium intybus* (Bais *et al.,* 2001) and enhanced rooting of *Cynara scolymus* (Saos and Hourmant, 2001).

4.5.1.4. Tryptophan

Tryptophan is an essential amino acid that actively participates in the primary and secondary metabolism pathways. Tryptophan (Trp) is a precursor for synthesizing various secondary metabolites, especially the auxins (IAA).

In the present study, the following concentrations of Tryptophan 20, 40, 60, 80 and 100 mg l^{-1} were supplemented in MS medium to study the its effect on *in vitro* morphogenic responses of *A. triplinervis* (**Table 13 & Plate 10**).

4.5.1.4.1. Leaves

The plants grown in the MS basal medium(control) had 10±0.7 green leaves per shoot (**Figure 17**), with a leaf area of 2.1255±0.16cm^2 (**Figure 18**). Conversely, plants grown in a nutrient medium supplemented with 20 mg l^{-1} had 14±0.7 bright dark green leaves per shoot, but the leaf area was reduced to 1.553±0.13cm^2. Plants grown in nutrient medium supplemented with 40 mg l^{-1} Trp had 12±0.7 dark green leaves per shoot, with a leaf area of 1.4795±0.13cm^2.

14±0.7 light green leaves per shoot were formed with a leaf area of 1.3686±0.13cm^2 in nutrient medium supplemented with 60 mg l^{-1} Trp. The number of leaves increased with an increase in the Trp concentration. In a nutrient medium supplemented with 80 mg l^{-1} Trp, 10±0.7 light green leaves were formed per shoot, with a leaf area of 1.437±0.09cm^2.

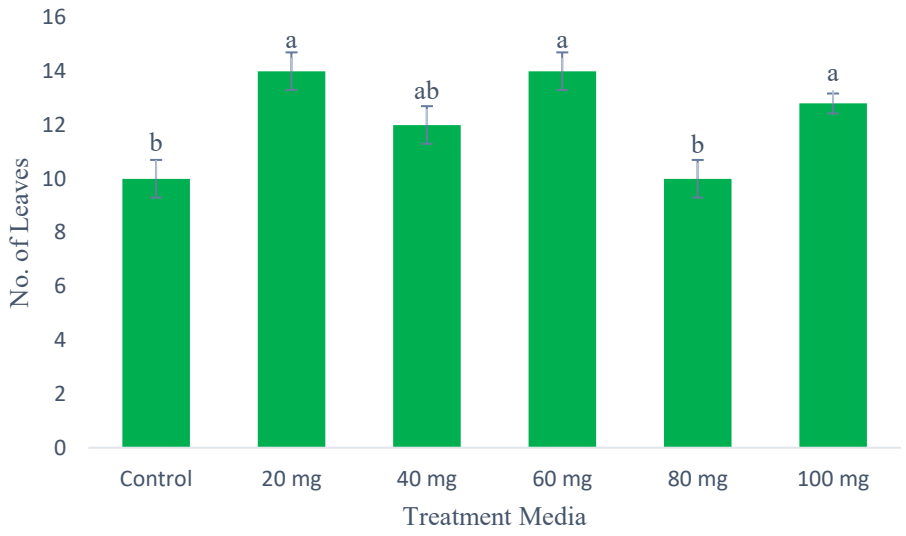

Figure 17: Effects of Trp on the no. of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE

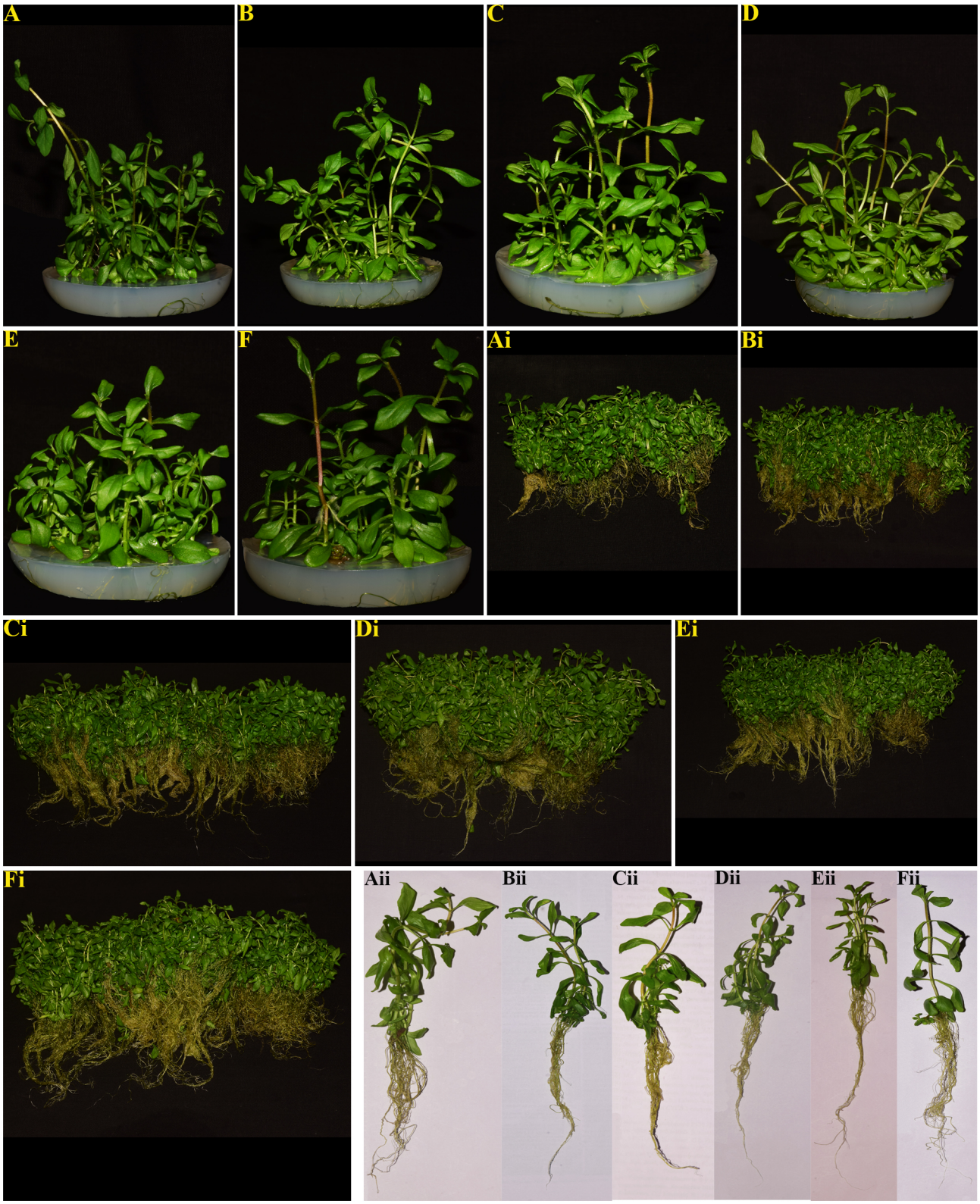

Plate 10: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 20 mg l^{-1} Tryptophan, **C)** 40 mg l^{-1} Tryptophan, **D)** 60 mg l^{-1} Tryptophan, **E)** 80 mg l^{-1} Tryptophan, **F)** 100 mg l^{-1} Tryptophan. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Fi**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Fii**.

Table 13: Effects of Tryptophan in morphogenic responses of *in vitro* cultures of *A. triplinervis*.

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	2.1255±0.16a	10±0.7b	2.5±0.17a	2.8±0.18c	3.6±0.16c	7.08±0.16d	84.272	6.2401
20 mg	1.553±0.13a	14±0.7a	1.8±0.20b	3.79±0.18b	5.4±0.70b	8.09±0.18c	83.8901	6.636
40 mg	1.4795±0.13a	12±0.7ab	1.9±0.23ab	3.79±0.15b	3.5±0.16c	8.89±0.19b	81.2555	6.7021
60 mg	1.3686±0.13a	14±0.7a	2.3±0.15ab	4.71±0.14a	6.8±0.33a	10.72±0.18a	99.7516	7.1805
80 mg	1.437±0.09a	10±0.7b	2.2±0.20ab	2.8±0.17c	6.8±0.33a	10.91±0.16a	74.7332	5.3694
100 mg	2.587±1.05a	12.8±0.37a	2±0.21ab	2.72±0.14c	6.3±0.26ab	7.5±0.28d	73.7076	5.978

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.

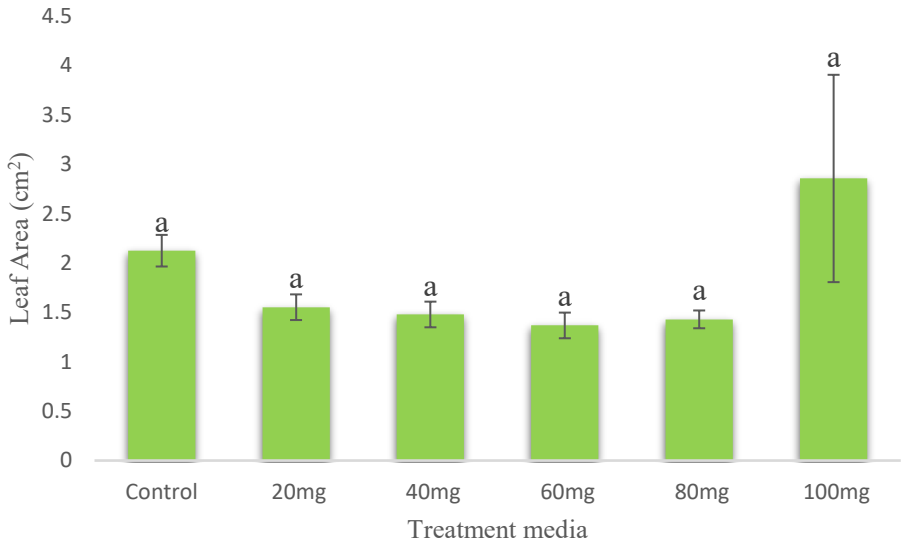

Figure 18: Effects of Trp on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE

In the nutrient medium supplemented with 100 mgl^{-1} Trp, 12.8±0.37 slightly dark green leaves with a leaf area of 2.587±1.05cm^2 were formed per shoot. The leaves of these plants had broad lamina and short petioles.

4.5.1.4.2. Shoots

In the control medium, the shoot number per culture was 2.5±0.17 (**Figure 19**). The average shoot length was 2.8±0.18cm (**Figure 20**) and the shoots were pale green. In the nutrient medium supplemented with 20 mg l^{-1} Trp, the shoot number (1.8±0.20) was lower than in the control medium. The stems were pale brownish in colour. In the nutrient medium supplemented with 40 mg l^{-1} Trp shoot number per culture was 1.9±0.23. The length of the shoots showed no significant variation, and the shoots were green.

In the nutrient medium supplemented with 60 mg l^{-1} Trp, a rise in shoot number (2.3±0.15) was observed. The average shoot length of the plants was 4.71±0.14cm. The stem's basal portion was pale green, while a reddish tinge was visible from the middle to the tip.

A slight decrease was observed in the number of shoots (2.2±0.20) and their length (2.8±0.17cm) in the medium supplemented with 80 mg l^{-1} Trp, compared to the plants formed in medium supplemented with 60 mg l^{-1} Trp. The shoots were pale green, and their internodes were shorter. Reduced shoot number (2±0.21) was observed in medium supplemented with 100 mg l^{-1} Trp. The average shoot length was 2.72±0.14 cm.

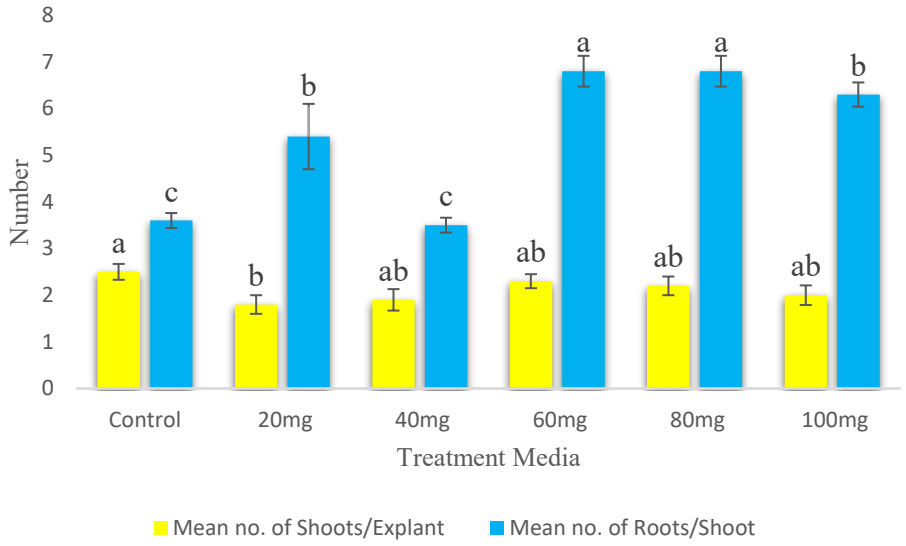

Figure 19: Effects of Trp on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

Roots

It was found that the plants formed in the control medium possess roots that are not only fewer in number but also shorter in length, measuring around 3.6±0.16 and 7.08±0.16cm, respectively (**Figure 20**). In contrast, Trp-induced cultures showed a more elaborate root system with slender green roots and whitish rootlets. Compared to the plants formed in the control medium, the plants formed

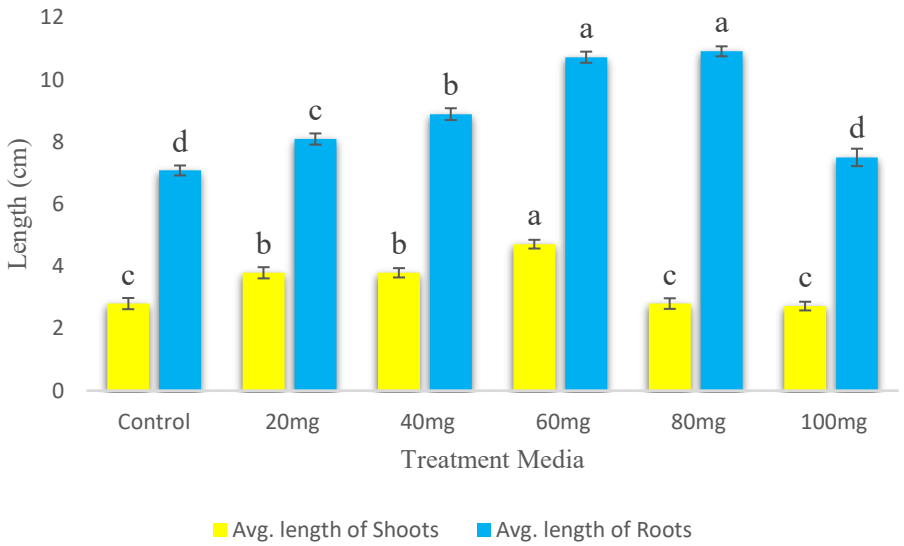

Figure 20: Effects of Trp on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

in the nutrient medium supplemented with 20 mg l^{-1} Trp produced a more significant number (5.4±0.70) of longer roots (8.09±0.18cm) (**Figure 19**).

In the nutrient medium supplemented with 40 mg l^{-1} Trp, less number of roots were formed (3.5±0.16), with an average root length of 8.89±0.19 cm. Nutrient medium supplemented with 60 mg l^{-1} Trp, was effective in promoting plant growth. The plants formed in this medium had an average of 6.8±0.33

roots per plant, with a maximum root length of 10.72±0.18cm. The roots formed in this medium were thin and green, and the root system was extensively branched with many delicate rootlets.

In the present study, supplementing the nutrient medium with the Trp was found to be useful in promoting plant growth. 80 mg l^{-1} of Trp in the medium was found to be the most effective in promoting root growth. In this medium, the plants had the highest number (6.8±0.33) and longest roots (10.91±0.16cm). The plants that were grown in the medium containing 100 mg l^{-1} Trp showed a significant decrease in both the number of roots (6.3±0.26) and their length (7.5±0.28cm) in comparison to the plants grown in the medium containing 80 mg l^{-1} Trp.

4.5.1.4.4. Biomass Production

The plants formed in the control medium and the plants formed in the nutrient medium supplemented with Trp exhibited significant differences in fresh and dry weights, with the most substantial biomass observed in nutrient the medium supplemented with 60 mg l^{-1} Trp (**Figure 21**). These findings suggest that Trp may hold promise to optimize biomass production in plant growth. However, in nutrient medium supplemented with higher Trp concentration (80 and 100 mg l^{-1} Trp) a significant decline in biomass production was observed.

In the present studies, supplementing the optimal concentration of Trp in the medium was found to have enhancing effects on biomass production, shoot induction, root induction and root elongation. Similar results were

reported earlier in many plants like the external supply of Trp to the medium enhanced the shoot regeneration in *Hypericum perforatum* (Erland & Saxena, 2019); Trp enhanced cell growth in cell cultures of *Catharanthus roseus* (Taha *et al.,* 2009) and Trp enhanced the production of a large number of micro shoots in the cultures of *Nasturtium officinale* (Klimek-Szczykutowicz *et al.,* 2021).

4.5.1.5. MS Medium Strength

Most plant tissue cultures were carried out in the MS (Murashige & Skoog, 1962) medium, a widely accepted culture medium (Fadel *et al.,* 2010). The higher salt content was the specialty of MS medium, and it contains higher

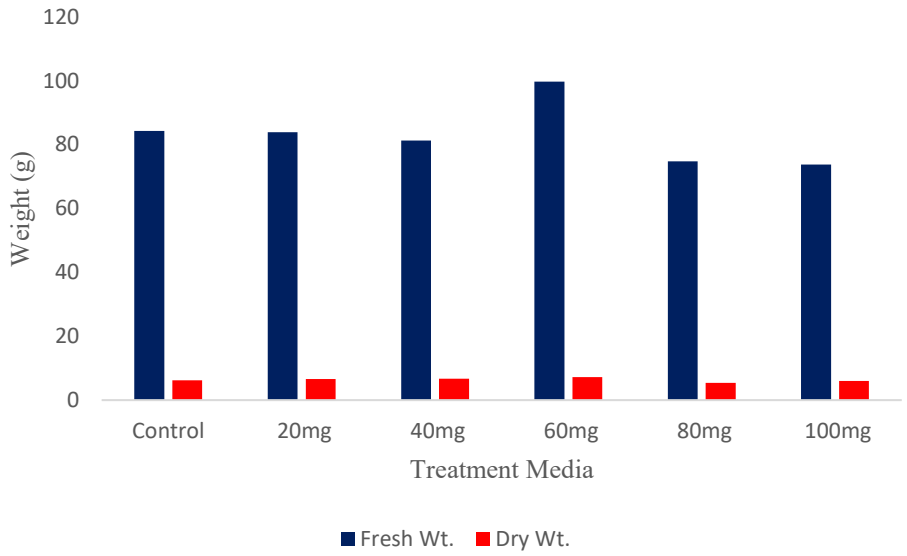

Figure 21: Effects of Tryptophan on the total biomass production in *in vitro* cultures of *A. triplinervis*

concentrations of nitrogen, potassium, and micronutrients like boron and manganese (Cohen, 1995). All the constituents in the media were essential for the growth and development of the plants. In the present study, different MS media strengths were applied as abiotic stress signals to the *in vitro* cultures of *A. triplinervis*. The following strengths of MS media were

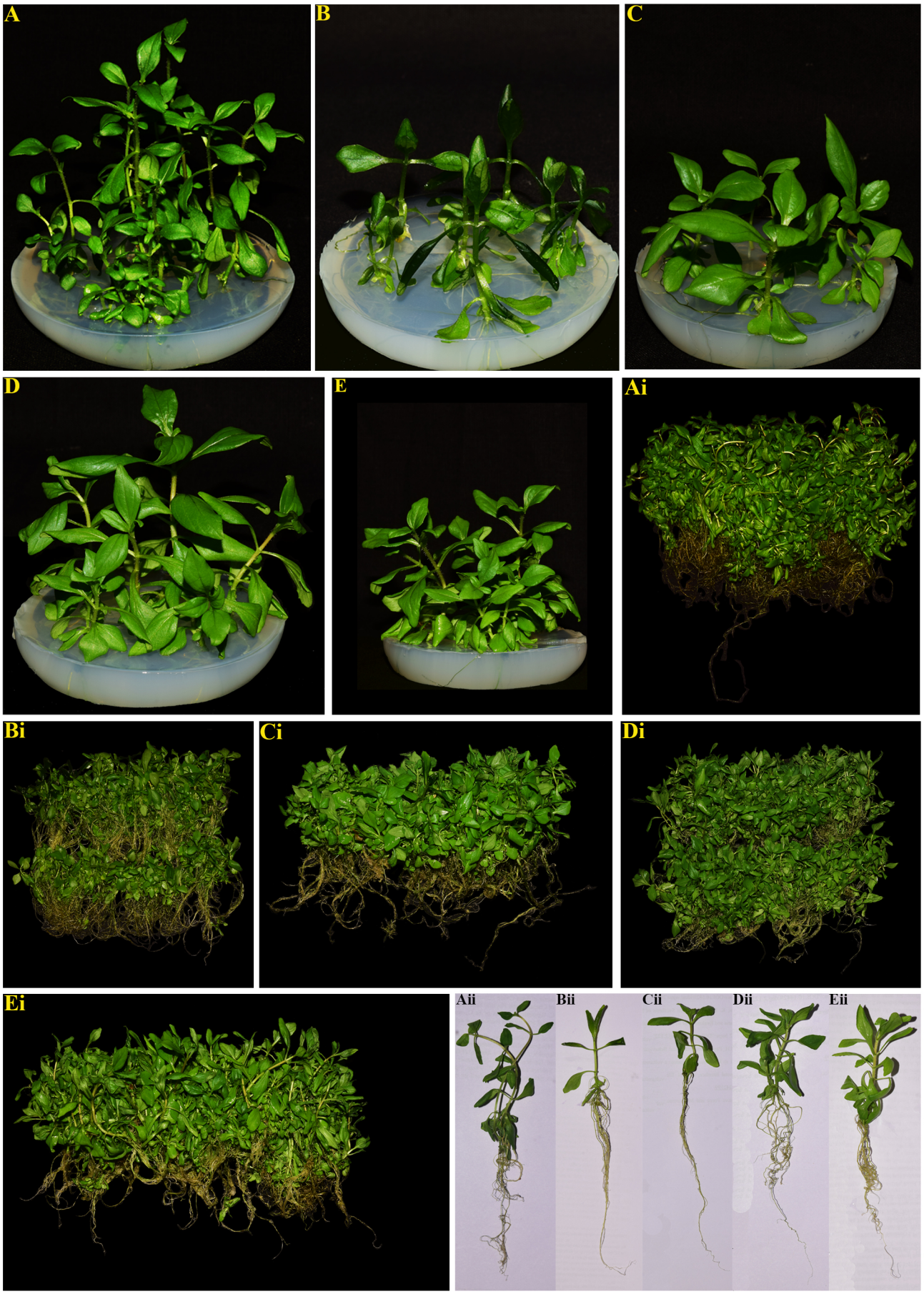

Plate 11: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** Full MS l^{-1} (control), **B)** $\frac{1}{6}^{th}$ MS l^{-1}, **C)** $\frac{1}{4}^{th}$ MS l^{-1}, **D)** ½ MS l^{-1}, **E)** $\frac{3}{4}^{th}$ MS l^{-1}. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Ai - Ei**. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Aii - Eii**.

used for the study: 1/6 MS l^{-1}, ¼ MS l^{-1}, ½ MS l^{-1}, ¾ MS l^{-1} and full MS l^{-1} (control). The effects of different MS media strengths in the morphogenic responses of *A. triplinervis* was evaluated with the help of various growth parameters (**Table 14 & Plate 11)**.

4.5.1.5.1. Leaves

In the present study, the full MS l^{-1} medium was used as the control. The plants formed in full strength MS medium, had 10.2±1.35 green leaves per shoot (**Figure 22**) and had a leaf area of 1.254±0.14cm^2 (**Figure 23**). However, the plants formed in the 1/6 MS l^{-1} medium had 8.2±0.66 pale green leaves per shoot and the lowest leaf area (1.146±0.15cm^2) compared to plants formed in other other strengths of MS media. Some of the leaves in this medium showed hyperhydricity. The plants formed in the ¼ MS l^{-1} medium had 8±0.7 light green-coloured leaves per shoot, tips and sides of the leaves were slightly yellowish. The leaf area (1.807±0.19cm^2) was high compared to the control and 1/6 MS l^{-1} media, but some of the leaves showed hyperhydricity. The plants formed in ½ MS l^{-1} medium had 9±1 pale green or green-coloured leaves per shoot and had the highest leaf area (1.999±0.13cm^2) compared to the other MS strength-induced media. The plants formed in the ¾ MS l^{-1} medium had 10.6±4 pale green-yellowish leaves per shoot, with a lower leaf area (1.33±0.12cm^2) than plants formed in the ½ MS l^{-1} medium.

Table 14: Effects of MS medium strength in morphogenic responses of *in vitro* cultures of *A. triplinervis*.

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
Full MS (control)	1.254±0.14b	10.2±1.35a	2.1±0.23a	3.81±0.14a	4.2±0.33a	5.98±0.16c	45.7631	4.1062
⅙ MS	1.146±0.15b	8.2±0.66a	1.5±0.17a	1.005±0.15c	3.1±0.50ab	6.95±0.20b	24.8858	1.8726
¼ MS	1.807±0.19a	8±0.7a	1.7±0.15a	1.49±0.15b	3.±0.29b	7.16±0.14ab	20.5212	1.7838
½ MS	1.999±0.13a	9±1a	1.6±0.16a	1.48±0.11b	2.5±0.17b	7.32±0.27ab	37.5943	2.5582
¾ MS	1.33±0.12b	10.6±4a	2.1±0.23a	1.88±0.13b	2.9±0.38b	7.7±0.19a	38.3122	3.1713

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.

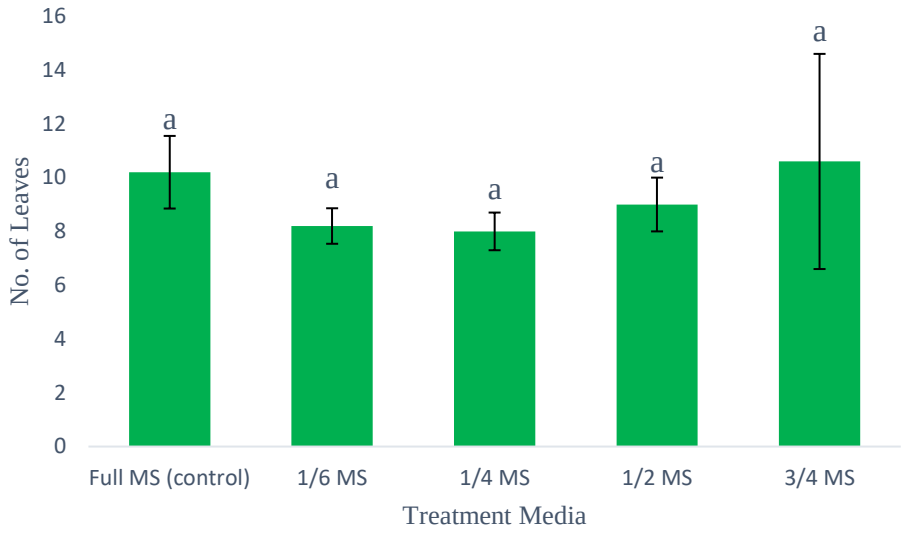

Figure 22: Effects of MS media strength on the no. of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

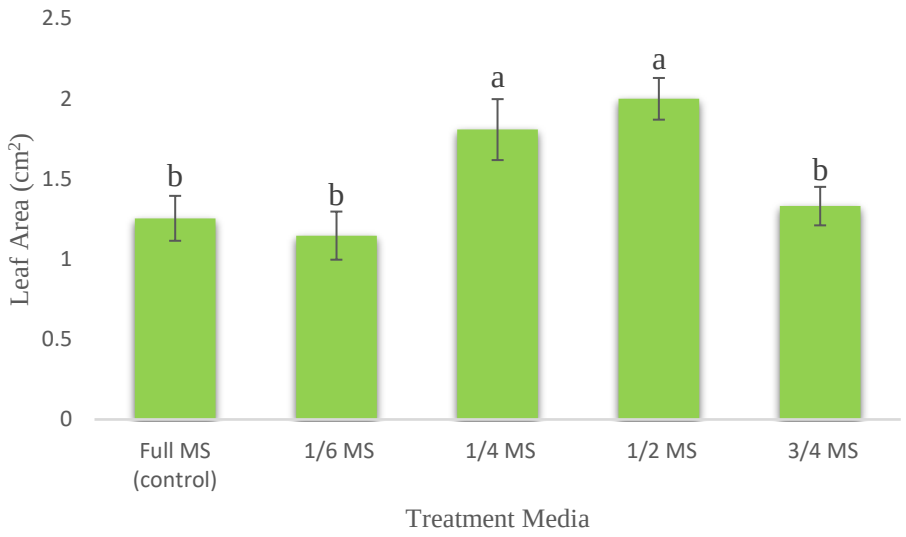

Figure 23: Effects of MS media strength on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

4.5.1.5.2. Shoots

In this study, different concentrations of Murashige and Skoog (MS) media were used to observe their effects on the growth and development of plants. The highest number of shoots (2.1±0.23) (**Figure 24**) was formed in full-strength MS l^{-1} medium, and the shoot length (3.81±0.14cm) (**Figure 25**) was also found highest in this medium. Less number of shoots (1.5±0.17) were formed in 1/6 MS l^{-1} medium, and in this medium the growth of the secondary shoot was either stunted or absent. These plants had a shoot length of 1.005±0.15cm, and their stems were pale green or whitish-green, with some of the shoots showing hyperhydricity. Hyperhydricity condition may be due to the abundance of ammonium ions (Pâques, 1991).

A slightly higher number of shoots (1.7±0.15) were formed in ¼ MS l^{-1} medium than in 1/6 MS l^{-1} medium, but their shoot lengths were shorter (1.49±0.15cm). The stems of these plants were whitish-green, and some of the shoots had a pale brown colour at the bottom. In contrast, the plants (shoot number - 1.6±0.16) formed in ½ MS l^{-1} medium had shoot length (1.48±0.11cm) similar to the plants formed in the ¼ MS l^{-1} medium. The stem colour of these plants ranged from whitish-green to pale red.

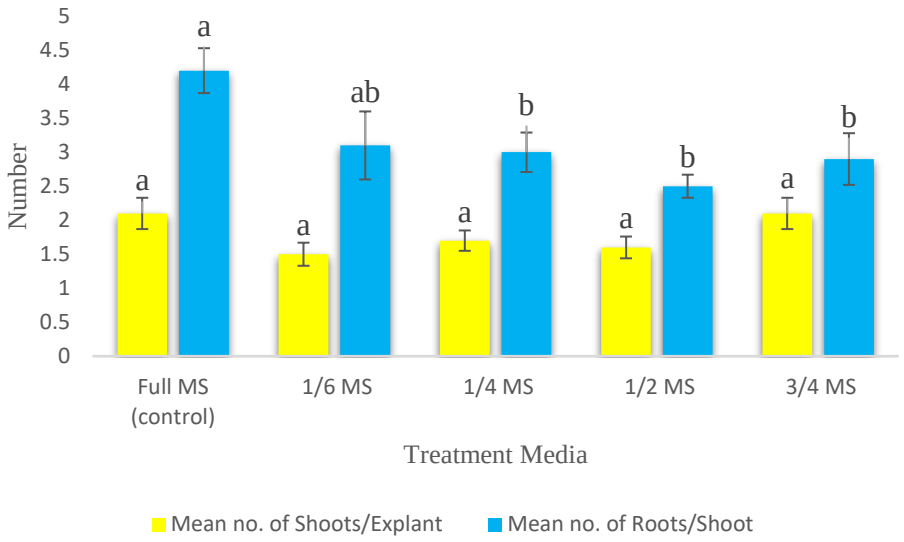

Figure 24: Effects of MS media strength on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

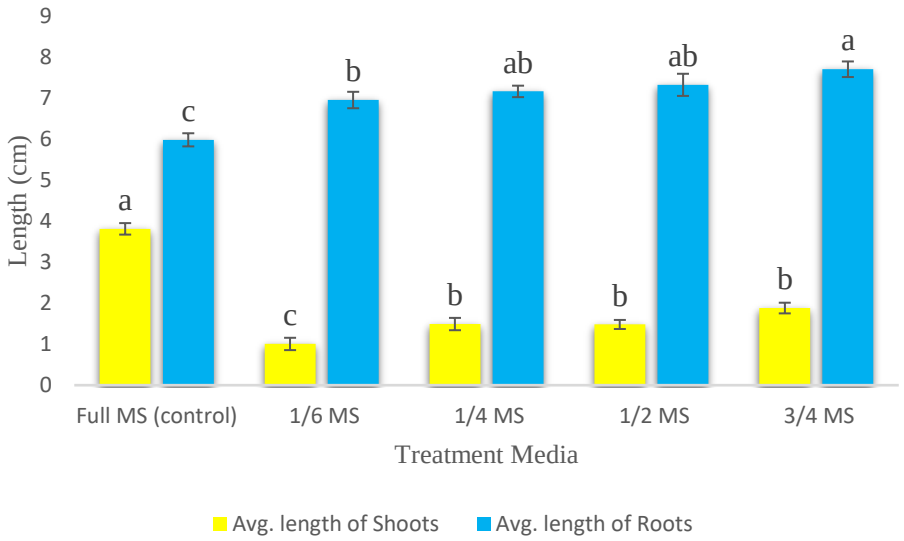

Figure 25: Effects of MS media strength on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

The plants formed in ¾ MS l^{-1} medium had a shoot number (2.1±0.23) similar to those formed in the control medium and higher than the plants formed in other lower strengths of MS media. These plants also showed a gradual increase in shoot length (1.88±0.13cm), and their stem colour ranged from whitish-green to pale red.

4.5.1.5.3. Roots

The plants formed in the control medium had the highest number of roots (4.2±0.33) (**Figure 24**) among the different cultures. However, the length of the roots of plants formed in the control medium (5.98±0.16cm) was the shortest compared to the roots of plants formed in other culture media. Plants grown in 1/6 MS l^{-1} medium had lengthier roots (6.95±0.20cm) (**Figure 25**) than the plants from the control medium. The roots were slender, green, and with white rootlets. In contrast, plants grown in 1/4 MS l^{-1} medium had fewer roots than those in 1/6 MSl^{-1} medium, but their roots were longer (7.16±0.14cm). Similar to the roots formed in 1/6 MS l^{-1} medium, these roots were slender, green, and with white rootlets.

