(**BIOTECHNOLOGICAL STRATEGIES FOR SUSTAINABLE PRODUCTION OF DIOSGENIN FROM *DIOSCOREA DELTOIDEA***)

A Thesis

Submitted in partial fulfillment of the requirements for the

award of the degree

of

DOCTOR OF PHILOSOPHY

in

(Botany)

By

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PUNJAB

2021

DECLARATION

I hereby declare that the thesis entitled, **"Biotechnological Strategies for the Sustainable Production of Diosgenin from *Dioscorea deltoidea*"** submitted for Ph.D. Botany, Degree to Department of Botany, Lovely Professional University is completely original work and all ideas and references have been duly acknowledged. The research work has not been formed the basis for the award of any other degree or fellowship previously.

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CERTIFICATE

This is to certify that the thesis entitled **"Biotechnological Strategies for Sustainable production of Diosgenin from *Dioscorea deltoidea*"**, which is being submitted by **Ms. Romaan Nazir** for the award of the degree of Doctor of Philosophy in Botany from the Faculty of Biotechnology, Lovely Professional University, Punjab, India, is entirely based on the work carried out by her under my supervision and guidance. The work reported, embodies the original work of the candidate and has not been submitted to any other university or institution for the award of any degree or diploma, according to the best of my knowledge.

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ACKNOWLEDGEMENT

First and foremost with a quite sincere and humble attitude, I bow before "Allah SWT" who blessed me with courage, dedication, patience, sincerity and strength to accomplish this piece of work. I am blessed and thankful to Almighty Allah for every good and bad happening, some were blessings and some were lessons.

"On my bad days I seek You,
On my good days I thank You,
On my great days I praise You,
But every day I need You,
Thank You for always being with me".

With deep sense, respect and obligation from the core of my heart, I wish to thank my Supervisor ***Dr. Devendra Kumar Pandey,*** *Department of Botany, Lovely Professional University for his endurance, intent supervision, deep attentiveness, valuable time and mentoring abilities, thereby providing me a free and pleasant atmosphere to work against all anomalous circumstances. I am truly very fortunate to have the opportunity to work with him and found his supervision to be enormously valuable.*

I would like to express my deep sense of gratitude to ***Dr. Vijay Kumar****, Department of Biotechnology, Lovely Professional University for his constant encouragement, valuable advises and the freedom of work he provided throughout my research work.*

This work would have been incomplete without the guidance of ***Dr. Suphla Gupta****, Principal Scientist, Associate Professor, Academy of Scientific & Industrial Research, Plant Biotechnology Division, CSIR-IIIM, Jammu for her sympathetic approach, encouragement and valuable suggestions during the lab oriented work. She allowed me to work in her lab (Plant Biotechnology Research Lab) and use her project equipment's. She provided a friendly atmosphere that helped me to achieve objectives related to tissue culture aspect of my study.*

I am indebited to my friends namely ***Rekha Goyat, Sandeep Kaur, Kajal Katoch, Kaya Puri*** *and* ***Bilques Farooq*** *for conceding a lot with their comfort only to make me feel comfortable, so I could complete my thesis with affluence. They make me believe that friendship is not about whom you have known the longest, it is about who walked into your life, said, "I am here for you", and "proved it"*

I would like to extend my warm thanks to all my research colleagues and friends ***Dr. Prabjot Kaur, Mr. Shahnawaz, Ms. Ayang, Aliya Tabasum*** *and* ***Amit Bhatt*** *for their co-operation, and friendly environment in the lab.*

I would like to thank the scholars of Plant Biotechnology Research lab namely ***Muzamil Yousuf and Muzaffar Malik*** *for their guidance and help. It was them who made me competent enough to perform the experiments related to the tissue culture study and genetic fidelity.*

I would like to show my gratitude to the entire family of ***Lovely Professional University*** *and* ***School of Bioengineering and Biosciences*** *for providing me a suitable research atmosphere to carry out my work in proper time.*

I would like to offer my special thanks to ***Mr. Rebel*** *for his guidance, encouragement, support and assistance. This work would have been incomplete without his support.*

*Finally, my deep and sincere gratitude to my loving and compassionate parents, the best one in this world (Alhamdulillah) –****Nazir Ahmad, Amina Akhter****. I am very fortunate to have you, the way you brought me up, the endless and unparalled love you bestowed on me, the self-determination you gave me, the equality you provided to me, there was not a single case in my life where I felt I have been given less prominenc., Only a fewer people are lucky enough to have such parents. I am forever indebted to my parents for giving me oppurtunities and experiences that have made me who I am.*

"No love is greater than mother's love and no care is greater than father's care".

Last but not least, I am thankful to my brother ***Saqib Hussain*** *for all the lovely fights we have and for being so giving and caring. "A brother is a friend given by nature and there can be no better companion than him".*

Romaan Nazir

Abbreviations

Abbreviations	Descriptions
2, 4- D	2, 4-Dichlorophenoxyacetic Acid
2ip	Isopentenyl Adenine
AAD	Absolute Average Deviation
ABA	Abscisic Acid
AC	Activated Charcoal
AFLP	Amplified Fragment Length Polymorphism
$AgNO_3$	Silver Nitrate
ANN	Artificial Neural Networking
ANOVA	Analysis of Variance
AP	Acclimatized Plant
ASN	$_L$-Asparagine Monohydrate
BA	N^6-Benzyladenine
BAP	6-Benzylaminopurine
BBD	Box-Behnken Design
BM	Basal Medium
BME	β-Mercapto ethanol
BP	Base Pair
$CaCl_2$	Calcium Chloride
CM	Cordyline Multiplication Medium
CM	Cold Maceration
CS	Cordyline Starting Medium
CTAB	Cetyl Trimethylammonium Bromide

DMRT	Duncan's Multiple Range Test
DNA	Deoxyribose Nucleic Acid
DNTPs	Deoxynucleotide Triphosphates
EPS	Exo-Polysaccharide
GC	Gas Chromatography
GC-MS	Gas Chromatography- Mass Spectrometry
GLN	AR, $_{L}$-Glutamine LR
H.P	Himachal Pradesh
HCL	Hydrochloric Acid
$HgCl_2$	Mercuric Chloride
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HRE	Heat Reflux Extraction
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
ICH	International Council for Harmonisation
ISSR	Inter Simple Sequence Repeats
J&K	Jammu and Kashmir
JA	Jasmonic Acid
KN	Kinetin
KNO_3	AR, Potassium Nitrate
LAF	Laminar Air Flow
LOD	Limit of Detection

LOQ	Limit of Quantification
LS	Linsmaier and Skoog
MAE	Microwave-Assisted Extraction
MD	Microwave Drying
MeJa	Methyl Jasmonate
$MgCl_2$	Magnesium Chloride
MLP	Multi-Layer Perceptron
MP	Mother Plant
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
NaOCl	sodium hypochlorite
NaoH	Sodium hydroxide
NH_4NO_3	Ammonium Nitrate
OD	Oven Drying
PBD	Plackett-Burman Deign
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PGR	Plant Growth Regulators
PLE	Pressurized Liquid Extraction
PVP	Polyvinyl Pyrrolidine
QTL	Quantitative Trait Locus
R^2	Determination Coefficient
RAPD	Random Amplification of Polymorphic DNA
RFLP	Random Fragment Length Polymorphism

RMSE	Root Mean Square Error
RSM	Response Surface Methodology
RT	Revised Tobacco
SA	Salicylic Acid
SD	Sun Drying
SD	Standard Deviation
SH	Schenk and Hildebrandt
SHD	Shade Drying
SPS	Sodium Hydroxide-Extracted Mycelia Polysaccharide
SSR	Single Sequence Repeat
TDZ	Thiadizuron
UAE	Ultrasound-Assisted Extraction
UPLC-DAD-MS	Ultra-performance Liquid Chromatography-Diode Array Detection-Mass Spectrometry
WPM	Woody Plant Medium
WPS	Water-Extracted Mycelia Polysaccharide

Abstract

India is named as the “world herbal garden” and known as one of the mega biodiversity centres of the world consuming 24% of world’s area with 8% global biodiversity. It has more than 45,000 medicinal plant species distributed over three environmental hotspots. A major portion of these medicinal plant species is used for pharmaceutical purposes in a range of ways in both modern and traditional medicinal systems however the medicinal traditional system of India still endure the leading herbal tradition. Around 70% population of the country are estimated to depend on the traditional medicinal system based on herbs and about 25,000 formulations based on plants were consumed in traditional medicine.

The genus Dioscorea holds a significant place in the world of commerce. The tubers of *Dioscorea deltoidea* commonly known as “Singli Mingli” are the prime sources of pharmaceutically important steroidal saponins as diosgenin, stigmasterol, campesterol and β-sitosterol. They are traditionally used for the treatment of piles, diarrhea, dysentery, abdominal pains, chronic liver disease, burns, wounds, sour throat, and anemia. Moreover, they also possess various biological properties for instance antimicrobial, antioxidant, anticancer, antidiabetic, antithrombosis and so on. Diosgenin derived from these plants is basically used in the preparation of anti-infertility drugs such as sex hormones (testosterone and supplements for bodybuilders to increase their muscles and testosterone level) and contraceptive pills.

The plant *D. deltoidea* is growing in great popularity and its demand is increasing day by day in both national and international market for having a very huge potential in the pharmaceutical industries and the traditional medicinal system. Due to certain reasons like excess exploitation, shifting of climate, unsustainable use, natural calamities and urbanization, this plant is degraded steadily. Thus, there is a critical requirement of conservation of the species, proper balance between supply and demand, to aware the residents for their commercial benefits and make attempts to permit its replenishment or its cultivation. Therefore, studies were started covering many features in various areas of the world. This encouraged me to initiate a study of the species by keeping various problems related with this unique plant system in view. Attempts have been made to display the data acquired thus in a rational and valuable way.

The huge adaptability of plants and the environmental and biological difficulties related to conventional cultivation has delivered abundant of the impetus behind using plant tissue

culture over past years. The tissue culture technology employs an exclusive range of *in vitro* methods comprising micropropagation which has arisen as an important utility for the mass production of elite genotypes in less period of time and enhanced secondary metabolites production by the use of elicitors and precursors. These biotechnological strategies have achieved the eminence of multibillion buck industry throughout the globe.

Keeping the economic significance of *D. deltoidea* and the potential of well-known biotechnological techniques in view, a comprehensive investigation was carried out regarding the micropropagation and large scale production of secondary metabolites by elicitation and precursor feeding. Further investigations were also performed to screen the elite population of *D. deltoidea*, optimization of drying methods, seasonal variation, extraction methods, solvents and other extraction parameters by using response surface methodology (RSM) and artificial neural network (ANN).

This thesis is organized into four key sections which cover four major objectives of the research and all these objectives are presented with validated results that are attained after experimentation. The four objectives with silent achievements of this study are described as follows:

- The HPTLC method was developed and validated for the simultaneous phytochemical screening of 24 accessions of *D. deltoidea* which were collected from north-western Himalayas of India which includes Jammu and Kashmir, Himachal Pradesh and Uttarakhand. Two bioactive compounds diosgenin and β-sitosterol were identified in the accessions and based on their content elite variety were screened. The HPTLC results revealed that the accessions from Gulmarg area of Baramulla district in J &K were screened as the elite variety of *D. deltoidea* as it contains the highest content of both bioactive compounds. Moreover, the HPTLC method was found simple, specific and sensitive and can be applied in quality control and standardization of drugs.
- Optimization of extraction parameters was carried out by using RSM and ANN modeling and it was found that solid: solvent ratio, particle size, extraction time and temperature were significant parameters for extraction of diosgenin. Furthermore, the optimal conditions for diosgenin extraction were found as solid: solvent ratio, 1: 45; particle size, 1.25 mm; extraction time, 45 min; temperature, 45 °C. The comparison between RSM and ANN was based on the values of R^2, RMSE and AAD obtained and ANN was found superior to RSM in terms of predicting abilities and accuracy.

Moreover, conventional and non-conventional extraction methods and different solvents were compared and observed that MAE was efficient extraction method than others and 50% ethanol better than other solvents. Furthermore, the effect of seasonal variation and drying process on diosgenin yield was checked and December month was found best harvesting season and shade drying best drying process.

- An efficient and improved regeneration protocol was developed for the micropropagation of an elite variety of *D. deltoidea*. For the explants surface sterilization, NaOCl (2%) for 2 min and 0.1% $HgCl_2$ for 3 min was found to be an effective treatment in respect of survival rate and establishment. Different PGRs and cost-reduction strategy such as the exclusion of agar were optimized for large scale production of *D. deltoidea*. Simultaneous shoots and roots were developed by direct organogenesis in liquid MS medium fortified with various BA and IBA combinations. Tuber formation was obtained in three different media however BAP containing media with 6% sucrose showed maximum number of tubers. Genetic and biochemical fidelity was performed by ISSR and HPTLC analysis and *in vitro* regenerants were found identical to the mother plant.
- Biotechnological strategies like elicitation and precursor feeding were carried out for enhanced production of diosgenin. MeJa and SA as elicitors and squalene, β-sitosterol and cholesterol as precursors were added in different concentrations to the liquid MS medium at different exposure times. The diosgenin content was improved in all treatments but content varied in each case. However, biomass accumulation was reduced in all elicitor and precursor treatments as compared to control cultures. The outcomes of the present study revealed that SA was found more significant elicitor in terms of diosgenin production and β-sitosterol as potent precursor for the production of highest diosgenin content.

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CHAPTER 1

INTRODUCTION

1.1Introduction

In global biodiversity, plants are considered important components and perform an essential role in ensuring ecological sustainability. The plant diversity possessing natural environments offers conditions for sustaining life and is vital for the welfare and sustenance of humanity. Plants offer the basic life-sustaining necessities of food and about 7,000 plant species are utilized for nutrition, shelter, fodder, fuel, fibre (Corlett, 2006). Apart from sustenance, various species of plants are enormous sources of medicines or drugs, specially used for the treatment of several diseases in both traditional and modern medicines (Bako et al., 2005). In actuality, pharmacological plants constitute an essential natural treasure and contribute considerably to the establishment of primary health care facilities, particularly in the emerging world (Yuan et al., 2016). UNESCO (1996) observed that in developing countries medicinal plants and traditional medicines are mostly used for the maintenance of good health. Moreover, in the emergent nations, more than 3.3 billion people exploit therapeutic plants consistently which means medicinal plants are considered as the "backbone" of traditional medicine (Davidson, 2000). Around 21,000 of plant species according to WHO have the properties for being utilized as therapeutic plants whereas, Rao et al. (2004) stated that more than 12,000 plant species possess medicinal properties.

1.1 Medicinal Plants in India

India represents Medicinal Garden of the World as it is bestowed with 12 mega biodiversity regions. The Indian biodiversity is unsurpassed because of the presence of 16 diverse agro-climatic and 10 vegetative regions. The existence of two 'biodiversity hotspots' enriches India's natural treasure. Studies have proposed that among 45,000 plant species, 15,000-20,000 are known for their pharmaceutical assessment (Sharma et al., 2008). India relishes the advantages of varied environment and circumstances, from mountainous in the Himalaya to tropical rainy in the south, dry in Rajasthan to different

physiography, plateaus, mountains and plains with associated valleys in North-eastern states. Such ideal climatic conditions (soil, temperature, precipitation and so on) have offered ascend to rich and diverse flora in the Indian subcontinent.

India is known for its traditional medicinal systems, more than 6000 medicinal species have been codified in Ayurveda (2000), Unani (1000), Siddha (1300), Homeopathy (800) and folk (4500) and about 40% of total herbal plants diversity of India is used in tribal medicines (Ved and Goraya, 2008). In India, Ayurveda and Unani are most advanced and broadly accomplished systems. India is on the verge of herbal revolution due to its rich herbal diversity and is capable of supplying herbal plant resources to meet its demand globally. Besides healthcare system, medicinal plants are also essential to lift the economy and play a substantial role in economic progress. In developed countries (United States) the estimation of plant drugs constitutes 25% of total drugs, whereas in emergent nations (India and China), the contribution is 80%. Hence, the economic significance of herbal plants is considerably more to India than the other countries. The total export share of traditional medicines is moderately low in India but is growing quickly. According to Wakdikar (2004), India shares only 1.6% of herbal medicines in this fast rising worldwide trade and exports 8.13% whereas China exports 28%. According to Dhanabalan (2011), Ayush suggested that after China India ranks globally as the second major exporter of herbal plants in 2009. As the Indian subcontinent is widely famous for its traditional facts and forest diversity, there is crucial necessity to relish these in both the nationwide and global perspectives for the profit of humanity.

Emblica officinalis (Amla) is most consumed raw material by domestic herbal industries of India, however *Psyllium husk*, *Senna alexandrina*, *Lawsonia inermis* and myrobalans constitute 70% of total export of raw plant material by volume (Ved and Goraya, 2008). According to Sharma et al. (2008), Opium, Ipecac and cinchona alkaloids, vinca extract, **diosgenin**/16 DPA, solasodine, menthol, Gymnema sylvestre herb and Lawsonia inermis (mehndi) are most exported pharmaceuticals of India. Various economically significant Indian medicinal plants are presented in Table 1.1.

Table 1.1: *Commercially important medicinal plants of India*

Plant species	Family	Common name	Medicinal uses	Reference
Andrographis paniculata	Acanthaceae	Kalmegh	Dyspepsia, stomachic, hepatoprotective, helminthic, bitter tonic, antipyretic	Agrawal et al. (2005) Okhuarobo et al. (2014)
Aconitum heterophyllum	Ranunculaceae	Atis	For curing stomach ache and fever, tonic, febrifuge, anti-cough, antioxidant, antifungal, anticholinesterane	Evans (2006) Ahmad et al. (2017)
Aconitum ferox	Ranunculaceae	Vatsnabha	Cardiac stimulant, used for rheumatic and inflammatory diseases	Agrawal et al. (2005)
Asparagus racemosus	Asparagaceae	Satavari	Galactogogue, diurectic, anti-dysenteric, nervine disorder	Singh and Geetanjali (2016)
Aegle marmelos	Rutaceae	Bael	Analgesic, anti-inflammatory, antipyretic, anti-cancer, anti-spermatogenic, radioprotective, anti-thyroid, anti-oxidative, antihyperglycemic	Rahman and Parvin (2014)
Allium sativum	Liliaceae	Garlic	Anticarcinogenic, antioxidant, antidiabetic, renoprotective, anti-atherosclerotic, antihypertensive	El-Saber et al. (2020)
Azadirachta indica	Meliaceae	Neem	Hypolipidemic, antifertility, antidiabetic, antipyretic, antiulcer, neuroprotective, cardioprotective, antileishmaniasis	Saleem et al. (2018)
Berberis aristata	Berberidaceae	Daru haridra	Hyperglycemia, antipyretic, astringent, hyperlipidemia, purgative,	Evans (2006) Rahimi-

			liver diseases	Madiseh et al. (2017)
Commiphora mukul	Burseraceae	Guggul	Hypocholesteremic, Hypolipidemic, Anti-inflammatory, Anti-rheumatic	Kumar et al (2020)
Crocus sativus	Iridaceae	Saffron	Used as colouring (food dye), spasmolytic, stimulant, antitumor, neuroprotective, Alzheimer's disease	Abu-Izneid et al. (2020)
Dioscorea deltoidea	**Dioscoreaceae**	**Singli-Mingli**	**Anti-microbial, orthopedic and metabolic disorders, oncology, dermatitis and autoimmune diseases**	**Chandra et al. (2013)** **Dangawal and Chauhan (2015)**
Embelica officinalis	Euphorbiaceae	Amla	Anticancerous, antidiabetic, antioxidant, antifungal, cardio, chemo, gastro, neuro and hepato protective	Gantait et al. (2021)
Glycyrrhiza glabra L.	Fabaceae	Mulethi	Hepatoprotective, antidepressant, antimicrobial, anticancer, gastro duodenal ulcers, hypolipidemic	Batiha et al. (2020)
Garcinia cambogia	Guttiferae	Kokum	Used in the treatment of obesity, ulcers, fungal diseases and are lipid-lowering agent	Kandya (2005)
Gymnema sylvestre	Asclepiadaceae	Gudmar	Cardiac stimulant, anti-diabetic, larvicidal, dyspepsia, dysentery	Khanna and Kannabiran (2007)
Holarrhhena antidysenterica	Apocynaceae	Kutuja	Used for the treatment of malaria, tuberculosis, pimples and is amoebicidal	Sinha et al. (2013)
Nardostachys grandiflora	Valerianaceae	Jatamansi	Hepatoprotective, antidepressant,	Sahu et al. (2016)

			anticonvulsant, cardioprotective	
Ocimum teniflorum	Lamiaceae	Holi basil	Aromatic, stimulant, tonic, oxidant, inflammatory and diabetic agent	Evans (2006)
Picrorhiza kurroa	Plantaginaceae	Kutki	Hepatoprotective, immunomodulatory, inflammatory, jaundice, periodic fever, nausea, bronchial asthma	Masood et al. (2015)
Plantaga ovate	Plantaginaceae	Isabgol	Used for inflammation, aphrodiasic, dysentery, purgative, emollient	Evans (2006) Kandya (2005)
Phyllanthus niruri	Phyllanthaceae	Bhumi amla	Anti-cancerous, Anti-oxidant, Anti-inflammatory, Anti-tumor, Anti-nociceptive Bronchitis, Anaemia, Leprosy, Asthma, Urinary disorders	Narenfra et al. (2012)
Rauvolfia serpentine	Apocynaceae	Sarpagandha	Insomnia, hypertension, dementia, epilepsy, hysteria, constipation, insanity, intestinal, cardiac and liver diseases. helminthic, sedative	WHO (1999)
Swertia chirata	Gentianaceae	Chirata	Anti-bacterial, Anti-fungal, Anti-inflammatory, Anti-hepatitis B, Skin diseases, Epilepsy, Anemia	Kumar and Van Staden (2016)
Saussurea lappa	Asteraceae	Kuth	Used for inflammation, arthrities, cytotoxic, aphrodisiac, carminative, antioxidant, antiulcer	Amara et al. (2017)
Solanum nigrum	Solanaceae	Makoi	Sedative, diaphoretic, diuretic, hydragogue, diarrhea, piles, fever, hypotension, antioxidant, antimicrobial, stomach	Hameed et al. (2017)

			ulcer, hepatoprotective	
Santalum album	Santalaceae	Chandan	Hepatoprotective, CNS, antibacterial, antiviral, anticancer, haemolytic, antipyretic	Sindhu et al. (2010)
Tinospora cordifolia	Menispermaceae	Giloe	Used for cancer, malaria, periodic, allergies, spasmodies, inflammation, leprotic, anti-oxidant	Singh et al. (2003)
Withania somnifera	Solanaceae	Ashwaga ndha	Antirheumatic, anti-inflammatory, anti-tumor, anti-stress, sedative, diuretic, rejuvenator, hemopoetic, hypotensive	Kandya (2005)

1.2 Secondary metabolites from medicinal plants

Secondary metabolites from plants have significant role in the life of human beings as they are used as food and medicinal sources. In developing and industrialized countries, the guarantee of the quality, safety and efficiency of herbal plants and drugs has now become crucial matter. From thousands of years medicinal herbs have been used for the conservation, flavor, cure and prevention of ailments. The awareness of their therapeutic properties has been conveyed over the centuries within and among human communities. The biological properties of plants are because of secondary metabolites formed through secondary metabolism are used globally for many purposes especially for infectious diseases treatment. These pharmaceutically important bioactive compounds are extracted and isolated from medicinal plants which are used for the synthesis of drugs. The ingredients in blood thinners, antibiotics, laxatives, anti-malarial drugs are isolated from plants. Different bioactive compounds possess different pharmaceutical activities such as terpenoids and flavonoids are known for anticancer, antibacterial, anti-inflammatory, antimalarial and antiviral properties whereas alkaloids mostly possess anesthetic activities. Phenolic bioactive compounds play significant role in free radicals neutralization. Because of these properties bioactive compounds are considered as rich sources of antioxidants and attained substantial role in development of modern drugs for various diseases like cancer, tumor, arthritis and hepatic diseases. Aside from medication, these phytochemicals were also used in cosmetics, fragrances and flavoring agents and many of them are already

known commercially both in medicines and food supplements. Thus in present era, bioactive compounds become alternative system for resolution of health problems all over the world.

A bioactive compounds qualitative and quantitative study mostly depends on the type of appropriate extraction technique (Smith, 2003; Sasidharan et a., 2011). Though plant materials contain less quantity of bioactive compounds and in the drug discovery process their extraction, purification and characterization still remain a great challenge. The crude extract of plants contains a combination of compounds (alkaloids, terpenoids, saponins etc.); so, the selection of more proper extraction procedure and more appropriate analytical technique is implemented in a way to attain best extraction, isolation, purification of the desired bioactive compound. The facts demonstrate that advancement of modern spectrometric and chromatographic techniques make secondary metabolites analysis simpler than earlier yet the achievement relies upon the extraction techniques, input factors and precise nature of different parts of plants (Poole et al., 1990). The main common parameters influencing the process of extraction are solvent, time, temperature, plant matrix, pressure (Hernández et al. 2009). Many extraction methods can be applied to extract bioactive compounds. Beyond the conventional extraction methods such as percolation, maceration, heat reflux, soxhlet, infusion, a wide range of non-conventional techniques have been developed in the previous years. The non-conventional methods are considered more environmentally friendly because of less use of organic and synthetic solvents, less time consumption, high yield and better extract quality. For the selectivity of phytochemical compounds and improvement of overall yield, microwave-assisted extraction (Kaur et al., 2019), ultrasound (Kulkarni and Rathod, 2014), supercritical fluids (Herrero et al., 2010), extrusion (Lusas and Watkins, 1998), enzyme disgestion (Puri et al., 2012), pulsed electric field (Roselló-Soto et al., 2015), ohmic heating (Lakkakula et al., 2004) and accelerated solvents (Nayak et al., 2015) have been considered as non-conventional techniques. But still conventional method Soxhlet is considered as a reference technique to relate success of recently developed method.

In the process of identification and characterization of bioactive compounds, separation of these phytochemicals from plant extracts is still a big challenge as a number of phytochemicals are present in plant extracts. Different techniques such as HPTLC, HPLC, TLC, UPLC, UFLC, GC-MS, LC-MS etc. have been used for the identification and

determination of these phytochemicals. According to World Health Organization, standard protocols should be followed for the standardization of herbal drugs.

1.3 Biotechnological approach for herbal/ medicinal plants

As medicinal plants signify reliable part of biodiversity, their consumption and conservation approaches needs strategic supervision for sustainability. Thus, organized attempts should not only be focused towards conservation of the plant populations but also uplifting the level of awareness for viable consumption of these plant species in drugs. Emerging approaches for long-term viable supply of medicinal plants is challenging; thus, it has been proposed that to meet future public diet and healthcare demand, combination of conventional techniques and biotechnology are important.

Biotechnological techniques not only provide rapid cloning and genotype conservation but also facilitate genetic alteration, expression and regulation of genes for an effective production of profitable natural constituents in large quantity or with enhanced properties (Tasheva and Kosturkova, 2012). Due to numerous benefits of biotechnology in various sectors of food, agriculture, forestry, pharmaceutical and others, the arena of biotechnology has become a centre of attraction for preservation and viable source of herbal plants. For the sustainable supply of medicinal plants or their raw material, several *in situ* and *ex situ* approaches such as *in vitro* methods, plant, seed and gene banks, botanical gardens and gene sanctuaries have been proposed for the conservation of critically threatened species of plants (Khan et al., 2012).

1.3.1 *In vitro* biotechnological methods

In the current emerging scenario of biotechnology, *in vitro* culture or plant tissue culture has become most promising biotechnological tool for conservation and sustainable production of economically important and endangered medicinal plants. It has gained huge importance in the mass propagation of ornamental, horticulture, medicinal and disease-free plant species, cryopreservation, plant improvement, plant breeding, production of economically important secondary metabolites and genetic transformation (Debnarh et al., 2006; Altpeter et al., 2016). Moreover, *in vitro* culture has nowadays a well-developed method to culture and study the biological activities of quarantined plant cells, organs, tissues, cell organelles and protoplasts under specifically organized chemical and physical conditions. Micropropagation is the base for all biotechnological researches as almost all applications of plant biotechnology eventually need the effective culture of plant cells, organs and tissues.

This method has various advantages over traditional or conventional vegetative promulgation via cutting, air-layering, grafting, seed and proliferation of huge number of virus free plants in less duration with great homogeneity (Garcia-Gonzales et al., 2010). *Coryodalis yanhusuo*, one of the essential therapeutic herb was promulgated via somatic embryogenesis from callus derived from tuber for production of pathogen-free tubers (Sagare et al., 2000).

The success rate of micropropagation depends on numerous elements like culture medium, medium composition, culture environment, plant growth hormones and genotype. The growth of techniques for fast *in vitro* clonal propagation of any plants may be a huge commercial significance to the industry. Certain callus cultures produce clones having inheritable features unlike from mother plants due to the probability of existence of somaclonal inconsistency (George, 1993), which causes the growth of economically significant enhanced varieties. From conservation perspective, the *in vitro* regenerants ought to have least somatic variations by micropropagation process by decreasing the subculturing number and shoot tip or axillary bud culture. Usually, somaclonal variations occur in callus formations or in cell suspension cultures which decrease the commercial importance of the *in vitro* regenerants (Borse et al., 2011). The presence of somaclonal variations during micropropagation, secondary metabolites production, and genetically engineered plants can cause immense commercial concerns and signifies a severe hindrance in the use of *in vitro* procedures for the phytochemicals production (Rahman and Rajori, 2001; Bhattacharya et al., 2016). Hence, to check somaclonal inconsistency in a cell culture, it is compulsory to screen and evaluate the genetic composition and constancy of the *in vitro* regenerants. The procedures involved in that method consist of the use of numerous methods to measure probable variations at altered levels (Bhattacharyya et al., 2015, 2017a; Bhattacharyya and Van Staden, 2016; Bose et al., 2016). In order to evaluate variations in chromosome number and ploidy, flow cytometry and chromosome counting are broadly used and various DNA-based molecular markers such as inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), random fragmented length polymorphism (RFLP), start codon targeted (ScoT) polymorphism and microsatellite markers have been effectively used to assess genetic fidelity of *in vitro* regenerants (Ahuja et al., 2002; Narula et al., 2007; Thankappan and Morawalla-Patell, 2011; Bhattacharyya et al., 2015; Stanišicet al., 2015; Bose et al., 2016).

Micropropagation has, thus, become a commercially beneficial initiative and offers clear benefits over conventional agricultural proliferation practices by assisting the production of huge numbers of identical plants perennially, the generation of pathogen-free plants and a significant improvement of multiplication rates (Debnarh et al., 2006). At present, an enormous number of protocols of micropropagation have been established for the pharmaceutical plants (Khatri et al., 2019; Bhusare et al., 2020; Jena et al., 2020), and also several commercially significant plants, such as *Agave salmiana* (Silos-Espino et al., 2007), *Cynara scolymus* (Comino et al., 2019), *Moringa oleifera* (Ridzuan et al., 2019) and *Stevia rebaudiana* (Alvarado-Orea et al., 2020). But, the high charges of *in vitro* methods in comparison to traditional methods and the impulsiveness of the requirements of the market have restricted the practice of *in vitro* methods at a profitable level (Debnarh et al., 2006; Methora et al., 2007).