The plants formed in 1/2 MS l^{-1} medium, produced the lowest number of roots (2.5±0.17) among the MS strength-induced cultures. However, compared to the lower two strengths of MS media, the roots in this medium were longer, measuring 7.32±0.27cm. All roots produced in this medium were green, including the rootlets. Plants grown in 3/4 MS l^{-1} medium had the longest roots among the MS strength-induced cultures, measuring 7.7±0.19cm. These roots were slender, green, and with white rootlets.

4.5.1.5.4. Biomass production

Results of the study showed that the full-strength MS l^{-1} medium induced the highest biomass production, both in terms of fresh and dry weights of harvested plants (Figure 16). Among all the different MS medium strengths tested, the the lowest biomass production was observed in ¼ MSl^{-1} medium.

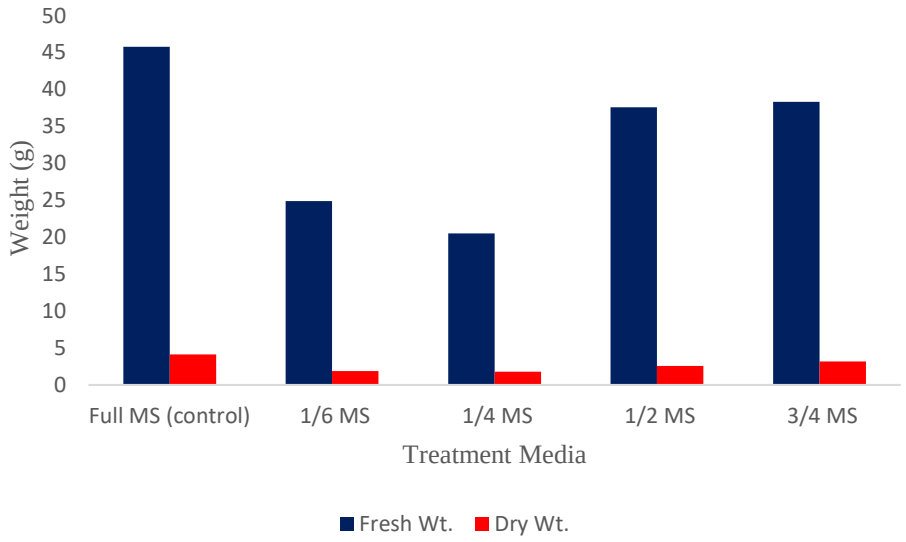

Figure 25: Effects of MS media strength on the total biomass production in *in vitro* cultures of *A. triplinervis*

The fresh weights of harvested plants from the ½ and ¾ MS l^{-1} medium were quite similar, but the dry weight of the plants harvested from the ¾ MS l^{-1} medium was slightly higher. Moreover, as the medium strength increased from ½ MS l^{-1} medium, there was a gradual rise in the fresh and dry weights of the plants were observed.

In the present studies full MS medium was the best for *in vitro* morphogenesis of *A. triplinervis*. This was corroborative with the results observed in the *in vitro* cultures of plants like *Tylophora indica* (Benjamin *et*

al., 1979) and *Pogostemon cablin* (Wan-Nurul-Hidayah *et al.* 2012) and *Rucus hypoglossum* L. (Abou-Dahab *et al.,* 2004). However, half-strength MS medium was found to be the best for *in vitro* morphogenesis in plants like *Mentha spicata* (Fadel *et al.,* 2010), *Ruscus hypoglossum L*. (Abou-Dahab *et al.,* 2004) and *Anacardium occidental*s L. (Mantell *et al.,* 1998; Boggetti *et al.,* 1999).

4.5.1.6. Jasmonic acid

Jasmonic acid (JA) is an endogenous signal molecule that induces plant resistance against diseases and herbivores and stimulates secondary metabolite pathways in plants (Wang *et al.,* 2004). JAs have several regulatory functions that affect the growth and development of plants, including axis elongation during embryogenesis, flower development, leaf senescence, root formation, growth of reproductive organs, and opening of stomata (Huang *et al.,* 2017; Lakehal & Bellini, 2019; Xu *et al.,* 2020; Ghorbel *et al.,* 2021).

In the present study, various concentrations of JA (1.25, 2.5, 5, 10, and 15 µM l^{-1}) were administered in the MS media to identify the morphogenic responses in the *in vitro* cultures of A. *triplinervis* (**Table 15 & Plate 12**).

4.5.1.6.1. Leaves

In the present study, the plants formed in the control medium (MS basal medium) had an average of 10.6±0.4 light green leaves per shoot (**Figure 27**) and had a leaf area of 1.7275±0.14 cm^2 (**Figure 28**). However, the plants formed in the nutrient medium supplemented with 1.25 µM l^{-1} JA, produced 11.6±0.92 dark green leaves on each shoot, with a reduced leaf area

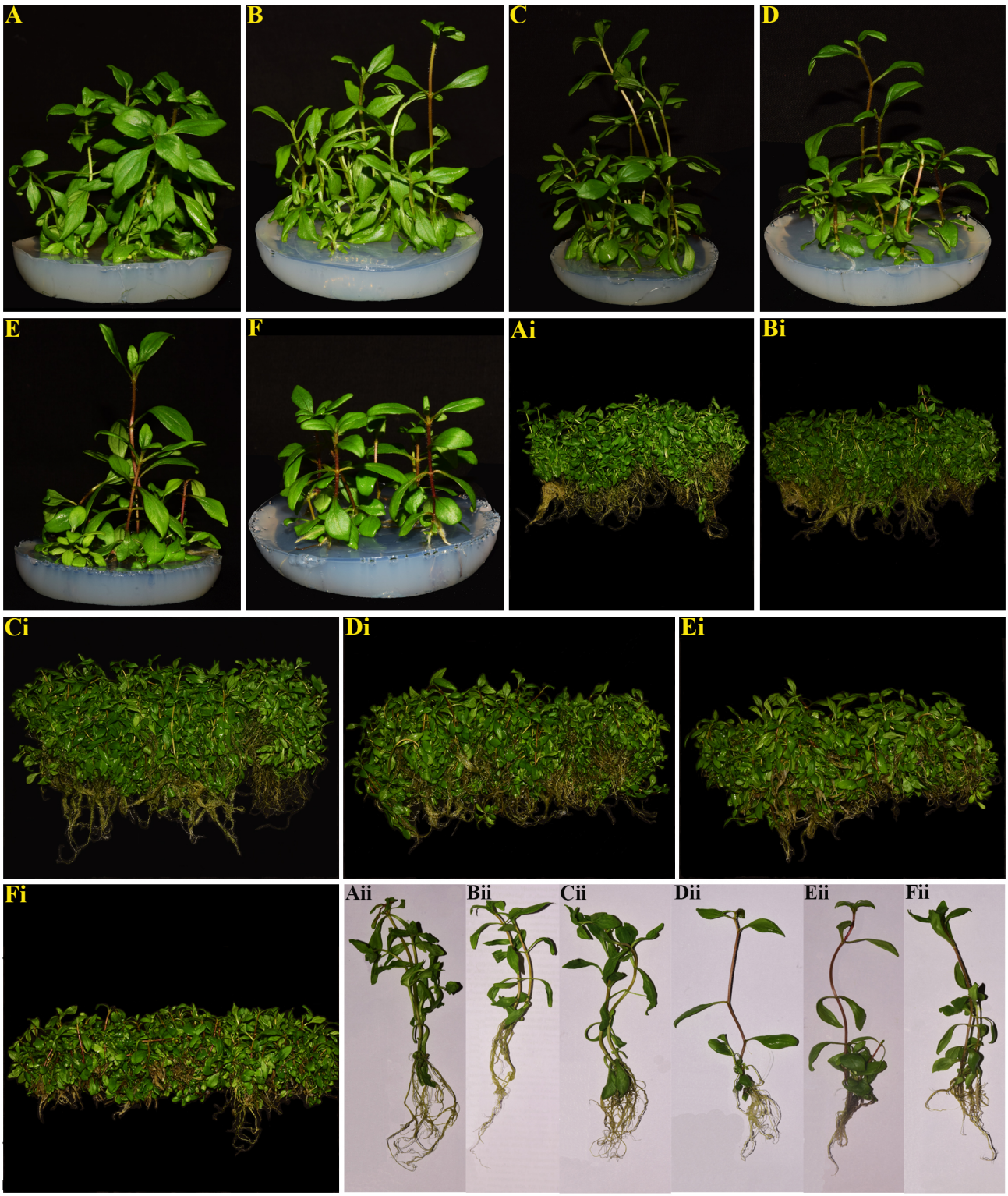

Plate 12: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 1.25 µM l^{-1} Jasmonic acid, **C)** 2.5 µM l^{-1} Jasmonic acid, **D)** 5.0 µM l^{-1} Jasmonic acid, **E)** 10 µM l^{-1} Jasmonic acid, **F)** 15 µMl^{-1} Jasmonic acid. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Ai - Fi**. Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Aii - Fii**.

of 1.221±0.14 cm^2. The lower leaves of these plants were arranged alternately and appeared thick and brittle. Occasionally, twin leaves were also observed. The application of 2.5 µM l^{-1} JA resulted in the plants with the highest leaf area of 1.957±0.15cm^2 and possessing 12±0.7 green leaves per shoot. However, in this medium some malformed leaves were also formed. Plants formed in nutrient medium supplemented with 5.0 µM l^{-1} JA had 10.6±0.4 small leaves (leaf area - 1.28795±0.09cm^2) per shoot. The leaves were green and slightly yellowish-green coloured.

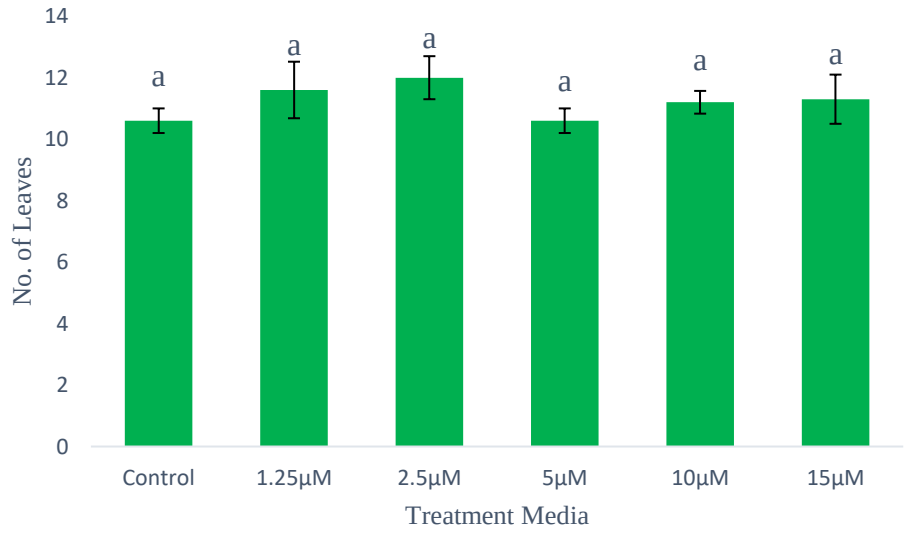

Figure 27: Effects of JA on the no. of leaves in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

Table 15: Effects of Jasmonic acid in morphogenic responses of *in vitro* cultures of *A. triplinervis*.

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	1.7275±0.14ab	10.6±0.4a	2.6±0.34a	3.94±0.16a	3.1±0.27c	5.87±0.13b	80.1641	5.9968
1.25 µM	1.221±0.14c	11.6±0.92a	2.3±0.34ab	3.79±0.16ab	3.6±0.16c	6.98±0.20a	69.5152	5.1664
2.5 µM	1.957±0.15a	12±0.7a	1.9±0.31ab	3.94±0.18a	5.5±0.22b	6.01±0.25b	58.7535	5.9272
5.0 µM	1.28795±0.09c	10.6±0.4a	2.4±0.34ab	3.32±0.18b	6.8±0.32a	4.87±0.17c	38.8108	3.7684
10 µM	1.448±0.10bc	11.2±0.37a	1.6±0.16b	2.42±0.21c	6.9±0.27a	4.64±0.27c	34.6579	3.0074
15 µM	1.207±0.10c	11.3±0.8a	2±0.21ab	2.38±0.19c	6.8±0.61a	1.83±0.11d	32.6839	4.0829

Means within a column followed by the same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.

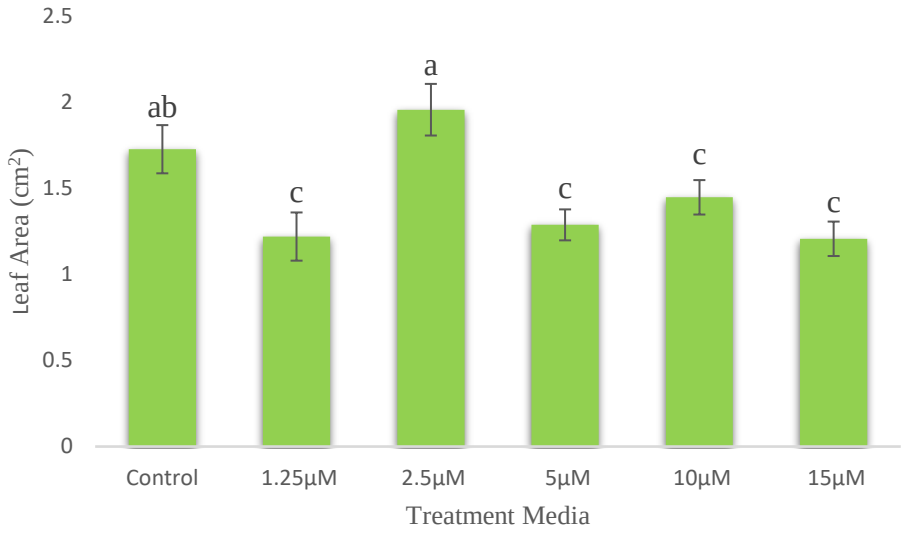

Figure 28: Effects of JA on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

The plants formed in nutrient medium supplemented with 10µM l^{-1} JA produced 11.2±0.37 dark green leaves per shoot, with a leaf area of 1.448±0.10cm^2. On the other hand, the plants formed in 15 µM l^{-1} JA-treated nutrient medium had the smallest leaf area (1.207±0.10cm2) among all the JA-treated cultures and had 11.3±0.8 yellowish green leaves per shoot.

4.5.1.6.2. Shoots

In the present experiment, 2.6±0.34 shoots **(Figure 29**) were formed per culture in the control medium with a length of 3.94±0.16cm. Shoot number slightly decreased (2.3±0.34) in cultures in which nutrient medium was supplemented with 1.25 µM l^{-1} JA and the average shoot length in this medium was 3.79±0.16cm (**(Figure 30**). The plants formed in this medium had pale reddish stems, while the plants formed in the control medium had pale green stems. Plants grown in the medium supplemented with 2.5 µM l^{-1}

JA had similar shoot lengths to the plants formed in the control medium, but their shoot number was significantly reduced (1.9±0.31). These plants had pale red stems and whitish-green shoots. The highest number of shoots (2.4±0.34) was formed in the medium supplemented with 5 µM l^{-1} JA, with a shoot length of 3.32±0.18cm. These plants had slender pale red or reddish-brown stems. The lowest number of shoots (1.6±0.16) with an average shoot length of 2.42±0.21cm was formed in the nutrient medium supplemented with 10 µM l^{-1} JA. These plants had pale red, or pale brown thick stems. The shortest plants (2.38±0.19cm) were formed in the nutrient medium supplemented 15 µM l^{-1} JA.

4.5.1.6.3. Roots

The lowest number of roots (3.1±0.27) ((**Figure 29**) was formed with an average root length of 5.87±0.13cm ((**Figure 30**) in plants formed in the control medium. The root system showed profuse branching. The roots were elongated, slender, green, with white rootlets. In the nutrient medium supplemented with 1.25 µM l^{-1} JA, a bunch of dark green roots with green rootlets were formed. In this medium, the number of roots increased to 3.6±0.16, and the root length (6.98±0.20cm) was the longest among all JA supplemented cultures. The roots formed in this medium were long, slightly thick and highly branched with prominent green root hairs.

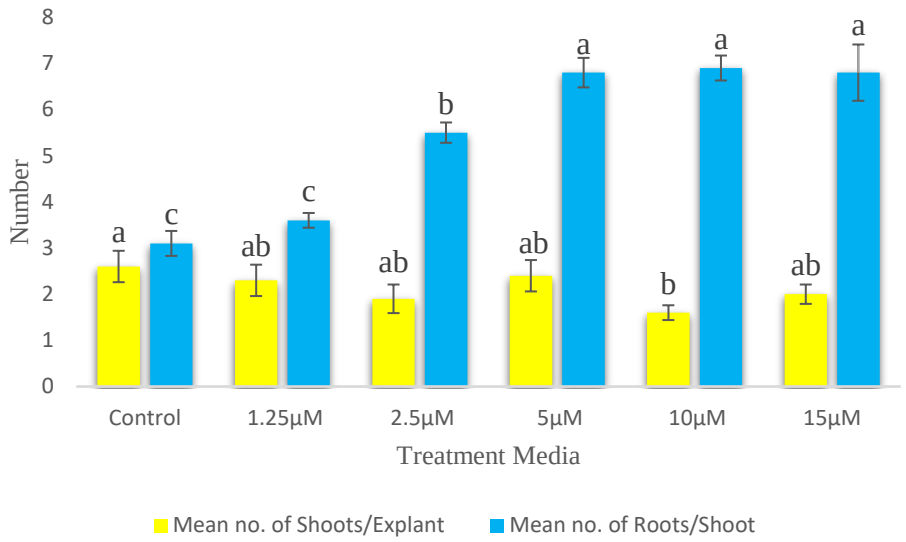

Figure 29: Effects of JA on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

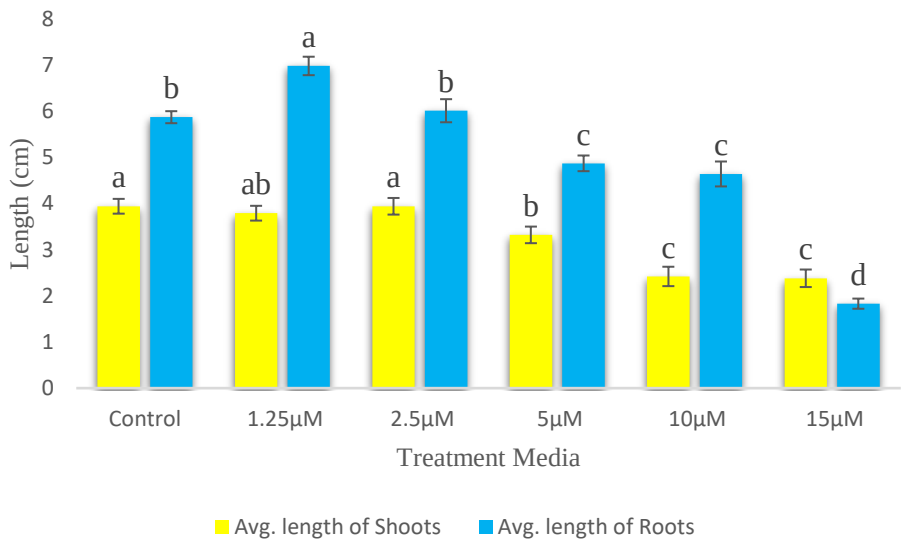

Figure 30: Effects of JA on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests.

In the medium supplemented with 2.5 µM l^{-1} JA, the number of roots increased to 5.5±0.22, with an average root length of 6.01±0.25cm. In the medium supplemented with 5 µM l^{-1} of JA, an increased number of roots (6.8±0.32) were formed. The roots (4.87±0.17cm) were shorter than plants from different cultures. The roots were white with abundant root hairs.

In the medium supplemented with 10 µM l^{-1} JA, the number of roots increased to 6.9±0.27 and the roots had an average root length of 4.64±0.27cm. The roots were thin and green, with root hairs. The root system was branched, and the basal portion of the roots appeared slightly thicker.

The highest number of roots (6.8±0.61) were formed in the medium supplemented with 15 µM l^{-1} JA. Roots, however, were short (1.83±0.11cm). Roots were thick, green-coloured, and had white rootlets, and the root system was not much branched.

4.5.1.6.4. Biomass Production

The presence of JA had an adverse impact on biomass production. The fresh and dry weights of the plants subjected to JA treatment were notably lower than those of the control plants **(Figure 31)**.

Application of 0.5 mM JA increased primary root growth and lateral root growth of *Brassica napus* but decreased shoot dry weight (Farhangi-Abriz *et al.*, 2019). Similar results showing the effect of JA were reported earlier in many plants like *Momordica charantia* (Alisofi *et al.*, 2020) and *Triticum aestivum* (Ilyas *et al.*, 2017). 0.1 mM JA was found to elevate the

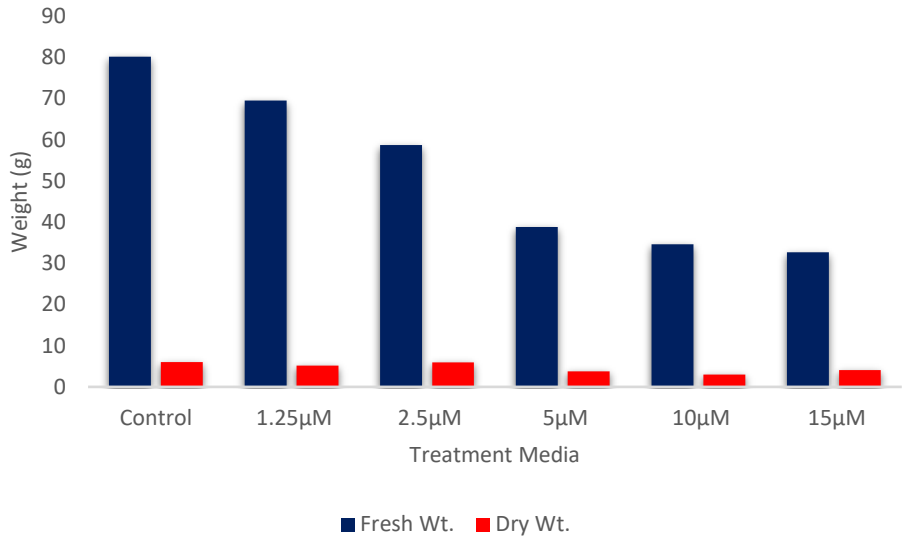

Figure 31: Effects of JA on the total biomass production in *in vitro* cultures of *A. triplinervis*

lengths of roots and shoots and dry weights in *Vicia faba* (Ahmad *et al.,* 2017). In *Catharanthus roseus*, the foliar application of JA enhanced the growth parameters (Al-Huqail & Ali, 2021). However, Hu *et al.* (2017) observed that JA application enhanced the leaf senescence in *Arabidopsis*.

Gadzovska *et al.* (2007) reported that the exogenous application of JA caused a negative impact on the growth and development in cell suspension cultures of *Hypericum perforatum*. They found that as the induction time increased, the cell viability was reduced (Gadzovska *et al.,* 2007). Creelman and Mullet (1995) and Krzyzanowska *et al.* (2011) found that cell growth was inhibited at higher concentrations of JA, and the biomass was reduced in *Glycine max* and *Mentha x piperita*. Similar to this observation, in the present study, it was found that as the concentration of JA in the medium increased, the growth and development of shoots were negatively affected, resulting in a reduction in biomass production in *A. triplinervis*.

4.5.1.7. Salicylic acid

Salicylic acid (SA) is an endogenous signalling molecule that reacts to biotic and abiotic stress in plants (Mendoza *et al.,* 2018). In the present study, five different concentrations of SA were applied in the MS medium. Their effects on the *in vitro* morphogenic responses of *A. triplinervis* were studied using various growth parameters **(Table 16** and **Plate 13)**. 1% $NaHCO_3$ solution was required for dissolving the SA due to its insolubility with water. The following concentrations of SA were used during the study; 10 µM l^{-1} – S1, 25 µM l^{-1} – S2, 50 µM l^{-1} – S3, 75 µM l^{-1} – S4 and 100 µM l^{-1} – S5. Also, individual concentrations of 1% $NaHCO_3$ solution were used (1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C1, 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C2, 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C3, 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C4 and 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C5) as the control for each treatment in addition to the MS basal medium control.

4.5.1.7.1. Leaves

In the experiment, the plants formed in the MS basal medium had 9.8±0.66 green leaves per shoot, and the leaf area was 1.039±0.11cm^2 (**(Figure 33**). Plants produced from the S1 medium had a comparable number of leaves (9.4±0.74) and area (1.087±0.07cm^2) to the control plants, while the plants from the C1 medium had a substantially higher leaf area (1.279±0.08cm^2) with 10.2±1.35 green leaves per shoot.

Plants formed in the S2 medium, developed 10.6±1.07 dark green leaves per shoot, which were smaller (0.9555±0.09cm^2) than those formed in S1 medium. Plants formed in C2 medium, developed 11.8±0.8 leaves per shoot, and had a large leaf area (1.5735±0.18cm^2) compared to all other tested media. The leaves at the top of the S2 plants were light green, while those at

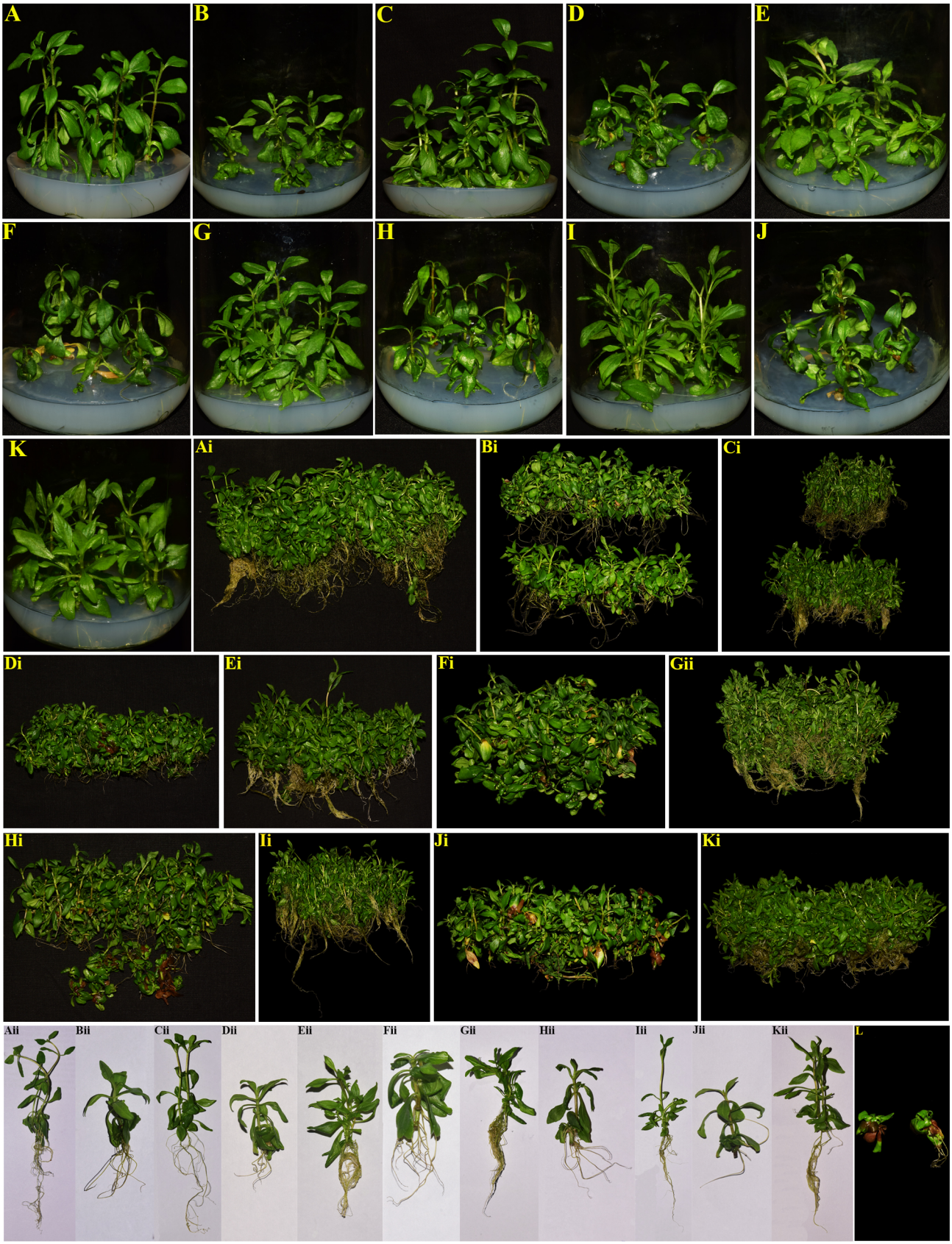

Plate 13: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 10 µM l^{-1} Salicylic acid, **C)** Control 1 (1 ml 1% NaHCO3 soln. l^{-1} MS Medium), **D)** 25 µM l^{-1} Salicylic acid, **E)** Control 2 (2.5 ml 1% NaHCO3 soln. l^{-1} MS Medium), **F)** 50 µM l^{-1} Salicylic acid , **G)** Control 3 (5.0 ml 1% NaHCO3 soln. l^{-1} MS Medium), **H)** 75 µM l^{-1} Salicylic acid, **I)** Control 4 (7.5 ml 1% NaHCO3 soln. l^{-1} MS Medium), **J)** 100 µM l^{-1} Salicylic acid, **K)** Control 5 (10 ml 1% NaHCO3 soln. l^{-1} MS Medium). Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Ki**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Kii**. **L)** Plants showing dried and curled leaves, short internodes and roots without rootlets.

Table 16: Effects of Salicylic acid in morphogenic responses of *in vitro* cultures of *A. triplinervis*

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	1.039±0.11bc	9.8±0.66a	2±0.21bcd	2.7±0.13a	4.4±0.27abc	6.28±0.17c	36.235	3.0112
10 µM SA	1.087±0.07bc	9.4±0.74a	1.5±0.17d	1.09±0.11d	3.4±0.16cde	4.01±0.17e	17.4699	1.8817
C1	1.279±0.08ab	10.2±1.35a	1.7±0.21cd	2.08±0.22b	3.2±0.29e	9.89±0.34a	30.2622	2.4599
25 µM SA	0.9555±0.09bc	10.6±1.07a	1.6±0.16d	1.67±0.11bc	5.3±0.26a	2.8±0.19f	22.9781	2.872
C2	1.5735±0.18a	11.8±0.8a	1.7±0.15cd	1.555±0.14c	4.9±0.43ab	5.93±0.14cd	30.4118	3.2114
50 µM SA	1.2115±0.09b	9±1.61a	1.5±0.17d	1.07±0.11d	3.7±0.40cde	1.8±0.25g	22.2467	1.8647
C3	1.289±0.13ab	11±1a	2.6±0.27a	2.04±0.12b	3.1±0.28e	6.06±0.20cd	31.0281	3.9381
75 µM SA	1.182±0.08b	12±0.7a	1.4±0.16d	2.03±0.12b	4.3±0.58abcd	2.78±0.13f	27.8128	2.8315
C4	1.197±0.13b	10±1.41a	2.3±0.15ab	2.56±0.20a	3.5±0.17cde	7.87±0.17b	27.0034	2.5405
100 µM SA	0.769±0.06c	9±1a	1.4±0.16d	1.83±0.15bc	4±0.33bcde	1.06±0.18h	26.0261	1.8172
C5	1.0565±0.16bc	12±0.7a	2.2±0.20ab	2.085±0.13b	3.3±0.26de	5.58±0.20d	54.626	4.6253

Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.
C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium.

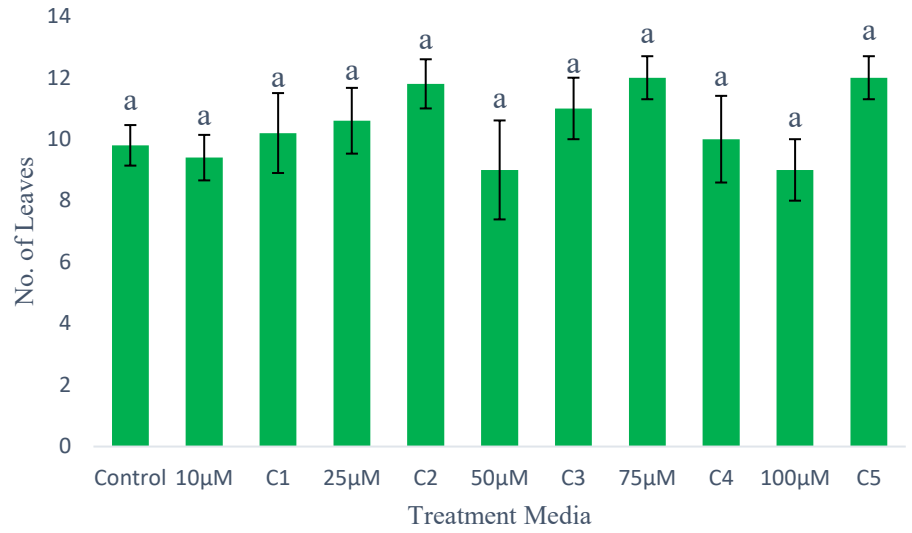

Figure 32: Effects of SA and 1% $NaHCO_3$ (C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium) on the no. of leaves in *in vitro* cultures of *A. triplinervis*.

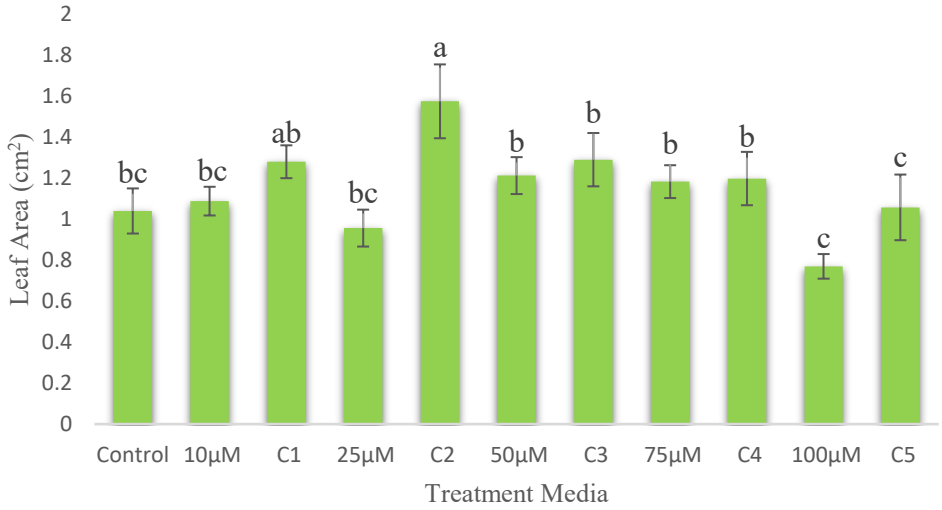

Figure 33: Effects of SA and 1% $NaHCO_3$ (C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium) on the leaf area in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

the bottom were dark green. In the S3 medium, the concentration of SA was high hence some plants formed in this medium had yellowish and dry lower leaves and some inoculated explants failed to grow. 9±1.61 number of leaves present per shoot. On average, the leaf area of the plants was 1.2115±0.09cm^2. The leaf area (1.289±0.13cm^2) of plants formed in C3 medium did not differ significantly from plants formed in S2 medium. But, the plants in the C3 medium has a leaf number of 11±1.