1.3.2. *In vitro* strategies for production of secondary metabolites

The *in vitro* culture of cells, tissues and organs of plants in aseptic circumstances provides a well-grounded technology for the natural products production from the plants. The micropropagation of plant organs generally roots or calluses can usually offer plant material proficient of producing bioactive compounds (Atanasov et al., 2015). Plant cell culture now signifies an effective method to produce numerous high value natural bioactive compounds (Fischer et al., 2015). The bioactive compounds production in *in vitro* culture relies on various factors, mainly nutrients for plant development. The optimal concentration of nutrients play important role in explants growth and bioactive compound accumulation (Fargoso Monfort et al., 2018). Cell suspension culture is considered one of the best techniques for the secondary metabolites production, the most eminent example is paclitaxel produced from species of *Taxus* (Atanasov et al., 2015). However, secondary metabolites production can be improved with some alterations to the culture media, like accumulation of elicitors and precursors, or environmental conditions like light, temperature (Figure 1.1).

Elicitors as plant defense compounds trigger the bioactive compounds production. Several types of elicitors can augment the bioactive compounds production, comprising plant cell wall components (pectin and cellulose), microbial constituents (chitin and glucan) and plant resistant signaling compounds (methyl Jasmonate salicylic acid and jasmonic acid). In biotic elicitors different fungal filtrates were used in *Azadirachta indica* root cultures to enhance Azadirachtin production (Srivastava and Srivastava, 2014). In *Oldenlandia umbellate*

cultures, anthraquinones production was elicited with yeast extracts, xylan and pectin while pectin resulted in best elicitation (Krishnan and Siril, 2018). The factors on which selection of suitable elicitor relies are the type of culture employed and the secondary metabolite being produced.

One more strategy is addition of precursors that are intermediates of the metabolic pathway of the desired metabolite. Precursors augmenting in the culture media can improve the production of the desired compound (Rao and Ravishankar, 2002; Hussain et al., 2012), and has been efficaciously utilized in numerous cases, comprising the production of flavonoids (Lu et al., 2011), withanolides (Sivanandhan et al., 2014), phenolic compounds (Świecaet et al., 2014) and triterpenoids (Watcharatanon et al., 2019). Before selecting the proper precursor it is necessary to view the whole biosynthetic pathway of the target compound.

Metabolic engineering, genetic transformation, hairy root cultures, bioreactor systems, endophytes and immobilization techniques are the other alternative approaches of biotechnology for the enhanced production of economically significant bioactive compounds.

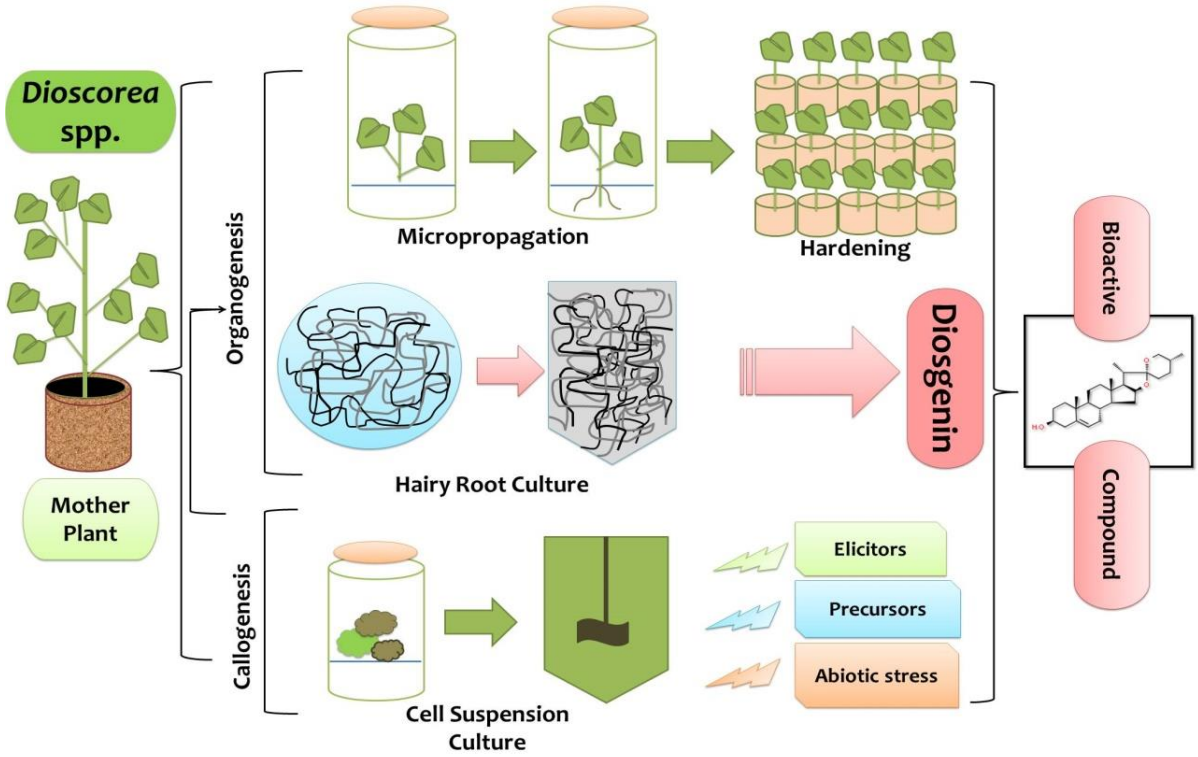


Figure 1.1: *Different biotechnological strategies for mass propagation and secondary metabolite production*

1.4 Aims and objectives of the study

Dioscorea deltoidea is an endangered high value therapeutic plant having huge economic significance. However, lot of research such as phytochemical screening for elite variety, production of plants and bioactive compounds through biotechnological techniques were still lacking. Therefore, keeping its economic significance and the potential of well-known biotechnological techniques in view, the current investigations were commenced for the development of alternate methods using biotechnological techniques for micropropagation, production of clonally identical *in vitro* regenerants, sustainable production of diosgenin by elicitation and precursor feeding and generation of molecular and chromatographic fingerprinting methods.

Major objectives of the study

- Collection of plants from different geographical locations of India and screening of elite variety of *Dioscorea deltoidea* on the basis of diosgenin and other related compounds by HPTLC method.
- Optimization of extraction methods on diosgenin yield from plants of *Dioscorea deltoidea*.
- Micropropagation of the elite variety of *Dioscorea deltoidea* for mass production.
- Application of elicitors and precursors for the enhancement of bioactive compound diosgenin in the plants.

CHAPTER 2

Review of literature

2. Review of literature

Dioscorea genus belonging to the family Dioscoreaceae are monocotyledons consist of more than 600 species (Coursey, 1969; Ayensu, 1972; Govaerts and Wilkin, 2007) and are divided into several sections on the basis of inflorescences, leaf and tuber morphology, stem twining, seed wings, formation of bulbils and bioactive compounds (Dahlgren et al., 2012). It is dispersed all over the tropical and sub-tropical areas of the world mostly in the Caribean and Central America parts, West Africa, South East Asia and Pacific Islands (Coursey, 1967; Prakash, 2011; Dangwal and Chauhan, 2015) and some species spread to temperate areas (Caddick et al., 2002). Around 50 species exhibit nutritional and industrial value (Han, 1995), among them 12 species are rich in nutritional value and 7 species possess steroidal saponins and sapogenins (diosgenin) which constitute two thirds of the total world production of corticosteroids and sex hormones (Ireland, 1981) and possesses several properties like antifungal, ant cancerous, antitumor, cytotoxic, cardiovascular, immunoregulatory and hypoglycemic (Lacaille-Dubois, 2005; Lacaille-Dubois and Wagner, 2000). In traditional Chinese medicine some *Dioscorea* species are used for the cure of rheumatism, cardio-cerebrovascular, cancer, gastropathy diseases (Lacaille-Dubois, 2002; Yang et al. 2006).

Dioscorea deltoidea is one of the most significant pharmaceutical species of Dioscoreaceae family due to the presence of steroidal compounds such as diosgenin, stigmasterol, β-sitosterol and campastrol (Stohs and El-Olemy, 1972). It is a perennial climbing herb growing 3 m in height contains ligneous irregular rhizomes or tubers. These tubers are traditionally used for piles, dysentery, chronic liver pain ailments and possess various properties such as antifungal, antibacterial, antioxidant, antimicrobial, antidiabetic, anticancerous, antithrombosis, antiaging and hypoglycemic activities (Subhash et al., 2012; Dangwal and Chauhan, 2015). It is distributed in Pakistan, china, Bhutan, India, Afghanistan and Vietnam and in India it is present in north-western Himalayas of Jammu

and Kashmir, Assam, Himachal Pradesh, Arunanchal Pradesh, Uttarakhand and Sikkim (Prakash, 2011).

Dioscorea deltoidea is called as Wild yam or Elephant's foot in English, Singly-mingly or Gun or Kin in Hindi, Varahikand in Sanskrit, Qanis in Urdu, Kildri Kreench in Kashmir, Kitra and Kniss in Punjabi, Sanjiaoyeshuyu in China and Tarul or Bhyakur or Ghunar in Nepal (Ali, 2012; Saikia et al. 2011).

2.1 Origin and Distribution of Genus Dioscorea

The Dioscorea genus consists of multi-species subsistence crops distributed in West Africa, South west Asia, south and Central America and Pacific Islands. *Dioscorea spp*. are inherent to warmer areas of both hemispheres, and in tropics some are considered as staple crops. It is assumed that *Dioscorea* spp. are originated from tropical regions of three distinct continents which are South-East Asia where *D.esculenta* and *D.alata* are native, Africa where *D. rotundata, D. cayenensis* and *D. dumetorum* are native and South America for *D. trifida*. Yams are main agricultural product and pivotal point of elaborate rites in New Guinea and West Africa. The most cultivated species of *Dioscorea* are *D. cayenensis, D. opposita, D. pentaphylla, D. alata, D. nummularia, D. bulbifera, D. esculenta, D. rotundata, D. japonica and D. trifida* (Lebot, 2009). *D. rotundata and D. cayenensis* are native to West Africa and are most significant in the matter of production and trade. The agronomy of these two species has extended to Caribbean and Pacific Islands and also to India (Abraham et al., 1989). In Caribbean Islands *D. trifida* is important which is American originated and *D. alata* as Asiatic origin has the broadest geographical distribution between food yams (Martin, 1969). Around 2,000 years ago *D. alata* was extended to Pacific Ocean and India (Coursey and Martin, 1970). It is assumed that *Dioscorea spp*. was moved to east to west whereas *D. esculenta* and *D. alata* extended westward to America and Africa and *D. cayenensis* and *D. rotundata* to America. In West African *Dioscorea* belt consisting Togo, Nigeria, Ghana, Republic of Benin, Coˆte d'Ivoire and Cameroon oldest *Dioscorea* cultures were believed and comprises biodiversity of *Dioscorea*. World's 90% of yams were cultivated in this belt in which *D. cayenensis* and *D. rotundata* accounted the most production. The distribution of Dioscorea genus is presented is Table 2.1 and Table 2.2.

India is rich in biodiversity and is distributed from Greater Himalayas to the plain of Ganga and Western to Eastern Ghats along with North-Eastern province. In India about 50 species of *Dioscorea* were identified. Among them 28 species occurred in North-Eastern region

(Sharma and Hore, 1995), 17 species to Western Ghats and 21 were found to exhibit ethno therapeutic significance. Some species are rare in existence like *D. deltoidea, D. orbiculata, D. prazeri* and *D. floribunda. D. prazeri* and *D. deltoidea* are threatened species and are listed in IUCN red list because of urbanization, over exploitation and illegal use for its economical pharmaceutical importance.

D. deltoidea is very important species of *Dioscorea* and is distributed in India, Pakistan, Afghanistan, Bhutan, China, Nepal, Cambodia, Thailand and Vietnam (Dangwal and Chauhan, 2015). In India, it is found in Indian Himalayan regions (IHR) such as Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, Arunanchal Pradesh, Assam and Meghalaya (Prakash, 2011) at an altitude of 550-3100 m (Stainton, 1988).

Table 2.1: *Worldwide distribution of Dioscorea species*

S.NO	Species	Distribution	Reference
1	*D. rotundata*	West Africa	Coursey (1976)
2	*D. cayenensis*	West Africa	Coursey (1976)
3	*D. dumetorum*	West Africa	Coursey (1976)
4	*D. trifida*	South America	Ayensu and Coursey (1972)
5	*D. esculenta*	Thailand, Indo-China, Philippines, New Guinea	Coursey (1976) Onwueme (1978)
6	*D. pentaphylla*	Tropical Asia, South-Eastern Asia, Tahati, Kauai, Savii, Molokai, Florida, Hawaii, North America	Ayensu and Coursey (1972)
9	*D. bulbifera*	East, West and South Africa, South Asia, Australia, Hawaii, Florida, South and Central America, Texas, Polynesia, Louisiana, Puerto Rico	Wilkin (2011) Mulaama (2004) Coursey (1967) Schultz (1993)
11	*D. deltoidea*	West Africa, Central America, India, Pakistan, Nepal, Vietnam, Thailand, China, Afghanistan	Prakash (2011) Tahir et al. (2016)

12	*D. belophylla*	Africa, Asia, Bangladesh, China, Pakistan, India, Nepal, Philippines, Myanmar, North and South America	Onwueme and Charles (1994) Govaerts (2013)
13	*D. hispida*	Indonesia, Malaya, Philippines, Bangladesh, Cambodia, China, New Guinea, Bhutan, Malaysia, Thailand, Fujian, Thailand, Vietnam, India, Africa, Laos, Myanmar	Nashriyah et al. (2011) Sharma and Bastakoti (2009)
14	*D. composita*	South America, Southern parts of Asia and Africa	Shen et al. (2018)
15	*D. wallichii*	South America, Central America and Caribbean, India, China, Bangladesh, Myanmar, Thailand	Lasser et al. (1974) Coursey (1967)
16	*D. zingiberensis*	China, Mexico	Zhang et al. (2018) Coursey (1967)
17	*D. floribunda*	Central America, Mexico	Coursey (1967)
18	*D. nipponica*	China, Japan, Korea	Yang et al. (2018)
19	*D. sylvatica*	East and South Africa	Wilkin (2011) Mulaama (2004)
20	*D. prazeri*	Africa, India, Burma	Coursey (1967)
21	*D. tokoro*	Japan	Tsukamoto and Ueno (1936)
22	*D. abyssinica*	Africa, Ethopia	Coursey (1967)
23	*D. mangenotiana*	Africa	Coursey (1967)
24	*D. polystachya*	China	
25	*D. pubera*	India, China, Central Nepal, Bhutan, America, Western Malaysia, Wet areas of Himalayas	Coursey (1967)
26	*D. villosa*	North America	Govaerts (2013)

27	*D. hirtiflora*	East and South Africa	Wilkin (2011) Mulaama (2004)

Table 2.2: *Distribution of Dioscorea species in India*

S. No	Species	Distribution	Reference
1	***D. deltoidea***	**Jammu and Kashmir, Himachal Pradesh, Assam, Uttarakhand, Arunachal Pradesh, Sikkim, Meghalaya**	**Prakash (2011)**
2	*D. alata*	Arunachal Pradesh, Assam, North Andaman Islands, Odhisa	Govaerts (2013) Kumar et al. (2012) Ghosh (2014)
3	*D. bulbifera*	Western Ghats of Maharashtra, North and South Andaman Islands	Gawande et al. (2015) Ghosh et al. (2014)
5	*D. pentaphylla*	Western Himalayas, Odisha, Nasik, Deccan, Rajasthan, North Andaman Islands	Kumar et al. (2012) Ghosh (2014)
6	*D. hispida*	Andhra Pradesh, Odhisha, Telangana, Jharkhand, North Andaman Islands	Kumar et al. (2012) Ghosh (2014)
7	*D. belophylla*	North Andaman Islands	Ghosh (2014)
8	*D. esculanta*	North and South Andaman Islands, Tamil Nadu	Ghosh (2014) Murugan and Mohan (2012)
9	*D. opppositifolia*	North Andaman Islands	Ghosh (2014)

10	*D. tomentosa*	North Andaman Islands	Ghosh (2014)
11	*D. wallichii*	North Andaman Islands	Ghosh (2014)

2.2 Morphology of *Dioscorea*

Dioscorea species are perennial plants, among which wild ones are either semi-perennials or perennials or annual and cultivated ones are annuals. Mostly, male plants are more than female plants. Generally, leaves are cordate, simple or acuminate borne on elongated petioles whereas in several species, they are palmate kind or lobed type with pointy tips (Okonkwo, 1985). Various leaf arrangements like opposite, alternate or both may found on similar stem depending on plant species. For example, simple cordate leaves are present in *D. rotundata* organized oppositely on the nodes whereas compound leaves are in *D. dumetorum* that are dissimilar from other species. *Dioscorea* leaves comprise of non-serrated lamina, reticulate veins and are non-pubescent. Leaf anatomy comprises of stomata that occurs on lower leaf epidermis (Okonkwo, 1985), but not in *D. bulbifera* in which stomata occurs on upper leaf epidermis (Onwueme, 1978). Depending on the growing condition and species, upper part growth of *Dioscorea* comprises of twining vines that are few meters in length. Spines on vines are common in wild species helps in the support of twinning and daunt animals (Okonkwo, 1985). Stems are cylindrical in most species whereas in *D. alata* consists of rectangular, stellate or polygonal structures having angular extension of membranous wings developing four sided cross section (Onwueme, 1978). Few *Dioscorea* species have dwarf plant structure. In *D. rotundata* dwarf genotypes were found having 1.4 m mean length of vine as comparison to non-dwarf species 19.8 m (Abraham et al. 1989). Basically *Dioscorea* species possess dioecious flowers having male and female flowers distinctly or on isolated plants. In *D. rotundata* hermaphrodite flowers are present on same spike (Sadik and Okereke, 1975a) but in *D. cayenensis* only male flowers occurs. Male and female flowers are borne from the axillary spikes of leaf axils. The male flowers are borne either terminally or axillary and are sessile, round and glabrous. The flowers comprises of calyx and corolla with three sepals and petals, organized habitually and virtually same in proportions and form, having three or six stamens (Onwueme, 1978). Mostly seeds are small and fruits are capsules in each capsule having different shaped wings in other species (Onwueme, 1978). Seeds are light and smooth and wings supported wind

dispersion. In *D. bulbifera, D. pentaphylla, D. alata, D. pubera* bulbils are present on axils and are adapted especially for vegetative propagation (Coursey, 1967).

Dioscorea plants have two underground structures i.e. storage tubers and fibrous roots that store starch. Roots growing from tubers are usually thin and short. The fibrous roots are normally flat but not in spinate vine cultivars. The tubers dimension, form and amount depend on genotype and species. The tubers of *D. rotundata* are generally large and cylindrical in shape having white flesh comprising of ovoid, four starch grains different from other species (Okonkwo, 1985).

2.3 Cytology of *Dioscorea*

Dioscorea genus is considered most challenging genera for cytogenetic and cytotaxonomic studies (Essad and Maunoury, 1984). In cultivated *Dioscorea* species various researchers previously stated the complications faced in chromosome counting (Miege, 1954; Baquar, 1980) and also in other species (Araki et al., 1983; Essad and Maunoury, 1984). The genus consists of Old and New world species. The basic chromosome number in Old world species are 10 whereas 9 in New World species (Orkwor et al. 1998). Currently, Yams are extensively considered as polyploids. *Dioscorea* species of Asia, Africa (52%) and America (13%) consists of basic chromosome number x = 10, whereas major American species show basic chromosome number x = 9 (Essad and Maunoury, 1984). Though, current data showed new basic chromosome number x = 6, x = 20 for yams and confronted past reports (Segarra-Moragues et al., 2004; Scarcelli et al., 2005; Bousalem et al., 2006). Presence of several levels of ploidy and absence of diploid relations to cultivated ploidy yams has convoluted yams study (Bousalem et al., 2006). However study on wild species ploidy level is intermittent and frequently conflicting. DNA content in *D. mangenotiana* Meige, *D. praehensilis* and *D. abyssinica* is same as reported by Hamon (1992) estimated by flowcytometry. But according to Essad and Maunoury (1984) *D. abyssinica* is tetraploid and *D. mangenotiana* and *D. praehensilis* are octoploids. *D. burkilliana* and *D. mangenotiana* were reported as tetraploid and hexaploid by Zoundjihekpon (1993).

In common food species chromosome ranges from 2n = 20 to 2n = 140. Extra chromosomes, B chromosomes or satellites sometimes larger than chromosomes occurs in Yams is usual (Essad and Maunoury, 1984). As compared to 2x, 6x, 8x genotypes tetraploid genotypes are habitually more frequent. In Yams mixoploid formation might be possible rarely. Dansi et al (2000b) reported 4x and 8x mixoploidy in 90 cultivars of two landraces in

Benin. Miege (1954) reported 2n = 36 and 54 chromosomes in *D. bulbifera* and Martin in 1974 reported 2n = 40, 60, 80 0r 100 chromosomes and 2n = 36, 40, 54 and 60 chromosomes in 10 varieties of Africa. In *D. esculenta* 2n = 40 chromosomes were reported (Martin, 1974), 2n = 90 (Bai and Jos, 1978) and Raghavan (1958) reported 90 and 100. In *D. cayenensis* 2n = 36, 54, 140 were reported (Miege, 1954) and in *D. alata* 30, 50, 70 chromosomes (Martin, 1976) and Sharma and De (1956) reported 52, 55, 66, 81 and 88 chromosomes. *Dioscorea* species of tropical areas contains highest number of chromosomes but smaller in size while temperate species have lowest chromosome number but larger in size (Orkwor et al. 1998). Table 2.3 shows chromosome numbers in *Dioscorea* spp.

Table 2.3: *Chromosome numbers in different species of Dioscorea*

S. No	Species	Chromosome Number (2n)	Reference
1	*D. bulbifera*	30, 40, 50, 60, 70, 80,100, 36, 54	Baquar (1980) Essad and Maunoury (1984)
2	*D. alata*	20, 30, 40, 50, 60, 70, 80	Sharma and De (1956) Hamon et al. (1992) Abraham and Nair (1991) Ramachandran (1968)
3	*D. esculenta*	30, 40, 60, 90, 80, 100	Miege (1954) Martin (1974) Ramachandran (1968) Raghavan (1958, 1959)
4	*D. rotundata*	40, 60, 80	Baquar (1980) Scarcelli et al. (2005)
5	*D. oppositifolia*	40, 138, 140, 142, 144	Araki et al. (1983) Ramachandran (1968)
6	*D. cayenensis*	36, 54, 60, 63, 66, 80, 120, 140	Miege (1954) Essad and Maunoury (1984) Baquar (1980)

8	*D. japonica*	40, 80, 100	Araki et al. (1983)
9	*D. transversa*	40	Essad and Maunoury (1984)
10	*D. hispida*	40, 60	Ramachandran (1968)
11	*D. dumetorum*	36, 40, 45, 54	Essad and Maunoury (1984)
12	*D. rubella*	30, 40	Raghavan (1958) Essad and Maunoury (1984)
13	*D. pentaphyllum*	40, 70, 80	Raghavan (1958, 1959) Essad and Maunoury (1984)
14	*D. spinosa*	40	Essad and Maunoury (1984)
15	*D. wallichii*	40	Ramachandran (1968)
16	*D. tomentosa*	40, 60	Essad and Maunoury (1984)
17	*D. schimperiana*	80	Baquar (1980)
18	*D. preussii*	40	Baquar (1980)
19	*D. mangetoniana*	40, 80	Lauzer et al. (1992)
20	*D. hirtiflora*	40	Baquar (1980)
21	*D. abyssinica*	40	Lauzer et al. (1992)

2.4 Genetic diversity assessment

Genetic diversity is defined as the quantity of genetic dissimilarity among individuals of population or species, genotype that develops new genotypes and offers adaptableness to unpredictable ecological surroundings (Brown, 2000). Such changeability between genotypes is expressed by the molecular, physiological, morphological, biochemical and cytogenetic traits (Ramanatha and Hodgkin, 2002). Therefore, wide germplasm characterization comprises of morphological depiction and also biochemical and molecular markers determination for genetic estimation (Mignouna et al., 2003).

Past studies of genetic diversity of *Dioscorea* species provided indulgent distribution and extend of diversity existing in both cultivated and wild genotypes. The species displayed significant diversity at intraspecific and interspecific levels (Okoli, 1991). In many countries cultivation diversity is improved further through constant domestication of wild species (Scarcelli et al., 2006). However, the level of genetic diversity of yams and their associations is yet to be studied in detail. On the basis of morphological characters, isozyme patterns and soluble protein profiles *Dioscorea* germplasm characterization has shown certain extent of variability (Ikediobi and Igboanusi, 1983; Dansi et al., 2000a). Different types of markers such as morphological, phytochemical, biochemical and molecular markers were used to study the genetic diversity of *Dioscorea* species.

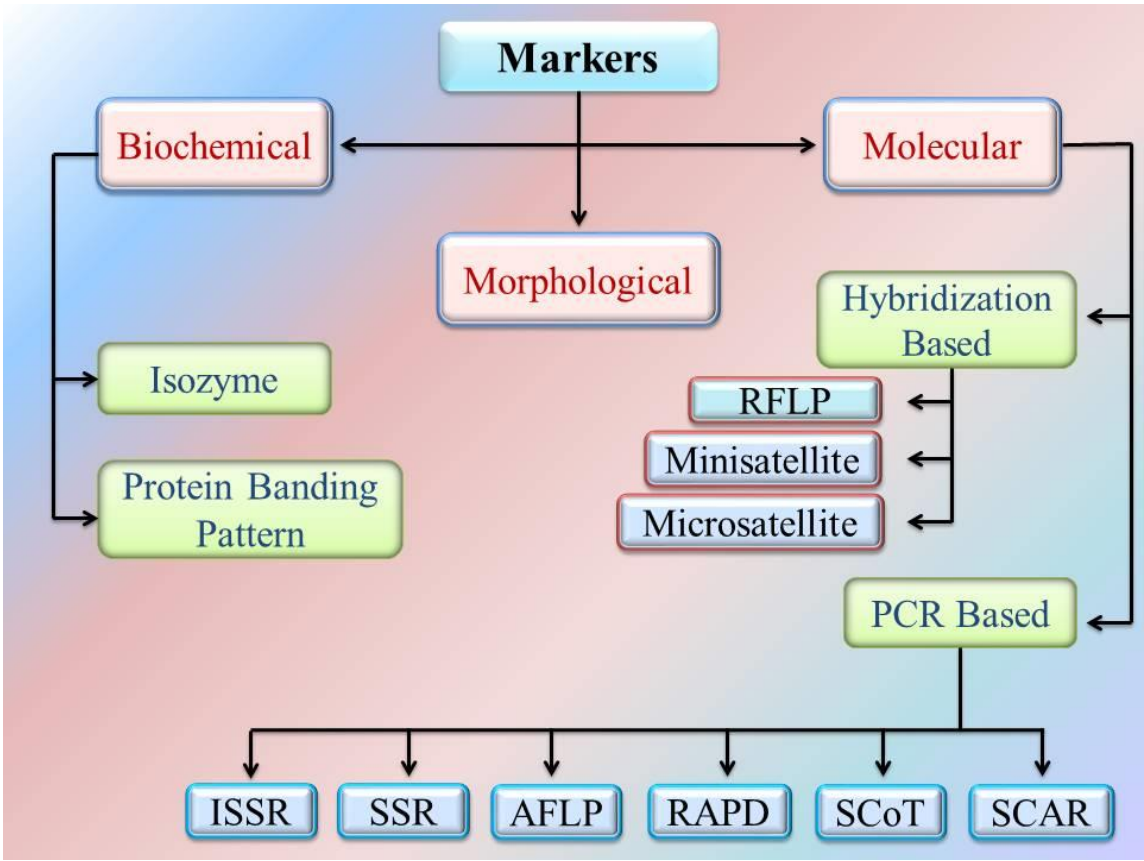


Figure 2.1: *Different markers used for genetic assessment*

2.4.1 Morphological Markers

Morphological or exomorphic description is among basic steps in the study of genetic diversity and utilised as a powerful tool in the cultivars classification as well as taxonomic study (Norman et al., 2011; Tamiru et al., 2011). Various studies of morphological diversity have carried out within and between *Dioscorea* populations to list present diversity

(Sastrapradja, 1982; Velayudhan et al., 1989; Lebot et al., 1998; Hasan et al., 2008). It provides inexpensive way of rapidly estimating the species that is why it is considered basic step for estimating species instead of going in deepness with biochemical or molecular characterization. Though, *Dioscorea* species are heterogeneous perennials having several overlying physiological, chemical and morphological features.

In previous studies 47 morphological traits were used for the assessment of 70 accessions of *D. alata* and it was found that characters like colour of petiole, shape, size, vein color of leaves, color and shape of aerial tuber, size, shape and flesh color of underground tuber showed most variability (Hasan et al., 2008). In another report 107 morphological characters were used for the assessment of 49 *D. alata* accessions and shape and margin of leaf, skin and flesh color of tuber, color of petiole wing, branching and shape of spine on stem characters were showed most differentiation (Anokye et al., 2004). Morphological inconsistency among 43 yam species of Kenyan by using 17 morphological variables was reported by Mwirigi et al. (2009). Velayudhan et al. (1989) identified 15 groups by the use of 22 morphological traits in 140 cultivars of India. Morphological traits like shape and color of leaf, stem color were usual variables studied by many researchers. Therefore, such variables might be announced as beneficial morphological markers for characterization and identification of *Dioscorea* species. Due to insufficient information on *Dioscorea* genotypes and their features confined their conservation, enhancement and leads to their genetic loss (Dansi et al., 2000). Due to ecological vitality that effected morphological characters, it is very essential to accompaniment morphological with molecular characterization.

2.4.2 Biochemical markers

Although morphological markers are effective but they show some disadvantages due to the ecological variations on expression of few morphological characters. These problems are solved by biochemical markers like isozymes and can simply be identified in a diversity of tissues by comparatively simple, fast and reasonable ways. In previous years, isozymes have been used widely in various plant breeding programs (Anderson et al., 1991; Manganaris et al., 1994; Degani et al., 1995) while in yams very few attempts reported (Hamon and Toure, 1990a, b). Isozymes analysis were used by Bressan et al. (2014) for defining yam cultivars into several species and clear genetic diversity were found between and within populations of yam species. Dansi et al., 2000 used isozyme polymorphism for the assessment of genetic variability in *Dioscorea cayenensis/Dioscorea rotundata* complex and 227 cultivars were

identified out of 467 accessions. Lebot et al. (1998) studied genetic variability between 269 cultivars of *D. alata* by isozymes and reported 66 isozyme phenotypes.

2.4.3 Molecular markers

With the initiation of molecular markers, a new generation of markers has been announced over the past two eras, which has transformed the whole scenario of biological sciences. Nowadays molecular markers play significant role in the determination of genetic diversity, genetic linkage maps construction, analysis of genetic relationship, identification of specific genes (Mignouna et al., 2002; Mignouna et al., 2003; Zhou et al., 2008). The assessment of genetic diversity of plants has become more reliable and simple because of molecular markers. Molecular marker methods are highly polymorphic for each loci and independent of environmental factors as compared to morphological or biochemical marker methods (Karp et al. 1997). Molecular markers are expensive but very effective for distinguishing between closely related genotypes. Being versatile tools, DNA-based molecular markers discover their own position in many fields of genetic engineering, physiology, taxonomy, embryology, etc. Various efforts have been made to explain the genetic diversity of *Dioscorea* species and genetic relationships within the species as listed in table. Variation assessments at molecular level have been achieved with random amplified polymorphic DNA (RAPD) (Dansi et al., 2000; Ahuja et al., 2002; Mignouna et al., 2003), inter simple sequence repeats (ISSR) (Zhou et al., 2008), simple sequence repeats (SSR)/ microsatellites (Mignouna et al., 2003; Obidiegwu et al., 2009; Otoo et al., 2015), amplified fragment length polymorphism (AFLP) (Mignouna et al., 2002; 2003; Malapa et al., 2005; Egesi et al., 2006; Mengesha et al., 2013), DS-PCR (Mignouna et al., 2005). Different markers used in genetic diversity assessment are displayed in Table 2.4.