In the S4 medium, the plants produced 12±0.7 dark green leaves per shoot. The leaf area (1.182±0.08cm^2) was slightly reduced compared to the S3 medium. Some shoots had yellowish lower leaves that eventually dried up in the medium. The plants formed in the C4 medium had 10±1.41 green leaves per shoot with a leaf area of 1.197±0.13cm^2.

In the S5 medium, the plants produced 9±1 green or dark green leaves per shoot. The lowest leaf area (0.769±0.06cm^2) value was observed in this medium, and most of the lower leaves turned yellowish and dried up. On the other hand, the plants formed in the C5 medium had 12±0.7 green, healthy leaves per shoot with a leaf area of 1.0565±0.16cm^2.

4.5.1.7.2. Shoots

In this study, the growth of *A. triplinervis* plants was observed in different *in vitro* media. Shoot length was highest in the control medium, measuring 2.7±0.13cm long (**Figure 35**). Shoots formed in this medium were whitish-green. On the other hand, the S1 medium produced shorter shoots (1.09±0.11cm) and fewer shoots (1.5±0.17) (**Figure 34**), and the C1 medium

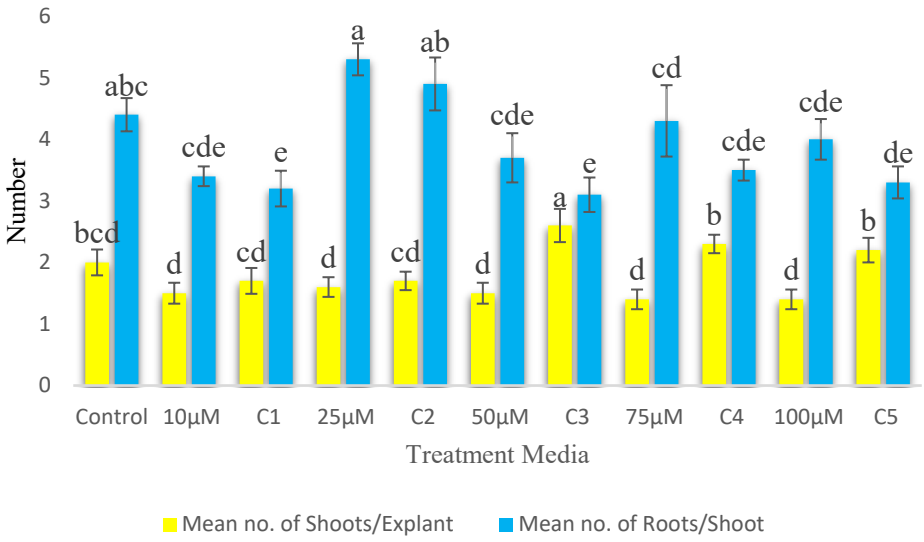

Figure 34: Effects of SA and 1% $NaHCO_3$ (C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium) on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

produced shoots that were the long (2.08±0.22cm) compared to the S1 medium. Internodes were long in the C1 medium.

Only a few shoots (1.6±0.16) were formed in the S2 medium the shoots were short in length (1.67±0.11bcm), with colour ranging from green to pale brown. Secondary shoots in this medium were often stunted or failed to grow. In C2 medium 1.7±0.15 shoots were formed, with a slightly lower length of shoots (1.555±0.14cm) than in the S2 medium. Shoots in the C2 medium ranged in colour from green to light green.

The plants formed in S3 medium produced fewer shoots (1.5±0.17) than the plants in C3 medium (2.6±0.27), and the length of their shoots (1.07±0.11cm) was shorter than that of the plants formed in C3

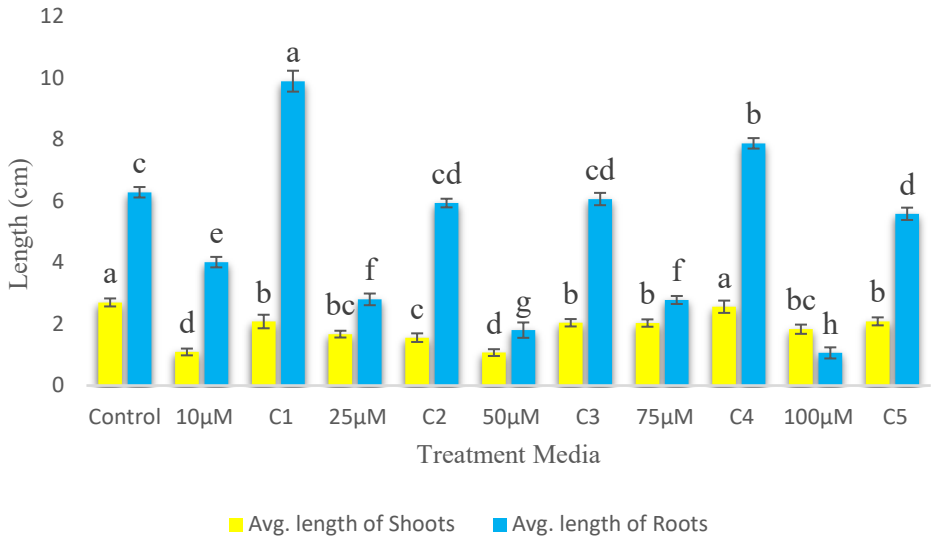

Figure 35: Effects of SA and 1% $NaHCO_3$ (C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium) on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

(2.04±0.12cm) medium. The plants formed in S3 medium had the shortest len gth among the SA-treated trials. The stems of the plants formed in S3 medium were brown or pale green, but the stems of the plants formed in C3medium were light green, with some having a pale red colouration in the lower portion of the stem.

There was a decrease in the number of shoots (1.4±0.16 - the lowest among all cultures) and shoot length (2.03±0.12 cm) in S4 medium, the shoot number (2.3±0.15) in C4 medium was higher with an average length of 2.56±0.20 cm. Plants formed in S4 medium had pale red-coloured stems, while C4 plants had mostly whitish-green stems.

Most of the explants did not grow in the S5 medium, and the secondary shoots either failed to grow or dried up. The number and length of shoots in S5

medium were very low compared to C5 and control media. The stem of the plants formed in S5 medium was pale red or green, with short internodes, while the stem of plants formed in C5 medium was pale green-coloured.

4.5.1.7.3. Roots

In the experiment, the plants formed in the control medium showed many roots (4.4±0.27) (**Figure 34**), but their length (6.28±0.17cm) was shorter than other cultures (**Figure 35)**. In the S1 medium less number (3.4±0.16), shorter (4.01±0.17cm) roots were formed in comparison to the control medium. Long, slender, green roots without rootlets were formed from the base of some of the shoots in S1 medium. On the other hand, roots were formed from all the shoots in the C1 medium, and the roots were long (9.89±0.34cm), thin, green coloured with white rootlets, but their number (3.2±0.29) was slightly less than the roots formed in S1 medium.

The plants formed in S2 medium had the highest number of roots (5.3±0.26) among all media containing SA, but their root length (2.8±0.19cm) was less than that formed in C2 medium (5.93±0.14cm). In S2 medium, the roots formed were creamy white or pale greenish and thin, while in C2 medium, the roots formed were slender green or pale green with whitish rootlets. In S3 medium, the number of roots produced by the plants was 3.7±0.40, whereas in C3 medium, the number of roots produced by the plants was somewhat lower (3.1±0.28). However, the root length was higher C3 medium (6.06±0.20cm) than S3 medium (1.8±0.25cm).

The number of roots (4.3±0.58) formed in plants grown in S4 medium was higher than in plants (3.5±0.17) formed in C4 medium. However, the lengthiest roots (7.87±0.17cm) were formed in C4 medium. The number of

roots of plants (4±0.33) formed in S5 medium was higher than plants (3.3±0.26). formed in C5 medium. Plants formed in S5 medium had the most petite root lengths among all the SA-induced cultures, with cream-coloured roots without rootlets. Roots formed in plants grown in C5 medium were green with white rootlets.

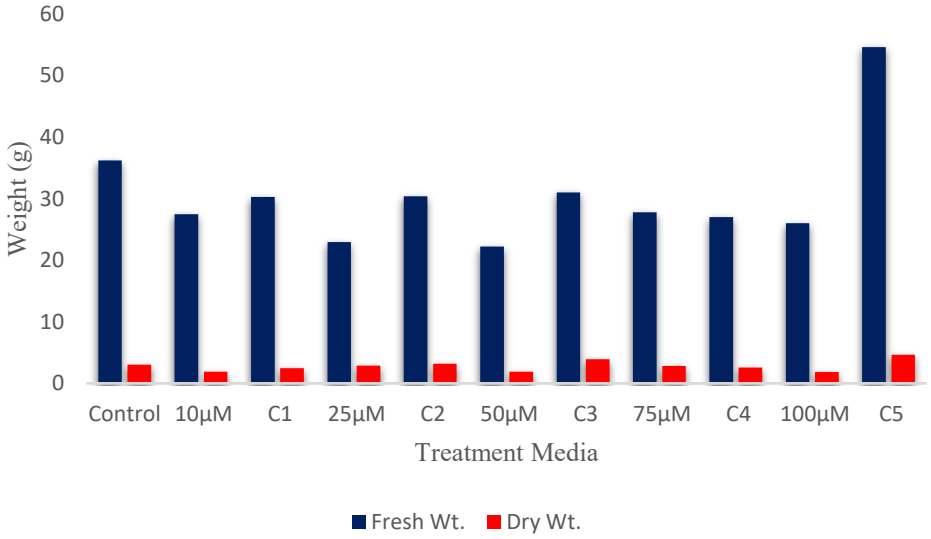

Figure 36: Effects of SA and 1% $NaHCO_3$ (C1 - 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C2 – 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C3 – 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium, C4 – 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium and C5 - 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium) on the total biomass production in *in vitro* cultures of *A. triplinervis*

The harvested plants formed in the control medium exhibited the second-highest biomass production (**Figure 36)**. The harvested plants formed in S1 medium showed a decrease in biomass production. On the other hand, the C1 medium produced plants with high biomass compared to S1 medium. The harvested plants from the S2 and C2 media had higher biomass than those formed in other media. Biomass production was higher in C3 medium than in S3 medium. There was no significant difference in biomass production in S4

and C4 media. In media containing higher concentrations of SA, biomass production was found to be considerably decreased. Higher biomass production was observed in plants formed in C5 medium than the plants formed in S5 medium.

Gadzovska *et al.,* (2013) earlier reported adventitious root formation in the SA supplemented *in vitro* cultures of *Hypericum perforatum*. Root growth and biomass were increased by SA in the root cultures of *Psoralea corylifolia* (Siva *et al.,* 2014). Shoot growth and production were higher with the effect of SA in the *in vitro* cultures of *Lavandula angustifolia* (Miclea *et al.,* 2020). SA promoted biomass accumulation of *Fagonia indica* (Khan *et al.,* 2019). SA increased the number of cells and dry biomass of *Achillea gypsicola* (Açikgöz *et al.,* 2019). However, SA inhibited the growth in *in vitro* cultures of *Stemona* sp. (Chaichana & Dheeranupattana, 2012).

In the present studies on *A. triplinervis* SA-induced media promoted the growth of roots. However, SA decreased shoot multiplication and growth. Furthermore, higher concentrations of SA in the medium resulted in stunted growth and decreased biomass production in the *in vitro* cultures of *A. triplinervis*.

4.5.1.8. Chitosan

The commercially produced deacetylated form of chitin is chitosan, a biopolymer found in the exoskeletons of insects and crustaceans, and in the fungal cell walls. The elicitation with the chitosan induced the production of enzymes participating in ROS scavenging systems such as catalase, SOD, peroxidase etc (Sathiyabama *et al.,* 2016).

MS media supplemented with five different concentrations (50 mg l^{-1} – S1, 100 mg l^{-1} – S2, 150 mg l^{-1} – S3, 200 mg l^{-1} – S4 and 250 mg l^{-1} – S5) of chitosan were prepared and nodal explants were inoculated in these media. Harvesting was done after 60 days of growth. Besides basal MS medium as the control, for each chitosan concentration a separate control medium was used. Because of the insolubility of the chitosan molecules in water, 1% acetic acid solution was required to dissolve them. So, 1% Acetic acid solution was also used as the control for chitosan-induced media, and the following concentrations were used as the individual controls: for 50 mg l^{-1} chitosan - 10 ml of 1% HAc soln, l^{-1} MS Medium (C1), 100 mg l^{-1} chit. – 20 ml 1% HAc soln, l^{-1} MS Medium (C2), 150 mg l^{-1} chi. – 30 ml 1% HAc soln, l^{-1} MS Medium (C3), 200 mg l^{-1} chit. – 40 ml 1% HAc soln, l^{-1} MS Medium (C4), 250 mg l^{-1} chit. – 50 ml 1% HAc soln, l^{-1} MS Medium (C5) (**Plate 14**).

Leaves

The study results showed that the control medium produced the best responses in all the selected parameters, except for the number of roots/shoots, as shown in **Table 17**. Plants grown in the control medium had the highest leaf area, at 1.494±0.22cm^2, and produced 10±0.7 green leaves per shoot (**Figure 37).**

Plants grown in the medium supplemented with 50 mg l^{-1} chitosan (S1) produced 9±0.44 yellowish-green leaves per shoot, with a leaf area of 1.363±0.07cm^2. In the 10 ml 1% HAc control medium (C1), the leaf area

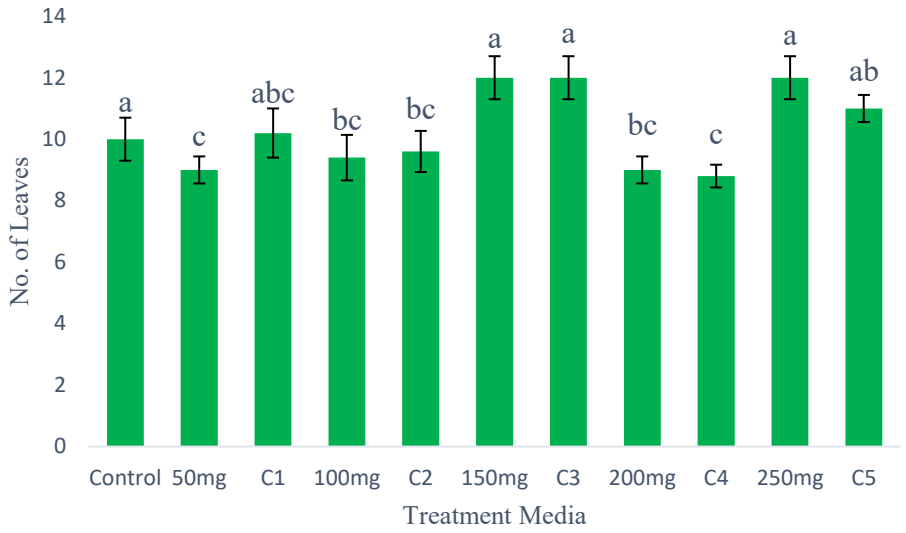

Figure 37: Effects of Chitosan on the no.of leaves in *in vitro* cultures of *A. triplinervis*; Chit., C1 – 10 ml 1% HAc soln, l^{-1} MS Medium, C2 – 20 ml 1% HAc soln, l^{-1} MS Medium, C3 – 30 ml 1% HAc soln, l^{-1} MS Medium, C4 – 40 ml 1% HAc soln, l^{-1} MS Medium, and C5 – 50 ml 1% HAc soln, l^{-1} MS Medium. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

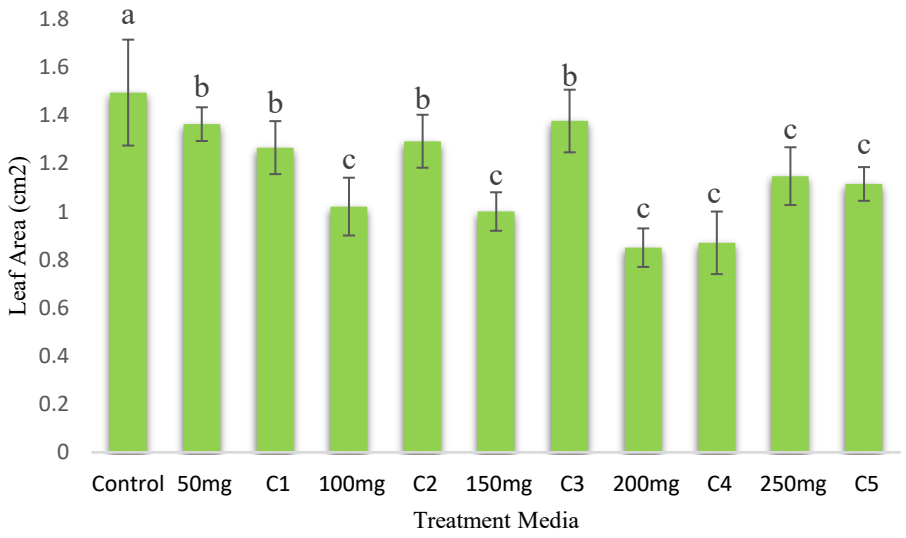

Figure 38: Effects of Chitosan on the leaf area in *in vitro* cultures of *A. triplinervis*; Chit., C1 – 10 ml 1% HAc soln, l^{-1} MS Medium, C2 – 20 ml 1% HAc soln, l^{-1} MS Medium, C3 – 30 ml 1% HAc soln, l^{-1} MS Medium, C4 – 40 ml 1% HAc soln, l^{-1} MS Medium, and C5 – 50 ml 1% HAc soln, l^{-1} MS Medium. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

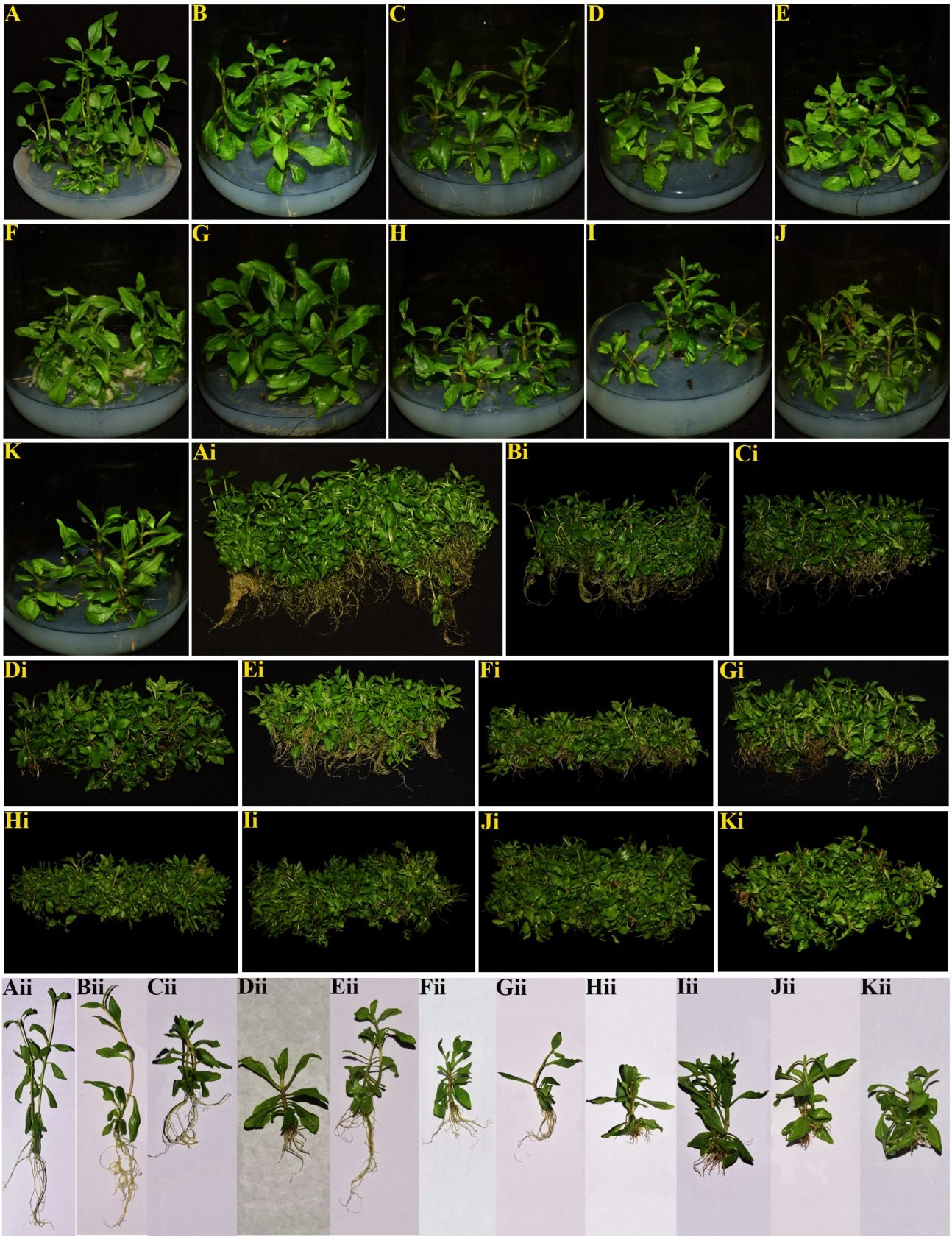

Plate 14: *In vitro* cultures of *A. triplinervis* in MS media containing; **A)** MS basal (control), **B)** 50 mg l^{-1} Chitosan, **C)** Control 1 (10ml 1% HAc soln. l^{-1} MS Medium), **D)** 100 mg l^{-1} Chitosan, **E)** Control 2 (20ml 1% HAc soln. l^{-1} MS Medium), **F)** 150 mg l^{-1} Chitosan, **G)** Control 3 (30ml 1% HAc soln. l^{-1} MS Medium), **H)** 200 mg l^{-1} Chitosan, **I)** Control 4 (40ml 1% HAc soln. l^{-1} MS Medium), **J)** 250 mg l^{-1} Chitosan, **K)** Control 5 (50ml 1% HAc soln. l^{-1} MS Medium). Harvested plants from different *in vitro* cultures of *A. triplinervis* after 60 days of growth period; **Ai - Ki**. Individual plants from different *in vitro* cultures of *A. triplinervis*; **Aii - Kii**.

Table 17: Effects of Chitosan in morphogenic responses of *in vitro* cultures of *A. triplinervis*

Stress treatments	Leaf area (cm^2)	No. of Leaves	Mean no. of shoot/explant (Mean±SE)	Avg. shoot length (cm) (Mean±SE)	Mean no. of root/shoot (Mean±SE)	Avg. root length (cm) (Mean±SE)	Fresh weight (g)	Dry weight (g)
0	1.494±0.22a	10±0.7abc	2.1±0.23a	3.21±0.17a	4.2±0.33e	7.03±0.25a	48.2938	4.5829
50 mg Chit.	1.363±0.07ab	9±0.44bc	1.7±0.15a	2.47±0.11b	4.4±0.45e	3.85±0.16c	49.9606	4.7492
C1	1.2655±0.11ab	10.2±0.8abc	1.0±0.16a	1.625±0.13c	3.0±0.23e	5.1±0.17b	45.0111	6.0134
100 mg Chit.	1.0205±0.12bc	9.4±0.74bc	1.7±0.15a	1.35±0.10cd	7.6±0.45bcd	1.2±0.12f	49.5643	5.0148
C2	1.292±0.11ab	9.6±0.67bc	1.6±0.16a	1.51±0.12c	11±0.37a	2.39±0.20e	33.0142	2.7458
150 mg Chit.	1.0±0.08bc	12±0.7a	1.8±0.13a	1.63±0.11c	11±0.60a	1.22±0.13f	20.5475	2.9354
C3	1.376±0.13ab	12±0.7a	1.7±0.15a	1.56±0.15c	11.7±1.14a	3.09±0.12d	34.6365	1.6075
200 mg Chit.	0.85±0.08c	9±0.44bc	1.7±0.15a	1.39±0.09cd	5.9±0.69de	0.57±0.11g	16.9573	1.7731
C4	0.876±0.13c	8.8±0.37c	1.8±0.13a	1.51±0.11c	6.6±0.54cde	0.65±0.09g	39.3276	2.2787
250 mg Chit.	1.147±0.12abc	12±0.7a	1.9±0.18a	1.255±0.09cd	9.2±1.61abc	0.61±0.11g	29.433	1.013
C5	1.1145±0.07abc	11±0.44ab	1.6±0.16a	1.09±0.11d	10±2.42ab	0.57±0.11g	34.271	2.1834

Means within a column followed by same letters are not significantly ($p < 0.05$) different as determined by Duncan's Multiple Range test.
C1 – 10 ml 1% HAc soln, l^{-1} MS Medium, C2 – 20 ml 1% HAc soln, l^{-1} MS Medium, C3 – 30 ml 1% HAc soln, l^{-1} MS Medium, C4 – 40 ml 1% HAc soln, l^{-1} MS Medium, and C5 – 50 ml 1% HAc soln, l^{-1} MS Medium

(1.2655±0.11cm^2) was reduced compared to the control and S2 plants. The plants produced 10.2±0.8 dark green leaves per shoot, with small basal leaves and large upper ones. In S1, pale brown-coloured shoots with shorter internodes were formed, giving the leaves a rosette-like appearance. In C1, the leaves were brown-coloured.

Plants grown in the S2 medium had 9.4±0.74 pale green leaves with small lower leaves, having a leaf area of 1.0205±0.12cm^2. In the C2 medium, 9.6±0.67 yellowish or pale green leaves per shoot were formed, but their leaf area (1.292±0.11cm^2) was slightly higher than plants formed in the S2 medium. The leaf area was lower in the plants grown in the S3 medium (1.0±0.08cm2) than in the C3 medium (1.376±0.13cm^2). In S3, shoots with 12±0.7 leaves were formed per shoot, with small green lower leaves, yellowish-green upper leaves, and larger middle leaves. Light green leaves were found in the C3 medium, with 12±0.7 leaves formed per shoot.

The plants with the lowest leaf area were formed in S4 and C4 media (0.85±0.08cm^2 and 0.876±0.13cm^2, respectively), and 9±0.44 (S4) and 8.8±0.37 (C4) yellowish-green small leaves were formed per shoot. In S5 and C5 media, the leaf area values did not show significant differences; in S5, leaves formed had a leaf area of 1.147±0.12cm^2, and in C5, leaves formed had a leaf area of 1.1145±0.07cm^2. In the plants formed in the S5 medium, 12±0.7 pale yellowish-green leaves were formed per shoot, while in the plants formed in the C5 medium, 11±0.44 pale green or yellowish-green small leaves were formed per shoot.

4.5.1.8.2. Shoots

The results showed that the shoot number (2.1±0.23) (**Figure 39**) and the shoot length were higher (3.21±0.17 cm) in the control plants than in other stress trials. It was found that an increase in chitosan concentration in the media decreased the length of shoots. The shoot number was slightly low in S1 when compared with the MS basal medium (1.7±0.15), and there was a reduction in shoot number in the C1 (1.0±0.16) medium. The shoot length was also low in S1 (2.47±0.11cm) and C1 (1.625±0.13cm) media.

The number of shoots in the S2 medium was similar to the S1 medium. In the S2 medium, most of the shoots had very short internodes. Shoots formed in the C2 medium (1.51±0.12cm) were slightly lengthier than the

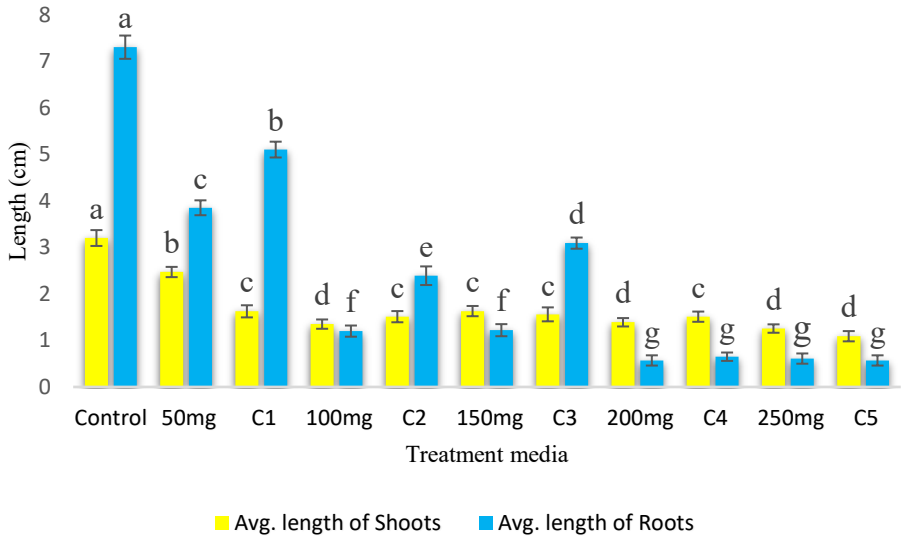

Figure 39: Effects of Chitosan on the numbers of shoots and roots in *in vitro* cultures of *A. triplinervis*; C1 – 10 ml 1% HAc soln, l^{-1} MS Medium, C2 – 20 ml 1% HAc soln, l^{-1} MS Medium, C3 – 30 ml 1% HAc soln, l^{-1} MS Medium, C4 – 40 ml 1% HAc soln, l^{-1} MS Medium, and C5 – 50 ml 1% HAc soln, l^{-1} MS Medium. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

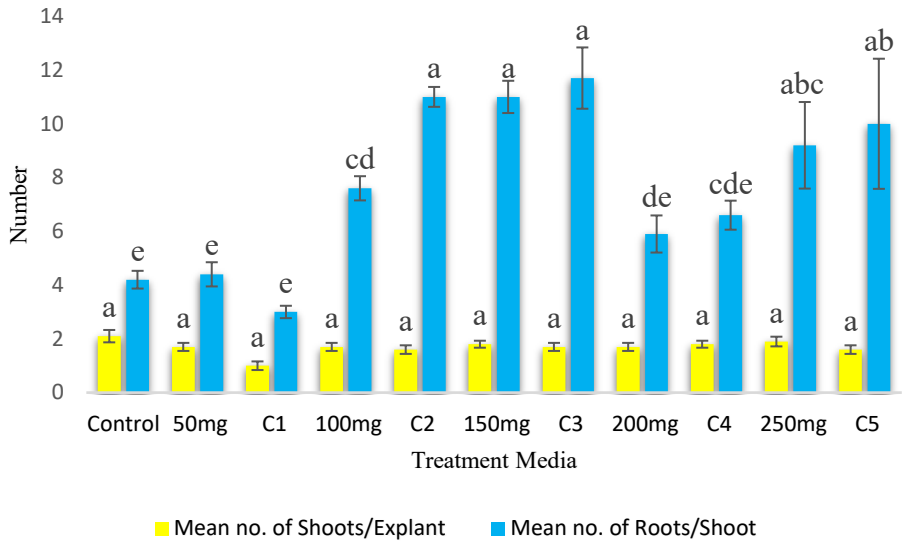

Figure 40: Effects of Chitosan on the average length of shoots and roots in *in vitro* cultures of *A. triplinervis*; C1 – 10 ml 1% HAc soln, l^{-1} MS Medium, C2 – 20 ml 1% HAc soln, l^{-1} MS Medium, C3 – 30 ml 1% HAc soln, l^{-1} MS Medium, C4 – 40 ml 1% HAc soln, l^{-1} MS Medium, and C5 – 50 ml 1% HAc soln, l^{-1} MS Medium. Different letters indicate statistically significant differences between means ($p<0.05$) corresponding to Duncan's Multiple Range tests. Error bars represent ±SE.

shoots formed in S2 (1.35±0.10cm) (**Figure 40**). The shoot number and length in the S3 and C3 media had no significant difference; they showed almost same heights.

The plants formed in the C4 medium exhibited short shoot lengths (1.39±0.09cm) and had shorter internodes. However, the shoots formed in the C4 medium were lengthier (1.51±0.11cm) than those formed in the S4 medium.

Several shoots (1.9±0.18) were formed in the S5 medium with a length of 1.255±0.09cm. The plants were formed in the C5 medium with a shoot length of 1.09±0.11cm and with much-shortened internodes; lesser number of shoots (1.6±0.16) were produced in this medium compared with the S5 medium.

4.5.1.8.3. Roots

The least number of roots were (4.2±0.33) formed in the control medium, but root length (7.03±0.25cm) was the highest in this medium. It was observed that the number of roots was increased in the chitosan stress-induced media and in the individual controls of 1% HAc **(Figure 39)**. The length of roots was decreased with an increase in the concentration of chitosan and acidity in the media. Among the plants formed from the SA-induced trials and their control media, the maximum root length was observed in the C1 medium (5.1±0.17cm) followed by the S1 medium (3.85±0.16cm) **(Figure 40)**. The root system had long green roots and white rootlets in both media. However, it was observed that the number of roots was less in both S1 (4.4±0.45) and C1 (3.0±0.23) media than in other SA-treated trial media.

In the S2 medium, the observed root number was 7.6±0.45, and a bunch of short, thick white coloured roots was formed. On the other hand, in the C2 medium roots with a length of 2.39±0.20cm and they were lengthier than in S2 (1.2±0.12cm). The C2 plants bear numerous whitish green roots. One of the highest numbers of roots (11±0.37) was formed in the C2 medium. Many whitish, thick roots were produced from the shoots in the S3 medium. Numerous roots were observed in the plants from the S3 (11±0.60) and C3 (11.7±1.14) media.

The results showed that the number and length of roots were less for the plants from the S4 and C4 media than in other SA stress trials. The root number for the S4 medium was 5.9±0.69, and for C4, it was 6.6±0.54. In the 200 mgl-1 chitosan concentration, a significant reduction in the length of the roots was observed: S4 - 0.57±0.11cm and C4 - 0.65±0.09cm. Most of the roots were

white-coloured. There was an increase in the number of roots in the S5 (9.2±1.61) and C5 (10±2.42) media. In the case of root length, for the plants of the S5 medium, it was measured as 0.61±0.11cm, but in the C5 medium (0.57±0.11cm), the length was similar to that of the S4 plants. The roots were white-coloured.

Biomass production

The plants harvested from the control, S1, and S3 media showed the highest biomass in terms of fresh and dry weight (**Figure 41**). Among the SA stress trials, the S4 (200 mg l^{-1} Chit.) medium showed the lowest biomass production. In the presence of higher chitosan concentrations, biomass

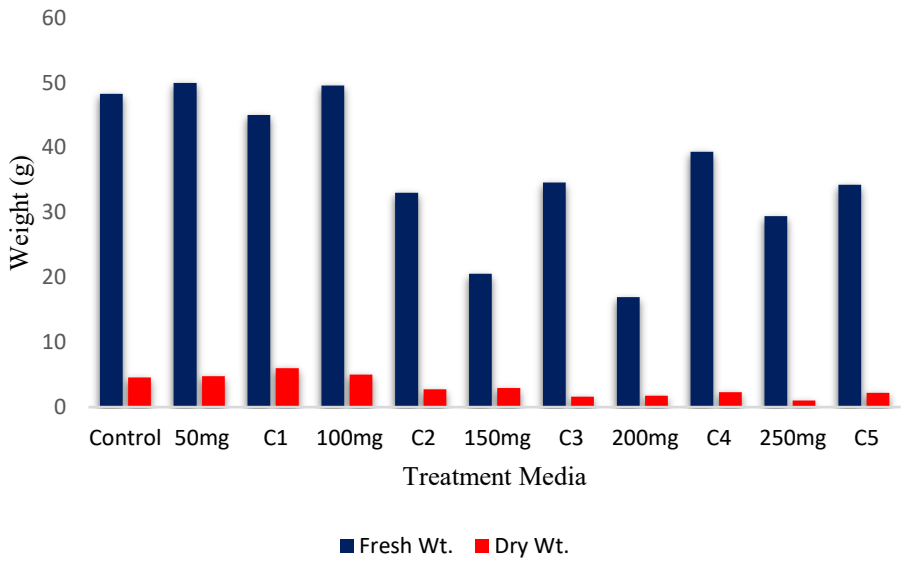

Figure 41: Effects of Chitosan on the total biomass production in *in vitro* cultures of *A. triplinervis*

production was reduced. Only the plants harvested from the S2 and S3 media exhibited higher biomass production than their control media (C2 & C3). In

all other individual controls, media showed higher biomass production than their respective stress trials. In the present study, 100 mg l^{-1} and 150 mg l^{-1} chitosan in the medium promoted biomass production. Similar results showing increased biomass production in the presence of chitosan in the medium were reported earlier in *Withania somnifera* (Shajahan, 2013).

In the present study, variable effects were observed depending on the concentration of chitosan in the medium. Similar results were reported earlier in several plants; *Silybum marianum* (Shah *et al.,* 2021) and *Morinda elliptica* (Chong *et al.,* 2005). Plants treated with chitosan solution were found to have shorter and thicker roots (El-Ghaouth *et al.,* 1993). Adding chitosan to the *in vitro* culture of *Hypericum perforatum* showed reduced biomass production and morpho-anatomical changes in the roots (Brasili *et al.,* 2016). The fresh and dry weight of roots and growth ratio was reduced with the increasing chitosan concentration from 0.2 mgl-1 to 0.8 mgl^{-1} (Baque *et al.,* 2012). El-Sayed *et al.* (2017) reported that 100 mM chitosan enhanced biomass production in the callus cultures of *Rumex vesicarius*. 1.75% chitosan increased the length of shoots, number of nodes, and shoot and root dry weight. The plant growth ceased in the presence of 2% chitosan, and the shoot length was decreased (Ait Barka *et al.,* 2004). In Dunal (Sivanandhan *et al.,* 2012) and *Hypericum perforatum* (Brasili *et al.,* 2014), the chitosan application enhanced the root biomass. Chitosan significantly affected the growth and development of orchid tissue culture (Nge *et al.,* 2006).

4.5.2. HPTLC study

4.5.2.1. NaCl

Plants can face significant challenges when exposed to salt stress, impacting their growth and the production of secondary metabolites (Eryilmaz, 2006).

The salt stress was induced in the *in vitro* cultures of *Ayapana triplinervis* with the application of different concentrations of NaCl to the culture medium. The following concentrations of NaCl were used during the studies: 10 mM l^{-1}, 25 mM l^{-1}, 50 mM l^{-1}, and 100 mM $l^{-1,}$ and the MS basal medium was used as control.

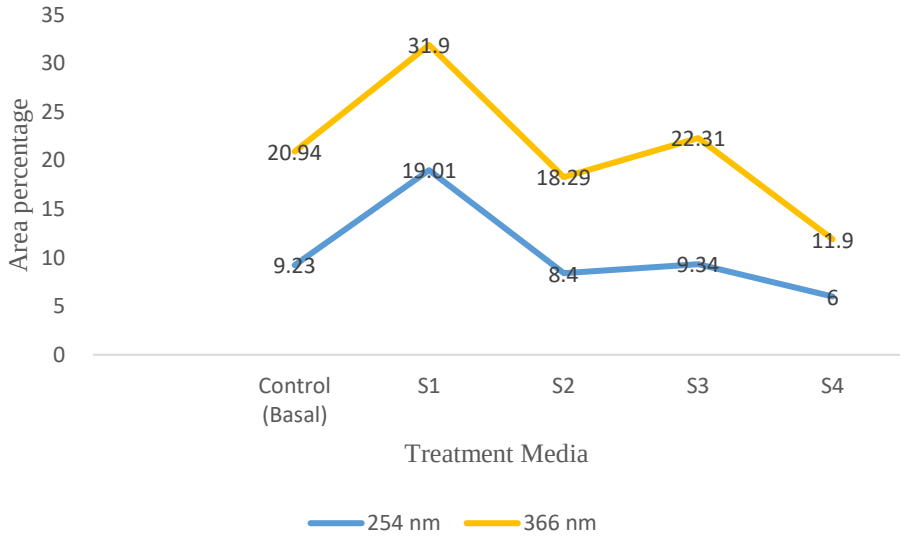

Figure 42: Area percentages of Ayapanin in *in vitro* NaCl-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (10 mM l^{-1} NaCl), S2 (25 mM l^{-1} NaCl), S3 (50 mM l^{-1} NaCl) and S4 (100 mMl^{-1} NaCl).

The work was conducted to study the effect of NaCl on the secondary metabolite production in *A. triplinervis* (**Plate 15**). In the HPTLC profile, at both 366 nm and 254 nm, the compound with Rf value 0.77 was identified as 'Ayapanin' by using the standard marker. In the chromatogram of plant methanolic extract, each Rf represents a different compound. Compared with the area percentage of the control culture, the 10 mM l^{-1}, and 50 mM l^{-1} NaCl-induced media showed an augmentation in the production of the compound 'Ayapanin' (**Figure 42**); a ten-fold increase was observed in the former. This unveils that the optimum concentration of NaCl has a positive effect on 'Ayapanin' production.

In the present study, 17 distinct compounds including the standard marker 'Ayapanin' were detected at a wavelength of 366 nm (**Table 18**). Examination of the HPTLC profile revealed that certain compounds were more abundant or absent in specific media, while others displayed an increase or decrease in response to salt stress induction. The compound with Rf 0.01 was exclusive to the control medium and not observable in trial media under salt stress induction. Two additional compounds with Rf 0.10 in 50 mM l^{-1} NaCl-induced media and with Rf 0.90 were formed in 100 mM l^{-1} NaCl-induced media. Several compounds were absent in the control medium, with Rfs 0.10, 0.30, 0.32, and 0.90.

Further investigation of the area percentages indicated that Rfs 0.30 and 0.32 were more prevalent with the increase in the concentration of NaCl, suggesting that these two may be significant compounds activated under salt stress conditions. Rfs 0.15, 0.23, 0.35, 0.55, 0.60, and 0.77 were present in all media, though their production was boosted in optimum salt concentration. On

the other hand, compound with Rf 0.35 had a higher area percentage in the control medium, with lower percentages observed in media with increased NaCl concentrations.

Rf 0.05 was absent only in the 50 mM l^{-1} NaCl-induced medium. At the same time, Rf 0.65 was absent in both the control and the 100 mM l^{-1} NaCl-induced medium. Rf 0.85 was more abundant in the 50 mM l^{-1} NaCl-induced medium than in other NaCl media, including the control, but not in the 100 mM l^{-1} NaCl medium. Compound with Rf 0.96 was absent in the 10 mM l^{-1} NaCl medium, but its area percentage gradually increased with increasing NaCl concentration in the nutrient media.

Table 18: Rf values and area percentages of methanolic extracts of NaCl induced *in vitro* cultures of *A. triplinervis* at visualized 366 nm.

Rf	Area Percentage				
(366 nm)	Control	S1	S2	S3	S4
0.01	4.32	-	-	-	-
0.03	2.33	-	-	0.22	-
0.05	0.42	1.14	0.28	-	1.04
0.10	-	-	-	0.40	-
0.15	2.12	5.59	4.42	2.53	2.79
0.23	11.51	9.48	11.13	11.98	12.66
0.30	-	18.28	24.77	25.46	32.28
0.32	-	14.58	16.75	16.83	17.24
0.35	35.52	5.28	6.69	6.34	5.58
0.55	1.79	2.53	3.38	2.39	1.71
0.60	9.10	7.30	6.82	5.25	5.69
0.65	-	1.14	1.45	0.96	-
0.77	20.94	31.90	18.29	22.31	11.90
0.85	1.23	1.23	1.07	2.07	-
0.90	-	-	-	-	2.64
0.96	0.56	-	0.92	1.28	4.45

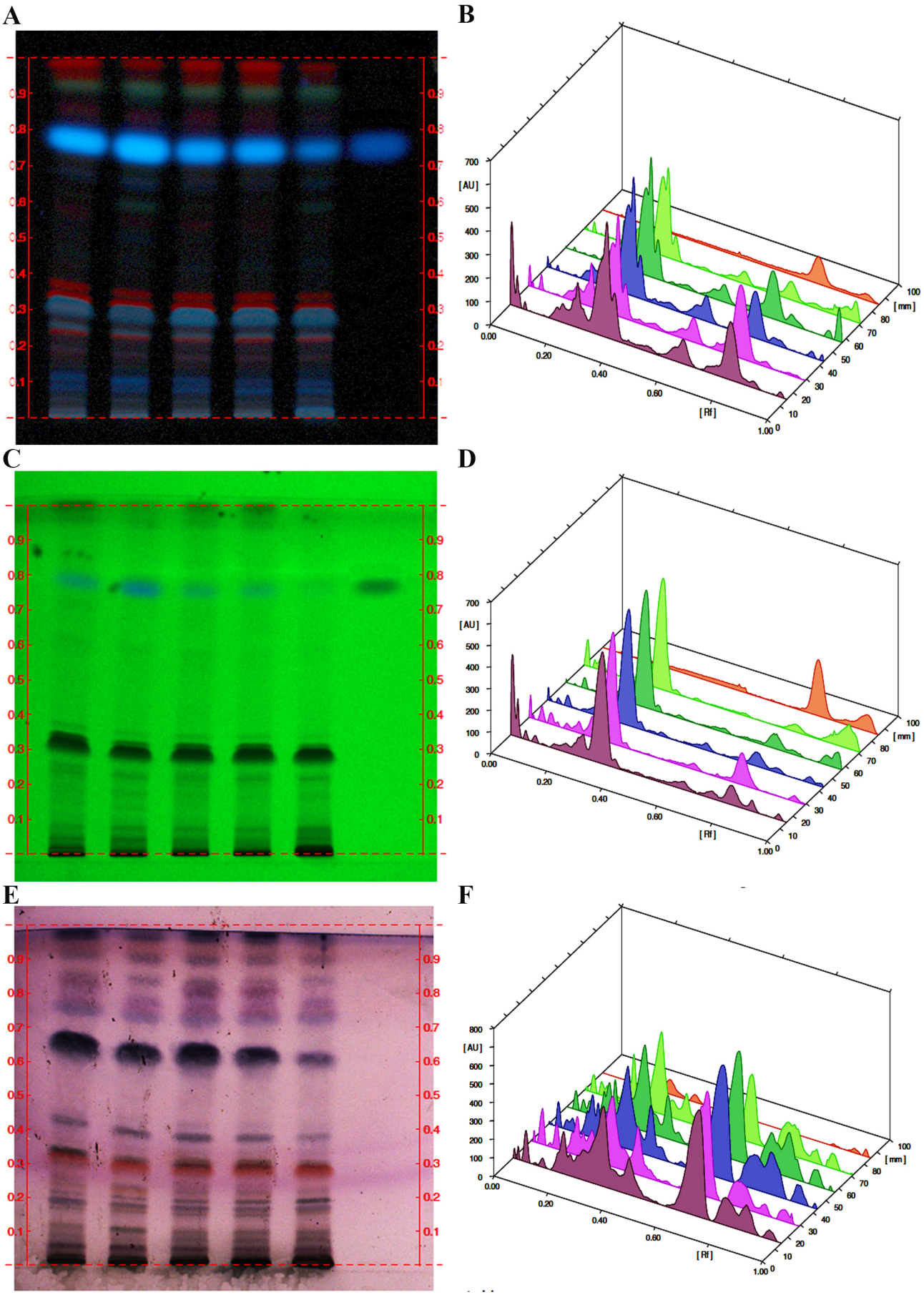

Plate 15: HPTLC Comparison of sequential methanolic extracts of *in vitro* NaCl induced and control cultures of *A. triplinervis*. (**Tracks:** 1. Control, 2. 10 mM l^{-1} NaCl, 3. 25 mM l^{-1} NaCl, 4. 50 mM l^{-1} NaCl, 5. 100 mM l^{-1} NaCl), 6. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** chromatogram and densitogram at 254 nm and **E&F.** chromatogram and densitogram after derivatization.

Compounds with 11 different Rfs were visualized at 250 nm (**Table 19**), and all the compounds were visible when the control medium was used, and the compound with Rf 0.20 was uniquely formed in the control medium only. Compound with Rf 0.01 was observed in the control medium and the 100 mM l^{-1} NaCl medium, but their area % was reduced at the high salt-containing medium. Rf 0.05 was not visible only in the 50 mM l^{-1} NaCl medium; it has a slightly higher area % in the 10 mM l^{-1} NaCl medium than the rest. Rfs 0.08, 0.17, 0.23, 0.30, 0.33, 0.60, 0.77, and 0.95 were visible in all the culture media used including the control. Out of these, the production of Rfs 0.08, 0.23, 0.30, 0.77, and 0.95 was increased with the increasing concentration of NaCl. Moreover, the compounds with Rf 0.30 and 0.95 have a remarkable increase in their production. The production of the compounds with Rfs 0.17, 0.33 and 0.60 were decreased in the NaCl-induced media compared with the control medium.

Table 19: Rf values and area percentages of methanolic extracts of NaCl induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

Rf (254 nm)	Area Percentage				
	Control	S1	S2	S3	S4
0.01	7.15	-	-	-	4.68
0.02	3.44	-	-	-	-
0.05	1.07	2.70	0.80	-	1.27
0.08	1.12	2.49	2.21	1.96	4.98
0.17	3.99	3.58	3.15	1.97	2.75
0.23	8.07	8.59	11.11	11.08	7.26
0.30	53.08	55.63	64.17	65.19	65.00
0.33	1.65	1.15	1.66	1.42	1.23
0.60	4.29	2.52	3.07	1.41	1.04
0.77	9.23	19.01	8.40	9.34	6.00
0.95	1.08	1.83	2.20	3.60	5.04

After derivatization, 16 compounds were observed at 550 nm (**Table 20**). The medium containing 50 mM l^{-1} NaCl contained more compounds than the other media. The compounds with Rfs 0.05, 0.73, and 0.97 were not visible in the control medium, and the compound with Rf 0.15 was absent in both the control and 10 mM l^{-1} NaCl containing medium. The compound with Rf 0.20 was present only in the control and 10 mM l^{-1} NaCl containing medium. Rfs 0.73 and 0.97 were visible only in the 10 mM l^{-1} NaCl and 50 mM l^{-1} NaCl-induced media. It was observed that the higher salt stress media had a slightly higher area percentage than other media in Rf 0.01. The rest of the Rfs 0.05, 0.08, 0.10, 0.18, 0.23, 0.30, 0.38, 0.65, 0.77, 0.85, and 0.95 were visible in all the culture media. The Rfs 0.08, 0.10, 0.38, 0.77, 0.85, and 0.95 showed significant variations with the changes in NaCl concentration in the medium. The production of compound with Rf 0.30 was increased at higher NaCl concentrations; the control and the 25 mM l^{-1} NaCl containing media shared similar area percentages. The area percentage of compounds with Rfs 0.05, 0.18, and 0.65 were reduced with the increase in salt concentration.

Similar studies showing the effect of NaCl in secondary metabolite production were reported earlier in many plants. Ben-Rejeb *et al.* (2022) reported that applying 100 mM NaCl in the microshoots of *Artemisia arborescens* has enhanced the accumulation of total flavonoids, polyphenols, condensed tannins, and volatile compounds. As per the report of Hawrylak-Nowak *et al.* (2021), treatment with 100 mM NaCl enhanced the accumulation of total phenolics, soluble flavonols, anthocyanins, and phenolic acids *in Melissa officinalis*.

Table 20: Rf values and area percentages of methanolic extracts of NaCl induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

Rf	Area Percentage				
(550 nm)	Control	S1	S2	S3	S4
0.03	0.44	-	-	1.22	1.79
0.05	2.45	3.21	1.41	0.82	1.43
0.08	0.13	0.25	0.63	1.02	1.17
0.10	0.78	2.31	1.01	1.95	0.18
0.15	-	-	5.32	3.43	5.50
0.18	6.38	6.00	2.36	3.10	5.50
0.20	1.85	2.17	-	-	-
0.23	6.43	4.88	6.43	6.22	4.35
0.30	23.58	20.28	19.24	21.60	24.28
0.38	7.43	10.17	6.29	5.91	6.85
0.65	34.31	33.00	32.76	30.01	21.13
0.73	-	-	-	-	9.68
0.77	8.56	9.90	10.28	10.47	7.86
0.85	5.81	1.94	9.44	10.14	2.67
0.95	1.46	2.27	2.90	2.68	3.32
0.97	-	-	-	0.18	-

In their study, Abdel-Farid *et al.* (2020) explored the impact of salt stress on Cucumber and Tomato and found significant changes in the secondary metabolite profile. Both plants showed increased total flavonoid production at increased salinity, while total phenolics content remained unchanged in Cucumber but increased in Tomato at 200 mM NaCl. Saponin production decreased at high salinity in Cucumber but was increased at 200 mM NaCl in Tomato (and reduced in 25 mM, 50 mM, and 100 mM NaCl).

Muchate *et al.* (2019) observed increased 20-hydroxyecdysone (20E) contents at higher salt stress in *Spinacia oleracea*. Özlem *et al.* (2018), found that in *in vitro* culture *of Rubia tinctorum*, 3 g l^{-1} NaCl was best in the maximum production of alizarin and purpurin (anthraquinone derivatives) and phenolic contents in the adventitious root cultures. Similarly, El-Shennawy *et*

al. (2017) found that rosmaric acid, total phenolics, and proline increased with salt-induced stress in *Mentha longifolia*.

Fatima *et al.* (2015), reported that the production of vinblastine and vincristine was augmented by the application of NaCl stress in *Catheranthus roseus*. The lower salt concentrations of 25 and 50 mM highly impacted the maximum yield of vinblastine and vincristine from the somatic embryo-generated leaves. While at higher concentrations, NaCl acts as an inhibitory in the alkaloid accumulation.

In *Gossypium hirsutum* L., salt stress elevated the production of gossypol, flavonoids, and tannic acid (Wang *et al.*, 2015). Sabir *et al.* (2012) noted that at higher salt concentrations, phenolics accumulated in *Withania somnifera*, with withanolide production enhanced only at 50 mM NaCl. However, higher salinity reduced the production of total phenolics, flavonoids, and tannins in Fenugreek calli (Hussein & Aqlan, 2011). Zhao *et al.* (2009) explored the effects of salt stress on *Thellungiella holophila* calli. They observed that glycine betaine, total flavonoid, and trehalose accumulation were higher in *Thellungiella*. *In vitro* cultures of *Solanum nigrum* exposed to 150 mM NaCl showed enhanced solasodine production, with a solid linear connection revealed through regression analysis (r=0.997, r2=0.994) (Bhat *et al.*, 2008).

The production of secondary metabolites mediated by NaCl (salt stress) positively affected the production of ‘Ayapanin’. Most of the compounds were present in the control and stress-induced culture media. Additionally, some new compounds were exclusively visible in certain stress media.

4.5.2.2 Sucrose

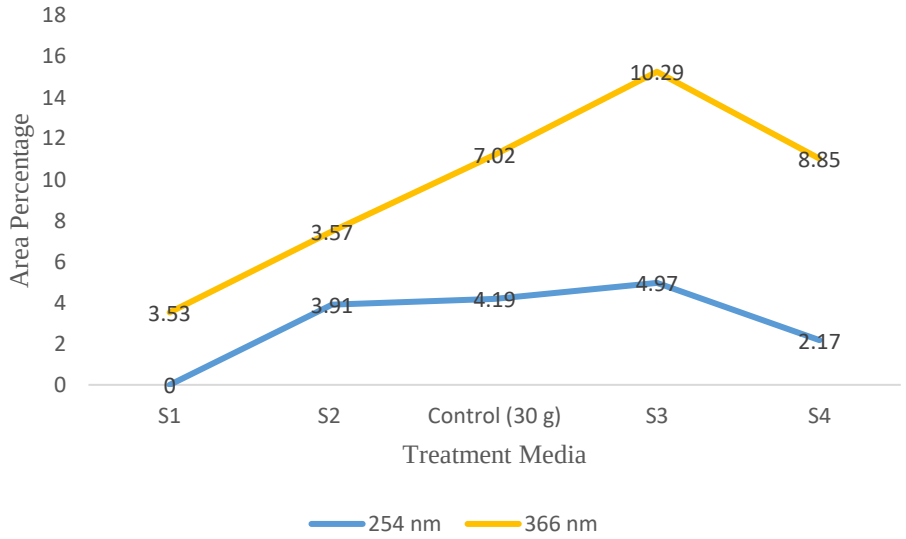

Figure 43: Area percentages of Ayapanin in *in vitro* Sucrose-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (10 g l^{-1} Sucrose), S2 (20 g l^{-1} Sucrose), S3 (40 g l^{-1} Sucrose) and S4 (50 g l^{-1} Sucrose)

Sucrose is the most important and widely used carbon source in plant cell tissue and organ culture media (El-Tahchy *et al.* 2011).

The present study analyzed the HPTLC profile of secondary metabolites (**Plate 16**) in *A. triplinervis* induced with different concentrations of sucrose as the stress signal. The following concentrations of sucrose were used for the study: 10 g l^{-1}, 20 g l^{-1}, 30 g l^{-1} (control), 40 g l^{-1} and 50 g l^{-1}.

Compound with Rf 0.83 was identified as the compound 'Ayapanin' with the help of the marker compound. Among the tested concentrations highest amount of 'Ayapanin' production was observed in medium containing 40 g l^{-1} sucrose (**Figure 43**). However, a fall was observed in 'Ayapanin'

production beyond the concentration of 40 gl-1 sucrose in the medium. In the medium containing 10 g l^{-1} sucrose, the compound was not visible after the visualization at 254 nm. The MS medium with 40 g l^{-1} sucrose was ideal for triggering the coumarin synthesis pathway.

Seventeen compounds of secondary metabolites were observed after visualizing the chromatogram at 366 nm. The area percentage values showed that sucrose concentration influenced the production of secondary metabolites. Two unique compounds were found, one with Rf 0.50 in 50 g l^{-1} sucrose containing medium and the other with Rf 0.80 in 10 g l^{-1} sucrose containing medium (**Table 21**).

The production of compound with Rf 0.50 was observed in the medium containing high concentration of sucrose. Compound with Rf 0.20 was produced only in media with 10 g l^{-1} and 20 g l^{-1} sucrose, and in media with increasing concentrations of sucrose, the compound was not observed. A higher area % value for compound with Rf 0.28 was found in the medium containing 20 g l^{-1} sucrose medium. Compound with Rf 0.30, was formed in media containing 10 g l^{-1} and 50 g l^{-1} sucrose.

The compound with Rf 0.48 was formed in control medium and in medium containing 40 g l^{-1}sucrose. Compound with Rf 0.68 was found to have a higher area % value in medium containing 50 g l^{-1} sucrose. Compound with Rf 0.57 was not formed in media containing low concentration of sucrose. In medium containing 10 g l^{-1} sucrose compound with Rf 0.73 was not formed. The production of this compound was found to be increased with the increasing sucrose concentration. The compound with Rf 0.90 was not formed in the control medium; in media containing low sucrose

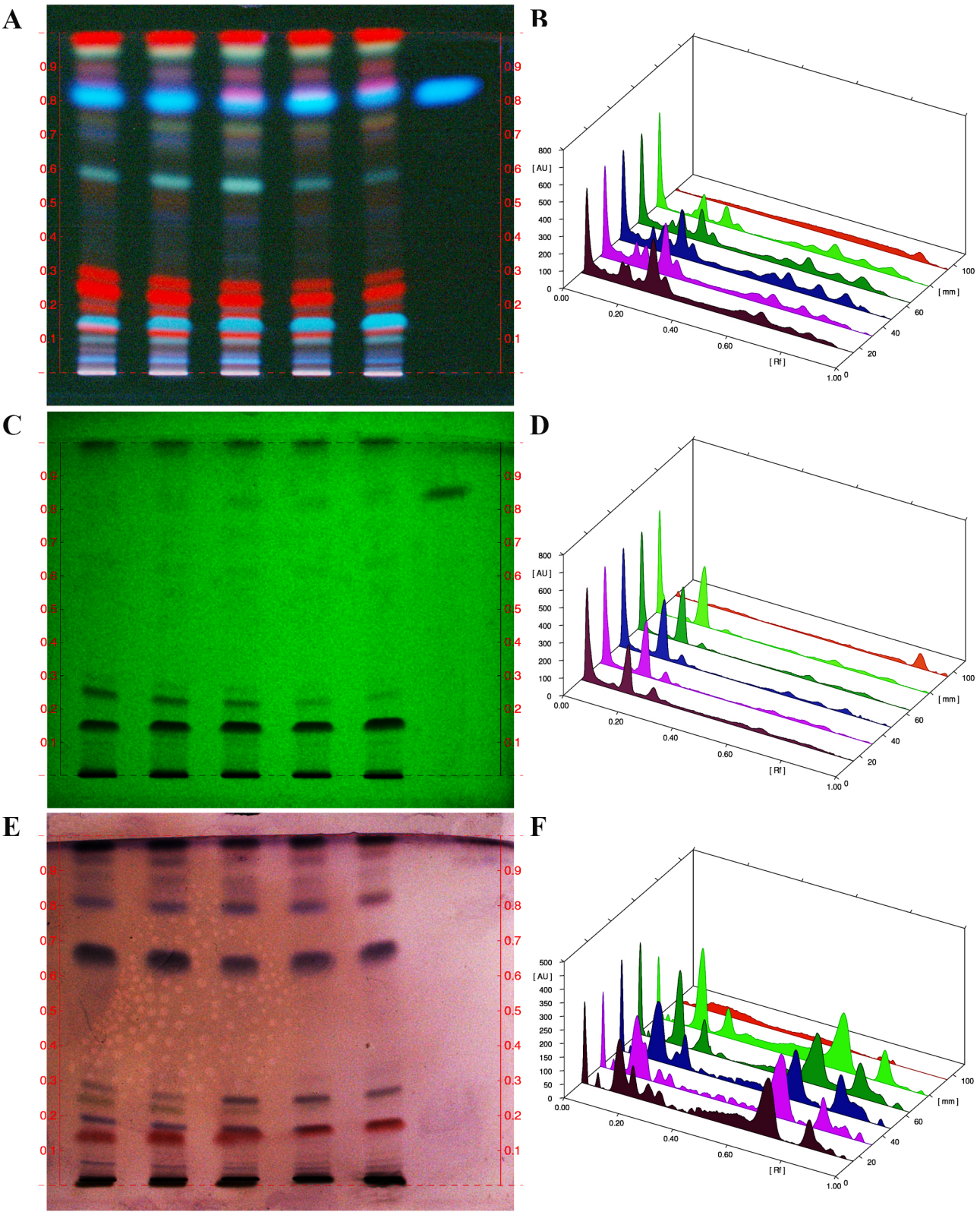

Plate 16: HPTLC comparison of sequential meathanolic extracts of *in vitro* Sucrose induced and control cultures of *A.triplinervis*. (Tracks: 1. 10 g l^{-1} Sucrose, 2. 20 g l^{-1} Sucrose, 3. 30 g l^{-1} Sucrose (control), 4. 40 g l^{-1} Sucrose, 5. 50 g l^{-1} Sucrose, 6. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** HPTLC Chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram after derivatization.

concentrations (10 g l^{-1} and 20 gl^{-1} sucrose), the compound was formed in low quantities, but in media containing higher sucrose concentrations (40 g l^{-1} and 50 g l^{-1} sucrose) the compound was formed in increased quantities.

Table 21: Rf values and area percentages of methanolic extracts of sucrose-induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

Rf (366 nm)	Area Percentage				
	Control	**S1**	**S2**	**S3**	**S4**
0.02	17.31	21.30	21.84	21.88	25.61
0.10	3.27	3.26	3.37	2.11	0.74
0.15	7.12	7.73	8.60	5.66	-
0.18	12.15	5.75	8.40	10.96	14.74
0.20	-	3.59	3.66	-	-
0.25	17.59	26.18	22.06	15.79	12.91
0.28	6.44	-	7.37	5.86	-
0.30	-	8.25	-	-	3.53
0.48	4.30	-	-	3.72	-
0.50	-	-	-	-	4.83
0.57	7.79	-	-	6.74	6.78
0.60	8.16	8.00	7.05	10.10	-
0.68	-	8.09	7.37	-	13.21
0.73	7.28	-	5.75	6.89	7.41
0.80	-	3.38	-	-	-
0.83	7.02	3.53	3.57	10.29	8.85
0.90	1.58	0.93	0.94	-	1.39

Compounds with Rfs 0.15 and 0.60 were not formed in the 50 g l^{-1} sucrose containing medium; the former was highly produced in the medium containing 20 g l^{-1} sucrose medium, and the latter had a prominent peak in the control medium. Compounds with Rfs 0.02, 0.10, 0.18, 0.25, and 0.83 (5 compounds) were produced in media containing all the concentrations of sucrose tested. The Compound with Rf 0.02 showed prominent peaks in the secondary metabolites profile obtained at 366 nm. A slight reduction in the production of the compound was found in the medium containing 40 g l^{-1} of

sucrose. In the 50 g l^{-1} sucrose containing media, a tiny peak was observed for the compound with Rf 0.10. The production of compound with Rf 0.18 gradually increased with the increasing sucrose concentration in the media. However, the production of compound with Rf 0.25 was reduced with the increasing sucrose concentration, and the compound with Rf 0.83 had a significant peak in the control medium.

The metabolite profile visualized at 254 nm exhibited only seven compounds (**Table 22**). In the control and in media containing 20 g l^{-1} sucrose all 7 compounds were formed. However, with increasing sucrose concentration, the peak size of compound with Rf 0.23 was decreased, and in the media containing very low and high sucrose concentrations, compound with Rf 0.23 was not formed. In the 40 g l^{-1} sucrose-stressed medium, the compound with Rf 0.28 was absent, but the compound was observed in medium containing lower concentration of sucrose.

Compounds with Rfs 0.01, 0.10, 0.15, and 0.62 were formed in all the media tested. compound with Rf 0.01 exhibited more significant peaks in the control and media containing higher concentration of sucrose.

Table 22: Rf values and area percentages of methanolic extracts of sucrose-induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

Rf	Area Percentage				
(254 nm)	Control	S1	S2	S3	S4
0.01	36.70	41.11	41.44	42.54	37.04
0.10	2.37	4.08	2.53	1.79	1.85
0.15	39.67	32.20	39.97	41.86	45.39
0.23	6.16	-	6.98	3.22	-
0.28	1.31	10.93	1.20	-	2.57
0.62	4.35	4.08	3.97	5.61	6.12
0.83	4.19	-	3.91	4.97	2.17

Presence of Sucrose in the media had an enhancing effect on the production of compound with Rf 0.01. For compound with Rf 0.10, production was highest in the medium containing low concentration of sucrose. The production of compounds with Rfs 0.15 and 0.62 was increased simultaneously with the increase in the concentration of sucrose applied in the media; the highest area percentage value was obtained in the medium containing 50 g l^{-1} sucrose.

Visualization of the methanolic extracts of the samples at 550 nm after derivatization, a profile of 16 compounds (**Table 23**) was identified. In the medium containing 20 g l^{-1}sucrose, two unique compounds with Rfs 0.10 and 0.18 were formed. In media containing 30 g l^{-1} (control) and 40 g l^{-1} sucrose compound with Rf 0.50 was not observed and highest area percentage value of this compound was observed in the medium containing 50 g l^{-1} sucrose. The compound with Rf 0.45 was not formed in medium containing 50 g l^{-1} sucrose.

The production of compounds with the Rfs 0.20 and 0.55 was found to be high in media containing low concentration of sucrose. Highest amount of compound with Rf 0.88 was formed in the control medium, and this compound was absent in media containing two extremities of the sucrose stress. Compound with Rf 0.90 was exclusively found in media containing 10 g l^{-1} and 20 g l^{-1} sucrose. Compound with Rf 0.95 was absent only in the 10 g l^{-1} sucrose-containing medium.

Table 23: Rf values and area percentages of methanolic extracts of Sucrose-induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

Rf (550 nm)	Area Percentage				
	Control	**S1**	**S2**	**S3**	**S4**
0.01	7.23	7.50	5.84	7.81	3.08
0.05	0.16	1.38	1.01	0.24	0.54
0.10	-	-	0.99	-	-
0.15	24.37	17.86	20.98	23.32	25.69
0.18	-	-	5.69	-	-
0.20	3.30	6.33	3.13	-	-
0.24	8.01	5.44	2.56	10.87	10.09
0.30	1.07	3.37	0.83	1.32	4.44
0.45	1.66	1.79	1.05	1.32	-
0.50	-	3.55	1.60	-	7.92
0.55	1.50	4.50	1.12	-	-
0.65	26.14	30.86	35.31	33.28	35.11
0.80	15.25	10.77	10.69	17.76	12.46
0.88	6.58	-	1.33	2.25	-
0.90	-	1.33	3.21	-	-
0.95	0.90	-	1.59	1.39	0.48

The rest of the compounds with Rfs 0.01, 0.05, 0.15, 0.24, 0.30, 0.65, and 0.80 (7 compounds) were produced commonly in all the media. The peak size of the compound with Rf 0.01 in media containing 10 g l^{-1}, 30 g l^{-1} (control), and 40 g l^{-1} sucrose, was nearly the same and was smaller in the 50 g l^{-1} sucrose containing medium. The production of the compound with Rf 0.05 became reduced with increasing sucrose concentration in the medium. Higher sucrose concentration in the media enhanced the production of compounds with Rf 0.15 and Rf 0.24. Media containing high sucrose concentrations augmented the production of compound with Rf 0.65 than the control medium. The highest production of compound with Rf 0.80 was

observed in the 40 g l^{-1} sucrose-stressed medium, and the reduced production resulted in a lower sucrose-stressed medium than other sucrose media.