Table 2.4: *Molecular markers used for genetic assessment in different Dioscorea species*

Plant Species	Markers	Achievements	References
D. opposita	ISSR	Evaluation of genetic diversity of *D. opposita*.	Zhou et al. (2008)
D. rotundata	AFLP	Genetic linkage map was constructed with AFLP marker in *D. rotundata* Poir.	Mignouna et al. (2002)

D. alata	AFLP and QTL analysis	Genetic linkage map was constructed with AFLP and QTL in *D. alata*	Mignouna et al. (2002)
D. cayenensis/D. rotundata	SSR	Determination of genetic diversity in *Dioscorea cayenensis/D. rotundata* core set	Obidiegwu et al. (2009)
D. alata	SSR	Determination of genetic identity, population structure and molecular categorization of *D. alata* L.	Otoo et al. (2015)
D. floribunda	RAPD	Determination of genetic stability of *D. alata* L. obtained from cryopreserved shoot tips.	Ahuja et al. (2002)
D. cayenensis/D. rotundata	RAPD	Identification of duplicates toward formation of an accurate core collection of Guinea yams.	Dansi et al. (2000)
D. alata L	AFLP	Intraspecific variability in *Dioscorea alata* L. was determined by AFLP marker.	Egesi et al. (2006)
D. alata L	SSR	SSR markers used for Genetic characterization of *Dioscorea alata* L germplasm.	Obidiegwu et al. (2009)
D. alata L. *D. mummularia* Lam. *D. transversa* Br.	AFLP	Determination of genetic diversity and genetic relatedness of *Dioscorea alata* L.	Malapa et al. (2005)

Dioscorea spp	AFLP	Genetic diversity and species delimitation was determined in cultivated and wild *Dioscorea* species by AFLP technique.	Mengesha et al. (2013)
D. rotundata	AFLP, RAPD and SSR Markers	Determination of genetic relationship, identification and discrimination of *Dioscorea rotundata*	Mignouna et al. (2003)
Dioscorea spp.	PCR (DS-PCR) and RAPD.	Analysis of genetic relationship of *Dioscorea* species.	Mignouna et al. (2005)
D. tokoro	AFLP	Construction of map and sex determination of *Dioscorea tokoro*	Terauchi and Kahl (1999)
D. alata L	SSR and morphological markers	Genetic diversity and structure of *D. alata* L. was analysed by SSR and morphological markers.	Siqueira et al. (2013)
D. alata L	RAPD ISSR	Determination of genetic diversity of *D.alata* L. by RAPD nad ISSR.	Rao et al. 2020
D. alata L	SSR	DNA fingerprinting of *D.alata* L. with the help of SSR markers	Siqueira et al. (2012)
D. dumetorum	AFLP and cpDNA	Assessment of genetic diversity of *Dioscorea dumetorum* by using AFLP and cpDNA	Sonibare et al. (2010)
Discorea species	AFLP	Analyses of genetic nature of *Dioscorea* species by using	Scarcelli et al.

		AFLP markers.	(2006)
D. rotundata	RAPD	Evaluation of genetic constancy of *in vitro* regenerants of *D. rotundata* after cryopreservation.	Mandal et al. (2008)
Dioscorea species	SSR	Evaluation of genetic diversity of *Dioscorea* spp. by SSR.	Muthamia et al. (2013)
D. trifida L.	SSR ISSR	Genetic diversity estimation of *Dioscorea trifida* L. by SSR and ISSR markers.	Nascimento et al. (2013)
D. bulbifera	SSR	Genetic diversity evaluation of *Dioscorea bulbifera* by SSR markers.	Osuagwu and Edem (2020)

2.5 Phytochemistry

The species of *Dioscorea* are well-known to possess a good amount of phytochemicals or bioactive compounds which are as saponins, alkaloids, glycoside steroids, flavonoids, tannins, anthraquinones, polyphenols, furanoid norditerpenes, withanolides, clerodane diterpenoids and phenols (Yang et al. 2014). However the most prominent phytochemicals of Yams are diosgenin and dioscorin. Various compounds found in *Dioscorea* spp. is presented in Table 2.5.

2.5.1 Diosgenin

Diosgenin is a bioactive steroidal sapogenin belonging to the triterpene group (Shah and Lele, 2012). Diosgenin is an important pharmaceutical compound gained great attention in pharmaceutical industries. It is used for the treatment of hypercholesterolemia, colon cancer, climacteric syndrome, leukemia, reduces cholesterol absorption and used as a preliminary substantial for the manufacturing of steroidal drugs like cortisone, sex hormones, oral contraceptives and other hormonal drugs (Lepage et al., 2010; Yan et al., 2015). In *Dioscorea* family, diosgenin and its glycosides are representative bioactive compounds. Around 137 types of *Disocorea* species comprising diosgenin in which 41 types comprise

1% diosgenin with abundant consumption value (Shen et al., 2018). In India, these species are the main sources of diosgenin and steroidal drugs production is totally based on it (Chaturvedi et al., 2007). Thus, essential approaches are required for the preservation and cultivation of *Dioscorea* species containing diosgenin to get such material. In India, *D. deltoidea* is the main source of diosgenin and highest diosgenin containing species. Singh and Chaturvarti were the first that reported diosgenin from *D. deltoidea* rhizomes (Shah, 2010). In China, *Dioscorea zingiberensis* and *Dioscorea opposite* are the sources of diosgenin production (Zhang et al., 2014). Fujii and Matsukawa (1935) were the first that discover diosgenin in *D. Tokoro* Makino, *D. esculenta*, *D. rotundata*, *D. dregeana* comprises diosgenin that possess antimicrobial, anti-inflammatory properties to gram negative and positive bacteria (Thajunnisha and Anbazhakan, 2013).

2.5.2 Dioscorin

Dioscorin is a yam storage protein present in several *Dioscorea* species and constitute 90% of extractable proteins of yams. It possesses many properties such as antioxidant, antihypertension invasion, trypsin inhibitor, immunomodulator and carbonic anhydrase (Harijono et al., 2013). *D. batatas*, *D. japonica*, *D. opposite*, *D. esculenta* and other many species of *Dioscorea* are sources of dioscorin.

2.5.3 Saponins

In many *Dioscorea* species more than 50 steroidal saponins of spirotane-, pregame- and furostane- type have been identified and categorised from 13 species specifically *D. deltoidea, D. nipponica, D. cayenensis, D. bulbifera, D. polygonoides, D. villosa, D. futschauensis, D. colletii, D. parviflora, D. spongiosa, D. zingiberensis, D. panthaica, D. pseudojaponica* (Santour et al., 2007). Saponins have several pharmacological properties such as antimicrobial (Lacaille-Dubois, 2002), anti-osteoporotic (Yin et al., 2003), anti-allergic (Tewtrakul and Itharat, 2006), anti-inflammatory (Tewtrakul and Itharat, 2006), cytotoxic, anabolizing, hormonal and immunomodulating actives (Lacaille-Dubois, 2002). Saponins are also good in the treatment of yeast and fungal infections.

2.5.4 Phytosterols

In many *Dioscorea* species phytosterols such as stigmasterol, β-sitosterol, campesterol and cholesterol are found in previous studies. Kadkade et al. (1983) reported campesterol, β-sitosterol, stigmasterol and cholesterol in *D. nelsonii, D. floribunda, D. bartletti* and *D.*

belizensis but cycloartenol in only *D. nelsonii* and *D. belizensis*. Osagie (1977) reported campesterol, β-sitosterol, stigmasterol and cholesterol in five *Dioscorea* species namely *D. bulbifera, D. dumetorum, D. rotundata, D. alata* and *D. cayenensis.* Stigmasterol, β-sitosterol and campesterol were also reported in *D. deltoidea* (Stohs and Olemy, 1972).

2.5.5 Flavonoids

Flavonoids are anti-oxidants present in many *Dioscorea* species. In *D. alata* 410. 52 mg/100 of flavonoids and in *D. cayenensis* 150.67 mg/100 flavonoids were reported and also showed free radical scavenging properties (Pushpanathan et al., 2013). Flavonoids were also reported in *D. deltoidea*, *D. bulbifera*, *D. pubera*, *D. glabra* (Subhash et al., 2012; Sheikh et al., 2002).

2.5.6 Tannins

Tannins are nontoxic compounds that produce physiological responses in animals. It exhibits many properties such as antioxidant, antifungal, anti hemorrhoidal and antidiarrheal (Lawal et al., 2014). Different cultivars of *D. bulbifera* possess phenols and tannins. *D. deltoidea* is also rich in tannins (Subhash et al., 2012).

Table 2.5: *Compounds present in Dioscorea Species.*

S. No	Species	Bioactive compound	Reference
1	*D. deltoidea*	Diosgenin, Dioscin, Flavonoids, Saponins	Asha and Nair (2005) Chauhan and Dangwal (2015)
2	*D. belophylla*	Flavonoids	Poornima and Ravishankar (2007)
4	*D. pentaphylla*	Phenolic compounds, Saponins, Flavonoids, Alkaloids, Terpenoids, Tannins	Kumar and Jena (2014) Prakash et al. (2014)
5	*Dioscorea spp.*	Saponin	Nayaboga et al. (2014)
6	*Dioscorea spp.*	Sapogenin	Martin (1969)
7	*Dioscorea spp.*	Cyanidin	Hou et al. (2000)
8	*Dioscorea spp.*	Allantoin Steroidal saponins	Lebot et al. (2019)

9	*D. alata*	Allantoin, Favonoids, Polyphenols, Alkanoids, Tannins	Lebot et al. (2019) Das et al. (2014)
10	*D. cyaenensis*	Dioscin, Gracillin, Allantoin, Protodioscin, Protogracillin	Lebot et al. (2019)
11	*D. villosa*	Allantoin	Lebot et al. (2019)
12	*D. rotundata*	Dioscin, Gracillin, Allantoin, Protodioscin, Protogracillin	Lebot et al. (2019)
13	*D. dumetorun*	Allantoin	Lebot et al. (2019)
14	*D. esculenta*	Dioscin, Gracillin, Allantoin, Protodioscin, Protogracillin, Stigmasterol, β-sitosterol, Diosgenin	Lebot et al. (2019) Olayemi and Ajaiyeoba (2007)
15	*D. pseudojaponica*	Dioscin, Gracillin, Methyl Protodioscin, Methyl Protogracillin	Yang et al. (2003)
16	*D. japonica*	Flavonoids, Polyphenols	Chiu et al. (2013)
17	*D. sparsiflora*	Diosgenin, Campesterol, Stigmasterol, β-sitosterol	Contreras-Pacheco et al. (2013)
18	*D. remotiflora*	Diosgenin, Campesterol, Stigmasterol, β-sitosterol	Contreras-Pacheco et al. (2013)

2.6 Analytical techniques for extraction and quantification

In medicinal plants the determination of secondary metabolites is the most important step and in sample preparation extraction and quantification are the two main steps.

2.6.1 Extraction techniques

Extraction of bioactive compounds is the main stage for separation, purification, isolation and recovery in the analysis of medicinal plants. Many steps can be used to extract the plant material. The extraction parameters (particle size, time, solvent composition, solid: solvent ratio, pH, temperature and extraction solvent) in any extraction method are the chief

parameters that define method efficacy and the final product properties. During the extraction procedures extraction solvents, sample pre-treatments and extraction methods were a few significant steps and holds importance in its application and for its activity.

In *Dioscorea*, different methods of extraction (conventional or non-conventional) were used for the extraction of diosgenin depicted in Table 2.6. From the existing literature, traditional methods for extraction of diosgenin from *Dioscorea* species consists of acid hydrolysis (Shah and Lele, 2012), maceration (Contreras-Pacheco et al., 2013), soxhlet (Zhang et al., 2009), heat reflux extraction (Wang et al., 2014). However, these techniques have some shortcomings like long extraction time, high energy and solvent consumption, expensive. Non-conventional methods on the other side green methods are environmental friendly techniques, uses less solvent and time, high extraction frequency (Takla et al., 2018; Tabani et al., 2018). Therefore, many green methods such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) and ultrasound assisted extraction (UAE) has been reported for the extraction of diosgenin from *Dioscorea* species. Xu et al. (2008) extracted diosgenin from Rhizoma *Dioscorea* by supercritical fluid extraction method used after acid hydrolysis. MAE technique was reported in the extraction of total saponins from *D. zingiberensis* (Ren et al., 2015). Apart from extraction techniques, solvent selection is also very important factor in the extraction process. Nowadays green solvents are more focused as they are environmental friendly as compared to organic solvents. Saponins are freely soluble in water as they are polar in nature and using water as an extraction solvent would be beneficial as it is cost effective, harmless and abundant. The most usual solvents used for saponins extraction are ethanol, methanol, aqueous alcohols and water whereas saponins solubility has also been reported in chloroform, ethyl acetate, ether, benzene or galacial acetic acid. In previous studies methanol, ethanol, petroleum ether, propanol, hexane, xylene and chloroform are used as solvents to extract diosgenin from *Dioscorea* species (Niño et al., 2007; Narula et al., 2007; Contreras-Pacheco et al., 2013; Wang et al., 2014).

Table 2.6: *Extraction techniques for bioactive compounds extraction in Dioscorea spp.*

Species	Extraction method	Solvent	Compound	References
Dioscorea species	Sonication Hydrolysis	Methanol HCL Chloroform	Diosgenin	Yi et al. (2014)
Dioscorea species	Sonication	65% ethanol	Steroidal saponins	Guo et al. (2016)
Dioscorea spp.	Maceration	Methanol	Dioscin, Gracillin, Allantoin, Protodioscin, Protogracillin	Lebot et al. (2019)
D. zingiberensis	Acidolysis Soxhlet	H_2SO_4 120# petrol	Diosgenin (1.26%)	Gong et al. (2011)
D. nipponica *D. panthaica*	Ultra sonication	Methanol	Diosgenin, Dioscin, Gracillin, Polyphyllin, Protodioscin, Pseudoprotodioscin, Pseudoprotogracillin	Tang et al. (2013)
Dioscorea alata Var purpurae	Hydrolysis	H_2SO_4 Hexane 70% Isopropanol	Diosgenin	Shah and Lele (2012)
Dioscorea polygonoides	Soxhlet	n-hexane Methanol n-butanol	Diosgenin (2.64%)	Niño et al. (2007)
D. zingiberensis	Ultra sonication Hydrolysis	95 % Ethanol H_2SO_4 Petroleum ether	Diosgenin	Li et al. (2012)
D. foribunda	Hydrolysis	HCL	Diosgenin	Mahato

D. deltoidea *D. prazeri*	Soxhlet	light petroleum		(1981)
D. esculenta	Soxhlet	n-hexane Methanol	Diosgenin Saponin Stigmasterol β-sitosterol	Olayemi and Ajaiyeoba (2007)
D. zingiberensis C.H. Wright	Ultrasonication Soxhlet Hydrolysis	120# petrol H_2SO_4	Diosgenin	Qiu et al. (2011)
D. alata	Shaking incubator	70 % Methanol	Phenolics	Dey et al. (2016)
D. japonica	Maceration	95 % Ethanol	Phenols Flavonoids	Chiu et al. (2013)
D. sparsiflora *D. remotiflora*	Soxhlet Maceration ultrasound maceration water bath	Petroleum ether Methanol 70% Ethanol 80% Ethanol 70% 2-propanol	Diosgenin Campesterol Stigmasterol β-sitosterol	Contreras-Pacheco et al. (2013)
Dioscorea zingiberensis C. H. Wright	IL-UMAE Heat reflux extraction Ultrasonic-assisted extraction Soxhlet	70 % Ethanol Petroleum Ether	Diosgenin	Wang et al. (2014)
Dioscorea bulbifera L.	Soxhlet Hydrolysis	Petroleum ether 2-Propanol	Diosgenin	Narula et al. (2007)

2.6.2 Analytical techniques

Various analytical techniques have been effectively utilized for the determination of secondary metabolites in *Dioscorea* genus as shown in Table 2.7. Estimation and detection of diosgenin have been done by High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), Gas chromatography-mass spectrometry (GC-MS), UV Spectrometry, Ultra Performance Liquid Chromatography (UPLC), Ultra-High Pressure Liquid Chromatography (UPHLC), High-Speed Counter Current Chromatography (HSCCC). In *Dioscorea*, Narula et al. (2003), Zhang et al. (2007), Gong and Huang (2011), Ghosh et al. (2014), Lu et al. (2016) have used HPLC while Avula et al. (2014) used UHPLC and Xu et al. (2008) used HSCCC for diosgenin analysis. However, HPLC combined with detectors are most commonly used techniques for diosgenin analysis (Patel et al., 2012). In this method, normal as well as reverse-phase modes are applied for isolation and purification of the samples on polarity basis. Nino et al. (2007) used HPLC-DAD-UV for diosgenin quantification from *D. polygonoids*.

In *Dioscorea*, HPTLC is now developing as very competent analytical method for secondary metabolites analysis. HPTLC in comparison to HPLC is rapid, precise, inexpensive and more reproducible for simultaneous valuation of several samples in precise time period. A few researchers have used HPTLC in *Dioscorea* species to evaluate diosgenin and other related compounds (Shah and Lele, 2012; Pandey et al. 2015; Roy and Geetha 2013). Several analytical methods with various settings have been used for qualitative and quantitative evaluation of diosgenin (Table 2.7).

Table 2.7: *Quantification techniques for diosgenin from Dioscorea species*

Species	Technique	Mobile Phase	Compound	References
Dioscorea spp.	UPHLC-DAD-MS	Acetonitrile-water	Diosgenin (14. 37 mg/g)	Yi et al. (2014)
Dioscorea spp.	HPLC-DAD-TOF/MS HPLC-QQQ/MS	Acetonitrile-formic acid	Steroidal saponins	Guo et al. (2016)
Dioscorea spp.	HPTLC	Chloroform: methanol: water (26:8:1)	Dioscin Gracillin	Lebot et al. 2019

			Allantoin Protodioscin Protogracillin	
D. zingiberensis	HPLC	Methanol: water (95:5)	Diosgenin (1.26%)	Gong et al. (2011)
D. zingiberensis	HPLC	Methanol: water (95:5)	Saponin Diosgenin	Li et al. (2010)
D. nipponica *D. panthaica*	UPLC-qTOF-MS	Water: Acetonitrile Water: Methanol	Diosgenin Dioscin Gracillin Polyphyllin Protodioscin Pseudoprotodioscin Pseudoprotogracillin	Tang et al. (2013)
D. polygonoides	HPLC GC-MS	Acetonitrile: water (90:10)	Diosgenin (2.64 %)	Niño et al. (2007)
D. zingiberensis	Spectrophotometer HPLC	Acetonitrile: water (90:10)	Diosgenin	Li et al. (2012)
D. sparsiflora *D. remotiflora*	GC-MS	-	Diosgenin Campesterol Stigmasterol β-sitosterol	Contreras-Pacheco et al. (2013)
D. bulbifera	HPLC	Petroleum ether-isopropyl alcohol (12:1)	Diosgenin	Narula et al. (2003)
D. japonica	HPLC	Water: methanol	Polyphenols Flavonoids	Chiu et al. (2013)

D. alata	HPTLC	Toluene: Ethyl acetate (7:3)	Diosgenin	Shah and Lele (2012)
D. zingiberensis	RP- HPLC-DAD	Water: Methanol (5:95)	Diosgenin	Qiu et al. (2011)
D. alata	HPLC- DAD GC-MS	Methanol: water	Phenolics	Dey et al. (2016)
D. zingiberensis	HPLC	Methanol: water (95:5)	Diosgenin	Gong et al. (2011)
D. prazeri *D. floribunda* *D. deitoidea*	HPLC	Petroleum: Isopropanol (12:1)	Diosgenin	Mahato et al. (1981)
Dioscorea alata L.	HPTLC	Toluene : Ethyl acetate (7:3)	Diosgenin	Pandey et al. (2015)
D. bulbifera	HPLC	Acetonitrile: water (90:10)	Diosgenin	Ghosh et al. (2014)
Dioscorea villosa	HPTLC	Toluene: Ethyl Acetate: Acetic Acid: Formic Acid (4: 3: 1:1)	Diosgenin	Roy and Geetha (2013)
Rhizoma dioscoreae nipponicae	RP-HPLC-UV		Diosgenin	Yang et al. (2013)
D. zingiberensis C. H. Wright	HPLC	Acetonitrile : water (95:5)	Diosgenin	Wang et al. (2014)
Dioscorea bulbifera L.	RP-HPLC	petroleum ether: 2-propanol (12:1)	Diosgenin	Narula et al. (2007)

2.7 Pharmacology

Dioscorea species possess various pharmaceutical properties like other medicinal plants. Various species of *Dioscorea* are already used in many traditional systems of medicines like Ayurveda. The ethno-pharmacological uses of *Dioscorea* spp. are also found in several complementary and alternative medicinal systems all over the world. Various important pharmaceutical activities of *Dioscorea* spp. and their compounds are given below.

2.7.1 Ethanobotanical uses

Dioscorea genus has comprehensive ethanobotanical uses all over the Tropics. Various reports are available on native claims on Yam Species globally. In South Thailand, *Dioscorea* species were used for the cure of warts by local people and also fever and asthma was cured by boiled *D. membranacea* tubers (Maneenoon et al., 2008). In Malaysia, local people used mucilage of *D. piscatorum* tubers for the fish poisoning. In India, paste of *D. prazeri* and *D. deltoidea* are used as shampoo and soap exterminated lice and allergies (Maneenoon et al., 2008; Mudasir, 2012; Kumari et al., 2012). In Cuba, native people uses *Dioscorea* to cure gastritis (Kadiri et al., 2014) and in Nepal, *D. deltoidea* rhizomes are boiled and used for gastric and blood dysentery problems (Dangwal and Chauhan, 2015). *D. hamitonii* tubers used in summer as body refrigerant and treats diarrhea and *D. oppositifolia* cures snake bites, swellings, scorpion stings (Dutta, 2015). Tuberculosis is treated with *D. bulbifera* and in cattles diphtheria is cured by *D. pentaphylla* tubers (Sharma and Bastakoti, 2009). Jaundice is treated with the juice of *D. wallichii* and *D. hispida* functions as a remedy for arrow poison (Swarnkar and Katewa, 2008; Sahu et al., 2010). *D. deltoidea* tubers are good for the cure and anticipation of many diseases such as metabolic and orthopaedic ailments, female reproductive system dysfunctions, oncology, autoimmune and skin ailments, cardiovascular and central nervous system diseases (Dangwal and Chauhan, 2015). Besides the traditional medicinal uses, numerous pharmacological and common values of *Dioscorea* species are reported by many researchers.

2.7.2 Pharmaceutical uses

Various researches revealed pharmaceutical importance and existing of polyphenolic compounds in *Dioscorea* species (Liu et al., 2011; Kumar and Jena, 2014). Several reports showed the antifungal, antioxidative, antimutagenic, immunomodulary and hypoglycaemic properties of *Dioscorea* species and are also essential components of nutritional supplements, cosmetics and pharmacological industries (Son et al., 2007; Black et al., 2007). Antimicrobial properties of *Dioscorea* are due to the presence of different kinds of phenolic components that occurs in tuber and other parts of plants (Sara et al., 2010). The peel extracts of D. alata possess antifungal property (Adkeriye et al., 1996).

Several researchers in different studies have revealed antimicrobial activity of various *Dioscorea* species like Xu et al. (2008) reported antibacterial activity of *D. zingiberensis;* tubers of *D. hamiltonii* with stem of *Azadirachta indica* stem shows antimicrobial property

(Kaladhar et al., 2010); *D. pentaphylla* shows both antifungal and antimicrobial properties as reported by Prakesh and Hosetti (2010). *D. villosa* tubers show antibacterial property against *P. aeruginosa*, *S. aureus*, *K. pneumonia*, *S. dysenteriae*, *V. cholera* and *E. coli* (Roy et al., 2012). *D. bulbifera* shows antibacterial activity against *A. niger, A. fumigatus, A. flavus, E. coli* etc (Seetharam et al., 2003). *D. deltoidea* extracts possess antimicrobial activity against three fungal strains and ten gram – and gram + bacteria (Chandra et al., 2013).

Dioscorea species have also shown antioxidant activities (Araghiniknam et al., 1996) and Hou et al. (2001; 2002) stated that tuber mucilage and dioscorin from *D. batatas* possess antioxidant property. *D. panthaica*, steroidal saponins were reported and its ethanol extract of tubers showed cytotoxic potential (Dong et al., 2004). Numerous segments mined from *D. bulbifera* showed anticancer activity (Yu et al., 2004). Earlier studies revealed that *D. bulbifera* and *D. esculenta* possess antitumor and antioxidant activities (Murugan and Mohan, 2012; Wang et al., 2012). *D. opposite* exhibited antitumor and antioxidant properties (Liu et al., 2016) and *D. oppositifolia* mucilage showed antimutagenic and antioxidant property (Zhang et al., 2016).

2.7.3 Pharmacologically significant bioactive compound

Dioscorea species has gained interest in both traditional and modern medicinal systems. Several types of bioactive compounds such as Diosgenin, Dioscorin, Dioscin, saponin, sapogenin, flavonoids, Allantoin, Phenolic compounds, cyanidin are found in the tubers as well as other parts of *Dioscorea* species presented in Table 2.8 with pharmaceutical activities. These pharmaceutically important compounds have native therapeutic uses between various races of indigenous of the world (Nayaboya et al., 2014). Martin (1969) reported diosgenin most important compound which is a sapogenin used in the steroidal drugs synthesis. In India *D. deltoidea* is reported as the main source of diosgenin (Asha and Nair, 2005). Biological activities shown by different *Dioscorea* species are displayed in Table 2.8.

Table 2.8: *Biological activities of Dioscorea species*

S. No	Pharmacological Activity	Species	Compound	Reference
1	Anti-cancer	*D. deltoidea* *D. villosa* *D. collettii* *D. alata*	Dioscin Diosgenin Saponin Flavonoids Phenolics Alkaloids	Aumsuwan et al. (2016) Hu and Yao (2001) Hu and Yao (2003) Das et al. (2014)
2	Anti-tumour	*D. bulbifera*	Diosbulbin B β-sitosterol	Wang et al., 2012
3	Anti-mutagenic	*D. pentaphylla*	Polyphenols Dioscorin Flavonoids	Prakash et al. (2014)
4	Anti-diabetic	*D. polygonoides* *D. bulbifera*	Diosgenin	Omoruyi (2006) Omoruyi (2008) Ghosh et al. (2014)
5	Anti-thrombosis	*D. zingiberensis* *D. hispida*	Diosgenin Steroidal saponins Phenolics	Zhang et al. (2013)
6	Anti-coagulation	*D. zingiberensis*	Steroidal saponins Diosgenin	Li et al. (2010) Gong et al. (2011)

7	Anti-bacterial	*D. bulbifera* *D. deltoidea*	Diterpenoids Saponin	Shen et al. (2002) Kuete et al. (2012)
8	Anti-fungal	*D. cayenensis* *D. deltoidea* *D. alata*	Saponins β-sitosterol	Shen et al. (2002) Santour et al. (2004) Chandra et al. (2013)
9	Anti-microbial	*D. deltoidea* *D. hispida*	Saponins Diosgenin Phenolics	Miah et al. (2018) Chandra et al. (2013)
10	Anti-oxidant	*D. belophylla* *D. batatas* *D. deltoidea* *D. bulbifera* *D. triphylla* *D. versicolor* *D. hispida* *D. esculenta* *D. japonica* *D. alata* *D. hirtiflora* *D. dumetorum*	Flavonoids Dioscorin Polyphenols Phenolics Diosgenin	Hou et al. (2001) Poornima and Ravishankar (2007) Son et al. (2007) Murugan and Mohan (2012) Chiu et al. (2013) Das et al. (2014) Miah et al. (2018) Adeniran and Sonibare (2017)
11	Anti-inflammatory	*Dioscorea spp*	Sapogenin Diosgenin,	Martin (1969) Olayema and

			Allantoin Diosgenin Saponin Stigmasterol β- sitosterol Phenolics Flavonoids Polyphenols	Ajaiyeoba (2007) Chiu et al. (2013) Mollica et al. (2013) Junchao et al. (2017)
12	Anti-aging	*D. villosa* *D. composita*	Diosgenin	Tada et al. (2009)
13	Immunomodulatory activity	*D. alata* *D. japonica*	Dioscorin	Lin et al. (2009)
14	Anti-allergic	*D. membranaceae* *D. quinqueloba*	Diosgenin Stigmasterol β-sitosterol Dioscoreanone Dioscorealide A Dioscorealide B Gracillin	Tewtrakul and Itharat (2006) Jegal et al. (2018)
15	Cytotoxicity	*D. panthaica* *D. collettii* *D. persimilis*	Dioscin Gracillin Saponins Protoneogracillin	Dong et al. (2004) Hu and Yao (2003) Ngan et al. (2020)
16	Anti-ulcer	*D. bulbifera*	Saponins	Dutta (2005)

			Diosgenin	
17	Skin infections	*D. bulbifera*	Saponins Diosgenin	Girachi et al. (1999)

2.8 Biotechnological strategies for *Dioscorea*

In *Dioscorea*, various challenges such as dormancy, dioecy, ploidy, diseases free tubers, lack of flowering, collection and selection are the main challenges. Regardless of every one of these confinements/issues, dealers or pharmaceutical organizations are gathering *Dioscorea* spp. from natural surroundings as a result of an absence of complimentary substitutes. With the increasing demand for *Dioscorea* spp. harvesting of these plants leads to extermination and reduction of these pharmaceutically important species. To overcome these problems, proficient biotechnological strategies such as advances in tissue culture, cell and molecular biology, genetic engineering specially transformation technology, have unlocked innovative possibilities for high production of drugs, nutraceuticals and other valuable constituents (Yaseen et al., 2009). In previous years, biotechnology offered great opportunity for the production of desired compounds and also conservation of significant plant resources (Rao and Ravishankar, 2002; Wilson and Roberts, 2004; Sujata et al., 2008). This includes elicitor and precursor feeding, high yielding cell suspension, tissue or organ cultures, bioreactors and other metabolic engineering processes.

2.8.1 Establishment of aseptic conditions, culture media and growth substances

For the successful establishment of cultures aseptic conditions, sterilization, explant selection, media composition and Plant growth regulators (PGRs) concentration were necessary. In *Dioscorea*, different types of explants and surface sterilizing agents have been used. Explants are generally contaminated due to presence of several microorganisms and to evade such surface contamination various sterilizing agents such as Tween-20, Bavistin, sodium hypochlorite, mercuric chloride, benomyl, ethyl alcohol, streptomycin and also some detergents were used by various researchers.