In the present study sucrose concentration in the media influenced the production of various compounds in the *in vitro* cultures of *A. triplinervis.* Similar results showing the influence of sucrose concentration in the production of various compounds were reported earlier in many plants like *Taraxacum officinale* (Martínez *et al.* 2023), *Stevia rebaudiana* (Ahmad *et al.* 2021) *Musa* species (Ayoola-Oresanya *et al.,* 2021),*Salvia leriifolia* (Modarres *et al.,* 2017), Amaryllidaceae species -*Narcissus pseudonarcissus*, *Galanthus elwesii*, and *Leucojum aestivum* (El-Tahchy *et al.* 2011) , *Hypericum perforatum* (Cui *et al.,* 2010), *Bacopa monnieri* (Naik *et al.,* 2010), *Eschscholtzia californica* (Park *et al.* 2006) , *Eleutherococcus sessiliflorus* (Shohael *et al.* 2006), *Ruscus hypoglossum* L.(Abou-Dahab *et al.* 2004), *Taxus chinensis* (Wang *et al.* 2000) *Taxus chinensis* (Wang *et al.* 1999), *Taxus chinensis* (Dong & Zong, 2001), *Taxus cupsidata* (Ketchum & Gibson, 1996), *Panax notoginseng* (Zhang *et al.,* 1996), *Aralia cordata* (Sakamoto *et al.,* 1993) *Populus* (Merillon *et al.,* 1984), and *Acer pseudoplatanus* L. (Phillips & Henshaw 1977) .

4.5.2.3. Putrescine

Polyamines are a group of positively charged, straight-chain polycationic compounds that include putrescine, spermidine, and spermine. They were discovered in prokaryotic and eukaryotic cells and are essential for various plant functions. Putrescine (Put), Spermidine (Spd), and spermine (Spm) are the three main forms of polyamines.

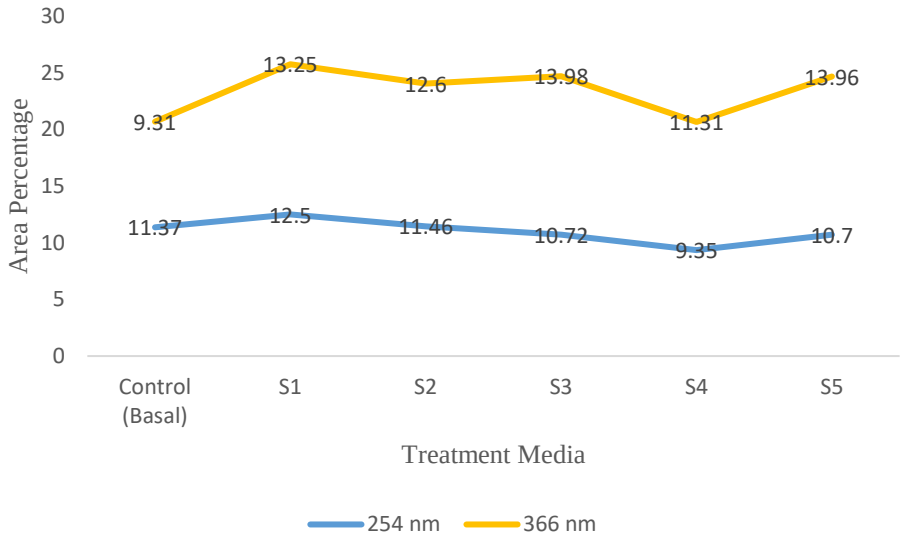

Figure 44: Area percentages of Ayapanin in *in vitro* Putrescine-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (20 mg l^{-1} Put.), S2 (40 mg l^{-1} Put.), S3 (60 mg l^{-1} Put.), S4 (80 mg l^{-1} Put.) and S5 (100 mg l^{-1} Put.).

The present HPTLC analysis investigated how different concentrations of Put affect the secondary metabolite production of *A. triplinervis* in the *in vitro* cultures (**Plate 17**). The study utilized five concentrations of Put: 20, 40, 60, 80, and 100 mg l^{-1}. The HPTLC analysis identified the compound with Rf 0.85 as 'Ayapanin' by comparing it with the peak of the marker compound. The area percentage values (**Figure 44**) obtained at 366 nm visualization showed that Put enhanced the production of the 'Ayapanin' compound. All media supplemented with Put showed higher values at 366 nm than the control medium. At 254 nm, in the medium supplemented with 20 mg l^{-1} Put an increased rate of the 'Ayapanin' production was observed when compared to the control, while a slight reduction was observed in the medium supplemented with 80 mg l^{-1} Put. Moreover, significant enhancement in the

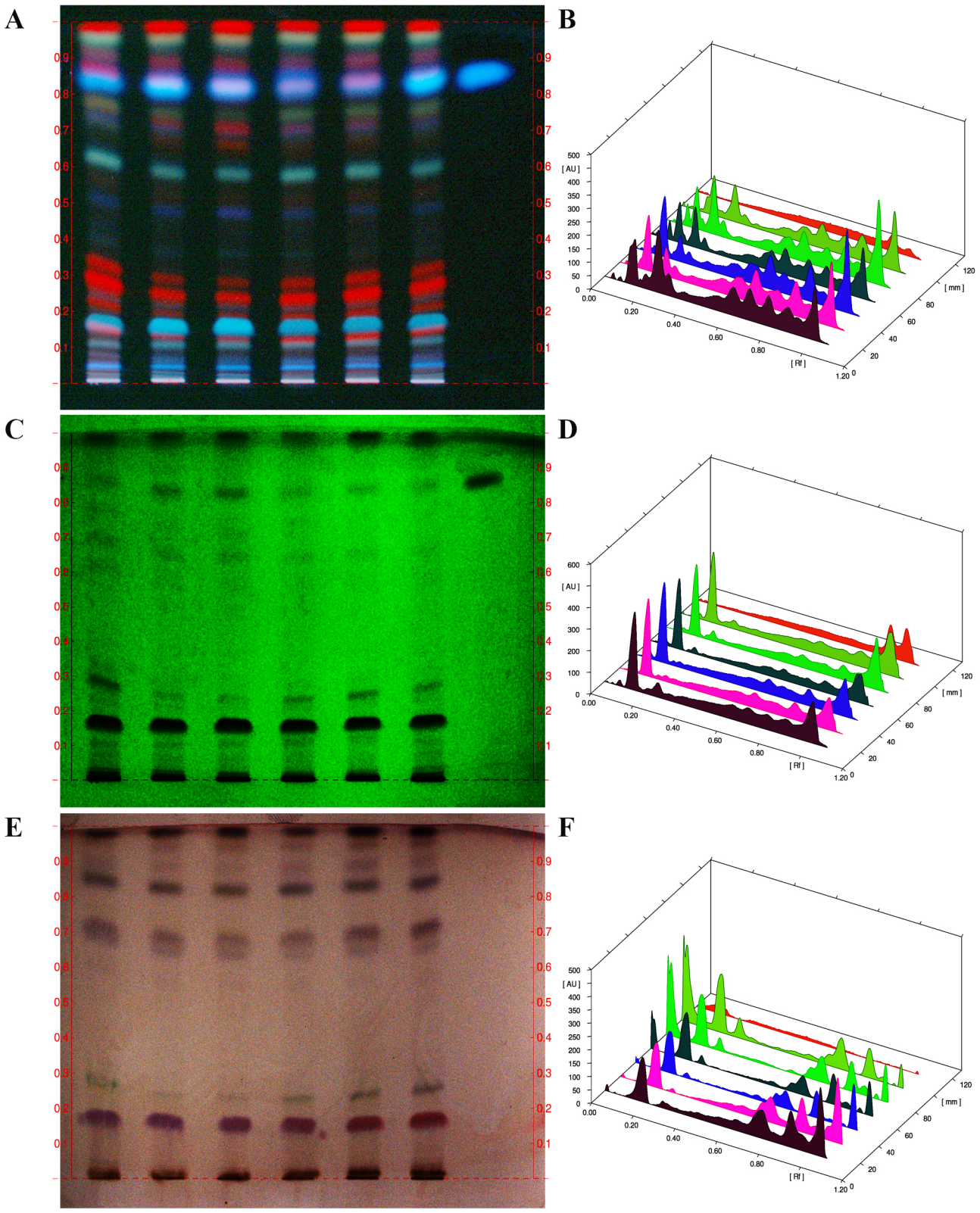

Plate 17: HPTLC comparison of sequential methanolic extracts of *in vitro* Putrescine induced and control cultures of *A. triplinervis*. (**Tracks:** 1. MS basal (control), 2. 20 mg l^{-1} Putrescine, 3. 40 mg l^{-1} Putrescine, 4. 60 mg l^{-1} Putrescine, 5. 80 mg l^{-1} Putrescine, 6. 100 mg l^{-1} Putrescine, 7. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** HPTLC chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram atfter derivatization.

area percentage values of ‘Ayapanin’ was observed in the Put-induced cultures compared to the control; except the culture with 80 mg l^{-1} Put.

Upon visualizing at 366 nm, 18 compounds (**Table 24**) were found in the secondary metabolites profile of *A. triplinervis* and the area percentage values indicated increased production of secondary metabolites in media supplemented with Put. Two unique compounds were identified: with Rf 0.01 in the 60 mg l^{-1} Put medium and with Rf 0.38 in the control medium. The compound with Rf 0.03 was only detected in control and 20 mg l^{-1} Put media. The optimum concentration of Put for the production of the compound with Rf 0.50 was found to be 20 mg l^{-1}. At higher concentrations, the production of the compound with Rf 0.50 decreased with increasing Put stress. The compound with Rf 0.50 was scarce in the control medium.

Compound with Rf 0.55 was absent in the media supplemented with 20 mg l^{-1} and 60 mg l^{-1} Put, while the remaining media showed approximately equal-sized peaks. Conversely, the compound with Rf 0.60 was not found in the extracts from the media-induced with 40 mg l^{-1} and 80 mg l^{-1} Put. However similar area percentages were observed for the compound with Rf 0.60 in other media. The compound with Rf 0.65 was not produced in either the control or the 60 mg l^{-1} Put media, while the remaining media had it in similar amounts, with the Put-induced media yielding the highest amount. The compound with Rf 0.70 was not produced only in medium supplemented with the 20 mg l^{-1} Put, while in medium supplemented with 60 mg l^{-1} Put a significant peak of this compound was observed.

Table 24: Rf values and area percentages of methanolic extracts of Putrescine induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

Rf (366 nm)	Area Percentage					
	Control	**S1**	**S2**	**S3**	**S4**	**S5**
0.01	-	-	-	0.30	-	-
0.03	0.47	0.31	-	-	-	-
0.08	0.40	0.44	1.39	0.31	1.31	1.35
0.15	11.03	12.96	14.25	13.70	14.1	14.31
0.22	2.66	7.40	6.52	-	6.52	6.71
0.25	16.03	3.05	3.51	14.27	3.51	3.53
0.30	4.77	2.69	2.23	3.17	2.51	2.31
0.38	1.85	-	-	-	-	-
0.50	-	5.10	2.94	2.54	2.93	3.93
0.55	6.11	-	5.46	-	5.47	5.73
0.60	10.23	10.66	-	10.12	-	-
0.65	-	12.66	11.34	-	11.35	11.31
0.70	9.89	-	6.12	11.90	6.13	6.31
0.75	9.99	8.41	4.30	8.84	4.30	4.35
0.85	9.31	13.25	12.60	13.98	11.23	12.5
0.90	3.27	5.50	3.82	-	3.51	3.30
1.00	13.98	17.56	21.63	20.79	21.10	21.53

In the 20 mg l^{-1} Put-induced medium compounds with Rfs 0.22 and 0.90 had significantly higher area percentage values than other media. The remaining seven compounds with Rfs 0.08, 0.15, 0.25, 0.30, 0.75, 0.85, and 1.00, were produced in all Put-induced and control media. Put stress positively impacted the production of compounds with Rfs 0.08, 0.15, 0.85, and 1.00. Compounds with Rfs 0.15 and 1.00 had higher area percentage values in Put-induced media than in the control medium. However, the production of compounds with Rfs 0.25, 0.30, and 0.75 decreased in the media supplemented with Put.

Twelve compounds were found in the chromatogram of the extracts visualized at 254 nm wavelength of UV (**Table 25**). The compound with Rf

0.60 was found only in the control medium and the compound with Rf 0.70 was found only 60 mg l^{-1} Put supplemented medium. The compound with Rf 0.25 was formed only in control and in the medium supplemented with 100 mg l^{-1} Put. However, the production of the compound with Rf 0.05 decreased with an increase in Put stress. The compound with Rf 0.10 was formed only in media containing higher concentrations of Put.

Table 25: Rf values and area percentages of methanolic extracts of putrescine-induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

Rf (254 nm)	Area Percentage					
	Control	**S1**	**S2**	**S3**	**S4**	**S5**
0.05	1.27	1.35	1.86	0.73	-	-
0.10	-	-	-	1.54	1.13	1.47
0.15	26.83	31.66	35.78	33.81	28.56	27.29
0.20	1.63	2.34	1.33	2.72	5.32	1.48
0.25	6.04	-	-	-	-	4.06
0.55	-	10.01	-	5.35	5.32	-
0.60	8.25	-	-	-	-	-
0.65	8.29	9.90	11.41	7.84	8.57	9.56
0.70	-	-	6.37	-	-	-
0.75	9.32	5.66	-	5.61	6.18	7.70
0.85	11.37	12.50	11.46	10.72	9.35	10.70
1.00	23.41	24.23	31.79	31.06	35.79	37.48

In the medium supplemented with 20 mg l^{-1} Put, a prominent peak for the compound with Rf 0.55 was observed. However, this compound was absent in media supplemented with 40 mg l^{-1} Put, 100 mg l^{-1} Put, and in the control medium. The compound with Rf 0.75 was present in all the media supplemented with Put and in the control except in medium supplemented with 40 mg l^{-1} Put. Compounds with Rfs 0.15, 0.20, 0.65, 0.85, and 1.00 were produced in all the Put-induced and control media. Put stress in the media positively affected the production of compounds with Rfs 0.15, 0.20, 0.65,

and 1.00. The compound with Rf 0.15 was produced in higher quantities in the medium supplemented with lower concentrations of Put, and the production of the compound with Rf 1.00 increased with an increase in the concentration of Put in the media. In the metabolite profile obtained large area percentages were found for compounds with Rfs 0.15 and 1.00.

Visualization of chromatogram at 550 nm wavelength of visible light, showed the presence of only nine compounds (refer to **Table 26**). All these compounds were visible in the medium supplemented with 80 mg l^{-1} Put. In the media supplemented with 80 mg l^{-1} and the 100 mg l^{-1} Put, the compound with Rf 0.03 was formed in significant quantities. However, the compound with Rf 0.95 was not formed in the control and 100 mg l^{-1} Put supplemented media, with the highest production observed in the medium supplemented with 20 mg l^{-1} Put. The control medium was found to be suitable for the production of the compound with Rf 1.00.

In all the Put stressed media and control medium compounds with Rfs 0.18, 0.25, 0.65, 0.70, and 0.85 were formed. Put stress positively impacted the production of compounds with Rfs 0.18 and 0.25 compared to the control medium. The highest peak for the compound with Rf 0.18 was noted in the 40 mg l^{-1} Put supplemented medium, and the 100 mg l^{-1} Put supplemented medium produced the compound with Rf 0.25 in more prominent quantities than other media. 20 mg l^{-1} Put supplemented medium effectively produced the compound with Rf 0.70, in the control medium also this compoundwas formed in sufficient quantity. However, increased Put concentration in the medium decreased the production of the compound with Rf 0.70. This could be because higher concentrations of Put stress negatively impacted the

biosynthesis of this compound or affected the intermediate steps of its formation.

Table 26: Rf values and area percentages of methanolic extracts of Putrescine induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

Rf (550 nm)	Area Percentage					
	Control	**S1**	**S2**	**S3**	**S4**	**S5**
0.01	0.62	0.51	0.98	11.83	10.75	8.75
0.03	-	-	-	-	21.55	23.00
0.18	20.22	28.26	42.69	36.66	26.12	26.51
0.25	2.02	1.33	3.14	3.58	4.65	7.32
0.65	5.51	5.84	9.17	5.04	4.21	4.32
0.70	22.08	30.11	18.22	16.79	14.73	16.49
0.85	24.87	23.19	23.63	21.10	13.23	11.97
0.95	-	6.76	2.18	4.10	3.84	-
1.00	24.69	-	-	1.50	0.94	1.64

The efficacy of putrescine in producing several secondary metabolites has been reported in several plants. Jaiswal *et al.* (2021) reported that inducing the putrescine on *Glycyrrhiza glabra* enhanced the contents of alkaloids. Esra *et al.* (2020) reported that various concentrations of putrescine increased capsaicin production in *Capsicum annuum*. The studies of Abd-Elbar *et al.* (2019) and Zeid *et al.* (2014) found that putrescine can enhance phenolic content, polyphenol oxidase, and PAL activity in *Thymus vulgaris*.

Aragão *et al.* (2017) reported that 2.5 mM putrescine increased endogenous polyamine content and changed the proteomic profile in *Cedrela fissilis*. Exogenous putrescine application increased the alkaloid compounds in *Datura stramonium* (Niakan *et al.*, 2015). Thiruvengadam and Chung (2015) reported that, in *Cucumis anguria*, putrescine significantly impacted the accumulation of flavonols, hydroxybenzoic acid, and hydroxycinnamic

acid derivatives. Diwan and Malpathak (2012) found that adding putrescine to the shoot cultures of *Ruta graveolens* increased the production of furanocoumarins such as psoralen, xanthotoxin, and bergapten. Kumar *et al.* (2008) reported that polyamines play a significant role in caffeine biosynthesis from *Coffea canephora*. In *Araucaria angustifolia*, 1.0 mM putrescine treatment leads to an accumulation of nitric oxide content in the embryonic cells (Silveira *et al.*, 2006). These reports were in agreement with the results of present experiments on *A. triplinervis*

In the present analysis conducted using HPTLC, several secondary metabolites, including 'Ayapanin was formed in Put supplemented media in the *in vitro* cultures of *A. triplinervis.*' Furthermore, most peaks had an excellent area percentage, while those with very small or negligible values were less prevalent. Based on this, it can be inferred that Put stress has enhanced the production of secondary metabolites in the cultures of *A. triplinervis*.

4.5.2.4. Tryptophan

Plants naturally produce a variety of secondary metabolites, many of which rely on Tryptophan (Trp) as an essential precursor.

The present study explored how different concentrations of Trp affect the production of *in vitro* secondary metabolites in *A. triplinervis* (**Plate 18**). The study used Trp concentrations of 20, 40, 60, 80, and 100 mgl^{-1}. Using a marker compound, the compound with Rf value of 0.85 was identified as 'Ayapanin' in the plant extract separated by HPTLC.

Visualization at 366 nm (**Figure 45**) demonstrated that Trp addition increased the production of 'Ayapanin' in the *in vitro* cultures of *A.*

triplinervis. The area percentage values showed that 'Ayapanin' production was high in Trp-treated media compared to the control medium and 'Ayapanin' production increased with an increase in the Trp concentration in the media. The highest production was observed in the 80 mg l^{-1} and 100 mg l^{-1} Trp-induced media. When visualized at 254 nm, the 100 mg l^{-1} Trp medium resulted in an approximately 10-fold increase in 'Ayapanin' production compared to the control.

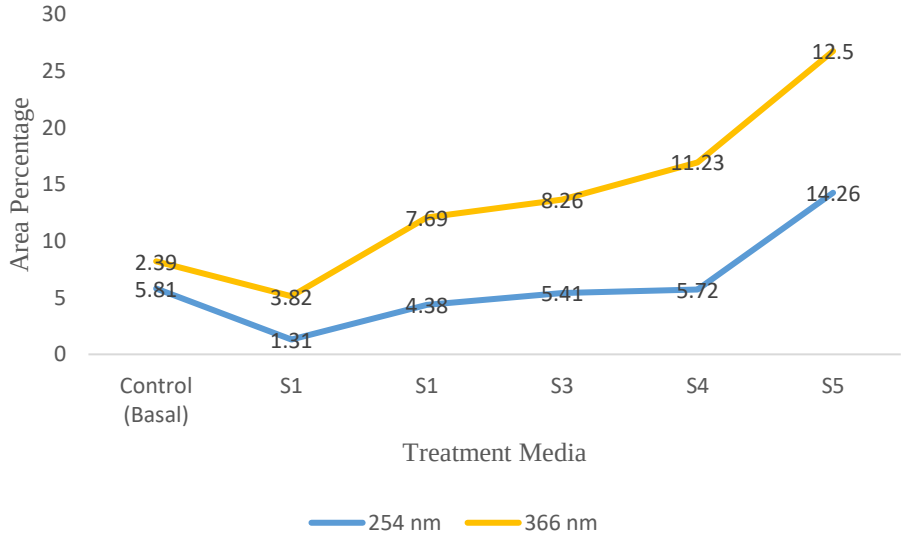

Figure 45: Area percentages of Ayapanin in *in vitro* Tryptophan-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (20 mg l^{-1} Trp.), S2 (40 mg l^{-1} Trp.), S3 (60 mg l^{-1} Trp.), S4 (80 mg l^{-1} Trp.) and S5 (100 mg l^{-1} Trp.).

The metabolic profile analyzed at 366 nm, showed 18 compounds (**Table 27**). Four compounds were unique to the control and 100 mg l^{-1} Trp media. Compounds with Rfs 0.30 and 0.68 were exclusively observed in the control medium and they had high area percentage values. These compounds were highly produced without the addition of exogenous Trp. Compounds with Rf 0.03 and 0.95, on the other hand, were produced only in the 100 mg l-

1 Trp supplemented medium. Compound with Rf 0.03 had a small peak, whereas compound with Rf 0.95 had a good area percentage value in the nutrient medium supplemented with high concentration of Trp.

The compound with Rf 0.40 was produced only in the nutrient medium supplemented with higher concentrations of Trp, indicating that an exogenous supply of high Trp may be necessary for its production. Compound with 0.45 had no significant difference in the area percentage values of the peaks in 80 mg l^{-1} and 100 mg l^{-1} Trp supplemented media. It was also produced in the 40 mg l^{-1} Trp supplemented medium, but the production was lower than in media supplemented with higher concentrations of Trp.

Compounds with Rfs 0.10 and 0.73 were absent in the control medium. The compound with Rf 0.10 was also produced in decreased amounts with increasing Trp content in the medium. The production of the compound with Rf 0.73 was observed only in the 40 mg l^{-1} Trp supplemented medium. The compound with Rf 0.01 was absent in control, 20 mg l^{-1}, and 100 mg l^{-1} Trp supplemented media but was produced in high quantity in the 40 mg l^{-1} Trp supplemented medium. With further increase in the concentration of Trp in the media, its production was found to be gradually decreased. The compound with Rf 0.15 was not formed in 100 mg l^{-1} Trp supplemented media but had good production in 20 mg l^{-1} Trp supplemented media. A further increase in Trp concentration in the media led to a gradual reduction in its production.

The compound with Rf 0.50, was not produced in the 20 mg l^{-1} Trp supplemented medium. However, the area percentage values of the peaks of the compound increased with the Trp concentration. The compound with Rf 0.50 was also found in the control medium.

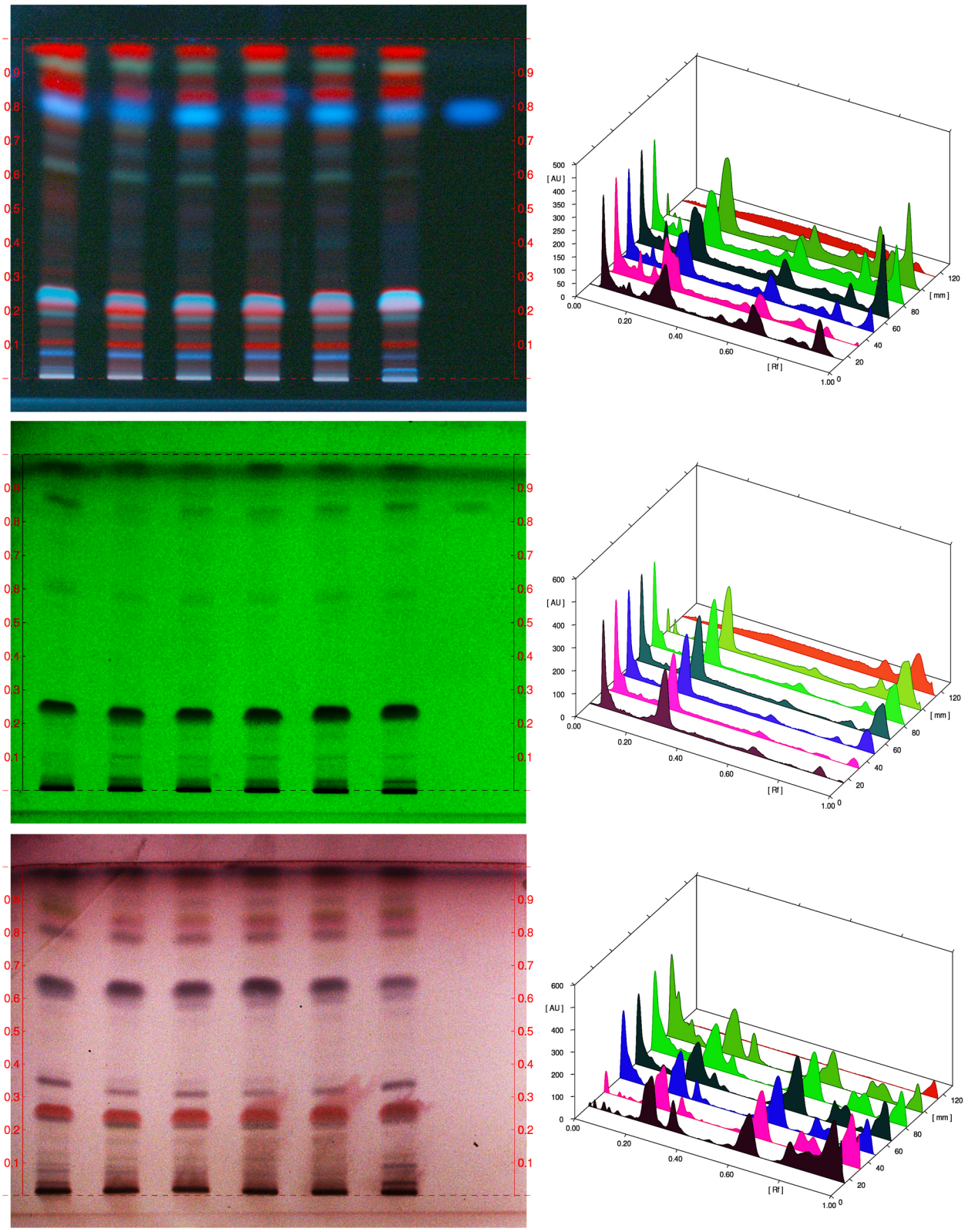

Plate 18: HPTLC comparison of sequential methanolic extracts of *in vitro* Tryptophan induced and control cultures of *A. triplinervis*. (**Tracks:** 1. MS basal (control), 2. 20 mg l^{-1} Tryptophan, 3. 40 mg l^{-1} Tryptophan, 4. 60 mg l^{-1} Tryptophan, 5. 80 mg l^{-1} Tryptophan, 6. 100 mg l^{-1} Tryptophan, 7. Ayapanin (marker compound)). **A&B.** HPTLC chromatgogram and densitogram at 366 nm; **C&D.** HPTLC chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram after derivatization.

Table 27: The compound with Rf values and area percentages of methanolic extracts of Tryptophan-induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

The compound with Rf (366 nm)	Area Percentage					
	Control	S1	S2	S3	S4	S5
0.01	-	-	20.70	19.70	12.80	-
0.03	-	-	-	-	-	0.23
0.05	20.55	23.80	-	-	-	-
0.10	-	3.38	2.62	2.99	2.25	1.03
0.15	2.21	6.64	4.41	5.47	2.85	-
0.18	2.82	6.98	3.32	4.77	3.31	0.98
0.25	2.71	30.30	31.75	32.81	24.80	26.12
0.30	27.73	-	-	-	-	-
0.40	-	-	-	-	2.73	2.39
0.45	-	-	2.47	-	3.63	3.72
0.50	3.56	-	4.58	4.74	5.88	6.07
0.60	3.64	14.37	14.46	13.40	13.49	9.48
0.68	16.35	-	-	-	-	-
0.73	-	6.16	3.07	7.87	7.21	6.61
0.80	3.34	-	4.23	-	5.69	5.35
0.85	2.39	3.82	7.69	8.26	11.23	12.50
0.90	14.70	-	0.69	-	4.12	7.74
0.95	-	-	-	-	-	17.78

The compounds with Rfs 0.80 and 0.90 were not formed in the 20 mgl^{-1} and 60 mgl^{-1} Trp supplemented media. The peak with the highest value for the compound was observed in media supplemented with higher concentrations of Trp. Highest production of the compound with Rf 0.90, was noticed in the control medium. The compound with Rf 0.05 was formed only in control and 20 mg l^{-1} Trp supplemented media. In media supplemented with higher concentrations of Trp, the compound was not formed. The compound with Rf 0.90 was formed only in media supplemented with Trp.

The remaining compounds, with Rfs 0.18, 0.25, 0.60, and 0.85, were produced in all the media used for the study. These compounds were well

produced in the Trp supplemented media rather than in the control medium. The compound with Rf 0.18 showed its maximum production in the 20 mg l^{-1} Trp supplemented medium and minimum production in the 100 mg l^{-1} Trp supplemented medium. In the medium supplemented 60 mg l^{-1} Trp the compound with Rf 0.25, was more effectively produced than other media used for the study. At certain concentrations, the external supply of Trp in the media was found to be beneficial in the production of the compound with Rf 0.25 or the pathways to produce the phytocompound. The production of the compound with Rf 0.60 was favoured by media supplemented with 20 mg l^{-1} and 40 mg l^{-1} Trp, whereas the production of the compound dropped in media supplemented with high concentrations of Trp.

The chromatogram analysis at 254 nm of UV wavelength, 11 compounds were observed (refer to **Table 28**). It was only in the medium supplemented with 20 mg l^{-1} Trp the compound with Rf value of 0.80 was formed. The compound with Rf 0.75 was formed only in media supplemented with lower concentrations of Trp (20 mg l^{-1} and 40 mg l^{-1}). Conversely, the compound with Rf 0.95 had prominent peaks and high area percentage values in the media supplemented with high concentrations of Trp. It was also observed that the external supply of Trp in the medium affected the formation of this compound. The control and 100 mg l^{-1} Trp supplemented media were ineffective in producing the compound with Rfs 0.10 and 0.15, and the maximum production of both compounds was observed in media supplemented with lower Trp concentrations.

Table 28: The compound with Rf values and area percentages of methanolic extracts of Tryptophan induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

The compound with Rf (254 nm)	Area Percentage					
	Control	**S1**	**S2**	**S3**	**S4**	**S5**
0.04	35.06	23.80	20.70	34.56	22.15	2.11
0.10	-	3.38	2.62	3.06	0.69	-
0.15	-	6.64	4.41	1.71	2.09	-
0.20	4.83	6.98	3.32	-	-	1.83
0.25	45.43	-	31.75	49.12	34.77	36.29
0.55	-	4.54	4.58	-	2.37	-
0.60	6.60	14.37	14.46	6.14	5.20	5.58
0.75	-	6.16	3.07	-	-	-
0.80	-	-	4.23	-	-	-
0.85	5.81	1.31	4.38	5.41	5.72	14.26
0.95	-	-	-	-	27.01	39.92

Additionally, the compound with Rf 0.55 was not produced in control, 60 mg l^{-1}, and 100 mg l^{-1} Trp supplemented media, but it was well produced in media supplemented with lower concentrations of Trp. The prominent peaks and area percentage values showed that 60 mg l^{-1} Trp supplemented medium was highly influential in producing the compound with Rf 0.25, and a prominent peak of this compound was also found in the control medium. The compound with Rf 0.25 was absent in the 20 mg l^{-1} Trp medium. The compound with Rf 0.20 was present in the 100 mg l^{-1} Trp supplemented medium but was absent in the 60 mg l^{-1} and 80 mg l^{-1} Trp supplemented media. The compounds with Rfs 0.60 and 0.85 were the only compounds visible in all media, including control and in media containing various concentrations of Trp. The 60 mg l^{-1} Trp medium was highly influential in producing the compound with Rf 0.60. However, a gradual reduction in the

production of this compound was noticed as the Trp concentration increased in the media.

Visualization of chromatogram at a wavelength of 550 nm, revealed a metabolite profile that comprised 14 compounds (**Table 29**). Most of the compounds were present in all the media used for the study. Compounds with Rfs 0.16 and 0.30 were absent in the 100 mg l^{-1} Trp supplemented medium. The highest production of the compound with Rf 0.16 was observed in the 80 mg l^{-1} Trp supplemented medium. Whereas, the highest production of the compound with Rf 0.30 was observed in the control medium.

Table 29: The compound with Rf values and area percentages of methanolic extracts of Tryptophan induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

The compound with Rf (550 nm)	Area Percentage					
	Control	S1	S2	S3	S4	S5
0.02	0.58	1.87	18.62	17.19	21.39	17.36
0.05	0.96	0.46	-	2.88	-	10.59
0.10	0.44	0.49	0.52	2.13	4.35	4.36
0.12	0.54	-	-	-	3.03	2.76
0.16	0.70	0.89	0.68	1.26	4.36	-
0.25	20.47	20.56	21.01	22.32	23.39	24.48
0.30	8.01	4.16	4.63	3.82	4.97	-
0.35	-	1.15	1.00	1.05	0.50	8.25
0.58	1.14	1.07	0.89	0.97	1.12	1.66
0.65	18.51	25.70	26.39	31.02	22.55	17.13
0.75	9.89	7.98	7.13	7.41	6.05	5.22
0.85	9.54	6.73	4.61	8.76	7.72	7.14
0.90	-	1.98	0.73	1.18	0.57	0.54
0.95	28.65	26.95	13.78	-	-	-

It was observed that in the control medium, compounds with Rfs 0.35 and 0.90 were not formed. When a higher Trp supplemented medium was used, the compound with Rf 0.35 produced the most, whereas the compound

with Rf 0.90 produced the most in a lower Trp medium. Both compounds were produced due to the external application of Trp in the growth medium. Additionally, the compound with Rf 0.05 was not detected in the 40 mgl^{-1} and 80 mg l^{-1} Trp supplemented media, and highest peak of this compound was observed in medium supplemented with 100 mg l^{-1} Trp. The medium supplemented with high concentration of Trp was effective in the production of the compound with Rf 0.05.

In media, supplemented with 20 mg l^{-1}, 40 mg l^{-1}, and 60 mg $l^{-1}$of Trp, the compound with Rf 0.12 was not formed. Additionally, the compound with Rf 0.95 was not present in the supplemented with higher concentration (80 mg l^{-1} and 100 mg l^{-1}) of Trp, and the compound exhibited a prominent peak in the control medium. Conversely, the other seven compounds, including the compounds with Rfs 0.02, 0.10, 0.25, 0.58, 0.65, 0.75, and 0.85, were formed in all media, including the control medium.

The external supplementation of Trp to the medium positively affected the production of compounds with Rfs 0.02, 0.10, 0.25, and 0.65. The higher concentrations of Trp showed a gradual enhancement in the production of compounds with Rfs 0.02, 0.10, and 0.25. The The compound with Rf 0.65 exhibited a higher area percentage value in the 60 mg l^{-1} Trp supplemented medium. Conversely, less amount of the compound with Rf 0.58 was produced in media supplemented with lower concentrations of Trp, while compounds with Rfs 0.75 and 0.85 showed maximum production in the control medium. The production of the compound with Rf 0.75 was decreased in media supplemented with higher concentrations of Trp, while the result was vice versa in the case of the compound with Rf 0.85.

In the present studies on *A. triplinervis* Trp was a crucial element that significantly enhanced the production of 'Ayapanin' and some other secondary metabolites. The influence of Trp in the production of phytocompounds were reported earlier in many plants like *Nasturtium officinale* (Klimek-Szczykutowicz *et al.*, 2021) and *Hypericum perforatum* L (Erland and Saxena, 2019). Knobloch and Berlin (1980), Whitmer *et al.* (2002), Taha *et al.* (2009), Van der Fits *et al.* (2000), and Verpoorte *et al.* (2002), were reported in *Catharanthus roseus.* The reports found that the addition of tryptamine or Trp in the nutrient media enhanced secondary metabolite production in plants. These reports were in corroborative with the results of present experiments on *A. triplinervis*

4.5.2.5. MS Medium Strength

The MS medium, developed by Murashige and Skoog in 1962, is a well-known medium for culturing higher plants. It contains various essential components necessary for plant growth and development. This study aimed to analyze the phytochemical effects of the modified strengths of the MS medium as stress signals in the *in vitro* cultures of *A. triplinervis*. The following strengths were used: 1/6 MS l^{-1}, ¼ MS l^{-1}, ½ MS l^{-1}, ¾ MS l^{-1}, and full-strength MS^{-1} (control).

In the present study, the HPTLC technique was used to analyze the effects of different MS media strengths on the *in vitro* production of secondary metabolites in *Ayapana triplinervis* (**Plate 19**). The compound with Rf 0.80, was identified as 'Ayapanin' or '7-methoxycoumarin', with the help of the marker compound (**Figure 46**). The area percentage values of the peaks

observed in HPTLC chromatogram showed that among the tested concentrations, the full-strength MS l^{-1} medium resulted in the highest

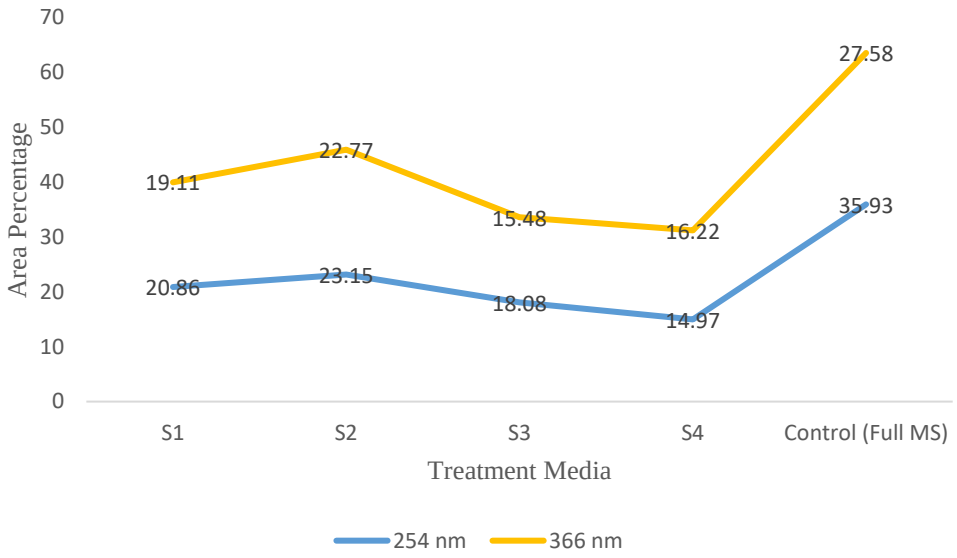

Figure 46: Area percentages of Ayapanin in *in vitro* MS Medium strength induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (1/6 MS l^{-1}), S2 (1/4 MS l^{-1}), S3 (1/2 MS l^{-1}) and S4 (3/4 MS l^{-1}).

production of 'Ayapanin.' This revealed that the optimal concentration of essential nutrients in the medium significantly affected the production of 'Ayapanin'.

Twenty-one secondary metabolite compounds were observed in HPTLC chromatogram at a wavelength of 366 nm (**Table 30**). Among the stress-inducing media, 1/2 MS l^{-1} produced significantly more compounds than the others. All the peaks formed in the media showed good area percentage values, with only a few exhibiting negligible values. Compounds with certain Rfs, were exclusively found in specific media: Rfs 0.03 and 0.07 in full MS l^{-1} (control), Rf 0.13 in 1/6 MS l^{-1}, and Rf 0.90 in 3/4 MS l^{-1}.

Compounds with Rfs 0.03 and 0.07, 0.13 and 0.90 had excellent area percentage values. Certain compounds were absent in the control medium, such as Rfs 0.05, 0.20, 0.40, 0.44, 0.60, and 0.65. Compounds with Rfs 0.03 and 0.07, 0.13 and 0.90 had excellent area percentage values and ccompounds with Rfs 0.05, 0.20, and 0.44 showed increased area percentage values in the stress media. The ccompound with Rf 0.05 was absent only in the control medium, this indicated that the specific concentration of essential nutrients in the medium significantly influences the production of various compounds.

Table 30: Rf values and area percentages of methanolic extracts of MS medium strength induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

Rf (366 nm)	**Area Percentage**				
	Control	**S1**	**S2**	**S3**	**S4**
0.01	0.08	0.08	0.09	0.01	0.19
0.03	0.54	-	-	-	-
0.05	-	0.16	1.03	1.24	1.03
0.07	0.61	-	-	-	-
0.10	8.96	-	1.75	2.28	3.69
0.13	-	3.41	-	-	-
0.15	9.94	15.06	18.35	22.74	19.24
0.20	-	-	5.19	8.10	13.87
0.25	22.33	5.69	1.33	1.62	3.75
0.29	8.41	1.46	0.22	0.40	0.16
0.40	-	-	5.63	3.92	1.52
0.44	-	2.43	6.27	6.69	-
0.50	1.63	6.98	5.76	6.49	13.54
0.55	2.96	10.50	14.32	14.45	12.47
0.60	-	-	3.47	1.76	1.04
0.65	-	5.01	-	3.34	-
0.70	3.48	12.22	9.93	3.65	5.94
0.80	27.58	19.11	22.77	15.48	16.22
0.85	3.17	4.60	3.89	4.06	3.19
0.90	-	-	-	-	4.13
0.95	9.41	13.28	-	3.60	-

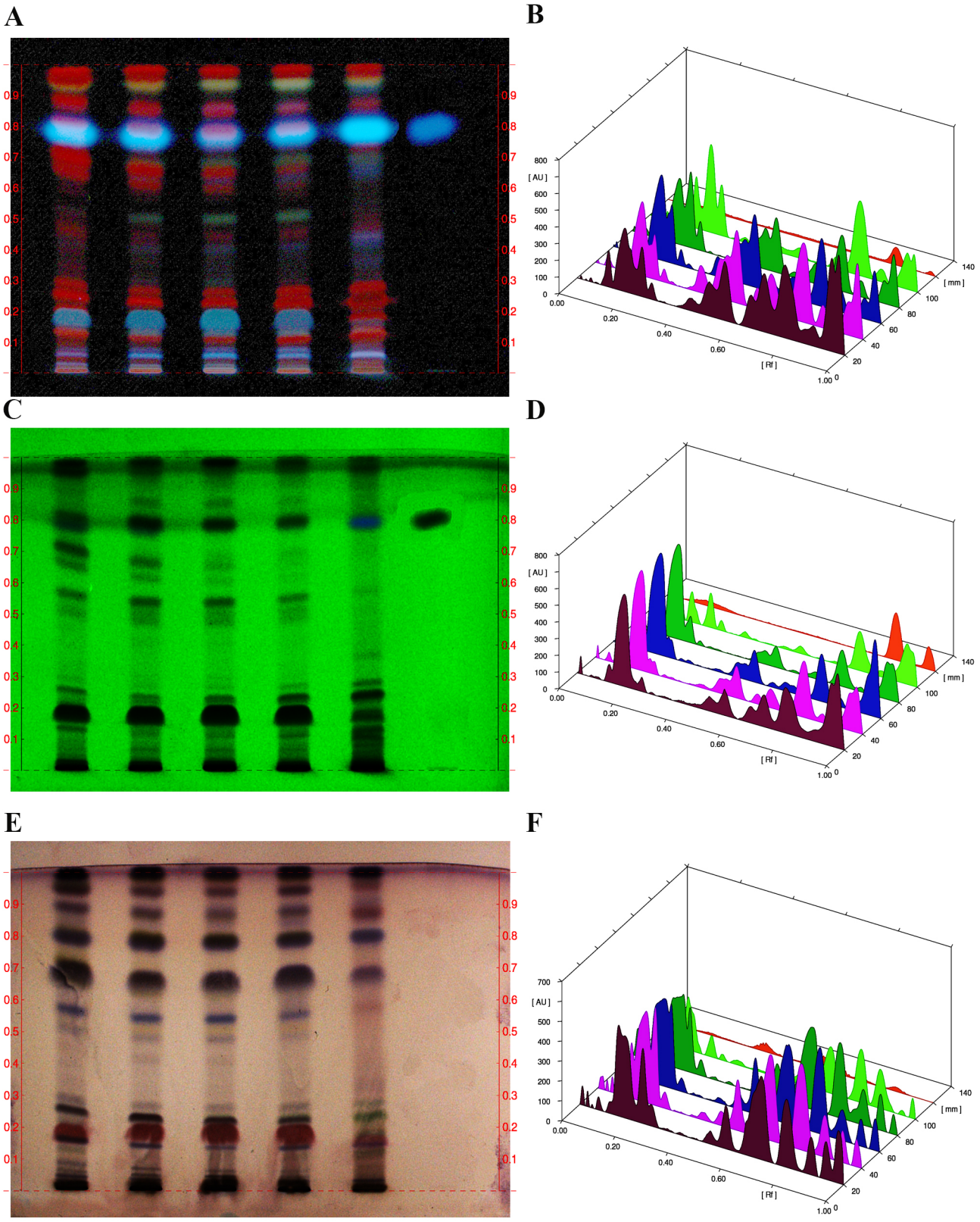

Plate 19: HPTLC Comparison of sequential methanolic extracts of *in vitro* MS media strength induced and control cultures of *A. triplinervis*. (**Tracks:** 1. ⅙ MS l^{-1}, 2. ¼ MS l^{-1}, 3. ½ MS l^{-1}, 4. ¾ MS l^{-1}, 5. Full MS l^{-1} (control), 6. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** chromatogram and densitogram at 254 nm and **E&F.** chromatogram and densitogram after derivatization.

The production of the compound with Rf 0.20 was higher in the 3/4 MS l^{-1} medium, and both Rfs 0.20, 0.40, and 0.60 were absent in the two extreme media strengths (1/6 MS l^{-1} and full MS l^{-1}) used in the study. However, the accumulation of compounds with Rfs 0.40 and 0.60 were reduced with the increase in nutrient strength of the media. The compound with Rf 0.44 was visible only at lower media strengths (1/6 MS l^{-1} - 1/2 MS l^{-1}), compound with Rf 0.65 was only produced in the 1/6 MS l^{-1} and 1/2 MS l^{-1} media. The production of the compound with Rf 0.95 was high in the 1/6 MS l^{-1} medium and the compound was also found in 1/6 MS l^{-1}, 1/2 MS l^{-1}, and the full MS l^{-1} media.

Compound with Rf 0.10 was absent only in the 1/6 MS l^{-1} medium, and the area percentage values of the peaks in the other media increased with increasing media strength. The other nine compounds with Rfs 0.01, 0.15, 0.25, 0.29, 0.50, 0.55, 0.70, 0.80, and 0.85 were produced in all the strengths of MS media used for the study. The production of compounds with Rfs 0.15, 0.50, and 0.55 increased with reduced strengths of MS media, but the production of Rfs 0.25 and 0.29 experienced a negative impact. The compound with Rf 0.70 was produced in high quantity in the 1/6 MS l^{-1} medium, and when media containing increased strength of nutrients were used, its production was found to be reduced.

Fifteen phytocompounds were observed in the analysis of chromatogram at 254 nm, (**Table 31**). Thirteen compounds, except compounds with Rfs 0.64 and 0.95, were found in the plant extract from the control medium. However, it was noticed that these compounds had higher area percentage values in the 1/6 MS l^{-1} medium. Compounds with Rfs 0.10

and 0.15 showed their presence only in the 1/6 MS l^{-1} and full MS l^{-1} strengths of the MS media. The compound with Rf 0.85 was not formed in 1/6 MS l^{-1} medium this may be due to the absence of a sufficient quantity of essential nutrients in the medium. However, in media containing increased strength of nutrients, this compound was formed.

Table 31: Rf values and area percentages of methanolic extracts of MS medium strength induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm.

Rf (254 nm)	Area Percentage				
	Control	**S1**	**S2**	**S3**	**S4**
0.01	0.25	0.97	0.60	0.53	0.34
0.05	1.22	0.17	1.20	0.65	0.84
0.10	6.98	0.25	-	-	-
0.15	5.58	2.49	-	-	-
0.18	15.68	31.66	43.64	53.05	47.79
0.22	17.14	2.18	1.62	3.77	6.26
0.27	3.77	0.22	0.32	0.39	0.73
0.38	2.81	0.30	1.01	0.81	-
0.50	1.61	4.77	6.40	3.93	6.29
0.55	4.81	6.71	9.22	9.92	6.52
0.64	-	4.14	2.42	2.66	-
0.68	1.17	9.91	6.74	1.98	2.61
0.80	35.93	20.86	23.15	18.08	14.97
0.85	3.04	-	2.07	2.45	1.05
0.95	-	15.04	-	-	12.02

The remaining ten compounds with Rfs 0.01, 0.05, 0.18, 0.22, 0.27, 0.38, 0.50, 0.55, and 0.68, were formed in all the media strengths used for the study. It was observed that the production of the compound with Rf 0.18 had high area percentage values in all the stress media and was significantly high in the 1/2 MS l^{-1} medium. The production of compounds with Rfs 0.50, 0.55, and 0.68 was enhanced in the various lower strengths of the MS medium. The area percentage values of the compounds with Rfs 0.22, 0.27, 0.38, and 0.80

showed that their production was maximum in the full MS l^{-1} (control) medium compared to other media.

Fifteen compounds were observed when the chromatogram was visualised at 550 nm (**Table 32**). All 15 compounds were present in the 1/6 MS l^{-1} medium.

Table 32: Rf values and area percentages of methanolic extracts of MS medium strength induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

Rf (550 nm)	Area Percentage				
	Control	S1	S2	S3	S4
0.01	1.33	0.96	-	-	-
0.03	1.38	0.58	0.93	2.98	2.16
0.05	0.53	0.11	0.34	0.35	0.10
0.10	-	0.94	0.12	-	-
0.15	22.61	12.74	4.21	-	-
0.18	-	12.51	20.07	27.14	25.85
0.23	5.08	9.72	9.66	6.77	6.21
0.27	1.04	2.36	1.64	1.42	1.69
0.30	1.74	0.31	-	-	0.33
0.49	-	1.92	2.16	2.50	1.72
0.55	6.70	6.81	7.56	8.88	7.40
0.68	22.66	26.34	21.86	22.25	26.99
0.80	20.33	14.19	19.21	17.85	15.83
0.88	15.79	5.28	6.72	5.91	7.20
0.95	0.77	4.97	3.63	3.05	4.27

The peaks with high area percentages were observed for compounds with Rfs 0.18 and 0.49 in all four altered MS media, except in the control. The compound with Rf 0.01 was found only in the 1/6 MS l^{-1} and control media, while the compound with Rf 0.10 was produced in noticeable amounts in the 1/6 MS l^{-1} and 1/4 MS l^{-1} media. The compound with Rf 0.15 was identified in the 1/6 MS l^{-1}, 1/4 MS l^{-1}, and full MS media, while the compound with Rf 0.30 was absent in the 1/4 MS l^{-1} and 1/2 MS l^{-1} media.

The remaining nine compounds with Rfs 0.03, 0.05, 0.23, 0.27, 0.55, 0.68, 0.80, 0.88, and 0.95 were produced in all the media used for the study. Compounds with Rfs 0.23, 0.27, 0.68, and 0.95 were produced more prominently in the 1/6 MS l^{-1} medium than in media with higher nutrient concentrations. The peaks with the highest area percentages were observed for compounds with Rfs 0.68 and 0.80 and were nearly equal in the 1/6 MS l^{-1} and control media. The compound with Rf 0.55 showed no significant differences in area percentage values; this showed that media strength did not significantly affect production. The production of the compound with Rf 0.88 gradually increased with the concentration of nutrients in the MS medium.

In the present study, the strength of MS media played a significant role in producing secondary metabolites in *A. triplinervis*. It was observed that the culture media with 1/6 MS l^{-1} and 1/2 MS l^{-1} generated compounds with significant peaks with noticeable area percentages. Additionally, supplementing the nutrients to an optimal level resulted in greater production of these compounds. Fadel *et al.* (2010) reported that altering the medium strength can increase the spearmint content in *Menta spicata* L. Abou-Dahab *et al.* (2004) reported a change in the production of carotenoids and indole compounds with a change in the strengths of media. These reports were in consonant with the results of present experiments on *A. triplinervis*

4.5.2.6. Jasmonic acid

Jasmonic acid (JA) is a plant growth regulator inducing stress responses (Shan *et al.*, 2015; Upreti & Sharma, 2016; Wang *et al.*, 2020).

The present study explored how different concentrations of JA influence the creation of secondary metabolites in the *in vitro* cultures of *A.*

triplinervis (**Plate 20**). The study employed five varying concentrations of JA: 1.25, 2.5, 5.0, 10 and 15 µM l^{-1}. With the help of the marker compound, the compound 'Ayapanin' was identified as a compound with Rf 0.85.

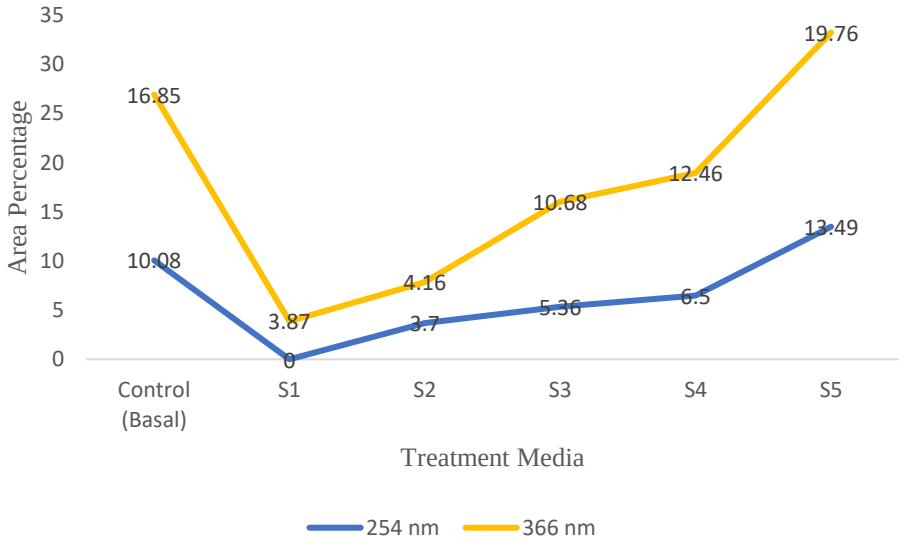

Figure 46: Area percentages of Ayapanin in *in vitro* Jasmonic acid-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (1.25 µM l^{-1} JA), S2 (2.5 µM l^{-1} JA), S3 (5.0 µM l^{-1} JA), S4 (10 µM l^{-1} JA) and S5 (15 µM l^{-1} JA).

A linear increase in the production of 'Ayapanin' was observed with the increasing concentrations of JA (Figure 40). The highest 'Ayapanin' formation was noticed in the 15 µM l^{-1} JA concentration. At 254 nm, production of 'Ayapanin' was not observed in the medium supplemented with 1.25 µM l^{-1} JA. However, a small peak was seen when visualised at 366 nm. A good area percentage value of 'Ayapanin' was found in the control medium. In the medium supplemented with 15 µM l^{-1} JA an enhanced production of 'Ayapanin' was observed.

In the HPTLC chromatogram of methanolic extracts of *A. triplinervis*, a metabolic profile consisting of 17 compounds was visualized at a wavelength of 366 nm (**Table 33**). Two unique compounds, with Rf 0.60 and Rf 0.95, were found in the 15 and 2.5 µM l^{-1} JA supplemented media. Similarly, two compounds, with Rfs 0.03 and 0.40, were produced only in the 15 and 10 µM l^{-1} JA supplemented media. The production of the compound with Rf 0.45 was not observed in the media supplemented with lower concentrations of JA (1.25 µM l^{-1} and 2.5 µM l^{-1}), but its production increased at higher concentrations of JA-induced media.

In contrast, the compound with Rf 0.70 was visible only in the 1.25 µM l^{-1} and 2.5 µM l^{-1} JA supplemented media, increased production was observed in the medium containing 2.5 µM l^{-1} JA medium. The compound with Rf 0.75 was not formed in the medium supplemented with 2.5 µM l^{-1} JA and the compound was produced in the highest amount in the control media than in media supplemented with JA. An increase in the production of the compound with Rf 0.75 was noticed with an increase in the concentrations of JA in the media. The compound with Rf 0.80 was produced exclusively in media supplemented with higher concentrations of JA and 10 µM l^{-1} was found as the optimum concentration of JA in the medium for the production of the compound. Additionally, the compound with Rf 0.90 showed a prominent peak in the 15 µM l^{-1} JA supplemented medium, and besides this medium, the compound appeared in the medium supplemented with 10 µM l^{-1} JA and in the control medium.

On the other hand, the compound with Rf 0.10 was formed in media supplemented with low concentrations of JA and the compound was not

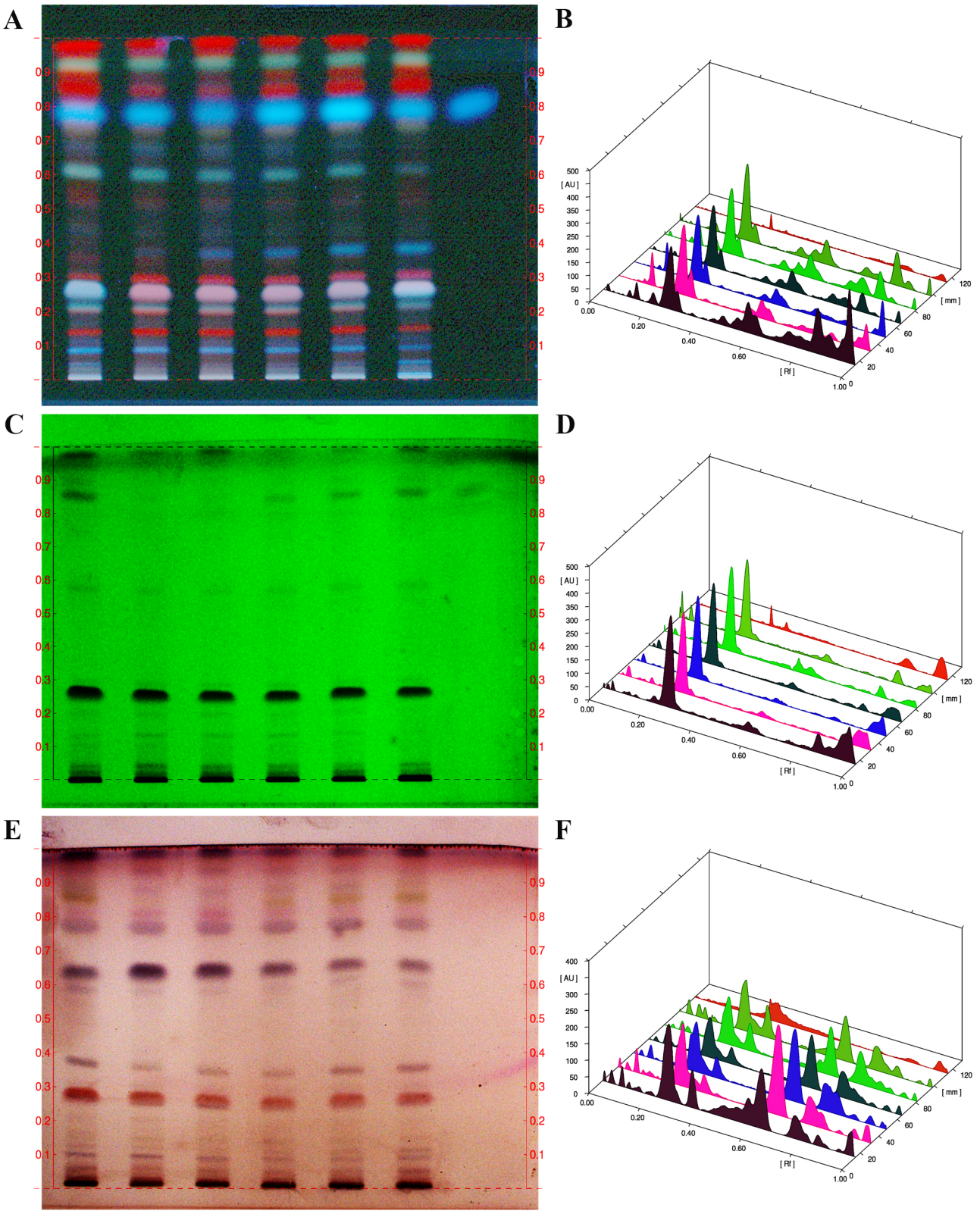

Plate 20: HPTLC comparison of sequential methanolic extracts of *in vitro* Jasmonic acid induced and control cultures of *A. triplinervis*. (**Tracks:** 1. 15 µM l^{-1} Jasmonic acid, 2. 1.25 µM l^{-1} Jasmonic acid, 3. 2.5 µM l^{-1} Jasmonic acid, 4. 5.0 µM l^{-1} Jasmonic acid, 5. 10 µM l^{-1} Jasmonic acid, 6. MS Basal (Control), 7. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.**HPTLC chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram after derivatization.

formed in media supplemented with higher concentrations of JA. The remaining compounds, with Rfs 0.15, 0.20, 0.25, 0.30, 0.50, 0.55, and 0.85, were visible in all the JA supplemented and control media. The lower JA concentrations were much more efficient in the production of compounds with Rfs 0.15, 0.20, 0.25, and 0.30, and the production of these compounds was positively affected by JA. The increasing JA concentrations reduced the production of these compounds. However, the production of compounds with Rf s 0.50 and 0.55 exhibited progressive augmentation with an increase in the concentration of JA in the medium, and the 5 µM l^{-1} JA-induced medium showed an increased production of the compound with Rf 0.55.

Table 33: The compound with Rf values and area percentages of methanolic extracts of Jasmonic acid-induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

The compound with Rf (366 nm)	Area Percentage					
	Control	S1	S2	S3	S4	S5
0.03	-	-	-	-	0.62	0.82
0.10	-	1.10	1.12	0.99	0.93	1.07
0.15	1.90	9.63	8.67	6.72	4.48	2.81
0.20	1.91	6.20	5.33	4.13	3.54	3.04
0.25	37.26	45.57	43.83	35.29	30.87	25.76
0.30	6.32	10.59	8.46	6.68	5.34	4.01
0.40	-	-	-	-	1.15	1.08
0.45	4.29	-	-	3.70	4.51	3.19
0.50	6.29	4.20	4.39	4.60	5.25	4.38
0.55	14.07	11.82	14.42	15.04	14.35	11.96
0.60	-	-	-	-	-	5.92
0.70	-	3.47	5.82	-	-	-
0.75	5.14	3.54	-	5.35	4.90	6.39
0.80	2.51	-	-	6.81	10.04	-
0.85	16.85	3.87	4.16	10.68	12.46	19.76
0.90	3.48	-	-	-	1.56	9.81
0.95	-	-	2.15	-	-	-

The metabolite profile visualized at 254 nm wavelength of UV light revealed only ten compounds (**Table 34**). Among them, one unique compound was detected in the 15 µM l^{-1} JA-supplemented medium (compound with Rf 0.10). The remaining compounds with Rf s, 0.15, 0.29, and 0.95, were not present in the 15 µM l^{-1} JA supplemented medium and the compound with Rf 0.15 was also absent in the control medium. The highest production of the compound with Rf 0.15 was observed in the 1.25 µM l^{-1} JA supplemented medium, the production of this compound gradually decreased with increasing concentrations of JA in the medium. The compound with Rf 0.29 was found only in the 1.25 µM l^{-1} JA supplemented medium and, in the control medium, and was not detected in any other media supplemented with JA.

Table 34: The compound with Rf values and area percentages of methanolic extracts of Jasmonic acid-induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

The compound with Rf (254 nm)	Area Percentage					
	Control	S1	S2	S3	S4	S5
0.02	4.31	-	-	0.77	1.41	1.06
0.05	4.66	3.67	3.40	3.19	1.61	1.94
0.10	-	-	-	-	-	1.60
0.15	-	2.10	2.08	1.92	1.03	-
0.20	2.17	3.10	3.85	3.48	3.34	3.79
0.25	63.86	64.88	81.56	78.03	65.00	69.15
0.29	2.43	2.64	-	-	-	-
0.60	7.25	5.57	1.66	7.25	8.14	7.41
0.85	10.08	-	3.70	5.36	6.50	13.49
0.95	5.24	18.03	-	-	4.09	-

The compound with Rf 0.95 was highly produced in the 1.25 µM l^{-1}JA supplemented medium and was absent in the media supplemented with

subsequent two higher JA concentrations. However, it was formed in the medium supplemented with10 µM l^{-1} JA and in the control medium. The highest area percentage of compound with Rf 0.02, was observed in the control medium. Compounds with Rfs 0.05, 0.20, 0.25, and 0.60 were also visible in all JA-induced media and control medium. The compound with Rf 0.05 was produced well in all media.

A decreased area percentage value of the compound with Rf 0.20 was observed in the control medium, but in the JA-induced media, the peaks of this compound had almost the same area percentage values. The compound with Rf 0.25 was produced highly with prominent peaks in all the media, and its maximum production was observed in the 2.5 µM l^{-1} JA supplemented medium. The next higher area percentage value of this compound was found in the 5.0 µM l^{-1} JA medium, and a significant difference was observed in the area percentage values between control and JA-supplemented media. Elicitation with JA enhanced the production of the compound with Rf 0.25.

Upon ANS-mediated derivatization, sixteen compounds were visualized at a wavelength of 550 nm in the secondary metabolites profile (**Table 35**). One of these compounds was unique to the 15 µM l^{-1} JA-induced medium (compound with Rf value of 0.48). In The 15 µM l^{-1} JA supplemented medium, compounds with Rf values of 0.10 and 0.80 were not formed. The compound with Rf 0.10 was also absent in the 2.5 µM $l^{-1}$and 15 µM l^{-1} JA supplemented media. The compound with Rf 0.80 was visible only in the 2.5 µM l^{-1} and 5.0 µM l^{-1} JA supplemented media, with significant production observed in the former medium. The compound with Rf 0.05 was

absent in the 2.5 µM l^{-1} and 5.0 µM l^{-1} JA supplemented media, with maximum production observed in the control medium.

The compound with Rf 0.15 was formed only in 15 and 1.25 µM l^{-1} JA- supplemented media. The compound with Rf 0.55 was visible only in the 10 µM l^{-1} JA supplemented medium and in the control medium, with the area percentage value reduced in the JA supplemented medium. The addition of exogenous JA reduced the production of the compound with Rf 0.95. In the control medium, the compound with Rf 0.95 was not formed, and the compound was produced only in the medium supplemented with JA. The compound with Rf 0.95 showed a high area percentage peak in the 15 µM l^{-1} JA-induced medium.

Table 35: The compound with Rf values and area percentages of methanolic extracts of Jasmonic acid-induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

The compound with Rf (550 nm)	**Area Percentage**					
	Control	**S1**	**S2**	**S3**	**S4**	**S5**
0.02	-	0.60	0.69	0.81	-	0.52
0.05	2.48	0.93	-	-	0.55	1.64
0.07	2.64	2.84	2.12	2.39	1.86	2.25
0.10	2.04	0.49	-	0.79	1.16	-
0.12	1.19	1.06	0.83	1.50	0.89	0.66
0.15	-	0.74	-	-	-	0.98
0.25	26.51	27.16	25.58	26.22	28.78	27.91
0.35	12.38	4.84	7.12	7.63	11.05	8.18
0.48	-	-	-	-	-	5.22
0.55	-	-	-	-	1.43	4.04
0.60	8.35	3.33	3.05	4.33	5.40	7.93
0.65	21.54	38.86	34.84	30.11	22.95	21.44
0.77	16.04	12.47	14.31	16.84	19.56	10.41
0.80	-	-	7.56	6.41	-	-
0.85	3.83	3.97	1.65	1.18	3.31	2.87
0.95	-	2.07	2.25	1.80	1.34	5.17

In all the media, including the control medium, the compound with Rf values of 0.07, 0.12, 0.25, 0.35, 0.60, 0.65, 0.77, and 0.85 were formed. The most prominent peaks in all the media were observed for the compound with Rf 0.25, and the JA-supplemented media showed no significant change in the production of this compound when compared with the control medium. More significant peaks were observed for the compound with Rf 0.65, with lower concentrations of JA (1.25, 2.5, and 5.0 µM l^{-1} JA) exhibited a positive effect in the production of this compound. However, production was reduced in the control and 15 µM l^{-1} JA supplemented media. In the case of the compound with Rf 0.77, increasing JA concentrations showed an enhanced production up to 10 µM l^{-1} JA medium, but in the 15 µM l^{-1} JA medium, a drop in the production was observed.