The most common media used for tissue culture of *Dioscorea* spp. is Murashige and Skoog (MS) media (Narula et al., 2007; Huang et al., 2009; Mahesh et al., 2010). All necessary plant nutrient components, PGRs, vitamins and carbon sources are present in nutrient

medium. However, other medium were also used by different researchers for the micro propagation of *Dioscorea spp*. Borges et al. (2004) used D-571 culture medium for the propagation of *D. alata* while as Kadota and Niimi (2004) used Linsmaier and Skoog media for micro propagation of *D. japonica.* The solidifying agent used mostly for the preparation of solid and semi-solid media is agar. Apart from culture media, PGRs type and concentration were very important for the success of tissue culture. Auxins and cytokinins concentration in the culture media regulates callus initiation and differentiation, shoot and root formation (Forsyth and Van; 1982; Behera et al. 2009). Callus formation is stimulated by gibberellins.

2.8.2 *In vitro* culture of *Dioscorea* spp.

The significance of *Dioscorea spp*. for agricultural and pharmaceutical industries requires its quick proliferation to give enormous number of plants. This issue might be understood by utilising *in vitro* culture, which is all the time functional for micropropagation of therapeutic plants (Bajaj et al., 1988). However Propagation method for *Dioscorea* through tuber fragments is an exceptionally moderate procedure and one tuber gave only 10 -15 plants every year (Lakshmi Sita et al. 1976). Even leaf cuttings gave only 30 % successful shooting and rooting. Hence in this circumstance, use of *in vitro* culture method appears to be useful for mass proliferation and regeneration of plants used for food and medicinal purposes.

In vitro culture of some economically essential Yam species has been attained through different explants by various researchers such as seeds (Helping et al., 2008), Nodes (Yan et al., 2002; Poornima and Ravishankar, 2007; Kumar et al., 1995), tubers (Xu et al., 2009; Kumar et al., 2017), immature leaves (Kohmura et al., 1995), meristem tips (Mitchell et al., 1995) and roots (Twyford and Mantell, 1996). Among these nodal segments were found most successful for direct and indirect organogenesis (Kumar et al. 2017). Various culture processes like callus and somatic embryogenesis, shoot and root cultures have been used in clonal propagation of *Dioscorea* species and in a moderately brief time huge number of plants were obtained from a single parent. The micropropagation of *Dioscorea* spp. is presented is Table 2.9.

2.8.2.1. Callus induction

In *Dioscorea* species, different growth hormones were used for callus initiation. Mahesh et al. (2010) reported that BA in combination with KN and 2ip showed callus induction in *D.*

wightii while Chen et al. (2003) reported that BA with NAA showed callus induction in *D. zingiberensis.* Shu et al. (2005) found BA and NAA good for primary callus initiation but BA (0.5 mg L^{-1}) + 2, 4-D (2.0 mg L^{-1}) showed best callus induction in *D. zingiberensis*. Some researchers have used IAA for callus initiation and found combination of IAA and BAP good for callus induction in *D. zingiberensis* (Heping et al. 2008). Effect of sodium nitroprusside (SNP) was studied and found that 40 µM SNP significantly stimulated callus induction in *D. opposite* (Xu et al., 2009). In a report, BAP (0.5 mg L^{-1}) with 2, 4-D (5 mg L^{-1}) showed callus formation from seeds of *D. balacanica* (Savikin-Fodulovic et al., 1998).

2.8.2.2 Shoot organogenesis

Plant tissue cultures are entirely reliant on PGRs for the initiation of adventitious shoots (Xu et al., 2009; Fan et al., 2011). Shoot morphogenesis and proliferation and cell division are particularly promoted by cytokinins and in various therapeutical plants combinations of cytokinins with auxins showed more shoot proliferation (Sengupta et al., 1984; Pant et al., 2010). Moreover auxin NAA regulates both organ growth and vegetative growth while as cytokinin BAP accelerates sprouting and cell division.

Many reports on shoot organogenesis in *Dioscorea spp*. are available and it is observed that proliferation was influenced by various factors which include PGRs presence or absence either individually or in combinations, explant type and genotype (Ondo et al. 2007; Staba 1982). In *Dioscorea,* combinations of BAP with KN and NAA or BAP alone are commonly used for the shoot formation and proliferation (Kadota and Niimi. 2004; Chen et al., 2007; Behera et al., 2009; Mahesh et al., 2010). In some reports, KN showed reduced multiplication rate in the clones of *D. cayenensis* (Ondo et al., 2010) and in *D. bulbifera* minimum shoot initiation was found (Forsyth and Staden 1982). Kadota and Niimi (2004) reported maximum shoot proliferation and survival in media augmented with NAA (0.01 mg L^{-1}) and BAP (0.5 mg L^{-1}). In *D. zingiberensis,* BA with low NAA concentrations showed positive influence on shoot initiation but inhibited elongation (Chen et al. 2003). In other report, two cytokinins (BA and KN) with NAA resulted in highest shoot proliferation rate in *D. oppositifolia* (Behera et al. 2009). However, in some studies of *Dioscorea* BA is reflected superior PGR as compared to KN in prompting greater mean number of axillary shoots (Yan et al., 2011). While as in other reports BA has adversely affected survival rate of explants (Martine and Cappadocia 1982).

Some growth inducers such as activated charcoal, zeatin, putrescine, sucrose, manitol, Thidiazuron etc were also used in Dioscorea genus. In *D. oppositifolia* and *D. pentaphylla* 0.3% activated charcoal was used for shoot multiplication (Poornima and Ravishankar 2007). In another report, 1.5% manitol along with BAP and activated charcoal high rates of regeneration and multiplication was reported in *D. alata* (Borges et al., 2004).

2.8.2.3. Root Induction

In vitro rooting efficacy was fond dependent on explant sources, plant species and growth hormones in previous reports. The *in vitro* regenerants proliferated by way of axillary segments might simply be transplanted and rooted and observed appropriate for ex rooting for mini tubers production. Auxin type and concentration affects root initiation with root numbers for each shoot. Behera et al. (2008; 2009; 2010), Poornima and Ravishankar (2007) reported NAA as an effective growth hormone for root initiation whereas Chen et al. (2003; 2007) have found IBA effective for rooting. In some cases ½ MS with NAA at the concentration of 2.0 mg L^{-1} was perceived effective for attaining maximum root formation in *D. alata* and *D. oppositifolia* (Behera et al., 2008; 2009). However in another study led by Poornima and Ravishankar (2007) reported best root formation in low concentrations of IBA and NAA. Thankappan et al. (2011) found no rooting in auxin free media and IBA with NAA in same concentrations induced rooting in 8-10 weeks. In a study, Ondo et al. (2007) revealed that number and length of roots had been increased when concentration of sucrose had been increased. Kadota and Niimi. (2004) found high rooting frequency in solid medium, liquid media produced high number of roots and gellan gum media produced longer roots.

2.8.2.4. Tuber Induction

In many species of Dioscoreacea *in vitro* propagation of tuberization has been reported by various workers (Ondo et al., 2007; Ng, 1988; Chu et al., 2002; Jean and Cappadocia, 1992). NAA and IBA promoted *in vitro* tuberization and growth of *D. composite* and also 80 – 100 g/L sucrose induced tuber development (Alizadeh et al., 1998). Forsyth and Van. (1982) reported that KN in higher concentrations (23.2- 46.4 µM) showed higher frequency of microtuberization in *D. bulbifera*. As compared to culture medium, TIS showed maximum fresh weight, diameter and number or micro tubers (Jova et al., 2005). In some cases it was reported that MS medium showed inhibitory effect on tuber formation (Mantell and Hugo, 1989). Various researchers have found sucrose significant for tuber formation. Jasik and

Mantell. (2000) reported 20g/L sucrose produced highest microtubers in number and size than 40g/L sucrose. Ng. (1988) reported that microtubers decreased in *D. rotundata* by the addition of sucrose (8 or 10%) and KN (2.5 µM) in MS whereas Mantell et al., 1978 found 2% sucrose produced highest microtubers in *D. alata* and *D. opposite* nodal culture. Kohmura et al., 1995 observed 6% sucrose with 8.0 µM BAP effective on tuberization while Chen et al., 2007 stated that sucrose helps in shoot and micro tuber induction by providing carbon source and energy. Polyamines effect was also studied by Onvono et al. (2010) on tuber formation and found that putrescine in low concentration (10^{-5}, 10^{-6} M) causes early tubers in the complex of *D. cayenensis – D. rotundata.*

Table 2.9: *In vitro culture of Dioscorea spp for mass production*

Species Name	Explant	MS Medium + PGR		Result/Achievement	Reference
		Shoot	Root		
D. alata	Nodal segments	IAA and KN (0.5 – 2.5 mg L^{-1})	IAA and KN (0.5 – 2.5 mg L^{-1})	Maximum shoot multiplication observed at KN (1.5 mg L^{-1}) + IAA (2 mg L^{-1}) shows highest shoot multiplication and best rooting at IAA (2.5 mg L^{-1}). Survival rate 85 to 87%.	Das et al. (2013)
D. zingiberensis	Stems	2.2, 4.4 µM BAP and 0.0, 1.1, 2.7 µM NAA.	4.9 µM or 9.8 µM IBA.	4.4µM BAP + 1.1 µM NAA showed shoot formation, IBA (8.9 µM) + NAA (5.4 µM) showed callus	Chen et al. (2003)

				formation and 22.2 µM BAP + 1.1 µM NAA showed root formation. 85% survival rate.	
D. wightii	Nodal segments	BA, KN, 2ip (0.5, 0.30, 1.75, 2.25, 5.00 µM)	BA, KN, 2ip (0.5, 0.30, 1.75, 2.25, 5.00 µM)	BA (0.15-1.75 µM), KN (0.75-5.0 µM), 2iP (0.150.30 µM) showed 100% callus initiation. BA (1.75µM) increased callus fresh mass.	Mahesh et al. (2010)
D. oppositifolia	Nodal segments	KN-BAP with IBA, IAA, NAA (0, 0.1, 0.5, 1.25, 2.5 or 5.0 µM)	NAA and BAP (0, 0.1, 0.5, 1.25, 2.5 or 5.0 µM)	BAP (0.5 mg L^{-1}) and NAA (0.1 mg L^{-1}) produced shoots and roots, 0.5 mg L^{-1} IBA produced callus and 2.0 mg L^{-1} NAA and 2.5 mg L^{-1} KN produced multiple shoots.	Maheswari et al. (2012)
D. alata *D. abyssinica*	Nodal segments		NAA (0.027, 0.27, 2.7, 27), BAP (0.22, 2.2, 22, 44), ABA (0.0038,	2.7 µM NAA showed highest number microtubers. 0.27 µM promoted callus growth. BAP (0.22 µM)	Jean and Cappadocia (1982)

			0.038, 3.8, 38)	showed tuber formation.	
D. belophylla	Nodal segments	BA (1-6µM), KN (1-7µM), NAA (1 µM), 1-8% sucrose	BA (3-5µM), KN (3-4µM), NAA (1 µM), 3-8% sucrose	BA (3 µM) + KN (4, 5µM) showed best shoot development and 4% sucrose showed best shooting. 3-8% sucrose showed best microtubers per culture	Thankappan and Abraham (2015)
D. abyssinia And D. mangenotiana	Nodal segments	NAA (0.5 µM) and BA (4.4 or 8.9 µM)	Sucrose (20, 40, 60, 80 g^{-1}), 2.71 µM NAA.	Sucrose (20,40,60,80 g L^{-1}) induced microtubers in *D.abyssinia* and sucrose (40,60 g L^{-1}) favoured tuberization in *D.mangenotiana*	Lauzer et al. (1992)
D. zingiberensis	Seed	BAP 0.2 mg L^{-1}	NAA 0.5 mg L^{-1}	For 16 h colchicine solution (0.3%) initiated maximum autotetraploid plants and initiation rate reached 36.7%.	Heping et al. (2008)

D. composita Hemsl	Nodal segments	NAA (1.25, 2.5, 5.0 µM), IAA(1.25, 2.5, 5.0 µM), IBA (1.25, 2.5, 5.0 µM), 2,4-D (1.25, 2.5, 5.0 µM), KN (1.25, 2.5, 5.0 µM), 2iP (1.25, 2.5, 5.0 µM), BA (1.25, 2.5, 5.0 µM), ABA (1.25, 2.5, 5.0 µM), AH (0.6, 0.9, 1.2 mM	NAA (1.25, 2.5, 5.0 µM), IAA(1.25, 2.5, 5.0 µM), IBA (1.25, 2.5, 5.0 µM), 2,4-D (1.25, 2.5, 5.0 µM), KN (1.25, 2.5, 5.0 µM), 2iP (1.25, 2.5, 5.0 µM), BA (1.25, 2.5, 5.0 µM), ABA (1.25, 2.5, 5.0 µM), AH (0.6, 0.9, 1.2 mM	Sucrose (8%) showed major component for microtuber induction. BA (1.25, 2.5 µM) showed strong inhibitory effect and NAA nad IBA (5.0 µM) indicated striking influence on microtuber induction and development.	Alizadeh et al. (1998)
D. forddii Prain et Burk	Nodal segments with axillary buds	BA (1.0 mg L^{-1}), NAA (0.1 mg L^{-1}) Sucrose (30 g l^{-1}), Agar (4.5 g l^{-1}), AC (1.5 g l^{-1})	BA (1.0 mg L^{-1}), NAA (0.1 mg L^{-1}) Sucrose (30, 50, 80 g l^{-1}), AC (1.5 g l^{-1}), JA (0, 0.22, 2.24 mg L-1), PBZ (0, 0.2,	MS + 1.0 mg L^{-1}BA, 0.1 mg L^{-1}NAA, 30 g l^{-1} sucrose and 1.5 g l^{-1} AC in liquid culture favoured axillary shoot production and tuber formation.	Yan et al. (2011)

			2 mg L-1)		
D. opposita	Tubers	BA (3 mg dm^{-3}), NAA (0.5 mg dm^{-3})	BA (3 mg dm^{-3}), NAA (0.5 mg dm^{-3})	Addition of SNP reduces browning and endorses higher *in vitro* proliferation frequency.	Xu et al. (2009)
D. cayenensis-D. rotundata	Nodal segments	JA (0.1, 1.0, 2.5, 5.0, 10.0 µM).	JA (0.1, 1.0, 2.5, 5.0, 10.0 µM).	JA (2.5 µM) optimum concentration resulting in 70 and 90% explants producing microtubers in the MS medium and the Tuberization medium.	Olivier et al. (2012)
D. alata	Nodal segments	BM + BAP (0.2)+ KN (0.5)+ TDZ (0.5) + IAA (0.5) IBA (0.5) + NAA (0.5) +2,4-D (2) + NH4NO3 (825) + KNO3 (950) + AgNO3 (10) +Gln (100) + Asn	BM + BAP (0.2)+ KN (0.5)+ TDZ (0.5) + IAA (0.5) IBA (0.5) + NAA (0.5) +2,4-D (2) + NH4NO3 (825) + KNO3 (950) + AgNO3 (10) +Gln (100) + Asn	BAP (0.2 ppm) + 2, 4-D (2 ppm) showed best results and similar callus induction was showed by explants.	Shah and Lele (2012)

		(100) + PEG (30000)	(100) + PEG (30000)		
D. opposita	Nodal segments	KT (0.5, 1.0, 2.0 mgL^{-1}), 2,4-D (0.02, 0.1, 0.5 mgL^{-1}), PP333 (0.01, 0.1, 1.0 mgL^{-1}), Thidiazuron (0.005 mg L^{-1}), Sucrose (30gL^{-1})	KT (0.5, 1.0, 2.0 mgL^{-1}), 2,4-D (0.02, 0.1, 0.5 mgL^{-1}), PP333 (0.01, 0.1, 1.0 mgL^{-1}), Thidiazuron (0.005 mg L^{-1}), Sucrose (30gL^{-1})	Sucrose (60 g L^{-1}) resulted highest microtuber yield.	Li et al. (2014)
D. bulbifera	Nodal segments	KN (5, 10 mgl^{-1}), sucrose 2, 5, 8%)	KN (5, 10 mgl^{-1}), sucrose 2, 5, 8%)	Sucrose alone is responsible for tuber initiation.	Forsyth and Van (1984)
D. alata	Vine nodes	KN (0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L^{-1}) + BAP (0.25, 0.5, 1.0, 1.5 mg L^{-1}) + NAA (0.25, 0.5, 1.0 mg L^{-1}) + Ascorbic acid (100 mg L^{-1})	½ MS + NAA or IBA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1})	MS + 2.0 mg-l KN +1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA sowed best shoot multiplication and ½ MS + 2.0 mg L^{-1} NAA shows best rooting. Survival rate 90%	Behera et al. (2010)
D. oppositifoli	Nodal segment	NAA (0.11 µM) + BAP	NAA (0.11 µM) + BAP	NAA (0.11 µM) + BAP (0.89 µM) +	Kohmura et al. (1995)

a L		(0.89, 8 µM) + sucrose (3, 6%)	(0.89, 8 µM) + sucrose (3, 6%)	sucrose (6%) showed effective shoot formation	
D. oppositifolia Linn *D. pentaphylla* Linn	Nodal segment	KN (2.32, 4.65, 9.29 µM) + BAP (2.22, 4.44, 8.87 µM)	NAA (0.54, 2.69)	MS + 8.8 µM BAP + 0.3% charcoal showed multiple shoots.	Poornima and Ravishankar (2007)
D. bulbifera	Axillary meristem	MS + IAA (5.00, 10.0 mg L^{-1}) + KN (0.05, 5.0 mg L^{-1})	MS + IAA (5.00, 10.0 mg L^{-1}) + KN (0.05, 5.0 mg L^{-1})	MS + IAA showed larger bulbils and 0.5 mg L^{-1} KN increased dry weight of bulbils. IAA and KN concentrations produced bulbils, shoots and roots.	Uduebo (1971)

2.8.3. Acclimatization and Hardening of Plantlets

Acclimatization and hardening of *in vitro* plants to the new environment is the most important task. In Dioscorea genus, various factors such as soil mixture, temperature, humidity play important role during acclimatization of *in vitro* regenerants. Kadota and Niimi. (2004) used vermiculite: soil mixture (1:1 v/v) for *in vitro* plantlets of *D. japonica* and 80% survival rate was observed. Behera et al. (2009) transferred *in vitro* raised plantlets of *D. oppositofolia* in pots containing soil: sand: manure (1:1:1) and 90% survival rate was observed. Bazabakana et al. (1999) used sand: compost: mould (1:1:2) and tubers were produced by acclimatized plants after 8 months. While Jova et al. (2005) transferred plants to soil rite: coir: peat moss for acclimatization.

2.9. Biosynthesis pathway of diosgenin

Diosgenin from *Dioscorea* species and other plants is considered as the precursor for the synthesis of various commercial steroidal compounds. Diosgenin is spirostan replaced at 3β position by hydroxyl group. According to Chappell et al. (1995) 3-Hydroxy-3-methylglutaryl-CoA is major compound in biosynthetic pathway of diosgenin and is generally derived from acetyl CoA by acetoacetyl CoA. Mevalonate is then formed from 3-Hydroxy-3-methylglutaryl-CoA via 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and is the major rate-limiting step in isoprenoid pathway. By several reactions isopentenyl pyrophosphate is formed from mevalonate. After that, isopentenyl pyrophosphate is converted to squalene 2, 3-oxide by various steps. From squalene, diosgenin formation was assumed via two different routes: one is from lanosterol via cholesterol and another one is from cycloartenol via sitosterol. Sterol-3-β-D-glucoside is formed from sitosterol by sterol-3-β-glucosyl transferase (STRL) and finally leads to the diosgenin synthesis (Chaudhary et al., 2015).

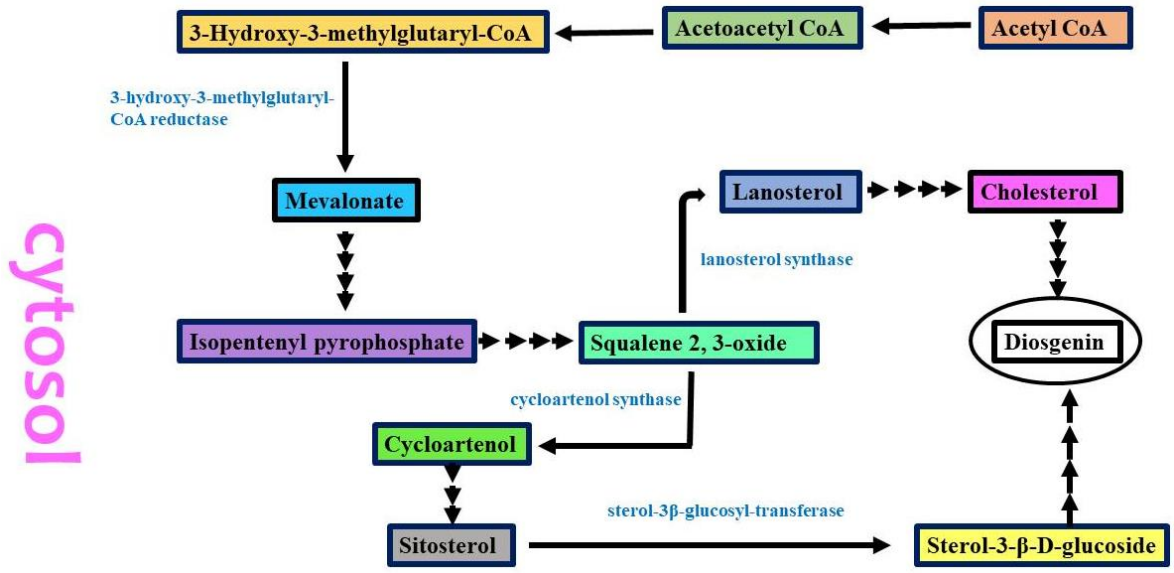


Figure 2.2: *Schematic representation of biosynthetic pathway of diosgenin*

2.10. Sustainable production of diosgenin by *in vitro* methods

D. deltoidea and other therapeutic species are eminent for their medicinal uses. The therapeutic property of *D. deltoidea* is due to the presence of secondary metabolite diosgenin. Local people are collecting this species from their natural habitat that causes its extinction. Hence, sustainable production of such important species is required to meet the demand of this compound in market. Biotechnological production of these pharmaceutically

important secondary metabolites is an alternate way for their sustainable production, however less awareness regarding genes, enzymes or intermediates involved in these metabolites biosynthetic pathways produced limited profitable success till date.

The production of diosgenin and other steroidal saponins in *in vitro* have been reported by many researchers in various species of Dioscoreaceae (Table 2.10). Diosgenin was first time isolated from callus and suspension cultures of *D. deltoidea* and maximum diosgenin (1.02%) were obtained within 3-4 weeks of suspension culture (Kaul and Staba, 1968). Narula et al. (2007) stated that addition of cytokinins and auxins increased diosgenin production and also promotes root and callus initiation. Chowdhary and Chaturvedi. (1980) reported that diosgenin yield was enhanced by the removal of KN from 1.3% to 1.6%. Carbon and nitrogen sources were also reported as important factors for the diosgenin production and in stationary phase of suspension culture diosgenin were obtained in *D. deltoidea* (Furmanowa et al., 1988).

The diosgenin content generated in tissue cultures is normally less but can be expanded by application of different precursors and elicitors (Radman et al. 2003; Namdeo., 2007). In *D. floribunda,* De and De. (2005) reported that the addition of 2-CEPA an ethylene agent increased the production of diosgenin. While Diarra et al. (2013) reported that ethylene improved diosgenin content in *D. zingiberensis.* Diosgenin production (72%) was enhanced by the treatment of autoclaved fungal mycelia in the cell suspension cultures of *D. deltoidea* while as laminarin, arachidonic acid and chitin showed no stimulating impact on the diosgenin content (Rokem et al., 1984). In another report palmarumycin C13 from *Berkleasmium* spp. Dzf12 enhanced diosgenin production in *D. zingiberensis* (Mou et al., 2015) while oligosaccharides were also reported as beneficial for diosgenin production (Li et al., 2012). Elicitors used for the production of diosgenin from *Dioscorea* spp. is presented in Table 2.11.

However investigations on secondary metabolite enhancement by various elicitors (biotic and abiotic) and precursors is quiet lacking in Dioscorea genus. Very few reports are available in this regard and needs to be studied for enhancement of diosgenin.

Table 2.10: *In vitro production of diosgenin*

Species	Culture	Medium + PGR	Diosgenin Content (%)	Reference
D. balcanica	Callus	MS + Myoinositol + Sucrose + Agar + 2, 4-D + BAP	0.67%	Savikin-Fodulovic et al. (1998)
D. bulbifera L.	Callus	MS + NAA or IAA + KN	12 mg g–1	Narula et al. (2007)
D. composita	Callus Suspension	RT + 2,4-D RT + 2,4-D	0.06 0.05	Mehta and Staba (1997)
D. composita	Callus	RT + BA	0.02	Heble and Staba (1980b)
D. deltoidea	Callus	RT + 2, 4-D + Cholesterol + Yeast extract	2.5	Kaul et al. (1969)
D. deltoidea	Suspension	RT + 2, 4-D + Cholesterol + Yeast extract	1.6	Kaul et al. (1969)
D. deltoidea	Callus	RT + 2, 4-D	0.35	Heble and Staba (1980b)
D. deltoidea	Suspension Callus	RT + 2, 4-D	0.22 1.0	Staba and Kaul (1971)
D. deltoidea	Hypocotyl origin callus Nodal origin callus	RT + 2, 4-D	0.18 0.26	Furmanowa et al. (1985a)

D. deltoidea	Stems with leaves	RT, SH, CM, CS	0.04-0.24	Furmanowa et al. (1985b)
D. deltoidea	Callus	RT + 2, 4-D + Cholesterol	1.66	Staba and Kaul (1971)
D. deltoidea	Callus	MS + 2, 4-D + Cholesterol + propionic acid + mevalonic acid	0.13 - 0.31	Sarkisova (1978)
D. deltoidea	Suspension	MS + 2, 4-D + KN	1.77-1.8	Tarakanova et al. (1979)
D. deltoidea	Callus	SH + IAA + KN + cholesterol	1.6 1.88 1.57 1.13	Chowdhury and Chaturvedi (1980)
D. deltoidea	Suspension	RT + 2, 4-D + Hydroxyurea	0.02	Heble and Staba (1980b)
D. deltoidea	Suspension culture	MS + 2, 4-D + sucrose	0.1 - 1.8	Tal and Goldberg (1982)
D. deltoidea	Suspension culture	MS + 2, 4-D + sucrose + KN03 + NH_4N0_3	3.8	Tal and Goldberg (1982)
D. deltoidea	Suspension	-	8	Tal et al. (1982)
D. floribunda	Callus	RT + 2, 4-D	0.295	Aminuddin and Chowdhary (1983)
D.	Callus	RT + 2, 4-D	1.33	Aminuddin and

floribunda				Chowdhary (1983)
D. spiculiflora	Callus Suspension	RT + 2, 4-D	0.09 0.035	Mehta and Staba (1970)
D. tokoro	Callus	LS + 2, 4-D + KN		Tomita and Uomori (1974)
D. deltoidea	Suspension culture	MS + 2,4-D + KN	7.2%	Rokem et al. (1984)
D. deltoidea	Callus	MS + 2,4-D + Sucrose	0.52%	Ravishankar and Grewal (1991)

Table 2.11: *Enhancement of diosgenin in in vitro by using different elicitors*

Plant species	**Medium**	**Elicitors**	**Achievements**	**Reference**
D. deltoidea	malt extract medium (malt extract, glucose, and peptone g/l).	Fungal mycelia Phyto-alexin elicitors (laminarin, arachidonic acid and chitin)	Autoclaved mycelia showed 72% increase in diosgenin production. Laminarin, arachidonic acid and chitin showed no stimulating effect on diosgenin level.	Rokem et al. (1984)
D. alata *D. caryenesis* *D. rotundata*	MS, WPM, ½ WPM, PGR free medium + sucrose + phytagel.	JA and MeJA	MeJa repressed microtubers differentiation in *D. alata* but in *D. caryenesis* and *D. rotundata* slight promontory effects on microtuberisation.	Jasik and Mantell (2000)
D.	MS + Morel vitamins +	Exogenous jasmonic acid,	JA (0.1 µM) and PUT (10 µM) accelerated tuber	Ovono et

cayenensis *D. rotundata*	sucrose + AC + Caldic agar.	Putrescine, Ornithine, Arginine	formation. JA (0.1-1 µM) as PUT (1 µM) increased tuber length and weight but JA (0.1 µM) and putrescine (1 µM) showed no positive effect on tuber formation.	al. (2010)
D. floribunda	MS medium 2,4-D and KN	2-Chloroethylphosphonic acid	2-CEPA (100 mg L^{-1}) increased diosgenin production but 2-CEPA (100 mg L^{-1}) increased diosgenin level to 72-fold.	De and De (2005)
D. zingiberensis	MS medium BA + NAA	Palmarumycin C13	Diosgenin yield increased to 1.4-fold in cultured plantlets at Palmarumycin C13 (60 -1) and 8.0-fold in cultured cells at Palmarumycin C13 (10 -1)	Mou et al. (2015)
D. zingiberensis	MS medium BA + NAA	Total oligosaccharide (TO) and oligosaccharide fractions (DP2-5, DP5-8, and DP8-12)	DP8-12 (20 mg L^{-1}) showed highest diosgenin content and yield by 4.76 and 5.65 fold and DP10 (6 mg L^{-1}) increased diosgenin content and yield to 7.24-fold and 9.33-fold.	Li et al. (2012)
D. zingiberensis	MS + BA	Ethephon	Ethylene (E3 40% ethephon) significantly promoted diosgenin biosynthesis.	Diarra et al. (2013)
D. zingiberen	MS medium BA + NAA	Oligosaccharides from *Fusarium*	DP5-8 (20 mg L^{-1}) showed maximum diosgenin yield by 5.65-fold (2.187 -1),	Li et al. (2013)

sis		*oxysporum* Dzf17	DP7 (6 mg L^{-1}) increased diosgenin level by 8.27-fold (3.202 mg L^{-1}) and when applied twice a week both diosgenin content and yield increased highest by 9.19- and 12.38 fold.	
D. zingiberensis	MS medium + 1.5 mg L^{-1} BA + 1.0 mg L^{-1} NAA	Oligosaccharides from Fusarium oxysporum Dzf17	DZf17 (30 mg L^{-1}) yields maximum diosgenin (5.25 - 1) that is 3-fold increase.	Zhang et al. (2009)
D. zingiberensis	MS medium BA + NAA	Polysaccharides (EPS, WPS,SPS) from *Fusarium oxysporium* Dz f17	WPS (20 g/L) showed maximum increase in cell dry weight, diosgenin content, diosgenin yield as compared to EPS and SPS.	Li et al. (2011)
D. zingiberensis	MS medium BA + NAA	Beauvericin	Dzf2 (100 mg L^{-1}) increased diosgenin yield by 1-fold in seedling culture and Dzf2 increased diosgenin by 3.6-fold in cell culture.	Yin et al. (2011)

CHAPTER 3

Collection of plants from different geographical locations and screening of elite variety of D. deltoidea on the basis of diosgenin and other related compounds by HPTLC method

3.1 Introduction

In the recent era, the rival in the usage of natural products, a blasting marketplace for herbal items and a rapidly developing consumer acknowledgement of complementary medication, it is imperative to instantly progress delicate modern standards for the superiority, security and adequacy of traditional medicine (Usmani et al., 2013). *Dioscorea deltoidea* belonging to the Dioscoreaceae family and genus *Dioscorea* is popularly known as "Singli-Mingli or "wild yam". *D. deltoidea* is a perpetual climbing plant that grows 3 m in height and is native to Asia (Ali, 2012). Caddick et al. (2002) described the genus *Dioscorea* comprises of 300-500 species, mostly found in South-east Asia, West Africa, parts of Central America and the Caribbean and the Pacific Islands. *D. deltoidea* is naturally distributed in Nepal, Afghanistan, Pakistan, Bhutan, Vietnam, China and India (Ali, 2012). In India, it is mostly found in Indian Himalayan regions (IHR) such as Jammu and Kashmir, Himachal Pradesh, Uttarakhand, Assam and Meghalaya at an altitude of 550-3100 m (Dangwal and Chauhan, 2015). Though, it is also cultivated in some parts of Punjab, Madhya Pradesh and Maharashtra.