Earlier studies on the effects of JA on phytocompound production have been reported in many plants, such as phenolics in *Celastrus paniculatus*, alkaloid contents in *Catheranthus roseus* (Al-Huqail & Ali, 2021), phenolics, flavonols, and flavols in *Hypericum perforatum* (Nabi *et al.*, 2021), various compounds in *Hypericum avicullariifolium* and *Hypericum pruinatum* (Cirak *et al.,* 2020), polyphenols and carotenoids in *Lavandula angustifolia* Mill (Miclea *et al.* 2020), triterpene in *Jatropha curcas* (Zaragoza-Martínez *et al.,* 2016), essential oils in *Ocimum basilicum* (Złotek *et al.,* 2016), phenolics and flavonoids in *Artemisia absinthium* L. (Ali *et al.,* 2014), bacoside in *Bacopa monnieri*, (Sharma *et al.,* 2014), plumbagin in *Plumbago rosea* (Silja *et al.* 2014), hypericin and pseudohypericin in *Hypericum hirsutum* and *H. maculatum* (Coste *et al.,* 2011), rosmaric acid in *Mentha piperita* (Krzyzanowska *et al.,* 2011), oleanolic in *Calendula officinalis* (Wiktorowska

et al., 2009). This may be because JA and its derivatives activate genes related to secondary metabolism, either directly or indirectly, acting as a signal molecule during the elicitation process (Gundlach *et al.,* 1992).

In the present experiments on *A. triplinervis,* the profile of secondary metabolites indicated a gradual progression in the production of 'Ayapanin' with the increase in the concentration of JA in the medium. Additionally, JA positively impacted the production of several other plant compounds.

4.5.2.7. Salicylic acid

SA is an endogenous hormonal signal molecule that enhances plant resistance to pathogens and facilitates the production of secondary metabolites (Wang *et al.,* 2004).

The objective of the present HPTLC study was to explore the impact of different concentrations of SA on the secondary metabolite production in *A. triplinervis* (**Plate 21**) *in vitro* cultures. To do so, the following concentrations of SA were selected - 10 µM l^{-1} SA (S1), 25 µM l^{-1} SA (S2), 50 µM l^{-1} SA (S3), 75 µM l^{-1} SA (S4), and 100 µM l^{-1} SA (S5). As SA is not soluble in water, a solution of NaHCO3 was prepared to dissolve it, and individual controls of NaHCO3 were included along with the basal MS medium control. The following concentrations were selected for the individual NaHCO3 controls – 1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C1, 2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C2, 5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C3, 7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C4 and 10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium – C5.

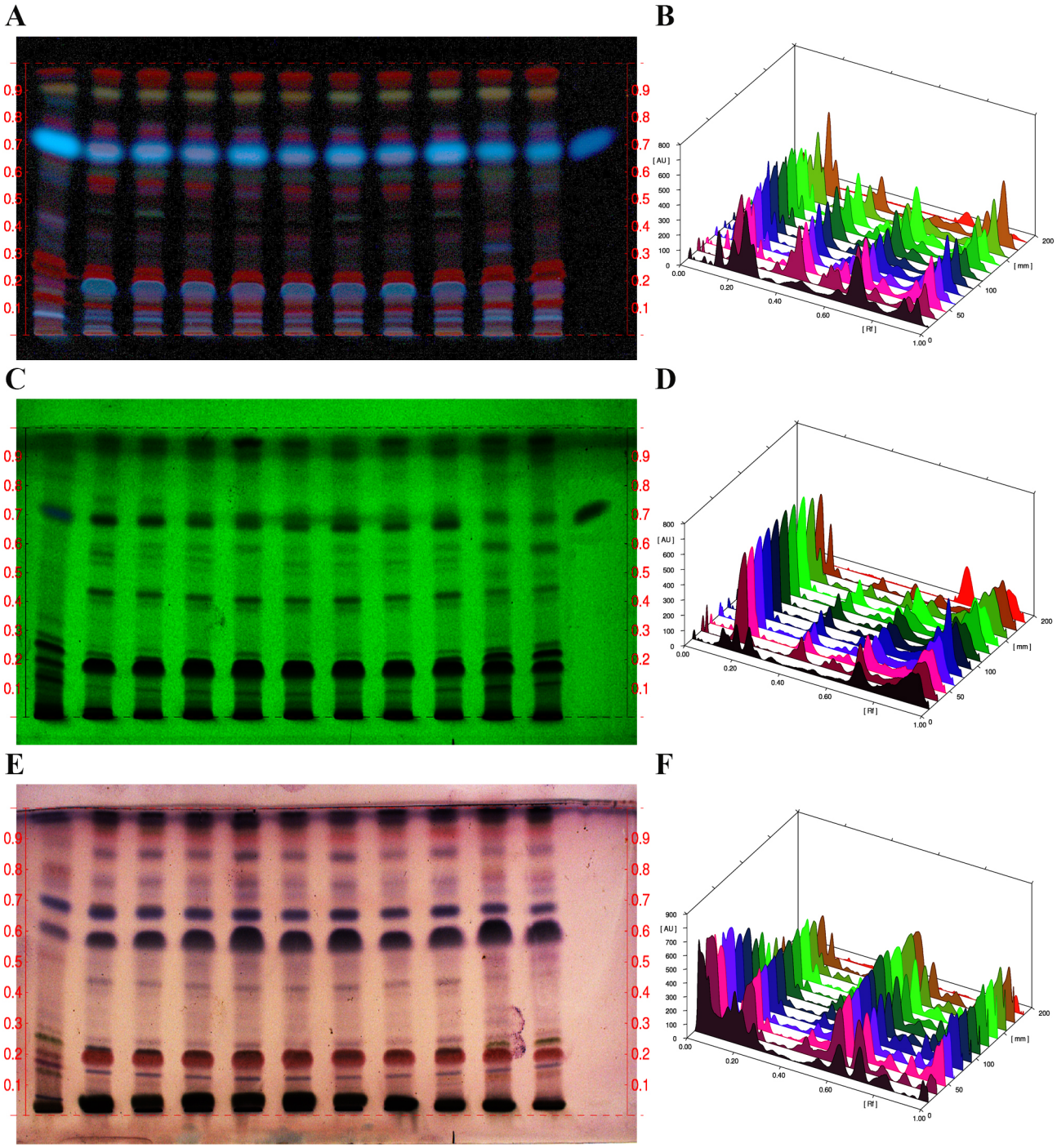

Plate 21: HPTLC comparison of sequential methanolic extracts of *in vitro* Salicylic acid induced, $NaHCO_3$ control and basal MS medium cultures of *A. triplinervis*. (**Tracks:** 1. MS basal control, 2. 10 µM l^{-1} Salicylic acid, 3. Control 1 (1 ml 1% NaHCO3 soln. l^{-1} MS Medium), 4. 25 µM l^{-1} Salicylic acid, 5. Control 2 (2.5 ml 1% NaHCO3 soln. l^{-1} MS Medium), 6. 50 µM l^{-1} Salicylic acid, 7. Control 3 (5.0 ml 1% NaHCO3 soln. l^{-1} MS Medium), 8. 75 µM l^{-1} Salicylic acid, 9. Control 4 (7.5 ml 1% NaHCO3 soln. l^{-1} MS Medium), 10. 100 µM l^{-1} Salicylic acid, 11. Control 5 (10 ml 1% NaHCO3 soln. l^{-1} MS Medium), 12. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** HPTLC Chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram after derivatization.

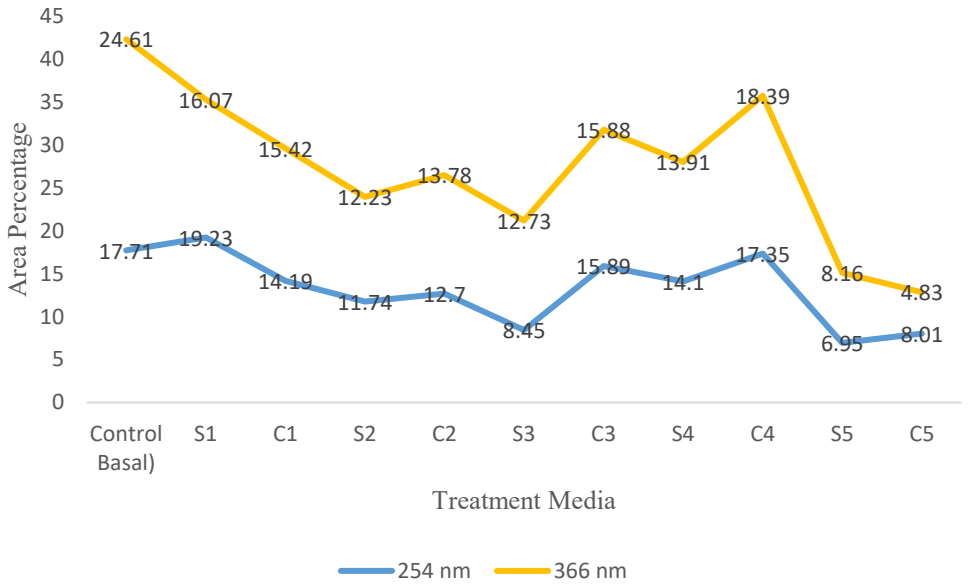

Figure 48: Area percentages of Ayapanin in *in vitro* Salicylic acid-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (10 µM l^{-1} SA), C1 (1 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium), S2 (25 µM l^{-1} SA), C2 (2.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium), S3 (50 µM l^{-1} SA), C3 (5.0 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium), S4 (75 µM l^{-1} SA), C4 (7.5 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium), S5 (100 µM l^{-1} SA) and C5 (10 ml 1% $NaHCO_3$ soln. l^{-1} MS Medium).

In HPTLC chromatogram the compound with Rf value of 0.70 was identified as 'Ayapanin' with the help of the standard marker compound (**Figure 48**). It was found that the production of 'Ayapanin' was higher in the MS basal control medium than in the elicited cultures. At 254 nm, a significant increase in the production of 'Ayapanin' in the S1 medium compared to the control was observed. A slight increase in the area percentage value in the S4 medium was also noticed. While the peaks of 'Ayapanin' in C2, C3, and C4 showed increased area percentage values compared to their respective stress media, a significant drop in both the S5 and C5 media was noticed. This showed that higher concentrations of SA in the media inhibited the production of 'Ayapanin'.

Visualization of chromatogram at 366 nm UV wavelength, revealed the presence of twenty-one compounds (listed in **Table 36**). Two unique compounds with Rf s - 0.13 and 0.76 - were exclusively found in the MS basal medium. The compound with Rf 0.08 was observed only in S5 and C5 media, while the compound with Rf 0.01 was identified only in the MS basal control, S1, and S2 and not detected in their corresponding controls or other media. The compound was not formed in media containing higher concentrations of SA. The compounds with Rf s 0.50 and 0.55 were absent in the MS basal control, S5, and C5 media, but were found in the rest of the SA media, their production was found to be gradually decreasing as the stress levels in the media increased. The compounds with Rf s 0.20 and 0.74 were present exclusively in the SA-induced cultures and their control media. The compound with Rf 0.20 showed a gradual increase with the increase in SA stress levels in the media. The compound with Rf 0.40 was absent only in the S1 media, and its production was found to be decreased with higher stress levels in the media. The compound with Rf 0.45 was present only in the basal control, S1, and S5, with higher values observed in the S1 medium.

The compound with Rf s 0.03 and 0.38 were not found in the C5 medium. The compound with Rf 0.38 was absent only in the C5 medium, and the lowest area percentage value was observed in the basal control medium. There was no significant difference in the area percentage values of the compound with Rf 0.38 in all the media used for the study. The production of the compound with Rf 0.10 was found to be lower at lower SA levels and was absent only in the S3 medium. The compound with Rf 0.60 exhibited a significant peak in the S5 medium. The compound with Rf 0.05 was absent in

the S1 and C5 media, while the compound with Rf 0.30 was highly produced in the basal medium. With the increase in SA stress levels, the production of the latter compound was found to decrease slowly and the compound was not found in the C2, S3, and C5 media.

Table 36: The compound with Rf values and area percentages of methanolic extracts of Salicylic-acid-induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

The compound with Rf (366nm)	**Area Percentage**										
	Control	**S1**	**C1**	**S2**	**C2**	**S3**	**C3**	**S4**	**C4**	**S5**	**C5**
0.01	0.82	0.59	-	0.50	-	-	-	-	-	-	-
0.03	0.12	0.65	0.13	0.10	0.28	0.40	0.11	0.09	0.10	0.20	-
0.05	0.76	-	0.34	0.33	0.48	1.90	0.52	0.28	0.22	0.41	-
0.08	-	-	-	-	-	-	-	-	-	0.31	0.61
0.10	0.32	1.27	1.81	2.01	2.24	-	1.77	2.06	2.41	4.25	5.69
0.13	7.10	-	-	-	-	-	-	-	-	-	-
0.18	8.65	20.08	16.08	18.81	25.13	28.17	25.56	20.22	16.44	13.02	10.56
0.20	-	5.25	5.75	6.94	4.17	2.86	2.55	7.29	7.81	14.55	17.52
0.25	19.74	1.22	2.04	0.45	0.27	0.33	0.36	2.94	2.80	4.44	6.57
0.30	5.61	2.48	0.92	0.76	-	-	0.79	0.58	1.22	0.50	-
0.38	1.04	6.30	5.01	5.58	5.63	5.03	6.45	5.35	6.03	3.98	-
0.40	1.94	-	13.07	12.19	12.13	11.85	15.30	11.79	13.03	8.94	3.94
0.45	4.52	13.32	-	-	-	-	-	-	-	-	9.39
0.50	-	2.34	1.39	1.27	0.50	1.09	0.87	1.35	0.96	-	-
0.55	-	5.07	2.33	2.66	1.31	2.82	2.44	3.06	2.66	-	-
0.60	4.86	4.25	5.01	5.53	-	3.56	3.42	4.05	5.59	9.02	4.74
0.70	24.61	16.07	15.42	12.23	13.78	12.73	15.88	13.91	18.39	8.16	4.83
0.74	-	4.47	4.96	4.49	4.34	4.32	5.18	4.37	4.31	3.05	4.05
0.76	3.58	-	-	-	-	-	-	-	-	-	-
0.90	9.21	6.18	8.26	8.89	9.62	9.23	7.17	7.63	6.40	12.83	10.81
0.95	7.12	10.16	17.02	14.54	17.04	15.71	11.62	15.03	11.25	15.69	21.29

The remaining five compounds with Rf s of 0.18, 0.25, 0.70, 0.90, and 0.95 were detected in all media, including the basal medium. Among them, the production of the compound with Rf 0.18 was positively affected by SA elicitation. Prominent peaks with higher area percentage values were observed for the compound with Rf 0.18 in the SA-treated media and their control media, with the S3 medium shown to be highly optimized for its production. The compound with Rf 0.25 was less produced in stress-induced media. In contrast, the production of the compound with Rf 0.90 increased with increasing SA concentration in the media. In SA-supplemented media and their control media, the compound with Rf 0.95 was formed in larger amounts than the basal control medium. The highest amount of this compound was formed in the C5 medium.

Visualization of the chromatogram at 254 nm wavelength (**Table 37**), showed 19 compounds. Among them, the compound with Rf 0.65 was formed only in the S3 medium. The compound with Rf 0.01 was absent in some control media (C1, C3, and C4) and negligible in the rest of the NaHCO3 control media. The compound with Rf 0.03 was present in media supplemented with low concentrations of SA, and was absent in media containing higher stress conditions. The compound with Rf 0.05 was not found in the S1, C4, and C5 media, while the compound with Rf 0.22 was only produced in the SA-induced and their control media and was highly abundant in the C5 medium. Higher concentrations of SA were favourable for the production of this compound. The compound with Rf 0.25 was found to be maximum in the basal control medium and was not detected in C3, C4, and S5. In S4, C4, and C5 media the compound with Rf 0.35 was not formed. In

the S4 medium the compound with Rf 0.40 was formed, which was absent in the basal control, S1, and C1 media.

Table 37: The compound with Rf values and area percentages of methanolic extracts of Salicylic acid-induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

The compound with Rf (254nm)	Area Percentage										
	Control	S1	C1	S2	C2	S3	C3	S4	C4	S5	C5
0.01	0.72	1.26	-	1.09	0.36	0.76	-	0.73	-	0.30	0.24
0.03	0.22	2.83	0.11	-	0.12	-	-	-	-	-	-
0.05	0.21	-	0.22	0.23	0.27	0.36	0.25	0.21	-	0.40	-
0.10	9.30	0.49	0.46	0.60	0.73	0.57	0.49	0.50	1.07	3.04	4.93
0.20	8.01	48.72	32.84	35.06	32.44	36.99	35.55	33.91	33.54	27.33	31.81
0.22	-	1.67	0.42	0.35	0.80	0.36	0.43	0.56	0.64	6.50	11.93
0.25	10.95	0.25	0.26	0.76	0.32	0.62	-	0.57	-	-	1.86
0.35	2.05	0.40	1.75	2.00	1.63	1.77	1.96	-	-	1.20	-
0.40	-	-	-	4.74	4.94	4.77	6.57	7.03	2.25	4.37	1.55
0.45	1.68	11.85	5.78	-	-	-	-	-	6.14	0.24	4.38
0.50	2.31	2.05	0.82	0.74	0.46	0.61	0.44	0.87	0.50	1.60	0.93
0.55	-	3.94	1.42	1.17	2.74	1.38	1.01	1.75	1.24	-	-
0.60	1.27	2.86	2.60	2.51	-	1.60	2.17	2.08	2.83	5.12	7.22
0.65	-	-	-	-	-	6.11	-	-	-	-	-
0.70	17.71	19.23	14.19	11.74	12.70	8.45	15.89	14.10	17.35	6.95	8.01
0.75	2.24	4.45	3.98	1.74	2.36	-	-	-	-	-	3.12
0.85	6.74	-	4.71	-	4.57	5.55	5.98	4.55	4.20	8.17	-
0.90	24.53	-	16.99	19.55	15.29	-	16.17	13.45	17.50	17.43	24.03
0.96	12.05	-	13.46	17.71	20.27	30.09	12.68	19.71	12.24	16.30	-

Prominent peaks were observed in the S1 medium for the compound with Rf 0.45, but the compound was absent in S2, C2, S3, C3, and S4 media. The compound with Rf 0.55 was not produced in the basal control or higher SA-treated media (S5) and its control (C5). A high amount of production of

the compound with Rf 0.55 was observed in the S1 and the production of this compound gradually reduced in the succeeding media. On the other hand, higher concentrations of SA enhanced the production of the compound with Rf 0.60, which was absent in the C2 medium but present in the S2 medium. The compound with Rf 0.75 was not produced in the S3, C3, S4, C4, and S5 media.

The compound with Rf 0.85 was absent in the S1, S2, and C5 media; the peak with the highest area percentage value of this compound was observed in the S5 medium. The basal control medium was also effective in the production of this compound. The compound with Rf 0.90 was not formed in the S1 and S3 media. The control medium exhibited a significant peak for this compound, and in the C5 medium, the area percentage value of this compound was almost the same as the value in the control medium. The compound with Rf 0.96 was another prominent compound. S3 was the optimal medium for the maximum production of this compound. In S1 and C5 media this compound was not formed.

Four compounds with Rf s 0.10, 0.20, 0.50, and 0.70 were formed in all the media tested. SA stress negatively affected the production of compounds with Rf s 0.10 and 0.50. Significant peaks were found for the compound with Rf 0.20 in the SA-treated and its control media, and the compound was produced in high amounts in media containing lower SA concentrations.

The chromatogram visualized after derivatization at 550 nm wavelength revealed the presence of 18 compounds (**Table 38**). The compound with Rf 0.03 was abundant in the S1 medium, while it was absent

in both S3 and C3 media. The S2 and S3 media significantly impacted the production of the compound with Rf 0.05. The basal control was determined to be the optimal medium for the production of the compound with Rf 0.15, and its presence was observed only in the S1 medium among the SA-treated media.

Table 38: The compound with Rf values and area percentages of methanolic extracts of Salicylic acid-induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

The compound with Rf (550nm)	Area Percentage										
	Control	S1	C1	S2	C2	S3	C3	S4	C4	S5	C5
0.03	22.67	26.29	8.87	8.08	3.94	-	-	9.20	2.86	2.74	1.44
0.05	12.50	-	15.81	18.50	12.81	17.33	8.24	-	-	-	-
0.13	7.55	0.24	2.43	1.17	1.74	1.48	1.09	1.82	1.84	3.45	2.91
0.15	10.78	2.12	-	-	-	-	-	-	-	-	-
0.20	7.00	20.25	18.73	15.31	14.84	16.76	15.63	20.52	17.25	16.73	13.81
0.25	6.38	1.23	1.88	0.98	1.49	0.76	1.86	1.78	2.71	2.82	5.50
0.27	-	-	-	-	-	-	-	0.37	0.20	1.55	0.75
0.30	1.15	0.32	0.11	0.49	0.23	0.41	-	0.31	0.39	1.28	0.27
0.40	-	-	-	-	-	3.48	3.06	1.28	1.16	-	-
0.45	-	4.84	4.30	2.48	3.06	-	-	3.62	3.26	3.25	6.21
0.50	-	2.18	2.74	3.27	3.38	3.61	3.54	4.90	2.16	5.47	7.82
0.60	4.24	20.15	22.54	21.26	24.67	23.24	26.75	30.21	24.47	38.57	36.11
0.65	10.56	15.16	12.86	10.20	9.57	9.22	10.88	13.61	11.91	10.00	9.51
0.70	9.46	-	0.85	1.28	2.49	1.39	-	-	-	1.18	2.16
0.75	-	3.48	3.82	2.64	4.01	2.69	5.20	3.56	4.77	3.72	3.83
0.85	6.65	2.60	4.39	3.12	4.33	3.59	4.48	3.95	4.95	2.39	2.39
0.90	0.26	-	-	2.45	4.08	-	4.49	4.87	6.75	5.51	3.09
0.95	0.26	0.79	-	7.36	9.25	15.71	12.93	-	15.09	-	-

The compound with Rf 0.27 was produced only in media supplemented with higher concentrations of SA. The compound with Rf 0.30 was absent only in the C3 medium, and the compound with Rf 0.40 was produced only in the S3, C3, S4, and C4 media, with its area percentage value decreased with an increase in stress levels in the media. The compound with Rf 0.45 peak was not observed in the basal control, S3, and C3 media; its prominent peak was found in the C5 medium.

Increasing the SA stress in the medium positively impacted the production of the compound with Rf s 0.50 and 0.75, which were absent only in the basal control medium. The basal MS medium was optimal for producing the compound with Rf 0.70, and this compound was absent in S1, C3, S4, and C4 media. The SA treatment was significant in the production of the compound with Rf 0.90; it had only a negligible peak in the basal control medium. In S3 and C4 media prominent peaks were found for the compound with Rf 0.95 and these compounds were absent in the C1, S4, S5, and C5 media.

The other six compounds - the compounds with Rf s 0.13, 0.20, 0.25, 0.60, 0.65, and 0.85 - were commonly present in all the media, and among them, the production of compounds with Rf s 0.13, 0.25, and 0.85 were negatively affected by the SA treatment. The production of these compounds was highest in the basal control medium. The SA stress, however, positively impacted the production of the compound with Rf s 0.20, 0.60, and 0.65. The compounds with Rf 0.20 and 0.65 were highly produced in the lower SA-induced medium. In contrast, the production of the compound with Rf 0.60 gradually increased with the increasing SA concentration in the medium.

In the present study, it was observed that the SA significantly impacted the production of various phytocompounds in the *in vitro* cultures of *A. triplinervis*. Similar reports showing the influence of exogenous application of SA in the production of phytocompounds in plants were reported earlier in several plants like ajmalicine and reserpine in *Rauvolfia serpentina* (Dey *et al.,* 2020), papaverine and noscapine from *Papaver armeniacum* (Sharifzadeh-Naeini *et al.,* 2020), plumbagin from *Plumbago zeylanica* (Roy & Bharadvaja., 2019), psoralen in *Psoralea corylifolia* (Siva *et al.,* 2014), hypericin pseudohypericin in *Hypericum pethe* (Gadzovska *et al.,* 2013), dicentrine in *Stephania venosa* (Kitisripanya *et al.,* 2013), aloe-emodin and chrysophanol in *Aloe vera* (Lee *et al.,* 2013), caffeic acid and salvianolic acid from *Salia miltiorrhiza* (Dong *et al.,* 2010), artemisinin in *Artemisia annua* (Pu *et al.,* 2009), glycyrrhizin in *Glycyrrhiza glabra* (Shabani *et al.,* 2009), 7-methyljuglone from *Drosera capensis* (Ziaratnia *et al.,* 2008), hyoscyamine and scopolamine in *Datura metel* (Ajungla *et al.,* 2009), taxol and cephalomannine in *Taxus chinensis* (Wang *et al.,* 2004), capsaicin in *Capsicum frutescens* (Sudha & Ravishankar, 2003), and alkaloids in *Catharanthus roseus* (Godoy-Hernández & Loyola-Vargas, 1997), sanguinarine and macarpine in *Eschscholzia californica* (Balažová *et al.,* 2019), amarogentin, swertiamarin, and mangiferin in *Swertia paniculata* (Kaur *et al.* 2020), polyphenols and lignin content in *Camellia sinensis* (Nechaeva *et al.,* 2020).

4.5.2.8. Chitosan

Chitosan (Chit.) is a biodegradable biopolymer with antibacterial properties and can elicit plant immunity responses (Alvarado *et al.,* 2019). Chitin, a polysaccharide that consists of N-acetyl-D-glucosamine, is commonly found in crustaceans, insects, marine diatoms, algae, fungi, and yeasts. Chit is a form of N-deacetylated chitin (Synowiecki & Al-Khateeb, 2003; Sato *et al.,* 2010). According to Xing *et al.* (2015), chitosan can trigger plant defense responses by accumulating pathogenesis-related proteins, defense-related enzymes, secondary metabolites, and complex signaling cascades. Chit-enhanced phytoalexin accumulation was studied by Hadwiger and Beckman (1980), Kenawy *et al.* (2005), Aziz *et al.* (2006), and Gai *et al.* (2019).

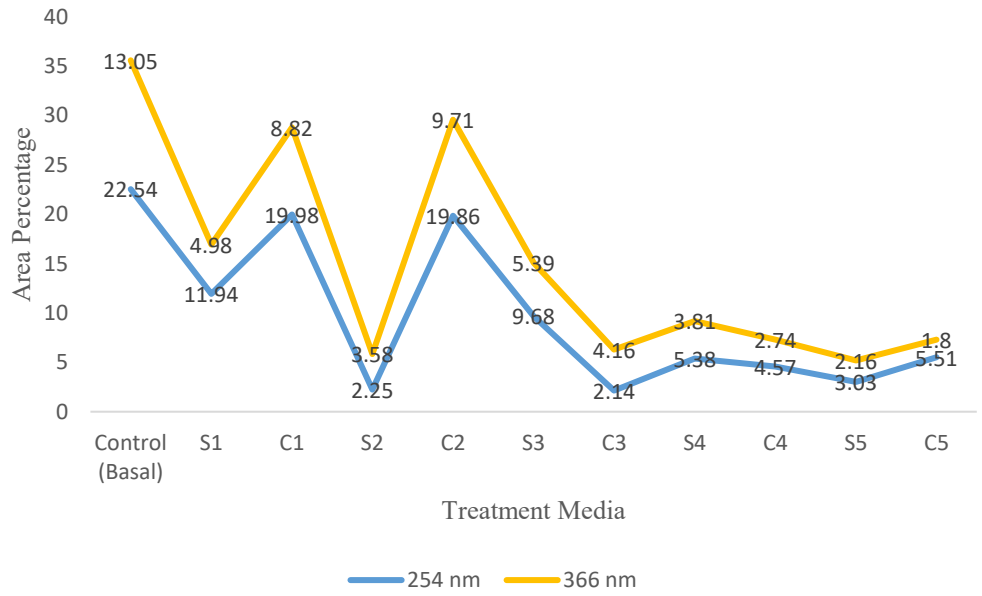

Figure 49: Area percentages of Ayapanin in *in vitro* Chitosan-induced cultures of *Ayapana triplinervis* visualized at 366 and 254 nm. S1 (50 mg l^{-1} Chit.), C1 (10 ml 1% HAc soln, l^{-1} MS Medium,), (S2 (100 mg l^{-1} Chit.), C2 (20 ml 1% HAc soln, l^{-1} MS Medium), S3 (150 mg l^{-1} Chit.), C3 (30 ml 1% HAc soln, l^{-1} MS Medium), S4 (200 mg l^{-1} Chit.), C4 (40 ml 1% HAc soln, l^{-1} MS Medium), S5 (250 mg l^{-1} Chit.) and C5 (50 ml 1% HAc soln, l^{-1} MS Medium).

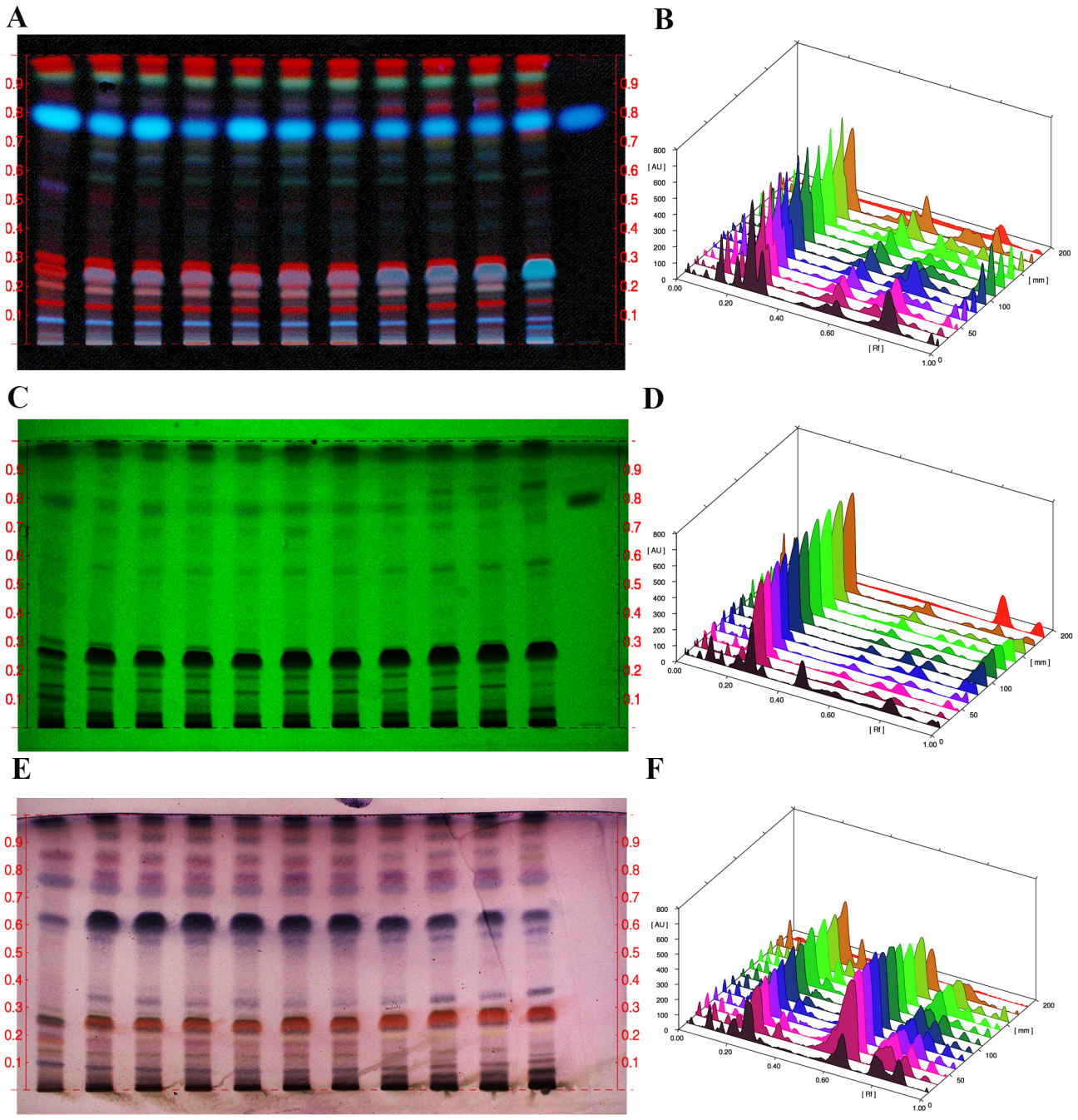

Plate 22: HPTLC comparison of sequential methanolic extracts of *in vitro* Chitosan induced, 1% Acetic acid control and basal MS medium cultures of *A. triplinervis*. (**Tracks:** 1. MS basal control, 2. 50 mg l^{-1} Chitosan, 3. Control 1 (10 ml 1% HAc soln. l^{-1} MS Medium), 4. 100 mg l^{-1} Chitosan, 5. Control 2 (20 ml 1% HAc soln. l^{-1} MS Medium), 6. 150 mg l^{-1} Chitosan, 7. Control 3 (30 ml 1% HAc soln. l^{-1} MS Medium), 8. 200 mg l^{-1} Chitosan, 9. Control 4 (40 ml 1% HAc soln. l^{-1} MS Medium), 10. 250 mg l^{-1} Chitosan, 11. Control 5 (50 ml 1% HAc soln. l^{-1} MS Medium), 12. Ayapanin (marker compound)). **A&B.** HPTLC chromatogram and densitogram at 366 nm; **C&D.** HPTLC Chromatogram and densitogram at 254 nm and **E&F.** HPTLC chromatogram and densitogram after derivatization.

The colloidal solution of chit was prepared with the 1% Acetic acid solution. So, in addition to the MS basal medium as the control, the 1% acetic acid solution was also given as individual control for the chitosan-induced stress media. The concentrations of chitosan selected for the present study; 50 mg l^{-1} (S1), 100 mg l^{-1} (S2), 150 mg l^{-1} (S3), 200 mg l^{-1} (S4) and 250 mg l^{-1} (S5) and the individual concentrations of control was selected as; 10 ml of 1% HAc soln, l^{-1} MS Medium (C1), 100 mg l^{-1} chit. – 20 ml 1% HAc soln, l^{-1} MS Medium (C2), 150 mg l^{-1} chi. – 30 ml 1% HAc soln, l^{-1} MS Medium (C3), 200 mg l^{-1} chit. – 40 ml 1% HAc soln, l^{-1} MS Medium (C4), 250 mg l^{-1} chit. – 50 ml 1% HAc soln, l^{-1} MS Medium (C5).

The HPTLC analysis aimed to examine how chitosan stress impacted the production of secondary metabolites in *Ayapana triplinervis* (**Plate 22**). With the help of the standard marker compound, Rf 0.78 was identified as 'Ayapanin.' The analysis revealed that chitosan stress negatively impacted the production of 'Ayapanin' or coumarin compounds. The area percentage values obtained from the MS basal medium control were higher than those obtained from the chitosan and 1% acetic acid solution cultures. The control had the highest area % value (**Figure 49**), with a slight increase observed at 50 mgl^{-1} Chit. These findings suggest that chitosan inhibits the production of 'Ayapanin' or coumarin compounds in the *in vitro* cultures of *A. triplinervis.*

After examination of TLC plates visualization at the wavelength of 366 nm, the profile of secondary metabolites revealed 21 compounds (**Table 39**). In the MS basal control medium, compounds with Rfs 0.05, 0.10, 0.34, 0.41, 0.50, 0.65, 0.83, and 0.94 were not detected. Compound with Rf 0.05 was found in only four media, namely C3, S4, C4, and S5, and its area percentage showed a slight increase at higher chitosan concentrations. Compound with Rf 0.10 was produced in all chitosan stress media, except for S4, and was absent in three individual control media, namely C1, C2, and C5. Compound with Rf 0.34 was not present in C2,

S3, C3, and C5 media, but it was visible in the lower and higher chitosan-containing media.

Compounds with Rfs 0.41 and 0.50 were present in all chitosan-induced and control media, except for S1. An enhancing effect of chitosan was observed at the production of compound with Rf 0.50. Compound with Rf 0.65 peaks had almost the same area percentage values in all the media used, but this compound was not formed in S3 medium. The area percentage values of Rf 0.83 were higher in media containing higher concentrations of chitosan, Compound with Rf 0.94 was not observed in the S1, C1, and MS basal control media, and the peak values were reduced at the higher stress concentrations.

Compounds with Rfs 0.06, 0.15, 0.20, 0.25, 0.55, 0.70, and 0.78 were formed in all the media. The area percentage values of compounds with Rfs 0.06 and 0.15 showed that their production decreased in chitosan stress induced media. A small amount of gradual reduction was observed in the production of these compounds. Increased production of compound with Rf 0.20 was observed in the S3 and S4 media, and the remaining concentrations of chitosan used in chitosan induced media showed no significant change in the production of chitosan when compared to the basal control medium. In the C5 medium, a considerable fall in the area percentage value of the compound was found.

The production of ‘Ayapanin’ decreased due to chitosan stress, but the production of compound with Rf 0.25 was enhanced. The compound with Rf 0.25 exhibits prominent peaks in all the media, and the maximum production was achieved in the S5 medium. The area percentage values of compound

with Rf 0.55 gradually increased with the increasing chitosan concentration. For compounds with Rfs 0.70 and 0.78, the highest area percentage values, were observed in the HAc control media than in the chitosan media.

The remaining compounds with Rfs 0.01, 0.02, 0.30, 0.60, 0.85, and 0.97 were not visible in several media. The production of compound with Rf 0.30 was absent in media containing higher concentrations of chitosan. The compound with Rf 0.60 was visible only in the basal control and the S1 media, and in S1, the production was found to be augmented. The area percentage values of compound with Rf 0.85 in the media containing lower stress concentrations were significantly higher than media containing higher stress concentrations. Compound with Rf 0.97 was formed in the MS basal control, S1, and C1 media and absent in all the other media.

Fifteen compounds were found after visualization at a wavelength of 254 nm (**Table 40**), with unique compounds with Rf values of 0.30 and 0.45 found exclusively in the MS basal control medium and compound with Rf 0.90 present only in S4. In the MS basal control and C5 media a compound with Rf 0.58 was formed, several media lacked compounds with Rfs 0.01, 0.85, and 0.97. Compound with Rf 0.01 was absent in C1, S5, and C5 media. In S3 medium compound with Rf 0.85 was not formed and the compound with Rf 0.97 was not formed in media containing higher concentrations of chitosan (higher than that in S2 medium). A significant peak for compound with Rf 0.97 was formed in the C2 medium, and least area % value was observed in S1 medium.

Table 39: Rf values and area percentages of methanolic extracts of Chitosan-induced *in vitro* cultures of *A. triplinervis* visualized at 366 nm

Rf (366 nm)	**Area Percentage**										
	Control	**S1**	**C1**	**S2**	**C2**	**S3**	**C3**	**S4**	**C4**	**S5**	**C5**
0.01	0.78	0.48	-	-	-	0.40	0.41	-	-	-	-
0.02	0.46	0.57	0.78	0.39	0.69	0.90	0.47	-	-	0.59	-
0.05	-	-	-	-	-	-	0.12	1.60	1.17	1.30	-
0.06	1.17	0.45	0.45	0.43	0.43	0.34	0.42	0.36	0.33	0.61	0.56
0.10	-	0.33	-	0.25	-	0.32	0.25	-	0.35	0.26	-
0.15	9.93	7.99	6.67	7.82	6.47	5.72	7.30	5.47	7.11	7.25	2.95
0.20	6.76	6.28	5.52	6.77	5.68	9.87	5.99	7.16	5.77	5.42	2.79
0.25	29.69	37.52	33.48	40.84	40.53	46.58	45.84	46.52	41.44	48.04	43.03
0.30	11.19	7.21	6.44	7.81	0.69	-	-	-	-	-	-
0.34	-	0.46	0.56	0.55	-	-	-	0.47	0.83	0.56	-
0.41	-	-	0.68	1.12	0.93	1.14	1.47	0.92	1.83	1.30	1.94
0.50	-	-	3.38	3.83	3.70	3.43	3.53	4.38	4.73	4.32	6.53
0.55	4.02	3.65	9.46	9.93	9.13	11.00	10.14	11.66	12.41	12.42	13.02
0.60	6.36	11.32	-	-	-	-	-	-	-	-	-
0.65	-	1.77	1.96	1.91	1.77	-	-	1.81	-	1.71	-
0.70	3.03	5.55	6.30	4.81	5.69	8.41	7.37	6.03	10.75	6.06	7.94
0.78	22.54	11.94	19.98	4.92	19.86	9.68	5.92	5.38	4.57	3.03	5.51
0.83	-	-	-	2.25	-	-	1.44	4.40	5.61	4.93	-
0.85	0.72	1.56	1.54	2.80	1.51	1.59	-	-	0.36	0.37	8.45
0.94	-	-	-	3.59	2.91	3.64	7.19	3.73	0.65	0.46	1.04
0.97	1.90	1.65	2.81	-	-	-	-	-	-	-	-

Table 40: Rf values and area percentages of methanolic extracts of Chitosan-induced *in vitro* cultures of *A. triplinervis* visualized at 254 nm

Rf (254 nm)	Area Percentage										
	Control	**S1**	**C1**	**S2**	**C2**	**S3**	**C3**	**S4**	**C4**	**S5**	**C5**
0.01	0.56	0.64	-	0.75	-	0.94	1.12	1.41	1.46	-	-
0.02	-	-	-	-	3.95	-	-	-	-	1.16	5.40
0.05	1.86	4.00	4.43	3.35	0.28	3.17	2.87	4.84	3.84	4.63	7.66
0.10	10.53	0.32	0.40	0.32	0.42	0.35	0.35	0.58	0.38	0.37	0.55
0.15	4.52	2.73		3.16	2.24	2.84	5.05	1.95	2.53	2.56	0.94
0.20	9.26	5.12	4.24	5.71	3.51	5.22	6.15	4.81	4.71	4.67	3.51
0.25	26.64	69.63	65.05	70.44	64.13	69.62	70.72	74.38	70.09	74.29	66.65
0.30	4.79	-	-	-	-	-	-	-	-	-	-
0.45	17.36	-	-	-	-	-	-	-	-	-	-
0.55	1.81	4.27	3.92	3.69	3.80	4.21	3.41	4.37	5.42	4.87	2.69
0.58	4.31	-	-	-	-	-	-	-	-	-	5.55
0.70	1.36	4.92	4.87	2.67	4.00	6.60	4.47	1.87	2.66	1.54	1.80
0.78	13.05	4.98	8.82	3.58	9.71	5.39	4.16	3.81	2.74	2.16	1.27
0.85	1.15	0.80	-	1.33	-	-	-	0.62	1.84	1.59	3.99
0.90	-	-	-	-	-	-	-	0.65	-	-	-
0.97	2.81	1.85	3.66	3.23	7.95	-	-	-	-	-	-

In all media, except C1, compounds with Rf 0.15. Rfs 0.05, 0.10, 0.25, 0.55, 0.70, and 0.78 were formed. The production of compounds with Rfs 0.05, 0.25, 0.55, and 0.70 was higher in chitosan-induced media than in the control. Compound with Rf 0.25 had high area percentage values in all the

media. A significant increase was observed in the production of the compound with Rf 0.25 in the stress media and its controls, this showed that chitosan enhanced the production of the compound with Rf 0.25. A higher area % value for compounds with Rfs 0.10 and 0.78 was observed in the MS basal control media than in the chitosan-induced media, and stress reduced the production of these compounds.

After undergoing ANS-mediated derivatization, 19 compounds were visible at 550 nm, eight of them were not present in the MS basal control medium (**Table 41**). These eight compounds had Rfs of 0.05, 0.08, 0.13, 0.75, 0.80, 0.85, 0.93, and 0.97. The production of compounds with Rfs 0.05 and 0.10 increased with an increase in chitosan concentration up to a certain limit in the medium, and these two were absent in some stress media and basal medium; the former was absent in C2, C3, and S4, and the latter in S1 and C1 media. However, production of compound with Rf 0.08 decreased with an increase in chitosan concentration.

Compound with Rf 0.75 was formed only in the HAc control media C3 and C5. This showed that HAc can also induce the formation of phytocompounds. On the other hand, compound with Rf 0.80 was formed only in S1 and C1, and its production decreased with increased chitosan concentration in the media. Compound with Rf 0.85 was not formed in media containing very low and very high concentrations of chitosan. Compound with Rf 0.93 was visible in all the media except the MS basal control, while compound with Rf 0.97 was found only in the S3 and C3 media.

Table 41: Rf values and area percentages of methanolic extracts of Chitosan-induced *in vitro* cultures of *A. triplinervis* after derivatization and visualized at 550 nm

Rf (550 nm)	Area Percentage										
	Control	S1	C1	S2	C2	S3	C3	S4	C4	S5	C5
0.02	1.06	1.22	1.21	0.95	0.24	0.30	0.30	0.91	-	-	1.84
0.05	-	0.22	0.41	0.15	-	1.43	-	-	1.14	1.70	5.44
0.08	-	2.11	1.67	1.23	1.53	-	1.40	1.38	1.56	1.38	-
0.10	6.78	-	1.10	0.14	-	-	-	-	0.23	-	2.09
0.13	-	-	-	1.39	1.12	2.68	2.09	2.21	1.34	2.18	0.66
0.15	2.76	1.27	0.29	0.68	2.49	2.58	3.08	0.39	0.43	0.79	1.76
0.20	8.65	3.47	1.16	3.58	-	-	-	1.58	-	-	0.49
0.25	23.31	18.45	17.39	17.57	17.30	21.08	20.32	26.67	27.45	28.65	35.38
0.30	1.78	3.20	3.93	2.26	4.05	2.33	2.12	3.57	5.56	4.02	-
0.35	0.48	0.62	0.72	0.25	0.86	0.33	0.59	0.52	-	-	8.68
0.58	1.81	6.82	6.41	5.68	7.60	10.98	11.37	6.53	6.81	7.70	7.66
0.63	22.87	40.60	41.48	39.78	40.79	33.92	36.87	30.49	26.30	27.90	18.84
0.75	-	-	-	-	-	-	7.70	-	-	-	4.97
0.78	16.81	8.93	15.20	9.47	10.42	15.40	5.04	17.28	18.97	16.94	6.11
0.80	-	5.33	5.97	-	-	-	-	-	-	-	-
0.85	-	-	-	8.19	5.86	6.47	6.28	2.28	4.04	-	0.88
0.88	12.81	6.16	-	-	-	-	-	-	-	1.98	-
0.93	-	1.05	2.53	1.66	2.54	1.29	1.66	1.14	1.54	1.00	0.53
0.97	-	-	-	-	-	0.65	0.54	-	-	-	-

In the S5 medium compounds with Rfs 0.02, 0.10, 0.20, and 0.35 were not formed. The compound with Rf 0.88 was present only in MS basal control, S1, and S5. All the other compounds (compounds with Rfs of 0.15, 0.25, 0.30, 0.58, 0.63, and 0.78) were present in all the media, including the MS basal control. An increase in chitosan stress enhanced the production of compounds with Rfs 0.25, 0.30, 0.58, 0.63, and 0.78 compared to the basal control medium. However, chitosan stress decreased the production of compounds with Rfs 0.15 and 0.88.

Chitosan-induced secondary metabolites elicitation was reported in several plants. Chitosan has been found to have a positive effect on the production of various compounds from different plants, such as; total flavonoids, phenols, terpenes, and salicylic acid in *Piper auritum,* vinblastine and vincristine from *Catharanthus roseus* (Pliankong *et al.,* 2018), hydrolysable tannin from *Phyllanthus debilis* (Malayaman *et al.,* 2017), curcumin in *Curcuma longa* L. (Sathiyabama *et al.,* 2016), dicentrine in *Stephania venosa* (Kitisripanya *et al.,* 2013), andrographolide from *Andrographis paniculata* (Vakil and Mendhulkar, (2013), withanolides from *Withania somnifera* L. (Sivanandhan *et al.,* 2012), artemisinin in *Artemisia annua* L. (Lei *et al.,* 2011), xanthone and 1,7-dihydroxyxanthone (euxanthone) from *Hypericum perforatum* (Tocci *et al.,* 2010), oleanolic acid *from Calendula officinalis* (Wiktorowska *et al.,* 2009), and paclitaxel from *Taxus canadensis* (Linden & Phisalaphong, 2000).

Shah *et al.* (2021) found that chitosan treatment can increase the accumulation of flavonoids, phenolic compounds, and silymarin in *Silybum marianum* (L.). Applying chit increases the contents of polyphenols,

anthocyanins, and tannins of *Vitis vinifera* L. ‘Touriga Franca’ and ‘Tinto Cão’ (Singh *et al.,* 2019). The flavonoids (rutin, neo-hespiridin, buddleoside, liquiritigenin, quercetin, isorhamnetin, kaempferol, iso liquiritigenin) synthesis in *Isatis tinctoria* L. hairy root cultures was augmented with the chit elicitation (Jiao *et al.,* 2018). Similarly, *Impatiens balsamina* suspension cultures treated with 50 mgl^{-1} chitosan have accumulated total flavonoids (Kasem, 2018). Chit induction increased plumbagin production in *Plumbago indica*'s root cultures (Jaisi & Panichayupakaranant, 2017; Gangopadhyay *et al.,* 2011).

Phenolic accumulation with chit-mediated elicitation has been reported by Brasili *et al.* (2016) in *Hypericum perforatum*. Sangeetha *et al.* (2016) in *Pseudarthria viscida*, Yin *et al.* (2012) in *Origanum vulgare*, Rahman and Punja (2005) in *Panax quinquefolius*, Romanazzi *et al.* (2002) in table grapes, and Nicholson and Hammerschmidt (1992). In *Vitis vinifera* cell cultures, a 5-fold increase of resveratrol was observed in 100 μg mL^{-1} chit-treated cultures compared to control cultures (Taurino *et al.,* 2015). Chitosan treatment increased the production of secondary metabolites in *Hypericum perforatum* roots, such as epicatechin, xanthones, and stigmasterols, by stimulating the phenylpropanoid and isoprenoid pathways (Brasili *et al.,* 2014).

In the present study *in vitro* chitosan stress on *A. triplinervis*, decreased the production of 'Ayapanin' and other coumarin compounds. However, some other phytocompounds showed increased production due to the stress. This may be due to the fact that chitosan promotes the production of some compounds and decreases the production of some other compounds.

CHAPTER 5

SUMMARY & CONCLUSION

The present research work revealed how abiotic stress signals affect in vitro morphogenic responses and secondary metabolites production of the important medicinal plant *Ayapana triplinervis*. The entire plant culture study was conducted using MS medium in an *in vitro* system.

Protocol development for direct and indirect organogenesis was an imperative step, and for this different concentration of growth regulators were used, either alone or in combinations. Nodal explants cultured in MS basal medium produced contamination-free plants and for further studies, explants collected from these plants were used. Direct organogenesis was obtained in MS medium supplemented with various concentrations and combinations of Kin, BAP, NAA and IBA. Among the PGRs Kin in a concentration of 0.5 mgl^{-1} was the best for the rapid multiplication of shoots from the single nodal explants. The *in vitro* rooting was established in IBA fortified medium and later the *in vitro*-grown plantlets were acclimatized to the field conditions and were grown well in the greenhouse.

The sterile leaf and internodal explants were cultured in MS medium supplemented with different combinations of TDZ, BAP, NAA and Kin for indirect organogenesis of *A. triplinervis*. The TDZ (1.0 mg l^{-1} and 1.5 mg l^{-1}) alone and TDZ (1.5 mg l^{-1} and 2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}) supplemented media was most effective for indirect shoot regeneration from the leaf explants. The internodal explant responded only in the medium containing TDZ (2.0 mg l^{-1}) + NAA (0.5 mg l^{-1}). The leaves collected from the *in vitro*-raised plants through indirect organogenesis and the mother plants were collected and analyzed for genetic fidelity using ISSR markers. Ten primers were tested for the annealing and among them, five were matched. After

testing with the five primers it was found that *in vitro*-raised plants were 100% genetically similar to the mother plant.

Different stress signals in different concentrations were used; NaCl, SA, Sucrose, MS medium strengths, Put, Trp, Chitosan and JA to study the morphogenic responses and secondary metabolites production in *in vitro* cultures of *A. triplinervis*. The HPTLC studies were used to analyze secondary metabolite profiles of the methanolic plant extracts and 'Ayapanin or 7-methoxycoumarin' was used as the marker compound during the HPTLC studies. Differences in the number and colour of bands were observed and each band or Rfs represents a phytocompound in the extracts. Unique compounds (bands or Rfs) were produced as a result of the application of elicitors in particular concentrations. Also, certain compounds were absent only in the control medium and present in the stress-induced media and some of them were present in particular concentrations of elicitors and in others they were not found.

Most of the growth parameters (length and no. of adventitious roots, fresh and dry weights) showed better morphogenic response than the control in medium supplemented with NaCl at 50 mM l^{-1}. The length of shoots and leaf area was higher in the 25 mM l^{-1} NaCl. The growth was retarded when the salinity concentration was increased beyond 50 mM l^{-1}. But NaCl at a concentration of 10 mM l^{-1} was the best in the production of 'Ayapanin'. The number of bands observed after visualizing in different wavelengths was; 17 at 366 nm, 11 at 254 nm and 16 at 550 nm.

The root number was increased simultaneously with sucrose concentration, but the length reached a maximum at 40 mgl^{-1}, and a fall-off

was seen at 50 mg l^{-1}. The best shoot proliferation was noticed in the 40 mg l^{-1} sucrose containing medium and this culture medium was also best in the production of 'Ayapanin'. The HPTLC analysis visualized 17 bands in 366 nm –, 7 bands in 254 nm and 17 bands in 550 nm.

Among the media supplemented with Put, the medium containing 60 mg l^{-1} Put was best for increased leaf area, increased length of shoots and adventitious roots. When the Put concentration was beyond 40 mg l^{-1} the leaf area, length of adventitious roots and biomass production was increased. The production of 'Ayapanin' in all the Put-induced cultures was enhanced as compared with the control medium. The results of the HPTLC analysis visualized 18 bands in 366 nm, 12 bands in 254 nm and 9 bands in 550 nm.

The growth parameters were enhanced in all the media supplemented withTrp. Especially in the growth and development of roots; the number and length of adventitious roots gradually increased with an increase in Trp concentration. The medium supplemented with 100 mg l^{-1} Trp was best for the maximum leaf area formation. The highest number and length of adventitious roots and biomass production was observed in the culture medium supplemented with 60 mg l^{-1} Trp. The accumulation of 'Ayapanin' was gradually increased with the increasing Trp concentration in the culture medium and reached the maximum in the medium supplemented with 100 mg l^{-1} Trp. The number of bands visualized after HPTLC analysis at 366 nm was 18, at 254 nm was 11 and at 550 nm was 14.

The number of shoots and length of adventitious roots increased in the ¾ MS l^{-1} medium than the control. But the maximum leaf area was observed in the ½ MS l^{-1} medium. Hyperhydric leaves were observed in the 1/6 MS l^{-1}

medium. The ‘Ayapanin’ content was high in the full MS medium and the number of compounds found after HPTLC analysis was 21 in 366 nm, 15 in 254 nm and 15 in 550 nm.

At the higher concentrations of JA, the number of adventitious roots was increased but their length was reduced. A gradual reduction in the number and length of shoots was observed. Medium supplemented with 2.5 µM l^{-1} JA was the best for the maximum leaf area formation. However biomass accumulation was decreased in the medium supplemented with higher concentrations of JA. There was an increase in ‘Ayapanin’ content in the medium supplemented with 15 µM l^{-1} JA. The secondary metabolites profile showed 17 bands at 366 nm, 10 bands at 254 nm 16 bands at 550 nm.

In SA-induced cultures, growth parameters varied but adventitious roots increased as concentrations of SA increased, while shoot numbers and length decreased. ‘Ayapanin’ content showed a gradual reduction but, some other unknown metabolites were enhanced. The number of bands obtained after the HPTLC study; was 21 at 366 nm, 19 at 254 nm and 18 at 550 nm. The highest number of compounds was also visualized in the SA-induced cultures.

Application of Chitosan reduced most of the growth parameters and produced stunted shoots with short, numerous adventitious roots. Chitosan favored root formation; the number of roots was high in media supplemented with Chitosan. The leaf area was reduced for the plants in stress-induced cultures and the biomass production also showed reduction. Levels of ‘Ayapanin’ were less in plants induced with Chit as compared to the control. The highest numbers of phytocompounds (21) were visualized at 366 nm in

the Chit-induced plant extracts. 15 bands were visualized at 254 nm and 19 bands were visualized at 550 nm.

In the secondary metabolites profile after the HPTLC analysis, the maximum number of compounds was observed in the *in vitro* cultures of *A. triplinervis* plants induced with SA and MS media strength. In the case of 'Ayapanin' content, the production was enhanced in the medium fortified with NaCl, Sucrose, Put and Trp than the control plants.

The present study effectively validated the effects of several abiotic stress signals at different concentrations in the *in vitro* morphogenic responses and secondary metabolite production of *Ayapana triplinervis*. The HPTLC study revealed a variety of bands in the secondary metabolites profile and variations were found in each stress signal and particular concentrations. The production of various phytocompounds was enhanced by the application of abiotic stress signals and this mode of work was novel for the plant *A. triplinervis*. Also, the indirect organogenesis from the leaf explants and their genetic fidelity analysis was a first-time study in *A. triplinervis*.

CHAPTER 6

RECOMMENDATIONS

The gene expression studies of coumarin synthase and PAL (Phenylalanine ammonia lyase) were not conducted in the present study. It is recommended to investigate gene expression using q-rtPCR of coumarin synthase and PAL l for the identification of the impact of different concentrations of stress signals in the production of 'Ayapanin'.

- Molecular studies are less explored in the plant *Ayapana triplinervis* so it is recommended to do molecular level studies to analyze the variations in gene expression of constitutive genes in response to stress signals.
- It is recommended to conduct phytochemical studies such as GC/MS analysis and NMR spectroscopic studies in the plant *Ayapana triplinervis* to identify unique phytocompounds formed in response to stress signals.
- A comparison study on *in vivo* and *in vitro* grown plants is recommended to find out variations in the accumulation of secondary metabolites.
- A study to validate secondary metabolites produced *in vitro* in different plant parts viz, leaves, stem and roots of *Ayapana triplinervis* can also be explored.

CHAPTER 7

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APPENDIX

International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 21-06-2021, Accepted: 06-07-2021, Published: 21-07-2021
Volume 6, Issue 4, 2021, Page No. 329-332

In vitro axillary bud multiplication of an important medicinal plant-*Ayapana triplinervis* vahl

Aparna Balakrishnan, Aswindas TP, Delse P Sebastian*, Satheesh George
Centre for Post Graduate Studies and Research in Botany, St Joseph's College (Autonomous), Devagiri, Calicut, Kerala, India

Abstract
An efficient protocol was developed for the micropropagation of *Ayapana triplinervis* through axillary bud multiplication using nodal explants. The excised nodal segments were inoculated to MS media supplemented with various concentrations and combinations of PGRs. Murashige and Skoog's medium fortified with 0.5 mgL^{-1} Kinetin was the best combination for the shoot proliferation of *Ayapana triplinervis.* An average of 15 shoots was formed from each explant within 60 days of incubation. Rooting was observed in all the media combinations tested; however most effective root formation was observed in MS medium supplemented with 1.0 mgL^{-1} IBA. The *in vitro* developed plantlets were hardened and successfully established in pots with 100% survival rate.

Keywords: axillary bud, *Ayapana triplinervis*, hardening, *In vitro,* kinetin

Introduction
Ayapana triplinervis Vahl. (Syn. *Eupatorium ayapana*, *Eupatorium tripliverve*) belonging to the family Asteraceae is an erect perennial herb with aromatic properties. It is familiar in India as well as in other tropical countries. The plant has antiseptic, stomachic, antitussive, diaphoretic, antiulcerous, hemostatic (Rajasekaran *et al*., 2010) [13], antitumerous, anticoagulant, hepatoprotective (Bose *et al*., 2007) [2], astrigen and emollient properties (Gauvin-Bialecki & Marodon, 2008) [7]. *A. triplinervis* contains a variety of coumarins; mainly 'ayapanin and ayapin', which have anticoagulant properties, so it is used as the precursor for drugs such as 'Warfarin' (Bose, 1937) [1]. The antiulcer effect and radical scavenging activity of *A. triplinervis* extract was used to treat against ulcerative colitis in mice (Krishnan *et al*., 2014) [9]. The active secondary compound present is 7- methoxy coumarin called as 'herniarin or ayapanin' and rest of the constituents found in leaves are 6, 7- dimethoxy coumarin (ayapin), carotene, vitamin C and stigmasterol (Bose & Roy, 1936) [3]. In addition to these, other coumarins like hydragetin, daphnetin, daphnetin-7-methyl ether dimethyl ether and umbelliferone are present in the plant (Chaturvedi & Mulchandani, 1989) [4]. The 7-methoxy coumarin has antitumerous activity and is toxic to multi drug resistant cancer cells (Kawase *et al*., 2005) [8] and inhibitory against chemicals dispensed from leukemic cells (Watanabe *et al*., 2005) [15], it also has anti-nociceptive activity (Cheriyan *et al*., 2017) [5]. Phytochemical analysis of *Ayapana triplinervis* revealed various phytochemicals except steroids and saponins (Mamatha & Thangavel, 2018) [10]. The objective of the present study was to develop an efficient protocol for the rapid propagation of this important medicinal plant using nodal segments.

Materials and Methods
Plant Material and Surface Sterilization
Nodal segments were collected from the shoots of *A. triplinervis* Vahl. Growing in the Botanical garden of St. Joseph's college (Autonomous), Devagiri, Calicut. The nodal segments (NSs) were thoroughly washed under running tap water for 30 minutes and then vigorously washed with Extran (5% v/v) for 10 minutes. Then the explants were rinsed with distilled water and were surface sterilized with 0.1 % (m/v) $HgCl_2$ for 8 minutes, followed by washing 3-4 times with sterile distilled water. After surface sterilization the nodes were trimmed at cut ends and reduced the size to 1 – 1.5 cm before inoculation to sterilized media.

Culture Medium
MS medium (Murashige & Skoog, 1962) [12] was used throughout the study and it was supplemented with varying concentrations and combinations of plant growth regulators (PGRs). 8 gL^{-1} agar was used to solidify the media and 30 gL^{-1} sucrose was used as the source of carbon. PGRs such as BAP, Kinetin (Kn), NAA and IBA in different concentrations and combinations were used. pH of the media was adjusted to 5.8 with 0.1 M NaOH and 0.1 M HCl, culture media were sterilized by autoclaving at 1 atmosphere pressure and 121°C for 20 minutes. After inoculation, the cultures were incubated at 25 ± 2°C with 16h photoperiod under white tube lights. These conditions were maintained throughout the multiplication procedure.

Shoot Multiplication
For axillary bud proliferation explants were cultured on MS media supplemented with different concentrations of PGRs like Kn, BAP, NAA and IBA, either individually or in combinations. A total of 14 combinations of PGRs with MS media were used. The best medium for axillary bud multiplication was selected based on the number of shoots formed per explant and the length of the shoots formed.

Rooting
For root induction, *in vitro* raised shoots were cultured on MS media supplemented with various concentrations and combinations of NAA/IBA.

Acclimatization
Fully grown *in vitro* raised plantlets were taken out from the culture vessels and washed with sterile distilled water for

removing the agar and then transferred to paper cups containing auotclaved sand: soil (1:1). The cups were covered with transparent polythene bags to maintain humidity and to prevent drying of the plants. The polythene bag was removed after 10 days. Finally, the plantlets were transplanted to the garden pots with potting mixture.

Statistical Analysis of Data

All the experimental trials were conducted with 12 replicas for each treatment. The observations of the cultures were done regularly and also every morphological change was recorded at regular time periods. The results were analysed statistically using SPSS Version 16.0 and data were compared using ANOVA and Duncan's multiple range test. Graph was constructed using MS Excel.

Results and Discussion

Shoot Multiplication

Nodal explants were cultured on MS media containing various concentrations and combinations of PGRs and the results were evaluated for 60 days of growth period (Table 1, Figures 1&2). 1 – 2 shoots (1.7±0.64) were formed from each explant in MS basal media without any plant growth regulators (control). When cultured in media fortified with cytokinins increased growth rate and increase in the number of shoots per explant were observed. Best axillary bud multiplication was obtained when cultured on MS medium fortified with 0.5 mgL^{-1} Kinetin, this combination induced the maximum multiplication of shoots (13.9±7.86) than other concentrations and combinations of PGRs. After 60 days of incubation, the number (13.9±7.86) and length of shoots (4.4±1.04cm) were high in 0.5 mgL^{-1} Kinetin fortified medium. However length of shoots (5.08±1.94cm) were highest in MS medium supplemented with 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} NAA, this could be due to the synergistic effect of cytokinin and auxin in shoot elongation. Previously Martin, 2003 reported synergistic effect of cytokinin and auxin in shoot elongation and axillary bud multiplication of *A. Triplinervis*. But in the present study highest shoot multiplication was observed in media supplemented with cytokinin alone. Kinetin in combination with auxins NAA and IBA induced the production of less number of shoots, large number of roots and callus formation. Similar results showing highest shoot multiplication of *A. Triplinervis* in medium supplemented with cytokinins alone was reported earlier by Usha & Karpagam, 2017 [14]. In the present study BAP with NAA combination was not effective in axillary bud proliferation (0.8±0.46) of *A. Triplinervis*, this combination only induced very low amount of callus (brown coloured). The MS medium containing 1.0 mgL^{-1} BA with 0.5 mgL^{-1} IBA induced the production of less number of shoots (1.5±0.57). At higher concentrations of NAA (1.0 mgL^{-1}) the number of shoots declined and a tuft of roots were formed from the basal cut ends.

Fig 1: *In vitro*, culture establishment of *A. triplinenervis*. (a) Habit; Culture established in medium containing (b) 0.5 mg La^{-1} Kinetin; (c) 0.5 mg L^{-1} BA: (d) 0.5 mg t^{-1} NAA: Acclimatized plantlets (e) in sterile soil al lab conditions and (f) well established plantlet in Garden pot.

Table 1: Effect of PGRs on shoot multiplication from nodal segments of *A. triplinervis* in MS medium

KIN (mgL^{-1})	BA (mgL^{-1})	NAA (mgL^{-1})	IBA (mgL^{-1})	No. of Shoot	Shoot length (cm)
0	0	0	0	1.7±0.64	4.8±1.16
0.5	0	0	0	13.9±7.86	4.4±1.04
0	0.5	0	0	0.9±0.63	4.7±2.05
0	0.5	0.5	0	0.8±0.46	3.04±1.66
0	1.0	1.0	0	1.1±0.63	5.08±1.94
0	1.0	0.5	0	1.7±0.93	3.5±1.51
0.1	1.0	0	0	3.2±2.09	1.7±0.97
1.0	0.1	0	0	18.1±11.85	0.6±0.35
0.5	1.0	0	0	3.08±2.18	2.3±0.71
1.0	0.5	0	0	2.4±1.32	1.5±0.58
0.5	0	0.5	0	1.9±1.11	3.2±1.39
1.0	0	0.5	0	0.9±0.57	4.2±2.3
0	1.0	0	0.5	1.5±0.57	2.9±1.05
0	0	0	0.5	1.4±0.78	4.2±1.54
0	0	0	1.0	1.4±0.64	3.6±1.37

Data in each column represents mean ± standard deviation. Significant differences denotes as $p< 0.05$ according to Duncan's multiple range test (Duncan, 1955) [6].

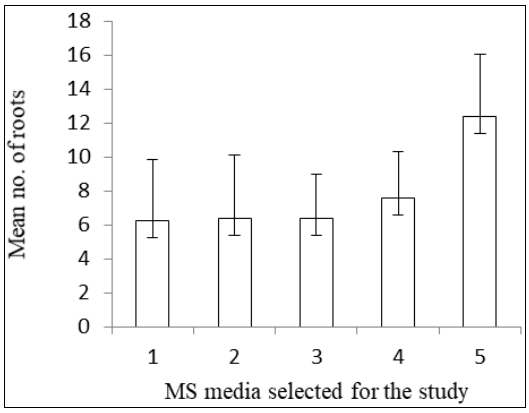

Fig 2: Root formation in *in vitro* plantlets of *A. triplinervis* in MS media with various PGRs (1) 0.5 mgL^{-1} NAA, (2) 1.0mgL^{-1} NAA, (3) 1.0 mgL^{-1}

Rooting

In the present study spontaneous root formation was observed from *in vitro* raised shoots of *A. triplinervis* in all the different combinations of media used. Rooting was visible after 5-6 days of inoculation and 90% of *in vitro* raised shoots produced 3-4 roots in the media used for shoot multiplication without the addition of auxins like NAA or IBA. This may be due to the presence of enormous amount of endogenous auxins within the explants. Slight brown callusing at the base with tuft of thin and hair like roots (6.36±3.74) were formed in MS medium supplemented with 1.0 mgl-1 NAA. MS media fortified with IBA improved the root growth and reduced the time duration for root induction. 1.0 mgL-1 IBA in MS medium was best for *in vitro* rooting (12.36±3.67) of *A. triplinervis.* Similar results showing efficiency of IBA in root induction of *A. triplinervis* was reported earlier by (Martin, 2003) [11].

Acclimatization

In vitro raised plantlets were successfully transferred to the *ex vitro* conditions and they showed good growth and stayed healthy. Their shoots flourished and leaves became bigger and greenish under field conditions. The plants produced by microprpagation were morphologically and physiologically similar to mother plants.

Conclusion

The protocol developed for the *in vitro* axillary bud multiplication of *A. triplinervis* is best and convenient for the rapid propagation of this important medicinal plant. MS medium fortified with 0.5 mgL^{-1} Kinetin was the best combination for the rapid shoot production of *A. triplinervis*. The present work developed an efficient protocol for the propagation of contamination free plants with in a limited time period. By using the *in vitro* raised plants other stress related studies such as enhancement and the assessment of secondary metabolite concentrations in the plant parts for the pharmaceutical pruposes of *A. triplinervis* could be conducted.

Acknowledgement

Authors greatly acknowledge the CSIR-HRDG, New Delhi for the financial assistance for the purchasing of chemicals and other equipment needed for completing the work.

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