The multi-therapeutic significance of *D. deltoidea* is attributed to its phytochemicals mostly diosgenin and gained much repute in pharmaceutical companies across the globe. The tubers of this plant have been used for a digestive disorder, abdominal pain, bone and joint disorder, skin diseases, cardiovascular and metabolic disorders (Ali, 2012, Mustafa et al. 2018). Moreover, *D. deltoidea* tubers are traditionally utilized for dysentery, piles, abdominal and chronic liver pain (Kumari et al 2012). Due to the presence of saponins in *D. deltoidea,* it is used for the manufacture of soaps and detergents (Muslim and Sikander, 2010). Furthermore, it is also supposed that the tubers of *D. deltoidea* possess other pharmacological activities such as antioxidant, antimicrobial, antifungal, stomachic and hypoglycemic properties (Chandra et al., 2012; Mustafa et al., 2018).

According to available literature, *D. deltoidea* contains diosgenin, β-sitosterol, stigmasterol, Campesterol, dioscin, 5 diene, dioscorin and 25-D-spirostan-3 (Stohs and El-Olemy, 1972; Dangwal and Chauhan, 2015). Furthermore, alkaloids, saponins, tannin, flavonoids, triterpenoids, carbohydrates, glycosides and sterol are reported in *D. deltoidea* (Chandra et al., 2012). Other compounds such as riboflavin, ascorbic acid, zinc, silicon, magnesium, aluminium, beta-carotene, potassium, silicon, thiamine, chromium, selenium, phosphorus, cobalt, tin, sodium, manganese are reported that makes this plant industrially important (Dangawal and Chauhan, 2015).

Diosgenin is a phytosteroid sapogenin (Figure 3.1) main compound of *D. deltoidea* is used for the preparation of sex hormones, corticosteroids, progesterone, and contraceptive pills (Ali, 2012; Dangwal and Chauhan, 2015; Tahir et al., 2016). Diosgenin has anti-diabetic activity, anti-tumour property, anti-thrombosis, anti-fungal, anti-microbial, anti-aging activity and stimulate osteogenic activity (Raju and Mehta, 2008; Alcantara et al., 2011; Dangawal and Chauhan, 2015). β-sitosterol is a phytosterol having the similar chemical structure as that of cholesterol (Figure 3.1) also possess a comprehensive array of pharmaceutical properties such as anticancer, anti-diabetic, prevent heart diseases, rheumatoid arthritis, hair loss, tuberculosis and hypercholesterolemia (Awad et al., 2007; Saeidnia et al., 2014; Rashed et al., 2020). The accessibility of marker compounds for the identification of specific components of any plant encourages the simple identity proof of herbal drug, though its non-accessibility shows extreme hindrances in the way of identification and authentication of herbal drugs. Taking into account these impediments, efforts have been made in the current study for the generation of multiple markers based fingerprinting patterns by HPTLC. In contrast with other chromatographic methods, HPTLC has many advantages such as versatile, sensitive, accurate, precise, cost-effective, less solvent consumption, minimum sample preparation, full optimization etc. makes it a powerful analytical tool. Nowadays HPTLC is considered as an advanced analytical technique possessing similar quality level as Gas chromatography (GC) and high performance liquid chromatography (HPLC). As of its various advantages, HPTLC is currently one of the most extensively applied techniques in the analysis of phytochemicals. HPTLC not only delivers fast identification and accurate quantifiable outcomes, but it also facilitates the identification of components existing in herbal drugs. Studies accompanied by many authors are revealing the statement that HPTLC may be commendably employed as a quality control instrument for fast confirmation from a wide range of herbal samples and the

fingerprinting profiles can be efficiently utilized for comparing a herbal sample from its commercial counterpart (Srivastava, 2011). Review of literature reveals that many workers have used HPTLC for the quantification of diosgenin in many *Dioscorea* species. Shah and Lele (2012) determined diosgenin content in *D. alata* by using HPTLC and toluene and ethyl acetate in the ratio of 7:3 v/v was used as mobile phase. Amir et al. (2012) developed and validated HPTLC method for the quantification of diosgenin content in the rhizome and *in vitro* culture of *D. deltoidea*. However, there is still a lack of proper screening system based on chromatographic fingerprinting and no simultaneous determination of diosgenin and β-sitosterol protocol is available. Considering its widespread application, pharmaceutical importance and the matter of impurities there is a strong requirement for the development of a consistent and affordable screening system on chromatographic fingerprinting. Considering these insufficiencies, the main objective of the current study was to develop a simultaneous HPTLC method for the identification and screening of the elite population of *D. deltoidea* on the basis of diosgenin and β-sitosterol.

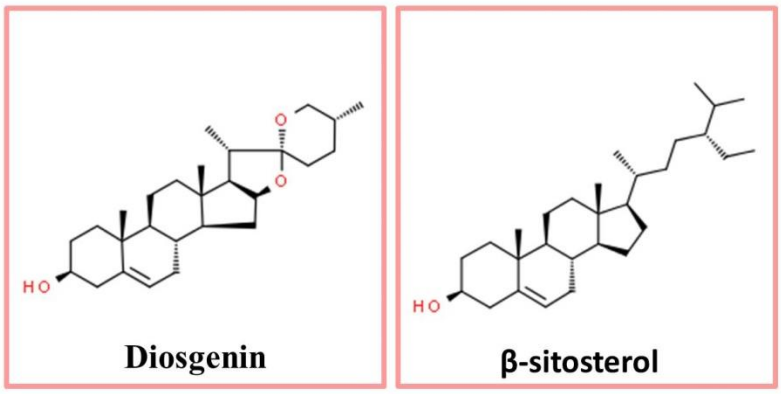


Figure 3.1: *Chemical structure of diosgenin and β-sitosterol*

3.2 Materials and methods

3.2.1 Chemicals and marker compound

Marker compound diosgenin (>99% purity) and β-sitosterol (>98% purity) were procured from Sigma-Aldrich Company, USA and were stored at -20 °C. Solvents such as methanol, ethanol, chloroform, hydrochloric acid (HCL) were of HPLC grade and were purchased from Himedia, India.

3.2.2 Collection of plant material

The plant material of *D. deltoidea* was collected from high altitudes of North-western Himalayan region of India especially from the areas of Jammu and Kashmir (J & K),

Himachal Pradesh (H.P) and Uttarakhand during the period of 2017-2018. All the accessions of *D. deltoidea* were collected during the maturity stage in the autumn season (November – December). The collection sites with altitudes and coordinates are presented in Table 3.1. In J & K, plants were collected from Yarikha, Gulmarg, Buniyar, Pahalgam, Dachigam and Ramban. In H.P, accessions were collected from the areas of Kullu, Kangra and Kinnaur and in Uttarakhand, accessions were collected from Dhungali, Kandoi and Kunwa.

Table 3.1: *Collection spots of plant accessions from different sites of North-Western Himalayas of India*

Plant accessions	Collection site	Elevation (m)	Geographical Coordinates
Y1	Yarikha, Baramulla , J &K	2560 m	34.074211, 74.428486
Y2	Yarikha, Baramulla, J &K	2600 m	34.076132, 74.435689
G1	Gulmarg, Baramulla, J &K	2640 m	34.074220, 74.429813
G2	Gulmarg, Baramulla, J &K	1590 m	34.202148, 74.348259
B2	Buniyar, Baramulla, J &K	1650 m	34.110082, 74.202730
B3	Buniyar, Baramulla, J &K	1690 m	34.101490, 74.200655
P1	Pahalgam, J &K	2740 m	34.016373, 75.318054
P2	Pahalgam, J &K	2780 m	34.046735, 75.295380
D1	Dachigam, J &K	2850 m	34.137184, 75.037750
D2	Dachigam, J &K	2900 m	34.137340, 75.037854
R1	Ramban, J &K	1156 m	33.242134, 75.233688
R2	Ramban, J &K	1200 m	33.231739, 75.209634
K1	Kullu, Himachal Pradesh	1280 m	31.957851, 77.109459
K2	Kullu, Himachal Pradesh	1320 m	31.954457, 77.103895
KA1	Kangra, Himachal Pradesh	1400 m	32.090940, 76.257700

KA2	Kangra, Himachal Pradesh	1450 m	32.109898, 76.304703
KI1	Kinnaur, Himachal Pradesh	2500 m	31.405933, 78.332567
KI2	Kinnaur, Himachal Pradesh	2550 m	31.650958, 78.475195
DH1	Dhungali, Chamoli, Uttarakhand	1600 m	30.392082, 78.332150
DH2	Dhungali, Chamoli, Uttarakhand	1650 m	30.391861, 78.332090
KD1	Kandoi, Uttarakhand	2100 m	30.819633, 77.754011
KD2	Kandoi, Uttarakhand	2150 m	30.812708, 77.761289
KN1	Kunwa, Uttarakhand	2640 m	30.807836, 77.810996
KN2	Kunwa, Uttarakhand	2700 m	30.810420, 77.8065472

3.2.3 Authentication of plant material

Plants were identified and authenticated by the taxonomists and Voucher Specimen No. 2614-KASH and 15062018 was submitted in KASH herbarium, Centre for Biodiversity and Taxonomy and School of Bioengineering and Biosciences, Lovely Professional University.

3.2.4 Sample preparation

The plant material of different accessions was collected and washed thoroughly under tap water and shade dried. The dried plant material was crushed into a fine powder with an electric grinder (Philips, India). Each sample (1.0 g) was macerated with ethanol (50%) at room temperature for about 24 h. After extraction, all samples were filtered with Whatman No.1 filter paper and concentrated to dryness at 40 ºC in a rotary evaporator (Buchi, R-300, India). The dried crude residue of all samples was then acid hydrolysed by adding 20 ml of 10% HCL in each sample and kept in a water bath for 1 h at 98 ºC. After hydrolysis, 15 ml chloroform was added twice to wash and form the two layers. The upper layer was discarded and the lower layer (chloroform layer) was collected and dried in a rotary evaporator. An appropriate amount of methanol was added to the dried residue, syringe filtered (Fisher Scientific, India) and used for HPTLC analysis for quantification.

3.2.5 Phytochemical analysis

3.2.5.1 Marker compound preparation

Marker compounds diosgenin and β-sitosterol (10 mg) were dissolved in methanol (10 mL) to prepare stock solution of both compounds(1 mg mL^{-1}) and 0.1 mg mL^{-1} concentration of diosgenin and β-sitosterol was used as the final concentration by dissolving 9 ml of methanol in 1 mL of stock solution.

3.2.5.2 Calibration curve preparation (Linearity)

Calibration curve of diosgenin and β-sitosterol were prepared by applying different volumes of standard solution (2, 4, 6, 8, 10 µl) to get the linearity range of 200-1000 ng/spot. Calibration graph was created with peak area versus concentration at 433 nm wavelength. With the help of regression equation and consistent peak area diosgenin and β-sitosterol yield in 24 accessions of *D. deltoidea* was estimated.

3.2.5.3 HPTLC Instrumentation

HPTLC instrument consist of Linomat-5 applicator CAMAG (Muttenz, Switzerland) fitted with Linomat-5 automatic sample applicator and CAMAG TLC scanner-3 ("Scanner_180710" S/N 180710 (2.01.02)) run by WinCATS software (version: 1.4.6.2002). The stationary phase comprised of pre-coated silica gel 60 F_{254} TLC plates (20 × 10 cm, E. Merck, Darmstadt, Germany). Each sample was applied to the plates as bands 6 mm wide, with 13 mm distance between tracks, via Linomat-5 automatic sample applicator fitted with a 100µl Hamilton syringe. Dosage speed from the Hamilton syringe was always 150 nL/s. Conditions for densitometric scanning were fixed at 4.00 x 0.30 mm slit dimension with scanning speed 20 mm/s and data resolution 100 µm/step. The HPTLC was performed at 24 ±2 °C temperature with 45% relative humidity in CAMAG twin trough glass chamber (20 cm x 10cm) saturated before for 20 minutes with mobile phase vapour.

3.2.5.4 Simultaneous quantification of diosgenin and β-sitosterol

The HPTLC method for the simultaneous quantification of diosgenin and β-sitosterol was developed and validated. Different mobile phases were tried to give clear separation and resolution of diosgenin and β-sitosterol. Each sample (3 µL) was applied on 20 × 10 cm precoated silica gel 60 F_{254} TLC plate. Among different solvent systems used for mobile phase, n-hexane: ethyl acetate: toluene (7.1:2:0.9 v/v) gave well resolute spots of both

compounds from crude samples of *D. deltoidea*. After development, plates were dried in hot air oven (Bionics Scientific, India) at 100 °C for 5 min and then scanned at 254 nm. After initial scanning, post- derivatization of plates with Anisaldehyde reagent (1 ml anisaldehyde, 20 ml glacial acetic acid, 170 ml methanol and 10 ml conc. sulphuric acid) was carried out and oven dried at 100 °C for 6 min. Plates were scanned at wavelength 433 nm after development of bands for both diosgenin and β-sitosterol.

3.2.5.5 Method validation

According to the guidelines of ICH, method validation was carried out on the basis of sensitivity, precision, specificity, accuracy, robustness and recovery presented in Table 3.2 (International Council for Harmonisation, 2005).

3.2.5.5.1 Sensitivity

The sensitivity of the method was determined by the use of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were evaluated on the basis of signal-to-noise ratio (S/N). LOD and LOQ were measured by the equations as 3 X N/B and LOQ= 10 X N/B, Where N represent standard deviation of the peak area of standard and B represents slope of calibration plot.

3.2.5.5.2 Specificity

With the help of peak purity and densitometric chromatogram of the marker compounds and samples, specificity was prudently examined. Both test samples and reference compound were compared on the basis of R_f values and were found similar in the premises of superimposable spectra. Developed method specificity was tested and not any impurities were spotted.

3.2.5.4.3 Accuracy

Recovery test was used to calculate the accuracy of the method. This included adding of known amounts of marker compound to one pre-analysed sample. At three levels (low, middle and high) three levels of concentration were tested. In triplicates samples were prepared and examined according to above mentioned method.

3.2.5.4.4 Precision

By the application of 50% of diosgenin in plant extract, precision was examined. For intra-day coefficient variation (% RSD) was tested and inter-day precision (n = 5) to compute the repeatability and reproducibility of the outcomes. Table 3.2 presents the data of precision and average recoveries.

3.3 Statistical analysis

The experiment were done in triplicates and expressed as mean ± standard deviation (SD). Statistical analyses were executed by one-way ANOVA followed by Tukey's test. Minitab 15 software was used for the statistical analysis and values of $p < 0.05$ were considered as significant.

3.4 Result and Discussion

3.4.1 Method development

The mobile phase ratio for HPTLC was optimized to achieve accurate and well-resolved peaks for diosgenin and β-sitosterol. The mobile phase n-hexane: ethyl acetate: toluene (7.1:2:0.9 v/v) showed good separation and symmetrical peaks at R_f value of 0.46 for diosgenin and 0.55 for β-sitosterol respectively (Figure 3.2).

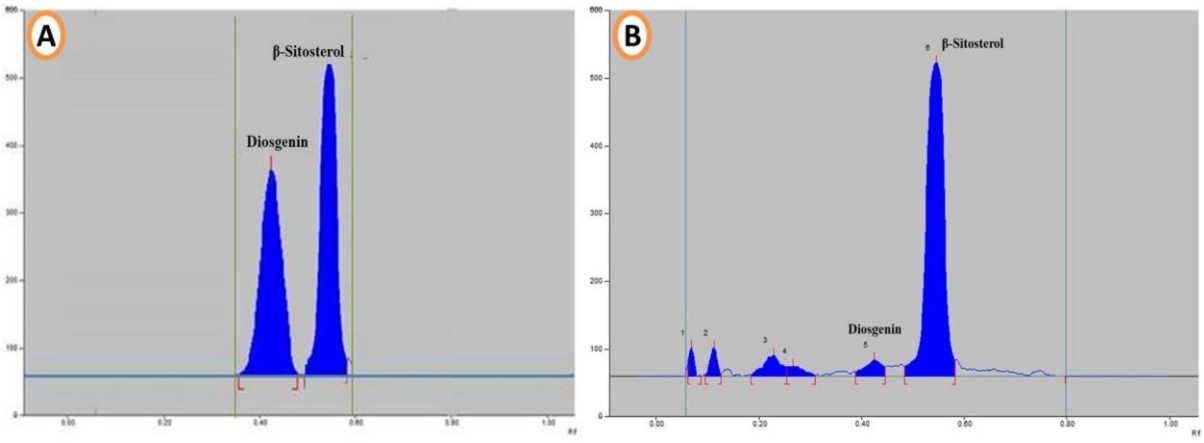


Figure 3.2: (A) *HPTLC densitogram of standard diosgenin and β-sitosterol* ***(B)*** *HPTLC densitogram of the D. deltoidea sample.*

3.4.2 Method validation

The linearity plot of peak area against amount of diosgenin and β-sitosterol was found linear in the range of 200-1000 ng/spot (Figure 3.3, Figure 3.4). Good linear relationship for the plot was confirmed by linear regression data (Table 3.2). The recovery trials are done at three different levels to set up the accuracy of the method. Precision was assessed by conducting repeatability and intermediate precision. LOD and LOQ were done by S/N ratio and as 20, 60 for diosgenin and 27, 68 for β-sitosterol. Table 3.1 presented the features of method validation for diosgenin and β-sitosterol quantification.

Table 3.2: *Method validation for diosgenin and β-sitosterol quantification*

S. No	Parameters	Diosgenin	β-sitosterol
1	Linearity range (ng/spot)	200-1000	200-1000
2	Correlation coefficient (r^2)	0.999	0.998
3	Regression equation	Y = 23.84X + 19265	Y = 25.39X + 17975
4	Calculated SD value	0.47	0.53
5	[b]Limit of detection (LOD) (ng) [3×SD/S]	20	27
6	[b]Limit of quantification (LOQ) (ng) [10×SD/S]	60	68
7	R_f	0.46	0.55
Precision and accuracy			
8	Intra-day RSD (%), n = 5	1.26	1.42
9	Inter-day RSD (%), n = 5	1.43	1.61
Recovery			
10	Amount of standard in plant samples (μg mg^{-1}) having maximum bioactive compounds	18.60	27.90

11	Amount of standards added in plant samples (µg mg^{-1})	9, 18, 27	14, 28, 42
12	Amount of standard found (µg mg^{-1})	27.63, 36.53, 45.63	42.11, 55.62, 69.68
13	Recovery (%)	100.10, 99.80, 100.06	100.50, 99.49, 99.68
14	Mean Recovery (%)	99.98	99.89

[a] Four concentration levels in triplicate.

[b] SD is the standard deviation of the blank response and S is the slope of the calibration plot

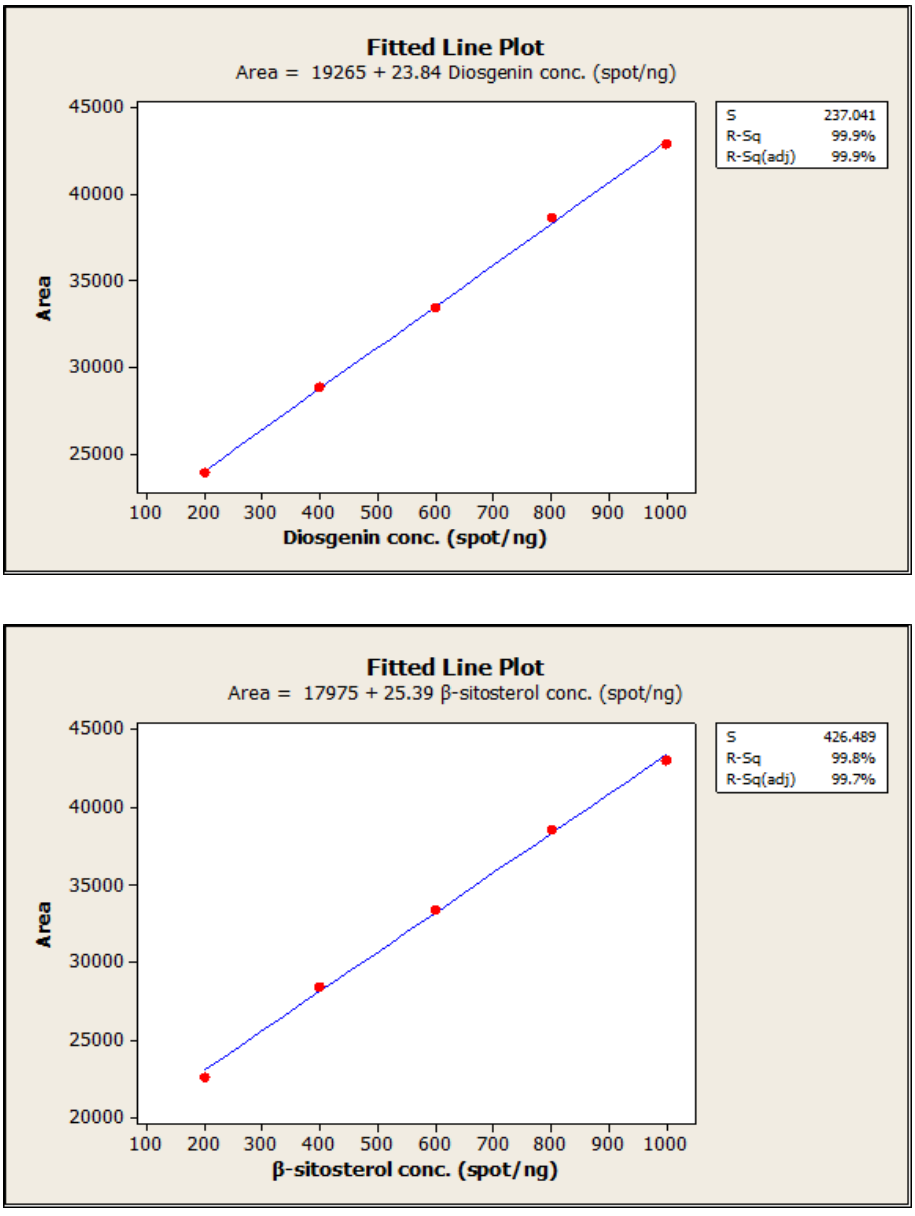


Figure 3.3: *The linearity graph of the standard diosgenin and β-sitosterol.*

3.4.3 Quantification of diosgenin and β-sitosterol

The phytochemical screening of elite genotype of *D. deltoidea* accessions from North-Western region of Indian Himalaya is not reported till date. Total 24 wild accessions of *D. deltoidea* have been collected from three states of India that comes under north-western Himalayan region which are as Jammu and Kashmir, Himachal Pradesh and Uttarakhand. The chemical screening of all the accessions was based on diosgenin and β-sitosterol. Diosgenin- a steroidal saponin is the specific bioactive compound of *Dioscorea* spp. HPTLC was employed for the qualitative and quantitative valuation of diosgenin and β-sitosterol in all accessions of *D. deltoidea*. Diosgenin and β-sitosterol peaks from the extracts of *D. deltoidea* samples were confirmed by matching their single spot at $R_f = 0.55$and 0.46 values with the peaks of standards (Figure 3.3). The quantity of diosgenin and β-sitosterol in plant samples of *D. deltoidea* was evaluated by applying the linear regression equation and the content was displayed in Table 3.3.

In this study, the phytochemical profile of 24 accessions of *D. deltoidea* by HPTLC technique revealed the presence of significant amounts of phytosterols identified as diosgenin and β-sitosterol. In all accessions both the compounds were found, however, their content varied from geographical region to other regions. The accessions collected from high altitudes showed a good quantity of diosgenin and β-sitosterol. In Jammu and Kashmir, accessions were collected from four different regions namely Baramulla, Dachigam, Pahalgam and Ramban. Among them, accessions collected from Gulmarg, Baramulla at an altitude of 2990 m showed highest β-sitosterol content (2.79%) and diosgenin (1.86%). At an elevation 2940 m, 50 m below it showed 2.73% of β-sitosterol and 1.82% of diosgenin. This area comes under Pir Panjal range of western Himalayas. The β-sitosterol content in plants collected from Yarikha, Baramulla at an altitude of 2560 m and 2600 m was 2.44% to 2.48% and diosgenin content ranges from 1.54% to 1.59%. However, when plant accessions were collected at an elevation of 1650 m and 1690 m from Buniyar, Baramulla, they showed a low range of β-sitosterol (1.94% to 1.99%) and diosgenin content (1.31% to 1.29%) as compared to plant accessions collected from high elevations. The plant accessions from Pahalgam and Dachigam also showed a good range of β-sitosterol (2.53% to 2.67%) and diosgenin in the range of 1.62% to 1.73%. On the other side, the collection site Ramban showed least β-sitosterol content (1.01% to 1.06%) and diosgenin (0.54% to 0.58%) than other collection sites of J & K state. From the collection sites of H.P state, the marker

compounds content varies from low altitude 1280 m to high altitude 2550 m and the content lies in the range of 1.11% to 2.38% in case of β-sitosterol and 0.76% to 1.16% in case of diosgenin. Kinnaur area (KI2) of H.P showed a good quantity of both maker compounds as compared to other areas of H.P. In Uttarakhand, plant accessions had been collected from Dhungali, Kandoi and Kunwa at an elevation of 1600 m to 2700 m. The β-sitosterol content obtained varies from 1.63% to 1.68% in plant accessions of Dhungali, 2.02% to 2.08% in Kandoi accessions and 2.41% to 2.47% in Kunwa accessions. Diosgenin content also varies in plant accessions collected from Dhungali (0.66% to 0.69%), Kandoi (1.07% to 1.11%) and Kunwa (1.39% to 1.42%).

Diosgenin is the main bioactive compound of *D. deltoidea* and was reported by many authors (Dangwal and Chauhan, 2015; Tahir et al., 2016). In a study, diosgenin and yamogenin were reported by Hussain et al. (1977) in *D. deltoidea*. Chandra et al. (2013) specified that the plants of *D. deltoidea* present in Uttarakhand were rich sources of diosgenin. Tomita and Uomori (1974) reported tokorogenin, diosgenin, stigmasterol, yonogenin, campesterol, cholesterol and β-sitosterol in the callus derived from *D. tokoro* seedlings. Stohs et al. (1969) also confirmed the presence of stigmasterol, cholesterol and β-sitosterol in suspension cultures of *D. deltoidea*. Diosgenin content in *D. deltoidea* and *D. prazeri* was reported in the range of 0.32-1% and in *D. alata* its content was 0.078% in the fresh tuber, 0.133% in the dried tuber and 0.048% in fresh callus determined by HPTLC technique by using mobile phase toluene: ethyl acetate in the ratio of 7:3 v/v (Shah and Lele, 2012). *D. deltoidea* is the highest diosgenin containing species with 1-8% of diosgenin present in its tubers, after that stigmasterol, campesterol, β-sitosterol, diosgenin; 25-D-spirostan-3, 5 diene8 are other compounds (Stohs and El-Olemy, 1972). In another study, diosgenin along with β-sitosterol and stigmasterol was isolated from *D. polygonoides* (McAnuff et al., 2005). β-sitosterol was also found in *D. opposita* along with eleven more compounds (Bai et al., 2008).

The variance in the amount of these bioactive compounds might be because of the variation in age of plants, or the physiological status of plants. Investigations accompanied by former authors have demonstrated that the amount of different phytochemicals relies on numerous factors, for example, physiological state, age of the plant and geographical location. The outcomes of our study are also in accordance with those attained by previous authors. Majority of the phytochemicals have been noted in high content from the high altitude

accessions collected from Gulmarg, Baramulla. In earlier reports, many workers have reported similar intra-specific alterations in the content of many bioactive compounds in various species (Maffi et al., 2001; Nikolova and Ivancheva, 2005; Monschein et al., 2009). It is eminent that many biological characters generally differ with elevation or altitude in plant species that possess huge altitudinal deviation, mostly as an effect of adaptation to alterations in several ecological factors. Increase in the quantity of diosgenin and β-sitosterol in high altitude accessions may be due to adaptation to variations in soil, temperature and soil.

Table 3.3: *Quantity of diosgenin and β-sitosterol in plant accessions collected from North-western Himalaya of India*

S. No	Plant accessions	Elevation (m)	Dry weight (DW %)	
			β-sitosterol	**Diosgenin**
1	Y1	2560 m	2.44 ± 0.13	1.54 ± 0.11
2	Y2	2600 m	2.48 ± 0.17	1.59 ± 0.13
3	G1	2940 m	2.73 ± 0.02	1.82 ± 0.03
4	G2	2990 m	**2.79 ± 0.06**	**1.86 ± 0.01**
5	B1	1650 m	1.94 ± 0.15	1.31 ± 0.40
6	B2	1690 m	1.99 ± 0.13	1.29 ± 0.30
7	P1	2740 m	2.53 ± 0.21	1.62 ± 0.35
8	P2	2780 m	2.59 ± 0.28	1.65 ± 0.35
9	D1	2850 m	2.62 ± 0.32	1.71 ± 0.04
10	D2	2900 m	2.67 ± 0.37	1.73 ± 0.03
11	R1	1156 m	1.01 ± 0.21	0.54 ± 0.02
12	R2	1200 m	1.06 ± 0.24	0.58 ± 0.36
13	K1	1280 m	1.11 ± 0.04	0.76 ± 0.32
14	K2	1320 m	1.26 ± 0.03	0.72 ± 0.13

15	KA1	1400 m	1.31 ± 0.23	0.79 ± 0.12
16	KA2	1450 m	1.39 ± 0.25	0.77 ± 0.22
17	KI1	2500 m	2.33 ± 0.17	1.13 ± 0.35
18	KI2	2550 m	2.38 ± 0.12	1.16 ± 0.21
19	DH1	1600 m	1.68 ± 0.09	0.66 ± 0.31
20	DH2	1650 m	1.63 ± 0.05	0.69 ± 0.20
21	KD1	2100 m	2.02 ± 0.03	1.07 ± 0.30
22	KD2	2150 m	2.08 ± 0.01	1.11 ± 0.46
23	KN1	2640 m	2.41 ± 0.04	1.39 ± 0.41
24	KN2	2700 m	2.47 ± 0.02	1.42 ± 0.36

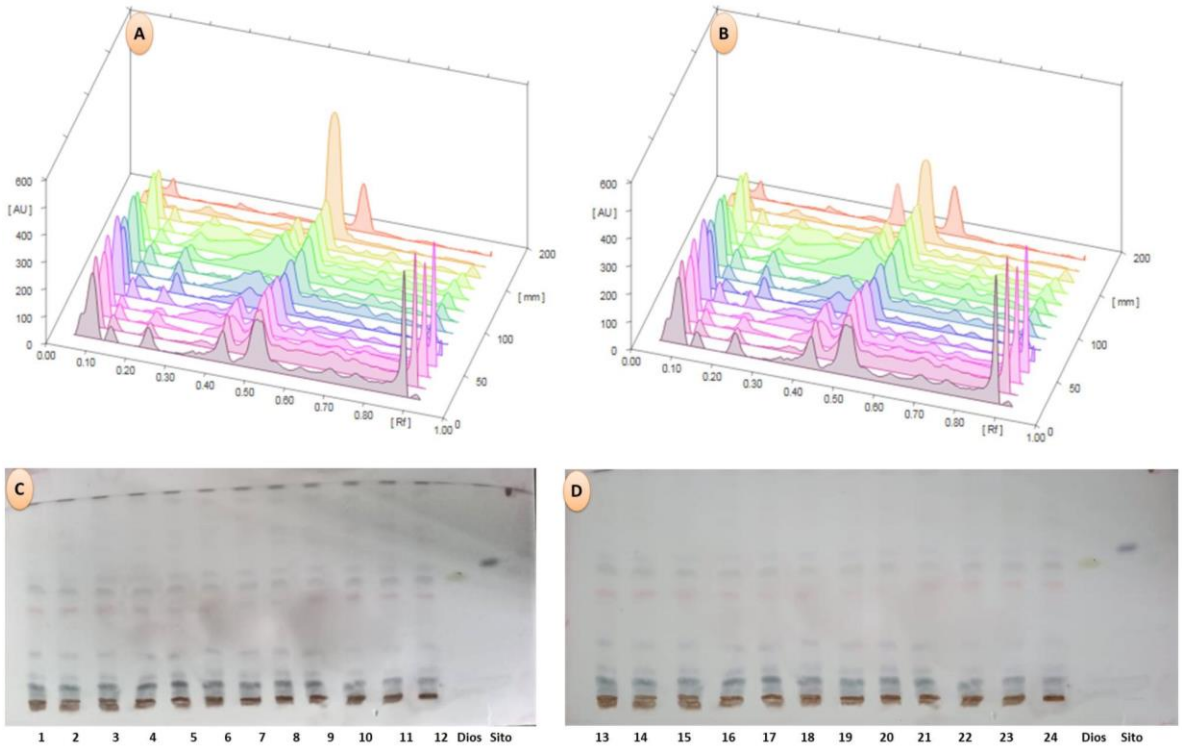


Figure 3.4: HPTLC finger printing *of different accessions of D. deltoidea compared with standard compounds (diosgenin and β-sitosterol)*

(A, B) *3-D densitometric chromatograms of plant samples of D. deltoidea*

(C, D) *HPTLC fingerprinting of marker compounds and plant samples*

3.5 Conclusion

In recent years, HPTLC fingerprinting has appeared as an imperative and efficient tool for the identification and authentication of pharmaceutical plants and standardization of herbal drugs. The HPTLC method developed and validated for the analysis of diosgenin and β-sitosterol in crude extracts of *D. deltoidea* in this study was found to be simple, precise and sensitive and can be used in quality control and standardization of drugs having *D. deltoidea* as principle component and also used by the laboratories for the analysis of diosgenin and β-sitosterol containing products. The profiles will also be beneficial to screen and differentiate the *D. deltoidea* from other species of the genus Dioscorea. The outcomes of our study can also be used for the screening and identification of elite populations whose promulgation whether commercial or *in vitro* might perform significant part in the enhancement of commercial trade across the world. Results revealed in our investigation comprising 24 accessions of *D. deltoidea* have resulted in the identification of an elite population with high diosgenin and β-sitosterol content. In the future, such promising populations may be utilised for mass cultivation.

CHAPTER 4

Optimization of extraction methods and parameters on diosgenin yield from D. deltoidea

4.1 Introduction

The bioactive compounds quality and quantity mainly depends on the selection of a suitable extraction method (Smith, 2003; Sasidharan et al., 2011). Basically, extraction is the initial part in the study of any medicinal plant however plays a very essential and critical part on the ultimate result. Sometimes extraction techniques are stated as sample preparation methods and according to Hennion et al. (1998) this portion of the study is maximum times ignored, usually carried out by inexperienced research workers. A study accompanied by Majors (1999) revealed that maximum research scholars consider sample preparation as the most important part of any analytical work.

Analysis of bioactive compounds has nowadays become easier due to development of spectrometric and chromatographic techniques however success still relies on extraction techniques and conditions, nature of extracting material and other parameters (Poole et al., 1990). The process of extraction is mostly affected by extraction time, temperature, plant matrix properties, solvent concentration, solid to solvent ratio and pressure (Hernández et al, 2009; Li et al., 2016; Ameer et al., 2017). Though the optimization of a single variable at a time is incapable to estimate the separate and joint influences of these parameters, therefore statistical modeling techniques such as Response Surface Methodology (RSM), Artificial Neural Networking (ANN) are better and gradually common techniques for simultaneous optimization of parameters. RSM is an assortment of mathematical and statistical modeling techniques used in various engineering applications to enhance and optimize processes, predict response and analyse interactions between independent variables and response (Karabegović et al., 2013). On the other side, ANN is a calculating system for nonlinear multivariate modeling ability to assess the response on the basis of trained data in the examined range (Rajković et al., 2013). RSM and ANN techniques have been recently applied simultaneously for the optimization of competent extraction of artemisinin from *Artemisia annua* (Pilkington et al., 2014), phenolic compounds extracted from *P. lentiscus*

by ultrasound-assisted extraction (UAE) (Dahmoune et al., 2015) and polyphenols from garlic (Ciric et al., 2020), chironji extracted from the fruit juice of *Buchanania Lanzan* by pectinase assisted extraction method (Pradhan et al., 2020), extraction of natural dye from the seeds of *Bixa orellana* and stevioside and rebaudioside-A from *Stevia rebaudiana* by microwave-assisted extraction method (Sinha et al., 2013; Ameer et al., 2017) and resveratrol extracted from *Polygonum cuspidatum* by enzyme-assisted ultrasonic extraction method (Lin et al., 2016).

Moreover, the extraction of bioactive compounds mainly depends on extraction techniques. Various conventional extraction methods such as maceration, soxhlet, digestion, infusion, heat reflux, percolation and decoction were used for the extraction of bioactive compounds but due to their low efficiency, high consumption of solvent, time and energy leads to the finding of innovative and efficient methods (Azmir et al., 2013; Belwal et al., 2018). So as to overcome these limitations, extraction technologies introduced efficient and innovative methods known as non-conventional methods providing a high yield, consumes less solvent, reduces time period and low energy consumption (Azmir et al., 2013; Mandal et al., 2015; Altemimi et al., 2017; Belwal et al., 2018). Microwave-assisted extraction (MAE) (Li et al., 2012; Sinha et al., 2013; Ameer et al., 2017), UAE (Dahmoune et al., 2015; Ciric et al., 2020) and pressurized liquid extraction (PLE) (Liazid et al., 2010; Mandal et al., 2015) are most commonly used non-conventional techniques that usually meet these necessities. Several studies have compared conventional and non-conventional extraction methods to check their efficacy on the yield of bioactive compound (Bagherian et al., 2011; Devgun et al., 2012; Vázquez et al., 2014).

In this study, we compare conventional extraction methods (Heat reflux, cold maceration) with non-conventional (MAE) method with different solvent compositions (ethanol 100%, 50% and water) with different extraction time. Also, comparison of different drying methods such as shade, sun, oven and microwave drying and seasonal variations on diosgenin yield were evaluated. Moreover, optimization of extraction parameters (solid: solvent ratio, solvent composition, particle size, extraction time, temperature, pH and extraction cycles) were studied by RSM, in which PBD screened significant parameters and BBD optimized significant variables to determine optimal levels and ANN model was developed for the prediction of the relationship between experimental or significant variables to obtain high diosgenin yield (response variable). Furthermore, valuation abilities and modeling efficacies of RSM and ANN were statistically compared on the basis of

coefficient of determination (R^2), root mean square error (RMSE) and absolute average deviation (AAD). To the best of our knowledge no comparison of conventional and non-conventional extraction methods, drying methods, seasonal variations and no comparison of RSM and ANN modeling techniques for optimization of extraction parameters for diosgenin yield from *D. deltoidea*.

4.2 Materials

4.2.1 Chemicals, marker compound and plant material

The materials required for this chapter has been discussed in details in Chapter: 3 (Section: 3.2.1-3.2.2).

4.3 Methodology

4.3.1 Modeling and optimization studies

Study was accompanied in two phases. Plackett–Burman design (PBD) is the first phase employed to screen the significant independent parameters and Box-Behnken design (BBD) is the second phase applied to check the optimal level and probable **c**ollaborations among significant parameters. Experimental design was set up in Minitab software.

4.3.1.1 Plackett–Burman design

PBD is an effectual system to select main factors and includes huge number of factors but moderately few runs (Asfaram et al., 2016). In this study, PBD was employed to screen and estimate the effect of seven parameters such as solvent composition, solid: solvent ratio, particle size, time, temperature, pH and extraction cycles. Total number of trials in PBD depends on the total number of variables (k +1). At two levels each variable is characterized, high and low represented as (+) and (−1), correspondingly. The coded level of each variable is presented in Table 4.1 and experimental design with 12 trials screening 7 variables is presented in Table 4.4. All trials were done in triplicates and regression analyses at 5% ($p < 0.05$) have been used to test the substantial elements.

This model depends on first-order model:

$$Y = \beta 0 + \sum \beta i Xi \quad (1)$$

Where expected target function is denoted by Y, scaling constant is β_0 and a regression coefficient is β_i and X_i is the coded independent variable.

Table 4.1: *Different variables with coded levels employed in PBD for screening of extraction parameters influencing diosgenin extraction*

Variable code	Variables	High level (+)	Low level (-)
X_1	Solvent Composition (Ethanol and water)	100	0
X_2	Solid: solvent ratio(g/ml)	1:60	1:30
X_3	Particle size (mm)	2.05	0.5
X_4	Time (min)	60	30
X_5	Temperature (ºC)	60	30
X_6	pH	9	5
X_7	Cycles/steps	3	1

4.3.1.2 Box-Behnken design

The variables with significant impact on diosgenin extraction, as screened by PBD were further optimized using BBD. The design consist of 27 runs where each variable was tested at three levels i.e., low, middle and high (-1, 0, +1) as shown in Table 4.2. According to the equation given below, the coding of variables was done (Maran and Manikandan, 2012):

$$xi = Xi - X0/\Delta Xi \qquad i = 1, 2, 3 \ldots, k \qquad (2)$$

Where x_i represents independent variable of coded value; X_i represents independent variable of actual value; X_0 is the independent variable of the actual value at the centre point; and ΔX_i is the independent variable of the step change value. The entire set of experiments was executed in triplicates and the mean response was used for analysis. In order to calculate the optimal levels second-order polynomial equation was applied given as below:

$$Y = \beta 0 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 5X5 + \beta 22X22 + \beta 33X32 + \beta 44X42 + \beta 55X52 + \beta 23X2X3 + \beta 24X2X4 + \beta 25X2X5 + \beta 34X3X4 + \beta 35X3X5 \qquad (3)$$

Where Y represents predicted response; β_0 shows modal constant; X_2, X_3, X_4 and X_5 are significant factors; β_2, β_3, β_4 *and* β_5 are linear coefficients; β_{22}, β_{33}, β_{44} and β_{55} are quadratic coefficients and β_{23}, β_{24}, β_{25}, β_{34}, β_{35} are the interactive coefficients. Minitab statistical software was used for the regression and ANOVA analysis of the data and to obtain quadratic polynomial model.

Table 4.2: *Significant variables with coded levels employed in BBD*

Variables	Coded levels			
	Codes	**+ 1**	**0**	**-1**
Solid: Solvent ratio (g/ml)	X_1	60	45	30
Particle size (mm)	X_2	2.05	1.25	0.5
Extraction time (min)	X_3	60	45	30
Temperature (ºC)	X_4	60	45	30

4.3.1.3 Artificial Neural Network Modeling

ANN is basically scientific simulations that lightly estimated the function of biological neural networks. In designing the ANN same experimental data as of RSM design has been used. In suggested method multi-layer perceptron model (MLP) was implemented by using MATLAB (The Mathworks Inc., 2012a) with four input neurons representing the solid: solvent ratio, particle size, extraction time and temperature, one hidden layer and output layer representing diosgenin production. To reduce the error and attain quicker convergence, back propagation algorithm was adopted by ANN in the training phase for model training and convergence deprived of any interval or damage. Without any negotiations, the achieved results are comprehensive and precise due to the contemplation of suitable neuron size. The MLP comprises of four inputs, one hidden and output layer for prediction. Log Sigmoidal function is used for training and validation analysis as per an activation element for non-linear output calculation. With the assistance of Marquardt algorithm training data is trained for synaptic weight adjustment and analysis and with the aid of 5-fold cross validation strategy, validation is sustained and executed. Figure 4.2 (B) portrayed the performance data attained over entire training data and fitted at best epochs for validation data being represented. Likewise, gradient loss and training state attained over entire ANN training is described in Figure 4.2 (B). The predicted output calculation is conveyed with the support of equation (4) as:

$$Y = f(A_z) = \sum_{p=1}^{m} w_{zp} * x_p + \theta_z \qquad (4)$$

Here, Y signifies the output attained from output layer, $f(A_z)$ signified the activation functions which is accountable for non-linear nature of model related with neuron z. w_{zp} signifies weight connection between neuron z and p. θ_z signifies the input bias and x_p demonstrated the inputs assumed to neuron p.

4.3.2 Optimization of drying methods, seasonal variation and potent part screening

Tubers of *D. deltoidea* were subjected to one of the following four drying methods which are as sun drying (SD), shade drying (SHD), oven drying (OD) and microwave drying (MD). In the SD method, samples were dried out in the sunlight and weighed every 2 hours till a constant weight was attained. For SHD method, samples were dried under shade and weighed every 2 hours weight to attain constant weight. In OD method samples were dried in a hot air oven at 60 °C (Bionics Scientific, India) and weighed every hour till constant weight was attained. For MD samples were dried in a microwave oven (IFB, Model-30SC3, India) at 80°C with power 180 W and after every 20 minutes samples were weight till constant weight was obtained.

Samples from four different harvesting seasons such as spring, summer, autumn and winter season were selected and optimized. Different plant parts such as fresh leaves, stems and tubers were screened on the basis of diosgenin content. All plant samples were extracted by the MAE method with solvent ethanol (50%) and analysis was done by HPTLC technique.

4.3.3 Optimization of extraction methods and solvents

Conventional and non-conventional extraction methods were executed using different solvents with different percentages for the diosgenin extraction. Cold maceration (CM), heat reflux extraction (HRE) as conventional methods and microwave-assisted extraction (MAE) as non-conventional with solvents 100% ethanol, 50% ethanol and water were tested as extraction techniques. The parameters of these methods are given in below Table 4.3.

Table 4.3: *Extractions conditions of three extraction methods*

Extraction Methods	Extraction Conditions			
	Solvent	**Solid to solvent ratio(g/ml)**	**Time**	**Temperature**
MAE	Ethanol (100%, 50%), water	1:30	1:30, 3, 5 min	900 W
HRE	Ethanol (100%, 50%), water	1:30	30, 60, 90 min	60 °C
CM	Ethanol (100%, 50%), water	1:30	12, 24, 36 h	30 °C

4.3.4 Sample preparation

Optimized extraction parameters, methods and other conditions were then incorporated to extract diosgenin. The sample preparation for this chapter has been discussed in Chapter 3 (Section 3.2.4).

4.3.5 Diosgenin analysis

Marker compound preparation, calibration curve, HPTLC instrumentation and method validation for this Chapter has been discussed in chapter 3 (Section 3.2.5). The mobile phase used was toluene: chloroform: acetone (2:8:2) saturated in CAMAG twin trough chamber.

4.3.6 Statistical analysis

The experiment were done in triplicates and expressed as mean ± standard deviation (SD). Statistical analyses were executed by one-way ANOVA followed by Tukey's test. Statistical analysis was done by software Minitab 15 and values of $p < 0.05$ were considered as significant.

4.4 Result and Discussion

4.4.1 RSM model

4.4.1.1 Screening of significant extraction parameters by PBD

PBD with relatively few numbers of runs was employed for the screening of significant parameters from multivariate system and provide a foundation for further optimization (Li et al., 2016). The particular design matrix and resultant % diosgenin yield acquired from *D.*

deltoidea tuber are displayed in Table 4.4. According to regression analysis (Table 4.5), particle size (P = 0.036), solid to solvent ratio (P = 0.018), extraction time (P = 0.001) and temperature (P = 0.049) showed positive effect as their P value is less than 0.05. Whereas solvent composition, pH and extraction cycles showed insignificant effect as their P values is greater than 0.05. The less value of P value and greater value of t value specify significant parameters and their important effect in the experiment (Guo et al., 2019). Hence, these four significant parameters were selected for further BBD design whereas the other parameters were set as solvent composition ethanol (50%), pH (5) and extraction cycles (1) respectively.

Table 4.4: *Yield of diosgenin from D. deltoidea tuber using different levels of extraction variables of Plackett-Burman design criteria*

Runs	$\mathbf{X_1}$	$\mathbf{X_2}$	$\mathbf{X_3}$	$\mathbf{X_4}$	$\mathbf{X_5}$	$\mathbf{X_6}$	$\mathbf{X_7}$	**(%)Diosgenin**
1	1	-1	-1	1	-1	-1	1	1.016
2	-1	-1	1	1	1	-1	-1	0.906
3	1	1	-1	-1	1	-1	-1	0.907
4	1	-1	1	1	-1	1	-1	0.909
5	-1	1	1	1	1	-1	1	0.477
6	1	1	-1	1	1	1	-1	0.964
7	1	-1	1	-1	1	1	1	0.953
8	1	1	1	-1	-1	-1	1	0.957
9	-1	1	1	-1	-1	1	-1	0.637
10	-1	1	-1	1	-1	1	1	0.464
11	-1	-1	-1	-1	1	1	1	0.758

12	-1	-1	-1	-1	-1	-1	-1	0.393

Table 4.5: *Regression analysis for prediction of significant extraction parameters by PBD*

Term	Effect	Coefficient	SE coefficient	*T*	*P*
Constant	0.14100	0.78250	0.02268	34.50	0.000
Particle size	0.17500	0.07050	0.02268	3.11	0.036
Solid: Solvent ratio	0.17500	0.08750	0.02268	3.86	0.018
Time	0.40367	0.20183	0.02268	8.90	0.001
Temperature	0.12333	0.06167	0.02268	2.72	0.049
Solvent composition	-0.07867	-0.03933	0.02268	-1.73	0.158
pH	-0.00433	-0.00217	0.02268	-0.10	0.928
Extraction cycles	0.02667	0.01333	0.02268	0.59	0.588

4.4.1.2 Effect of significant variables (BBD)

Investigational design is comprehensively utilized for knowing the influence of parameters in a procedure to diminish the number of tests, material assets and time. Moreover, the experiment implemented on the outcomes effectively acknowledged, and thus the test errors are reduced. The impacts of variations in the test variables are measured by the statistical procedures and their mutual interactions through the investigational design (Ekren and Ekren, 2008). BBD is one of the most efficient and most applied designs (Jalilian et al., 2020). In this scheme, four parameters at three level (solid: solvent ratio (60, 45 and 30 g/ml), particle size (2.05, 1.25 and 0.5 mm), extraction time (60, 45 and 30 min), and temperature (60, 45 and 30 ºC) was utilised to optimize and examine the independent variables influence on diosgenin of *D. deltoidea* tubers extract obtained by solid-liquid extraction method and the results along with experimental values and predicted values achieved by model equation were presented in Table 4.6. The maximum yield was obtained in run 27 (1.204 %) and lowest in run 10 (1.116 %).

Table 4.6: *BBD criteria of extraction variables with corresponding experimental and predicted value*

Run order	Factors				Diosgenin (% dry weight)		
	Solid: solvent ratio (X_2)	**Particle size (X_3)**	**Time (X_4)**	**Temperature (X_5)**	**Experimental**	**Predicted**	**ANN**
1	30	0.50	45	45	1.129	1.128	1.129
2	60	0.50	45	45	1.153	1.152	1.153
3	30	2.00	45	45	1.152	1.150	1.152
4	60	2.00	45	45	1.137	1.135	1.137
5	45	1.25	30	30	1.128	1.127	1.128
6	45	1.25	60	30	1.132	1.129	1.132
7	45	1.25	30	60	1.147	1.147	1.147
8	45	1.25	60	60	1.155	1.153	1.155
9	30	1.25	45	30	1.155	1.156	1.155
10	60	1.25	45	30	1.116	1.117	1.118
11	30	1.25	45	60	1.135	1.134	1.133
12	60	1.25	45	60	1.183	1.182	1.183
13	45	0.50	30	45	1.128	1.127	1.128
14	45	2.00	30	45	1.134	1.135	1.134
15	45	0.50	60	45	1.137	1.136	1.137
16	45	2.00	60	45	1.132	1.133	1.138
17	30	1.25	30	45	1.119	1.119	1.119
18	60	1.25	30	45	1.137	1.137	1.137
19	30	1.25	60	45	1.135	1.137	1.135
20	60	1.25	60	45	1.125	1.127	1.125
21	45	0.50	45	30	1.144	1.145	1.144
22	45	2.00	45	30	1.133	1.133	1.134

23	45	0.50	45	60	1.150	1.152	1.15
24	45	2.00	45	60	1.169	1.170	1.168
25	45	1.25	45	45	1.202	1.202	1.191
26	45	2.00	45	60	1.201	1.202	1.192
27	**45**	**2.00**	**45**	**60**	**1.204**	**1.202**	**1.192**

Multiple regression analysis was employed on experimental results, providing second-order polynomial equation:

$$Y = 1.2023 + 0.00217\,X2 + 0.00133\,X3 + 0.00192\,X4 + 0.01092\,X5 - 0.03187\,X22 - 0.029125\,X32 - 0.04025\,X42 - 0.02300\,X52 - 0.00975\,X2X3 - 0.00700\,X2X4 + 0.02175\,X2X5 - 0.00275\,X3X4 + 0.00750\,X3X5 \quad (5)$$

The effect of solid: solvent (X_2), particle size (X_3), time (X_4) and temperature (X_5) on the extraction of diosgenin (Table 4.7). Regression coefficients had shown significant positive linear effects of the four variables (X_2, X_3, X_4, and X_5) that are shown in Table 4.7. Among four parameters, temperature has revealed maximum effect on diosgenin yield, by giving 0.01092 value of linear coefficient accompanied by solid: solvent ratio (0.00217), time (0.00192) and particle size (0.00133). The interactive effect of particle size and time (X_{25}) had significant effect on diosgenin whereas time and temperature (X_{45}) were not significant on diosgenin yield. So, only interaction between of solid: solvent and particle size (X_{23}), solid: solvent and time (X_{24}), solid: solvent and temperature (X_{25}), particle size and time (X_{34}) and particle size and temperature (X_{35}) were shown in the model regression equation (4). For all the responses quadratic effect of variables such as solid: solvent ratio (X_2), particle size (X_3), time (X_4) and temperature (X_5) were seen to be significant.

The determination coefficient (R^2) for the model (eq.5) was 0.996 proposes that 0.4% of the variation in response cannot be clarified and 99.6 % of deviation detected in diosgenin yield is subtle to the four variables selected for BBD. According to Chen et al. (2009) R^2-value greater than 0.9 in a regression model is considered as possessing high correlation. Therefore the present R^2-value showed brilliant fit among experimental and predicted responses (Table 4.6 and 4.7). Hence it was reasonable to use regression equation (eq.5) for the analysis of interactive trends in the response.

Table 4.7: *Regression analysis of BBD criterion data for diosgenin extraction from D. deltoidea tuber*

Model parameters	Regression coefficient	S.E.Cofficient	*T*	*P*
Constant	1.20233	0.001207	996.418	0.000
Solid: Solvent	0.00217	0.000603	3.591	0.004
Particle size	0.00133	0.000603	2.210	0.047
Time	0.00192	0.000603	3.177	0.008
Temperature	0.01092	0.000603	18.094	0.000
Solid:Solvent^2	-0.03187	0.000905	-35.221	0.000
Particle size^2	-0.02913	0.000905	-32.183	0.000
Time^2	-0.04025	0.000905	-44.476	0.000
Temperature^2	-0.02300	0.000905	-25.415	0.000
Solid:Solvent × Particle size	-0.00975	0.001045	-9.330	0.000
Solid:Solvent × Time	-0.00700	0.001045	-6.699	0.000
Solid:Solvent × Temperature	0.02175	0.001045	20.814	0.000
Particle size ×Time	-0.00275	0.001045	-2.632	0.022
Particle size × Temperature	0.00750	0.001045	7.177	0.000
Time × Temperature	0.00100	0.001045	0.957	0.357

ANOVA analysis specifies the linear, interactive and quadratic relationship among the independent variables on their dependent variables (Kim et al., 2003). Adequacy of the model was confirmed by Fisher's statistical test for ANOVA and results presented in Table 4.8. Model values obtained by the ANOVA describe whether this model is fit for the variation found in diosgenin extract. If the significance in F-Test is found at the 5% level ($P < 0.05$), then the model can clearly explain the variations and is fit for the analysis. The F-value of model (258.41) indicates model is significant. The F-value of lack of fit is 2.05 (*P*-value: 0.373), which implies that it was not significant to the pure error. Therefore, it could be concluded that the model denoted by eq.5 was fit to the observed results acquired by BBD.

Table 4.8: *Analysis of variance for diosgenin extraction from D. deltoidea by BBD norm*

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	14	0.015802	0.015802	0.001129	258.41	0.000
Linear	4	0.001552	0.001552	0.000388	88.82	0.000
Square	4	0.011523	0.011523	0.002881	659.48	0.000
Interaction	6	0.002728	0.002728	0.000455	104.08	0.000
Residual Error	12	0.000052	0.000052	0.000004		
Lack-of-Fit	10	0.000048	0.000048	0.000005	2.05	0.373
Pure Error	2	0.000005	0.000005	0.000002		
Total	26	0.015855				

Figure 4.1 A-F presented the contour plots of various levels of particle size, solid: solvent ratio, time and temperature on diosgenin yield. Figure 4.1 (A) displays the interaction of temperature and solid: solvent ratio (X_5X_2) and it was observed that maximum diosgenin yield was found at extraction temperature 45°C and solid: solvent ratio 1:45 g/ml. It indicated that both these variables strongly effected diosgenin yield. Figure 4.1 (B) shows interaction of temperature with particle size and time (X_5X_3, X_5X_4) and at 45°C with 1.25 mm particle size maximum yield obtained. Figure 4.1 (C-E) shows interaction of time with temperature, particle size, solid: solvent ratio (X_4X_5, X_4X_3, X_4X_2) and Figure 4.1 (F) shows interaction of particle size and solid: solvent ratio (X_3X_2). On the basis of diosgenin yield, the optimal extraction conditions (solid: solvent ratio, 1: 45; particle size, 1.25 mm; extraction time, 45 min; temperature, 45 °C) were evaluated by using model equation and resolving the regression equation.

Similar optimization approaches by various researchers has been used to extract various compounds such as anthocyanins and flavonols from blackcurrant marc (Li et al., 2016), samara oil from *Acer saccharum* (Chen et al., 2017), flavonoids from *Cyclocarya paliurus* (Hu et al., 2019); phytonutrients from food (Belwal et al., 2020).

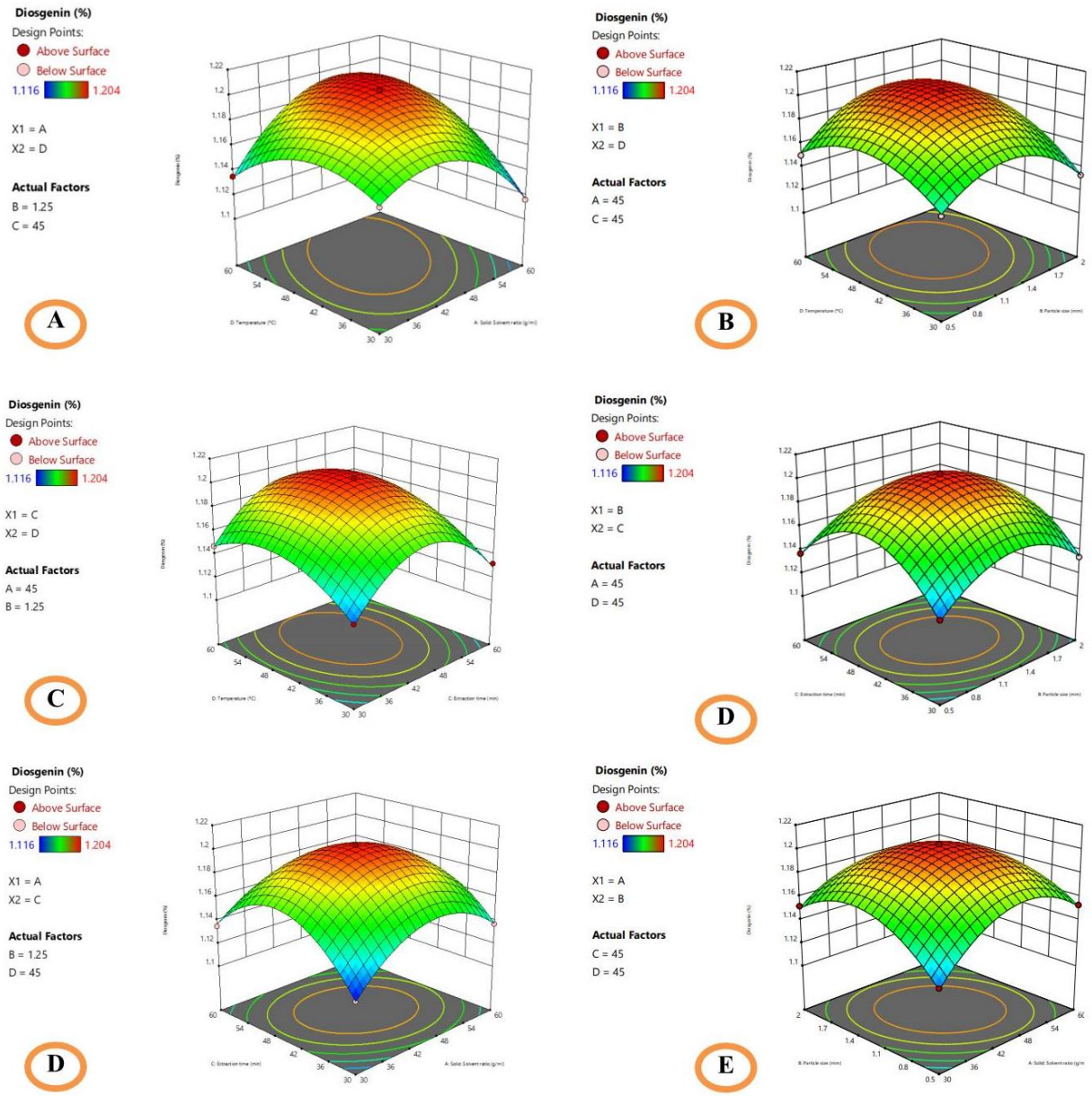


Figure 4.1: *Contour plot for diosgenin extraction yields: the interaction between*

A *interaction between temperature and solid/solvent ratio*

B *interaction between temperature and particle size*

C *interaction between temperature and time*

D *interaction between time and particle size*

E *interaction between time and solid/solvent ratio*

F *interaction between particle size and solid/solvent ratio.*

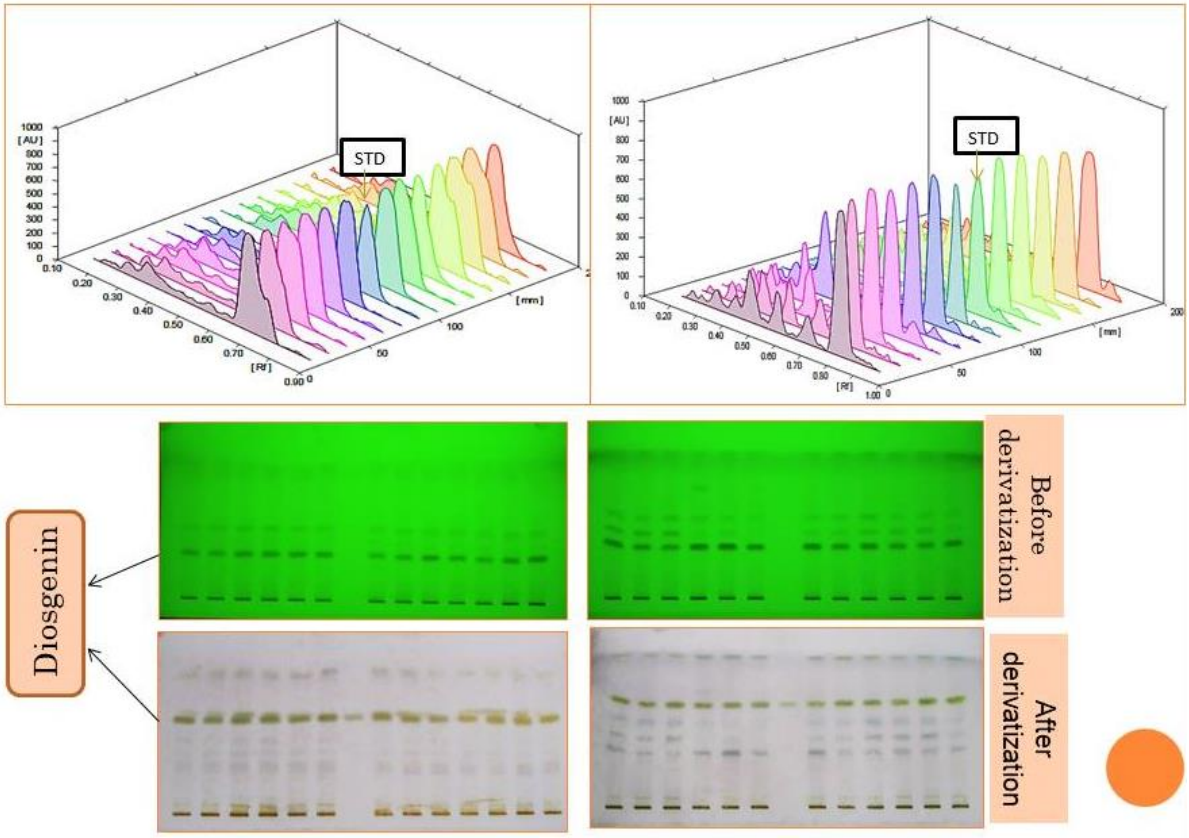


Figure 4.2: *HPTLC fingerprinting and chromatograms of BBD samples*

4.4.2 ANN modeling

Recently ANN has appeared as a sophisticated optimization and simulation tool that exhibits great potential due to its robust prediction and assessment abilities. Several studies have proposed that ANN is superior to RSM regarding predictive abilities (Ciric et al., 2020; Pradhan et al., 2020). Hence, a non-linear association among four input variables and responses was defined by emerging an ANN model by feed forward back propagation algorithm and topology optimization process. In this study, network was created by using BBD experimental data consist of one input layer (X_2, X_3, X_4, X_5), one hidden layer and output layer (diosgenin yield %). The number of neurons in this work was already decided by BBD for both input and output layers. The complete data set of 27 runs were distributed into 3 sets with 19 points for training, 4 points for validation and 4 points for testing the dataset. The experimental data versus the computed ANN data in training, testing and validation networks is presented in Figure 4.2 (A). The performance data attained over entire training data and fitted at best epochs for validation data being epitomized in Figure 4.2 (B). Likewise, gradient loss and training state attained over entire ANN training is described with the assistance of Figure 4.2 (B). Best validation performance for the

optimization of diosgenin was observed at epoch 15. The experimental outcomes applied for RSM was also applied to predict the optimum design of ANN (Table 4.6). In the experimental design, the selecting of appropriate numbers of neurons in the input, hidden and output layers were limited. In hidden layer number of neurons was selected when lowest error of predictive models attained. Initially, ANN model was acquired with least dimension and errors in training and testing by optimizing neural network. The data apportioning (training, testing and validation) were accomplished to evade extreme training and over-parameterization. The goodness of fit between the experiential and predicted response data form ANN are displayed in Figure 4.2 (A) with correlation coefficients of 0.998% for extraction yield. Greater correlation coefficients reveal the consistency of the predictive models by ANN.

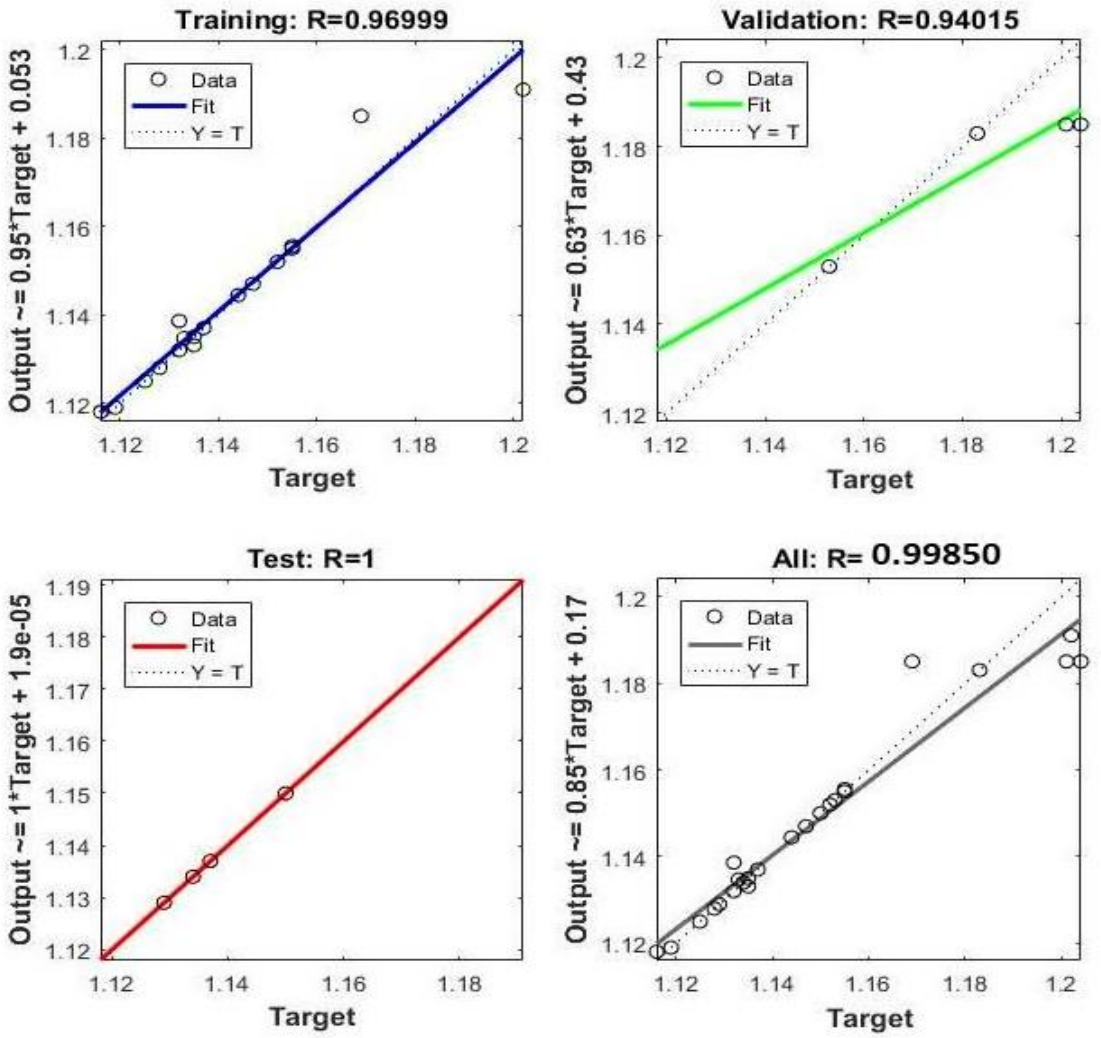


Figure 4.3 (A): *Correlation between experimental versus computed yield of ANN*

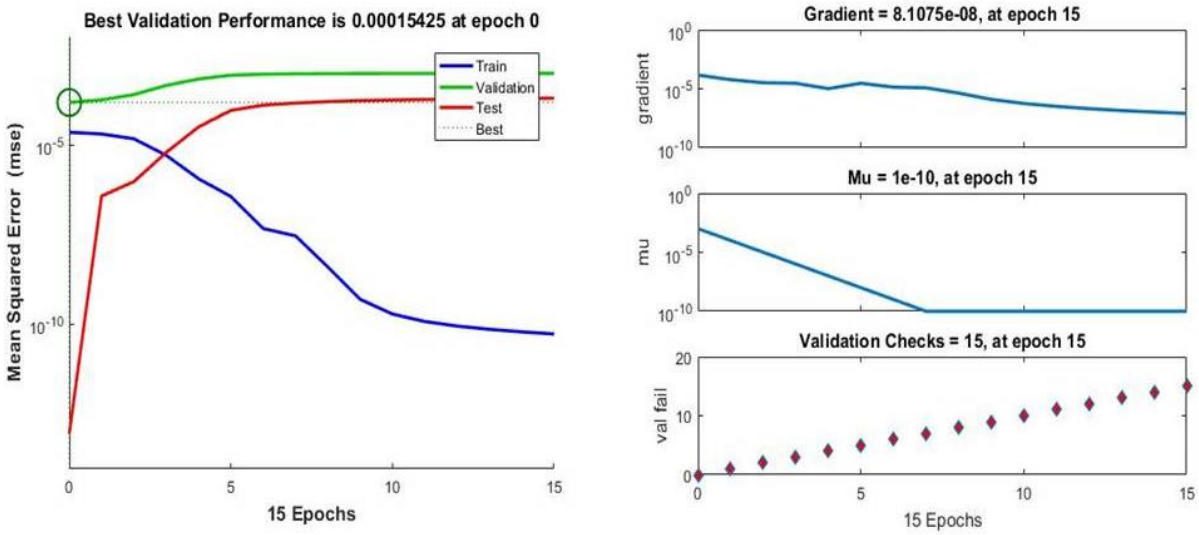


Figure 4.3 (B): *Performance data obtained (ANN) over entire training data and gradient loss for diosgenin*

4.4.3 Comparison between RSM and ANN models

RSM and ANN modeling methods were compared on the basis of their predictive performance and valuation abilities. Table 4.6 displays the predicted values of RSM and ANN. Between experimental and predicted values less deviation was noticed in the ANN model than RSM model. Moreover, comparison between these two models was also made on the basis of R^2, AAD and RMSE as shown in Table 4.9.

$$RMSE = \left(\frac{1}{n}\sum_{i=1}^{n}(Y_{pred} - Y_{exp})2\right)^{1}/_{2} \quad (6)$$

$$AAD = \left(\frac{1}{n}\sum_{i=1}^{n}\left(\frac{Y_{pred}-Y_{exp}}{Y_{exp}}\right)\right) \times 100 \quad (7)$$

$$R^2 = 1 - \sum_{i=1} n\left(\frac{Y_{pred}-Y_{exp}}{Y\_exp}\right) \quad (8)$$

The value of AAD must be very lesser while value of RMSE must be near to zero for a good model. High RMSE and AAD values signify greater probabilities of errors in prediction. The AAD value (0.0184) and RMSE value (0.0012) for ANN was smaller than AAD value (0.0186) and RMSE value (0.0013) for RSM (Table 4.9). Moreover, ANN has larger R^2 value than RSM model. The outcomes revealed that ANN has considerably enhanced predictive capability in contrast to RSM, which is correlated to its universal capability to estimate non-linear systems, however RSM is effectual by means of the nature of model is

limited to second order polynomial regression. Being independent of experimental design, ANN is better at computing many responses in single run than RSM that involves multiple runs with standard experimental design for multi-response optimization (Dadgar et al., 2015; Hammoudi et al., 2019). Therefore, ANN model is more consistent and precise in case of predictive capacity and fit to the target responses (yield %), for diosgenin extraction optimization as compared to RSM.

RSM and ANN have also been compared for effective extraction of natural dye from *Bixa orellana* (Sinha et al., 2013), artemisinin from *Artemisia annua* (Pilkington et al., 2014), stevioside and rebaudioside-A from *Stevia rebaundiana* (Ameer et al., 2017), oleonolic acid from *Ocimum sanctum* (Khamparia et al., 2020), polyphenols from garlic (Ciric et al., 2020), and also reported that ANN to be superior than RSM model in case of predictive capability.

Table 4.9: *Comparison of Predictive capacity of RSM and ANN models*

	RSM	**ANN**
Coefficient of determination (R^2)	0.9968	0.9985
Root mean square error (RMSE)	0.001374	0.001295
Absolute average deviation (AAD)	0.018653	0.01847

4.4.4 Screening of potent part

In order to check the potent source of bioactive compound diosgenin in *D. deltoidea*, different fragments for instance leaves, stem and tubers were screened. The samples of these three parts are extracted with ethanol (50%) by MAE method and HPTLC was executed for the estimation of diosgenin. HPTLC fingerprinting and chromatogram shows the presence of diosgenin in different plant part samples displayed in Figure 4.3. The HPTLC outcomes indicated that maximum diosgenin amount was found in tuber and the leaf contains minimum content of diosgenin presented in Table 4.10. The order of diosgenin content in these parts are as follows tuber (1.15%) > stem (0.32) > leaf (0.22%). So, on the basis of results it was clear that tuber was the potent part as it contains highest diosgenin content.

Table 4.10: *Diosgenin content in different parts of D.deltoidea*

Plant part	Diosgenin (%)
Leaf	0.22 ± 0.01
Stem	0.32 ± 0.01
Tuber	1.15 ± 0.01

* Values are expressed as mean ± SD; SD = standard deviation and n=3.

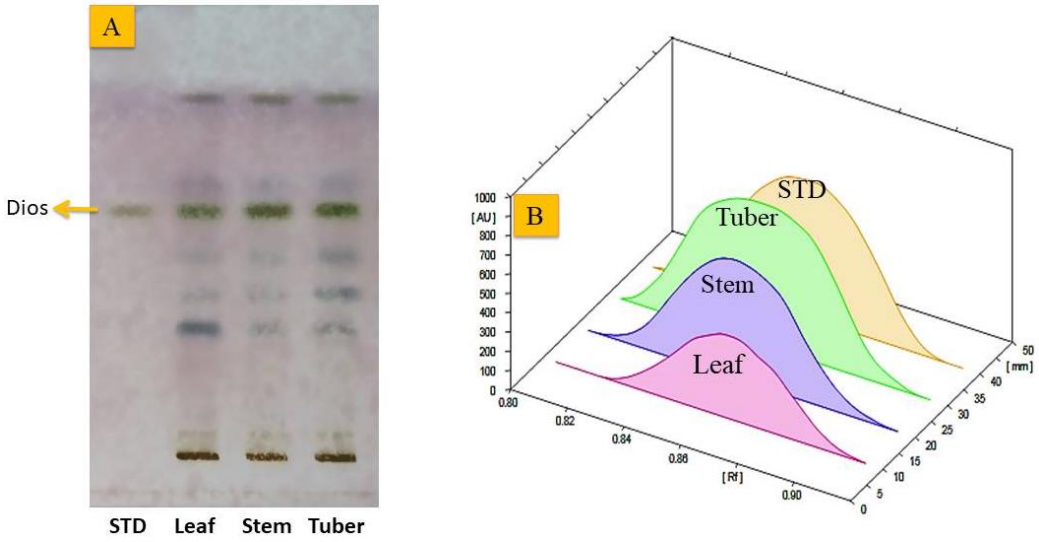


Figure 4.4: A *HPTLC fingerprinting and* **B** *chromatogram of diosgenin in different plant parts*

4.4.5 Optimization of drying processes and seasonal variation

In this study, different drying methods and seasonal variations are evaluated and to the best of our knowledge no optimization studies on drying methods and seasonal variations were accompanied on *D. deltoidea.*

4.4.5.1 Effect of drying methods on diosgenin

The supremacy of rare drugs extremely depends on their drying method after harvesting. In this study, the effect on bioactive compound content in *D. deltoidea* tuber powders dried by different drying process was evaluated. The extracts of different drying methods samples

were generated by MAE method with solvent ethanol (50%) and estimation was carried out by HPTLC analysis. Figure 4.4 displays HPTLC fingerprinting and chromatogram of marker compound diosgenin along with different samples. As shown in Table 4.11 diosgenin content was highly influenced by the drying processes. Maximum diosgenin content was observed in SHD samples and minimum in sun dried samples. MD also showed good content of diosgenin followed by OD method. The order of drying processes according to diosgenin content is as follows SHD (1.43%) > MD (1.17%) > OD (1.13%) > SD (1.12%). The reason SD showed least diosgenin yield may be the degradation of phytochemicals due to direct exposure of sunlight. SHD has been widely used for the drying process as it does not degrade the eminence and amount of phytochemicals present in the plant material. Moreover, it is the cheapest and easiest drying method as compared to other methods. So the present investigation revealed that shade drying is superlative, efficient and cost-effective drying method to attain maximum phytochemicals amount from the plants. Comparable studies were also reported that shade drying method is superior to other drying methods including microwave drying, oven drying, sun drying and other methods (Ennajar et al., 2010; Manika et al., 2013).

Table 4.11: *Yield of diosgenin in D. deltoidea by different drying processes*

Drying method	Diosgenin (%)
Fresh	0.78 ± 0.01
Sun drying	1.12 ± 0.01
Shade drying[a]	1.43 ± 0.01
Oven drying[b]	1.13 ± 0.01
Microwave drying[c]	1.17 ± 0.01

[a] At room temperature.

[b] At 60°C.

[c] At 80°C (180 W).

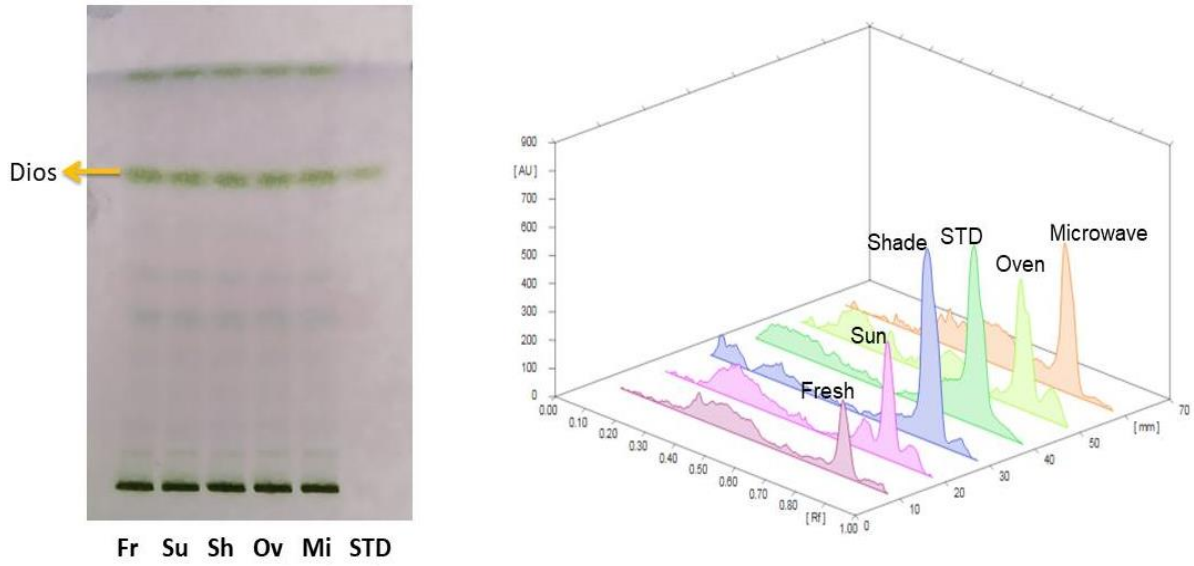


Figure 4.5: *represents HPTLC fingerprinting and chromatogram of different drying processes (sun, shade, oven, and microwave)*

4.4.5.2 Influence of seasonal variation on diosgenin

As no reports are available on the seasonal variation on diosgenin content till date, hence to check the seasonal variation four seasons spring (March), summer (June), autumn (September) and winter (December) were selected and diosgenin amount was estimated three monthly. HPTLC fingerprinting and chromatogram are presented in Figure 4.5. As shown in Table 4.12 huge variation was observed in the amount of diosgenin harvested in different seasons. The quantity of diosgenin was found maximum in the month of December in the winter season (1.19%) and minimum amount was attained in the month of June of the summer season (0.38%). After December, March yielded second highest diosgenin content (1.14%) followed by September (0.61%). The kinetics of diosgenin content is probably linked with the expression of numerous genes at several developmental phases of the plant or to the environmental components rising from seasonal variations. From this study, it was clear that seasonal variations play a significant role on the content of diosgenin and it is highly recommended to harvest the plant on the right season to obtain the bioactive constituents at its most. Manika et al. (2013) studied seasonal variations on the yield of gymnemagenin from *Gymnema sylvestre* and suggested summer season (April-July) to be best for its harvesting. Sridhar et al. (2018) also investigated seasonal variations on the content of conophylline in *Tabernaemontana divaricate* and found that conophylline content was higher in the samples harvested in August.

Table 4.12: *Seasonal variation on diosgenin content in D.deltoidea tubers*

Month	Diosgenin (%)
March	1.14 ± 0.01
June	0.38 ± 0.01
September	0.61 ± 0.01
December	1.19 ± 0.01

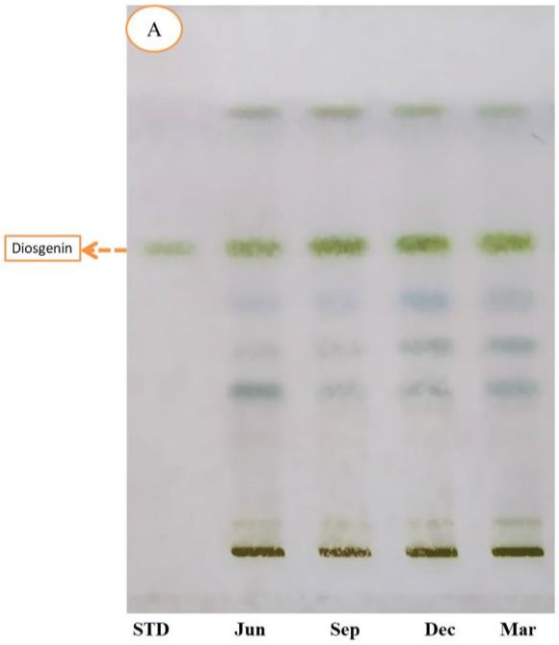


Figure 4.6: *HPTLC fingerprinting after derivatization*

4.4.6 Optimization of extraction methods and solvents

The rehabilitated interest in plant-derived drugs has prompted an expanded requirement for effective extraction techniques. Moreover, the qualitative and quantitative investigations of secondary metabolites from plants mainly depend on the selection of a suitable extraction method (Smith, 2003; Sasidharan et al., 2011). Therefore in this study comparison of conventional extraction methods with non-conventional methods, for example, cold maceration (CM), heat reflux extraction (HRE) and microwave-assisted extraction (MAE) methods and solvent selection (ethanol (100%), ethanol (50%) and water) has been performed to determine the proficient method for diosgenin extraction from *D. deltoidea* tubers and till now no comparison of different methods on this plant has been reported. Figure 4.6 (A, B) shows the presence of diosgenin in HPTLC fingerprinting, densitometric

chromatograms and overlay spectra conquered from the studied from the extraction methods and solvents. The extraction efficiency of nine experiments was between 0.46 to 0.99% as represented in Table 4.13. In HRE method, three different time periods (30, 60, 90 min) and three solvents (ethanol 100%, 50%, water) were optimized and results showed that ethanol (100%) as a solvent at first two time slots 30 min and 90 min increased diosgenin yield from 0.65% to 0.68% then remained constant to 0.68% when extraction time reached to 90 min. In case of ethanol (50%) both 30 min and 60 min caused increase in diosgenin yield from 0.77 to 0.79 %, however slight decrease (0.78 %) was observed at 90 min of extraction time. However, water as a solvent gave low diosgenin yield in the range of 0.54 to 0.59%. In CM methods, all three solvents with different extraction timings were tested and it was found that ethanol (50%) yielded high diosgenin (0.77%) at 24 and 36 h while ethanol (100%) and water yielded less yield of diosgenin compared to ethanol (50%). The extraction time 24 h showed good result in all these three solvents and yielded high diosgenin in comparison to them. The diosgenin yield obtained in the CM method was in the range of 0.46% to 0.48% in water solvent, 0.63% to 0.66% in ethanol (100%) and 0.74% to 0.77% in ethanol (50%). In MAE method, ethanol (100%) yielded diosgenin 0.85% at 1:30 min, 0.88% at 3 min and 0.85% at 5 min. Similarly, ethanol (50%) yielded 0.95% at 1:30 min, 0.99% at 3 min, 0.96% at 5 min and solvent water yielded 0.81% at 1:30 min, 0.84% at 3 min and 0.82% at 5 min. In each case, as the extraction time increased to 5 min gradual decrease was observed in diosgenin content. From the above study, it was clear that MAE a non-conventional method at extraction time 3 min with solvent ethanol (50%) showed highest diosgenin yield as compared to conventional methods HRE and CM.

The non-conventional methods are less time and solvent consuming, low of cost and environmental friendly as compared to conventional ones which require longer extraction time, costly, huge solvent consumption and unsustainable. Several researchers have used conventional and non-conventional extraction techniques for the extraction of various bioactive compounds (Dabiri et al., 2005; Devgan et al., 2012; Zeidan et al., 2014; Deng et al., 2015; Caldas et al., 2018; Rocchetti et al., 2019; Brás et al., 2020). Total saponins from *D. zingiberensis* were extracted by MAE and conventional methods and it was found that MAE extracted maximum saponins content in short time and more effectively than ultrasonic extraction, soxhlet and HRE (Ren et al., 2015). Saponins and phenolics have been extracted by MAE method from Fenugreek seeds at 2.84 min (Akbari et al., 2019). Pan et al. (2003) extracted caffeine and polyphenols from the leaves of green tea and obtained high

yield at 4 min by MAE method than other methods for 20 h at room temperature. Several researchers have also revealed MAE as the best extraction method and green solvent as eco-friendly (Lesellier et al., 2012; Kaur et al., 2019).

Table 4.13: *Influence of various extraction techniques and solvents on diosgenin content of D.deltoidea*

Extraction Method/ Solvent	Time	Diosgenin (%)
HRE with 100% Ethanol	30 min	0.65 ± 0.01
	60 min	0.68 ± 0.01
	90 min	0.68 ± 0.01
HRE with 50% Ethanol	30 min	0.77 ± 0.01
	60 min	0.79 ± 0.01
	90 min	0.78 ± 0.01
HRE with water	30 min	0.54 ± 0.01
	60 min	0.57 ± 0.01
	90 min	0.59 ± 0.01
CM with 100% Ethanol	12 h	0.63 ± 0.01
	24 h	0.66 ± 0.01
	36 h	0.66 ± 0.01

CM with 50% Ethanol	12 h	0.74 ± 0.01
	24 h	0.77 ± 0.01
	36 h	0.77 ± 0.01
CM with water	12 h	0.46 ± 0.01
	24 h	0.48 ± 0.01
	36 h	0.47 ± 0.01
MAE with 100% Ethanol	1:30 min	0.87 ± 0.01
	3 min	0.88 ± 0.01
	5 min	0.85 ± 0.01
MAE with 50% Ethanol	1:30 min	0.95 ± 0.01
	3 min	0.99 ± 0.01
	5 min	0.96 ± 0.01
MAE with water	1:30 min	0.81 ± 0.01
	3 min	0.84 ± 0.01
	5 min	0.82 ± 0.01

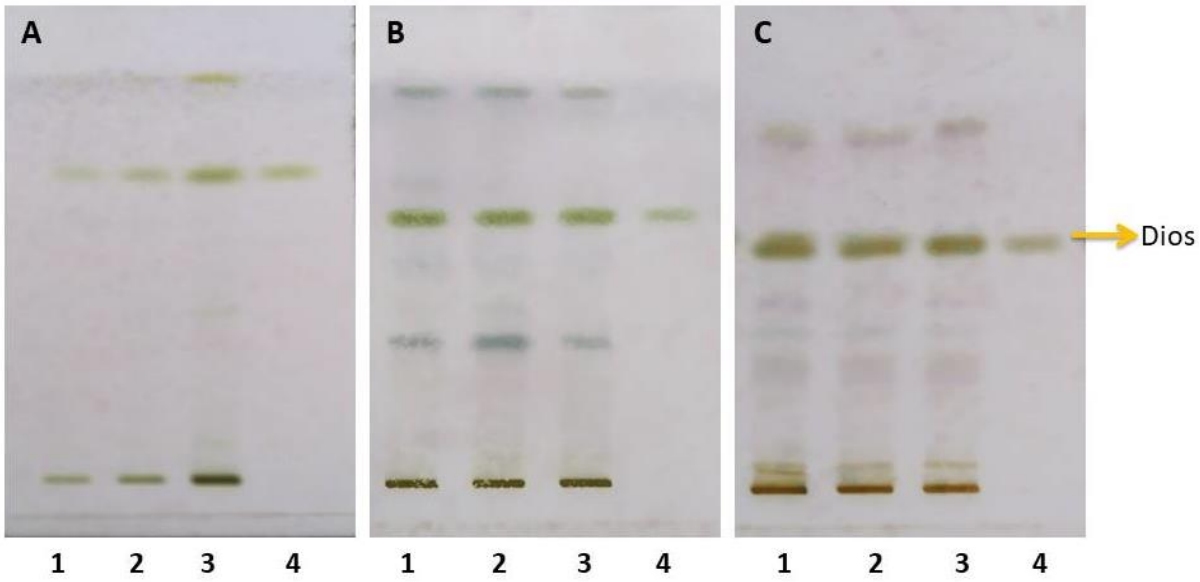


Figure 4.7 A: *HPTLC fingerprinting **A** represents CM (24 h) **B** represents MAE (3 min) **C** represents HRE (60 min) with 1 as water, 2 as ethanol (100%), 3 as ethanol (50%) and 4 as standard*

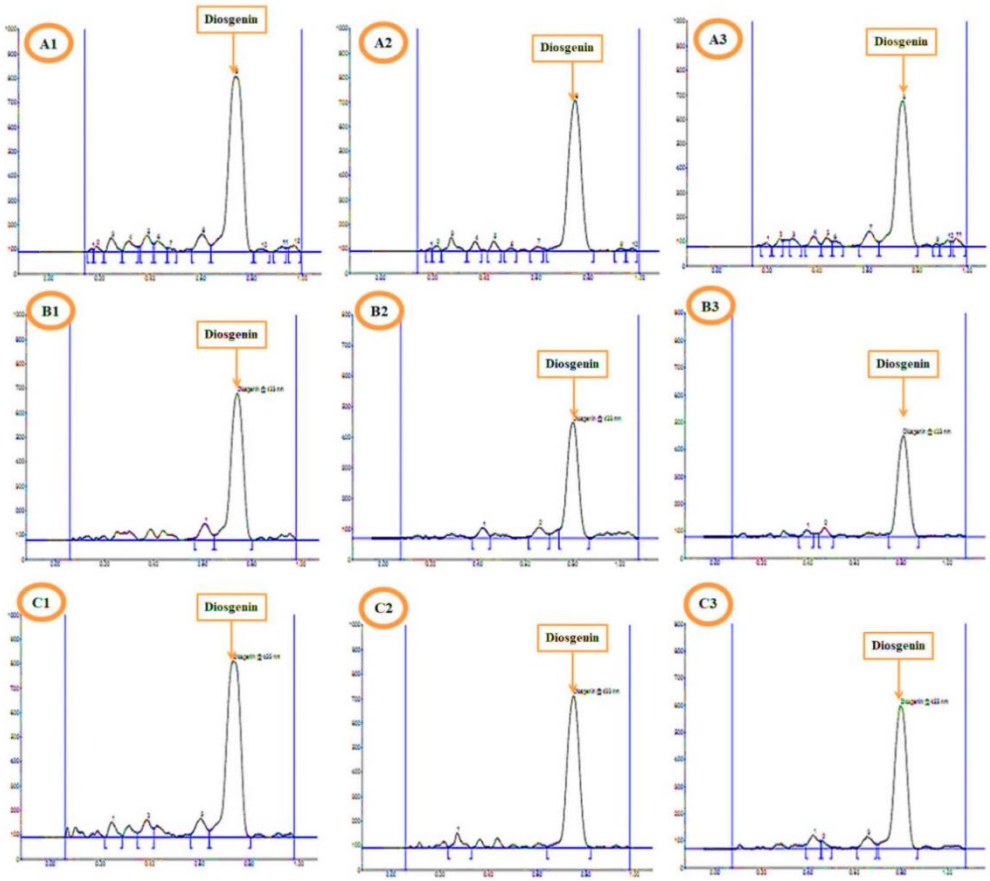


Figure 4.7 (B): *Densitometric chromatograms and overlay spectra signifying the influence of extraction techniques viz. MAE (A1- Ethanol 50%, A2- Ethanol 100%, A3- Water), HRE (B1- Ethanol 50%, B2- Ethanol 100%, B3- Water), CM (C1- Ethanol 50%, C2- Ethanol 100%, C3- Water) on yield of diosgenin.*

4.4.7 Method validation

Table 2 and 3 presented the features of method validation for diosgenin quantification. Good linearity-range of r^2 (correlation coefficient) was observed to be 200-1000 ng/spot for diosgenin with reference to peak area (Figure 4.7). To determine the proper sensitivity of the method, LOD and LOQ of diosgenin were estimated and are shown in Table 4.14.

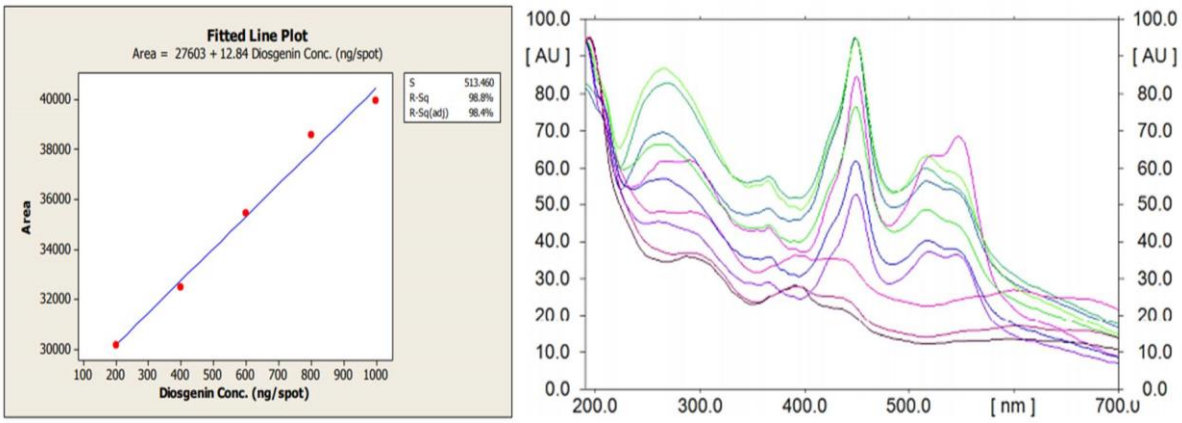


Figure 4.8: *Calibration plot and spectra for diosgenin*

Table 4.14: *Method validation for diosgenin quantification*

S. No	Parameters	Value
1	Linearity range (ng/spot)	200-1000
2	Correlation coefficient (r^2)	0.988
3	Regression equation	Y = 12.84X + 27603
4	Calculated SD value	0.42
5	[b]Limit of detection (LOD) (ng) [3×SD/S]	20
6	[b]Limit of quantification (LOQ) (ng) [10×SD/S]	60
7	R_f	0.78
Precision and accuracy		
8	Intra-day RSD (%), n = 5	1.34
9	Inter-day RSD (%), n = 5	1.56
Recovery		
10	Amount of standard in plant samples (µg mg^{-1}) containing highest bioactive compounds	12.04

11	Amount of standards added in plant samples (μg mg^{-1})	6, 12, 18
12	Amount of standard found (μg mg^{-1})	18.15, 23.98, 29.98
13	Recovery (%)	100.6, 99.75, 99.80
14	Mean Recovery (%)	100.05

[a] Four concentration levels in triplicate.

[b] SD is the standard deviation of the blank response and S is the slope of the calibration plot

4.5 Conclusion

In this present work, RSM and ANN, conventional and non-conventional extraction methods were compared for their predictive abilities, susceptibility analysis and optimization efficacy in diosgenin extraction optimization. Moreover, the influence of different drying methods and seasonal variations on diosgenin yield was also evaluated. PBD screened seven parameters among which four are found significant which are further studied by BBD modeling. The effect of solid: solvent ratio, particle size, extraction time and temperature were studied by RSM and ANN. The values of R^2, RMSE and AAD were used for the comparison of RSM and ANN methods. The ANN model was found superior to RSM in terms of predicting abilities and accuracy.

Drying processes has significantly affected bioactive content, among four drying methods shade drying yielded significantly higher diosgenin yield than SD, OD and MD. Hence shade drying is most proficient and cost-effective drying method. Seasons have also shown variation in the diosgenin yield, however, tubers harvested on their maturity stage during December yielded maximum diosgenin. In extraction methods, MAE showed very good yield of diosgenin in less time (3 min) as compared to CM and HRE. Furthermore, MAE could be used as a competent and eco-friendly alternate to conventional methods to extract the desired compound from plant matrices for their utilization in various industrial areas.

Therefore, in this current investigation we succeeded in determining efficient extraction method and conditions, drying methods and seasonal variations on diosgenin yield from *D.deltoidea*. Significantly, this is the first study on *D.deltoidea* that provides efficient, cost-effective, green technology based sustainable production of medicinally important bioactive compound diosgenin.

CHAPTER 5

Micropropagation of Elite Variety of D. deltoidea for Mass Production

5.1 Introduction

Plant tissue culture offers an alternate means of fast propagation and production of biomass and secondary metabolites of therapeutically important plant species. Clonal propagation by plant tissue culture which is known as micropropagation is defined as the true-to-type propagation of particular genotypes and can be accompanied in short space and time. A single explant can be multiplied into a huge number of plants in quite less period and space regardless of the season. By the process of micropropagation, threatened, endangered and rare plant species have effectively propagated and preserved due to high degree of multiplication and fewer demands of primary plants and space, however, affected by the presence of growth hormones (Hussain et al., 2012).

Plant growth hormones play important role in the establishment of particular type of growth in the cultured cells or tissues, which might be caused by increase of a particular chemical present in them. When different hormones individually or in combinations were added to the medium result in the maintenance of particular and stable organic and inorganic substances in the developing tissue and cause the cells/tissues to form callus and then segregate into shoots or roots or decease (Haq and Dahot, 2007). In different plant hormones, auxins and cytokinins are the main hormones to establish *in vitro* cultures as the former is mostly required for root formation and later for shoot formation and buds growth (North et al., 2012). Altering and changing the auxins and cytokinins concentration alterations the development activities of plant cultures (Ngomuo et al., 2013). However, optimal concentration and good combination of auxins and cytokinins in the culture medium determines effective plant regeneration. The cytokinin-auxin interaction controls vital morphological processes which include the appearance and development of meristem. According to Su et al. (2011) auxins in low concentration and cytokinins in high concentration in *in vitro* culture initiates shoot multiplication.

Cytokinins are basically diverse group of N^6 –a substituted purine derivative that promotes cell division in plants but later found natural hormone important for normal growth and

development of plant. Cytokinins also effect on many other developmental processes of plants such as leaf senescence inhibition, cell enlargement, development of chloroplast and mobilisation of nutrients and shoot and root branching (Mok, 1994). In a series of investigations accompanied by Skoog and Miller (1957) revealed that the cytokinin to auxin ratio in culture media affects the shoot and root formation in the tissue culture process. However, in some cases development of callus tissues occurs without the cytokinins in medium (Sunderland and Wells, 1968). Cytokinins are found very helpful in endorsing direct or indirect shoot induction. Shoot regeneration was induced from superficial meristem by the accumulation of cytokinins and later root regeneration was acquired from callus (Paterson and Rost, 1981). Auxins on the contrary are essential part of culture media broadly used in tissue culture for micropropagation process and causes wide range of activities in plants. The most significant activity of auxins in *in vitro* cultures are enhance the production of shoot and root, initiation of cell division either in callus or defined organs, differentiation and elongation of cells or conductive tissues, and prompting apical dominance (Singh et al., 2016).

The regularly used auxins in *in vitro* cultures are 2, 4-dichlorophenoxyacetic acid (2,4-D) frequently used for callus and suspension cultures, 1-naphthaleneacetic acid (NAA) used for organogenesis and mostly for root formation, indole-3-acetic acid (IBA) mostly used for shoot multiplication and root formation (Hussain et al., 2012). On the other hand, the common cytokinins are N^6-benzylaminopurine (BAP) and KN used for dominance of apical meristem and induction of both axillary and adventitious shoot formation. However, most commonly preferred cytokinin is BAP (Ngomuo et al., 2013).

The production of genetically identical plantlets through *in vitro* culture is considered a main advantage. Genetic homogeneity among the *in vitro* regenerants is greatly required for clonal propagation and conservation of elite genotypes having superior features. Though, many reports have revealed the presence of variation among *in vitro* plantlets which is known as somaclonal variation. These variations are ascribed to dissimilarities in physiological, epigenetic and genetic superiority and are reliant of genotype and culture conditions (da Silva et al., 2007). These variations can be investigated through various methods such as evaluating alterations in morphological characters, cytogenetical evaluation for studying numerical and structural difference in chromosomes, biochemical and DNA based molecular markers. Though, investigators depend more on molecular markers as the variations identified by morphological, cytological and biochemical analysis might be

affected by developmental and environmental factors (Alizadeh et al., 2015). Genetic fidelity can be evaluated by many molecular markers such as Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), and so on. However, ISSR is the most commonly used molecular marker as it is cost-effective, non-radioactive nature and requires less amount of DNA (Pathak and Dhawan, 2012). Similarly many biochemical factors have been compared between *in vitro* regenerants and mother plant.

A rapid glimpse at the existing reports on micropropagation *D. deltoidea* clearly exposes a clear paucity of reports on both direct and indirect organogenesis. Taking into consideration the gaps left sincere efforts have been made for the development of effective and improved protocol of micropropagation using explants of elite variety of *D. deltoidea* (G2) as revealed in **Chapter 3**. In the current study, attempts have also been made to assess the genetic and biochemical fidelity of the *in vitro* regenerants by using ISSR and HPTLC analysis. The biochemical fidelity was assessed by the presence of diosgenin which is the main bioactive compound of *D. deltoidea*.

5.2 Materials and Methods

5.2.1 *In vitro* studies

The most significant aspect of *in vitro* studies is the maintaining of cultures in aseptic conditions. The aseptic conditions or techniques used in *in vitro* culture of *D. deltoidea* are as follows:

- Mother plant source.
- Sterilization of glassware and other equipments.
- Preparation and sterilization of nutrient media.
- Preparation of aseptic conditions in laminar air flow.
- Selection and surface sterilization of explants.
- Inoculation and maintenance of cultures.

5.2.1.1 Mother Plant source

In this study, the elite variety of *D. deltoidea* reported in our Chapter 3, which was collected from Gulmarg area of Baramulla district of Jammu and Kashmir at an altitude of 2990 m was used as a mother plant source for all *in vitro* investigations and genetic stability studies.

Leaf, stem and tuber of the plant were separated and washed thoroughly to remove the field contamination.

5.2.1.2 Sterilization of glassware and other equipments

All the glassware's including flasks, petri plates, test tubes, beakers, culture bottles, forceps and scalpels are cleaned before their usage. At first they were splashed with liquid detergent followed by water to eradicate the traces of detergent. After that they were dipped in chromic acid and a few hours later they were washed with double distilled water. Finally, they were disinfected by drying in hot air oven at 60-80 °C.

5.2.1.3 Preparation and sterilization of nutrient media

Murashige and Skoog (1962) (MS) supplemented with $CaCl_2$ and vitamins available in readymade packets (Hi-Media) were used in this work. The media was prepared by dissolving MS media (4.9 g) and 30 g of sucrose (3%) in 1 litre of double distilled water and was continuously stirred till it dissolves completely. Heat stable growth hormones were added to the MS media in desired amounts and pH was set at 5.8 by adding 1N NaOH (Sodium hydroxide) or HCl (Hydrochloric acid). Then agar 0.7% (7 g) was added to the media and heated to dissolve. The media was then poured into sterilized conical flasks, petri plates and test-tubes and finally sterilized by autoclaving at temperature 121 °C and 15 psi for 15 minutes.

5.2.1.4 Preparation of stock solution of plant growth hormones

The main hormones used in this study are auxin and cytokinins. Different concentrations of BA, IBA, NAA, IAA, 2, 4-D and TDZ were prepared and added onto the MS media for the induction of callus, shoot and root. All PGRs are not soluble in water, so they are dissolved in relative solvent and then double distilled water was added to make the required volume. Solubility of these PGRs was presented in Table 5.1. Stock solution was prepared as mg L^{-1}. BA, NAA, IBA, IAA, 2, 4- D (100 mg) was dissolved in 2 ml of 1N NaOH and TDZ in 2 ml of 1N KOH and made up to 100 ml by the addition of double distilled water and stored in the refrigerator at -20 °C for further use.

Table 5.1: *Plant growth hormones stock solution preparation*

Plant hormone	Formula	Molar mass (g/mol)	Solubility
BA	$C_{12}H_{11}N_5$	225.2492	1N NaOH/ EtOH
IBA	$C_{12}H_{13}NO_2$	203.24	1N NaOH/ EtOH
IAA	$C_{10}H_9NO_2$	175.184	1N NaOH/ EtOH
NAA	$C_{12}H_{10}O_2$	186.2066	1N NaOH/ EtOH
2, 4- D	$C_8H_6O_3Cl$	211.04	1N NaOH
TDZ	$C_9H_8N_4OS$	220.25	1N KOH/ DMSO

5.2.1.5 Preparation of aseptic conditions in laminar air flow (LAF)

Before inoculation, LAF was thoroughly sterilized by wiping with ethanol (70%), followed by UV treatment for 15 minutes. Mouth and nose were covered by a mask and hands were sterilized with ethanol (70%). All equipments such as forceps, scalpel, scissor, surgical blades etc, were dipped in ethanol (70%) and flame sterilized. Inoculation should be done near the flame and during inoculation cotton plug or cap of culture test-tubes/ conical flasks was kept in hand. The flasks or test-tubes were recapped and wrapped with parafilm after the inoculation and incubated in the culture room.

5.2.1.6 Selection and surface sterilization of explants

Explants such as leaf, nodes, internodes and tuber were selected for the *in vitro* regeneration of *D. deltoidea*. The explants were surface sterilized for the removal of contamination. In this study, commonly used surface sterilizing agents such as bavistin, ethanol, mercuric chloride and sodium hypochlorite were used with various exposure durations (Table 5.2). Initially all explants were thoroughly washed in running tap water followed by drenched in tween-20 for 30 min. The tween-20 solution was discarded and explants were rinsed with running tap water thrice. The washed explants were then taken to laminar air flow chamber to proceed the further surface sterilization process in which various sterilizing agents with different time intervals were employed to standardize the sterilization protocol are discussed below:

- **Treatment-1:** Washed explants were treated with mercuric chloride ($HgCl_2$) for 5 minutes and then splashed with autoclaved distilled water thrice.

- **Treatment-2:** Washed explants was sterilized with sodium hypochlorite (NaOCl-2%) for 5 min and then cleaned with autoclaved distilled water thrice to eliminate the bits of bleach.
- **Treatment-3:** 2% of NaOCl was used to wash the explants for 2 min accompanied by washing with autoclaved distilled water twice. Then $HgCl_2$ for 3 min was used to sterilize the explants and washed with autoclaved distilled water thrice.
- **Treatment-4:** Firstly explants were sterilized for 3 min with NaOCl, then splashed with autoclaved distilled water twice, accompanied by $HgCl_2$ for 4 min, then again rinsed with autoclaved distilled water thrice.
- **Treatment-5:** In this process, firstly splashed explants were sterilized with bleach for 2 min, and then splashed with autoclaved distilled water twice, accompanied by sterilization with $HgCl_2$ for 3 min then again rinsed with autoclaved distilled water twice and finally sterilized with ethanol (70%) for 2 min. After that explants were splashed with autoclaved distilled water thrice and are ready for inoculation.
- **Treatment-6:** In this process, firstly washed explants were treated with bleach for 3 min, and then washed with autoclaved distilled water twice, followed by sterilization with $HgCl_2$ for 4 min then again washed with autoclaved distilled water twice and finally sterilized with ethanol (70%) for 3 min. After that explants were washed with autoclaved distilled water thrice and are ready for inoculation.

Table 5.2: *Concentration, exposure time and preparation of selected sterilization agents*

S. No	Sterilization agents	Concentrations (%)	Treatment duration (min)	Preparation
1	Mercuric chloride ($HgCl_2$)	0.1	3	1 g $HgCl_2$ dissolved in 1000 ml of distilled water
			4	
			5	
2	Sodium hypochlorite (NaOCL)	2	2	50 ml of NaOCl in 100 ml of distilled water
			3	
			5	
3	Ethanol	70	2	70 ml ethanol in 30 ml distilled water
			3	

5.2.1.7 Inoculation and maintenance of cultures

Leaf segments, nodal segments, internodes and tuber explants were cut into proper size (1-1.5 cm) and inoculated on the MS medium. Nodal and internodes were cultured vertically and leaf segments were inoculated with their abaxial and adaxial surface touching the media. At an interval of 4 weeks explants are sub-cultured.

The cultures were incubated under aseptic conditions in the culture room with fluorescent tubes at (40-50 µmol m^{-2} s^{-1}) and temperature maintained at 25 ± 2 ℃. The cultures were monitored and interpretations were prepared from the second day of inoculation. Parameters such as callus, number of shoots and roots, length of shots and roots, culture response etc. were noted.

5.2.1.8 Direct shoot initiation and multiplication

The explants of *D. deltoidea* were inoculated on MS media augmented with several concentrations of plant hormones such as BA individually and in combinations with IBA, NAA, and TDZ for shoot induction (Table 5.3). After the development of shoots on shoot induction medium, cultures were transferred to shoot multiplication media supplemented with different concentrations of BA and IBA and were transferred on the particular media for auxiliary shoot propagation.

5.2.1.9 Root and tuber induction

The well-developed shoots were transferred on the rooting media. For rooting, solid MS and liquid MS augmented with different PGRs such as BA, NAA and IBA with different concentrations were used either individually or in combination. For tuberization, BAP in the range of 0.15 to 2.0 mg L^{-1} alone and in combinations with different concentrations of NAA (0.1 – 1.0 mg L^{-1}) and KN (0.25 – 5 mg L^{-1}) were used in liquid MS medium (Table 5.3).

5.2.1.10 Callus formation

The explants were cultured on callus initiation media augmented with different concentration of 2, 4-D, NAA and IAA.

Table 5.3: Different *PGRs used for direct and indirect organogenesis with concentration*

Plant growth hormone (PGR)	Concentration (mg L^{-1})	Culture
BA	1.0, 1.5, 2.0, 2.5	Shoot induction, multiplication, root and tuber formation
IBA	0.5, 1.0, 1.5, 2.0	Shoot induction, multiplication, root and tuber formation
IAA	0.5, 1.0, 1.5, 2.0	Callus induction
NAA	0.5, 1.0, 1.5, 2.0	Callus, shoot, root and tuber induction
2, 4- D	0.5, 1.0, 1.5, 2.0	Callus induction
TDZ	0.5, 1.0, 1.5, 2.0	Shoot induction
KN	0.25, 0.5, 1, 2.5, 5.0	Tuber formation

5.2.1.11 Acclimatization or hardening of plants

The well developed *in vitro* plants with shoots and roots were removed from the flasks and were rinsed thoroughly with autoclaved distilled water to eliminate the bits of agar. The washed *in vitro* plantlets were moved to plastic pots comprising vermiculite for primary hardening and were kept in growth chamber with relative humidity 70-80% and temperature 28 ºC. Later they were shifted to clay pots containing soil: sand: manure (1:1:1) for acclimatization and were shifted to green house where they grew under natural photoperiod conditions after two weeks of period.

5.2.2 Genetic fidelity assessment

5.2.2.1 Plant material

Leaf samples were randomly selected from *in vitro* plantlets and naturally grown mother plants (*ex vitro*) for the isolation of genomic DNA to carry out ISSR analyses.

5.2.2.2 Genomic DNA extraction

Cetyl trimethylammonium bromide (CTAB) extraction method was used for the extraction of genomic DNA (Doyle and Doyle, 1990). The procedure is discussed below:

- ♣ 500 mg fresh and young leaves were pulverized in mortar and pestle into fine powder by using liquid nitrogen.
- ♣ 5 ml pre-warmed extraction buffer (Table 5.4) was added to the pulverized material and was shifted to eppendrof tubes.
- ♣ The tubes were incubated in water bath for 1 hour or 45 minutes at 65 ℃ and mixed slightly after every 10 minutes.
- ♣ The tubes with homogenized mixture were then centrifuged at 10,000 rpm for 15 minutes at 4 ℃ to settle down the cell debris.
- ♣ The supernatant was transferred to new tube and equal volume of Chloroform: Isoamyl alcohol (C: I, 24:1 v/v). Samples were mixed and at 10,000 rpm were centrifuged for 10 minutes.
- ♣ The above step (Chloroform: Isoamyl alcohol step) was again repeated and aqueous part was shifted to new eppendrof tubes
- ♣ Equal volume of 70% chilled isopropanol was added in the eppendrof tubes, mixed gently and kept for overnight at -20 ℃.
- ♣ These tubes at 10,000 rpm were centrifuged for 10 minutes to pellet out the DNA.
- ♣ Pellet was rinsed with ethanol (70 %) and at 10,000 rpm was centrifuged for 10 minutes.
- ♣ Supernatant was discarded and pellet was dried and dissolved in 30 µL Milli-Q (MQ).

Table 5.4: *DNA Extraction buffer concentration*

Chemicals	**Required concentration**	**Working concentration (50 ml)**
Tris base (pH 8.0)	1 M	5 ml
EDTA	0.5 M	5 ml
NaCl	5 M	15 ml
CTAB	10 %	15 ml
β-Mercapto ethanol (BME)	4 %	2 ml

5.2.2.3 Purity and quantification of isolated DNA

The purity or quality of isolated genomic DNA was tested in 0.8% agarose gel by electrophoresis and DNA bands were detected in Geldox (BioRad). The quantity of DNA was determined by using UV- spectrophotpmeter.

5.2.2.4 PCR amplification by ISSR primers

Primarily seven ISSR primers (Sigma-Aldrich) were screened for ISSR analysis (Table 5.5) and only five primers revealed reproducible and distinct amplification using PCR (Table 5.13). The PCR was carried out in 20 µL volume of reaction mixture comprising of 1.0 µL of DNA template (50 ng), 0.13 µL primer (15 ng), 2.5 µL of 10x PCR buffer, 1.5 mM $MgCl_2$, 200 µM each dNTPs and 0.5 U Taq DNA polymerase (Bangalore Genei, India). The amplification was executed in a thermal cycler (MultiGene™ Gradient, Labnet International Inc.). The amplification reaction comprised of initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation for 45 sec at 94 °C, annealing at 55 °C foe 30 sec, extension at 72 °C for 2 min with final extension at 72 °C for 10 min. The amplified products were separated by electrophoresis on 1.5 % agarose gel in 1 × TAE buffer (pH 8.0). The gels were visualized and photographed by gel documentation system (Bio-rad, USA). The amplicons size was evaluated by comparing with 100bp DNA ladder (GeneRuler Thermo Scientific, USA). Only those reproducible bands having same migration were considered homologous bands and counted for analysis regardless of their intensity.

Table 5.5: *Sequence details of ISSR primers*

S. No	Primer code	Primer sequence (5′-3′)
1	ISSR 10	TCTCTCTCTCTCTCTCG
2	ISSR 11	ACACACACACACACACT
3	ISSR 12	ACACACACACACACACC
4	ISSR 13	ACACACACACACACACG
5	ISSR 15	ATGATGATGATGATGATG
6	ISSR 17	GGAGAGGAGAGGAGA
7	ISSR 20	AGAGAGAGAGAGAGAGCT

5.2.3 Biochemical fidelity assessment

The biochemical fidelity was assessed on the basis of diosgenin content present in the samples of *in vitro* plants and *ex vitro* mother plant.

5.2.3.1 Plant sample preparation, extraction and HPTLC instrumentation

The *in vitro* and *ex vitro* grown plants were separated into stem, leaves and roots and under tap water were rinsed carefully to eradicate the impurities. The plant sample preparation, extraction and HPTLC instrumentation has been discussed in Chapter 3 (Section 3.2.4; 3.2.5.3).

5.2.4 Statistical analysis

The trials were achieved in triplicates with minimum 12 explants per treatment. Observations were recorded and data was analysed by means of SPSS 16.0 software (SPSS Inc., Chicago, USA) and mean values are expressed as mean ± SE and significance level at 5 % for each trial was done by Duncan's Multiple Range Test (DMRT).

5.3 Result and Discussion

5.3.1 *In vitro* Micropropagation of *D. deltoidea*

In this study, different explant such as leaf, nodes, internodes and tubers were used to establish effective protocol for the *in vitro* micropropagation of *D. deltoidea*. The explants tried on MS media fortified with different concentration of plant hormones responded differently and the media without any plant hormone was used as control. The influence of various plant hormones on callus formation, shoot initiation, shoot multiplication and root formation is as follows:

5.3.1.1 Surface sterilization

The different explants were sterilized with many surfactants for proper sterilization of explants to standardize the surface sterilization protocol for the Micropropagation of *D. deltoidea*. In the surface sterilization, six different treatment processes were tried (Treatment-1, 2, 3, 4, 5 and 6). Results were recorded on the basis of contamination, survival and necrotic percentage.

In treatment-1, only $HgCl_2$ was applied to sterilize the explants for 5 min and results showed that contamination was moderate, necrotic and survival rate was found less (Table 5.6). Treatment-2 in which NaOCl 2% for 5 min was used showed maximum contamination

89.30% among all treatments with 12.26 ± 0.20 necrotic percentage and survival rate 5.73 ± 0.15%. The combination of NaOCl for 2 min and $HgCl_2$ for 3 min in Treatment-3 showed less contamination percentage 8.50 ± 0.20, less necrotic rate 3.60 ± 0.20% and good survival percentage 91.40 ± 0.30. In Treatment-4, sterilization of explants with NaOCl (3 min) and $HgCl_2$ for 4 min showed 11.30 ± 0.20 of contamination percentage with the necrosis 5.50 ± 0.20% and 71.36 ± 0.30% survival rate. In Treatment-5 explants were first sterilized with NaOCl for 2 min, $HgCl_2$ for 3 min and ethanol for 2 min showed the percentage of 58.43 ± 0.25% survival of explants with necrotic percentage 7.56 ± 0.20 and contamination percentage 17.56 ± 0.25. In Treatment-6, explants were sterilized with NaOCl for 3 min followed by $HgCl_2$ for 4 min and ethanol for 3 min showed 21.73 ± 0.15 of contamination percentage with the necrosis 9.73 ± 0.15% and 47.33 ± 0.25% survival rate. Among all the treatments, Treatment-3 showed maximum survival rate with less contamination and necrosis rate. The results of all treatments were represented in Table 5.6 and Figure 5.1. On the basis of less percentage of contamination and necrosis and high survival rate of explants, Treatment-3 was found most suitable protocol for the surface sterilization of explants of *D. deltoidea*.

Many researchers have used $HgCl_2$ for the surface sterilization of explants at various time exposures to acquire maximum aseptic culture for different species. Chen et al. (2007) surface sterilized tender stem cuttings of *D. nipponica* with 75% alcohol for 30-60s and 0.1% $HgCl_2$ for 3-5 min. Chu et al. (2002) used 80% ethanol for 2 min, 50% NaOCl for 30 min and 1.0% $HgCl_2$ for 1 min in the case of *Dioscorea* species. Shah and Lele (2012) surface sterilized leaf and nodal cuttings of *D. alata* with 70% ethyl alcohol for 30s to 1 min and 4% NaOCl for 1 to 5 min to establish aseptic culture. Guo et al. (2006) used 70% ethanol for 30s and 5.4% NaOCl for 20 min for surface sterilization of *Saussurea involucrata* seeds. In *Andrographis echioides,* 0.1% $HgCl_2$ for 2 min was used for surface sterilization (Savitikadi et al., 2020) and in *D. deltoidea*, 0.1% $HgCl_2$ for 3 min was used to sterilize nodal segments (Nazir et al., 2020). In many Zingiberaceae species, bleach, ethanol and $HgCl_2$ as effective surface sterilizing agents have been used (Shirgurkar et al., 2001; Salvi et al., 2002; Yusuf et al., 2007).

Table 5.6: *Effect of surface sterilization processes*

Sterilization process	Contamination of explants (%)	Necrotic of explants (%)	Survival of explants (%)
Treament-1	38.46 ± 0.30^{b}	12.26 ± 0.20^{a}	61.73 ± 0.15^{c}
Treament-2	89.30 ± 0.20^{a}	10.50 ± 0.20^{a}	3.63 ± 0.25^{e}
Treament-3	8.50 ± 0.20^{a}	3.60 ± 0.20^{e}	91.40 ± 0.30^{a}
Treament-4	11.30 ± 0.20^{cd}	5.50 ± 0.20^{d}	71.36 ± 0.30^{b}
Treament-5	17.56 ± 0.25^{c}	7.56 ± 0.20^{c}	58.43 ± 0.25^{d}
Treament-6	21.73 ± 0.15^{e}	9.73 ± 0.15^{bc}	47.33 ± 0.25^{de}

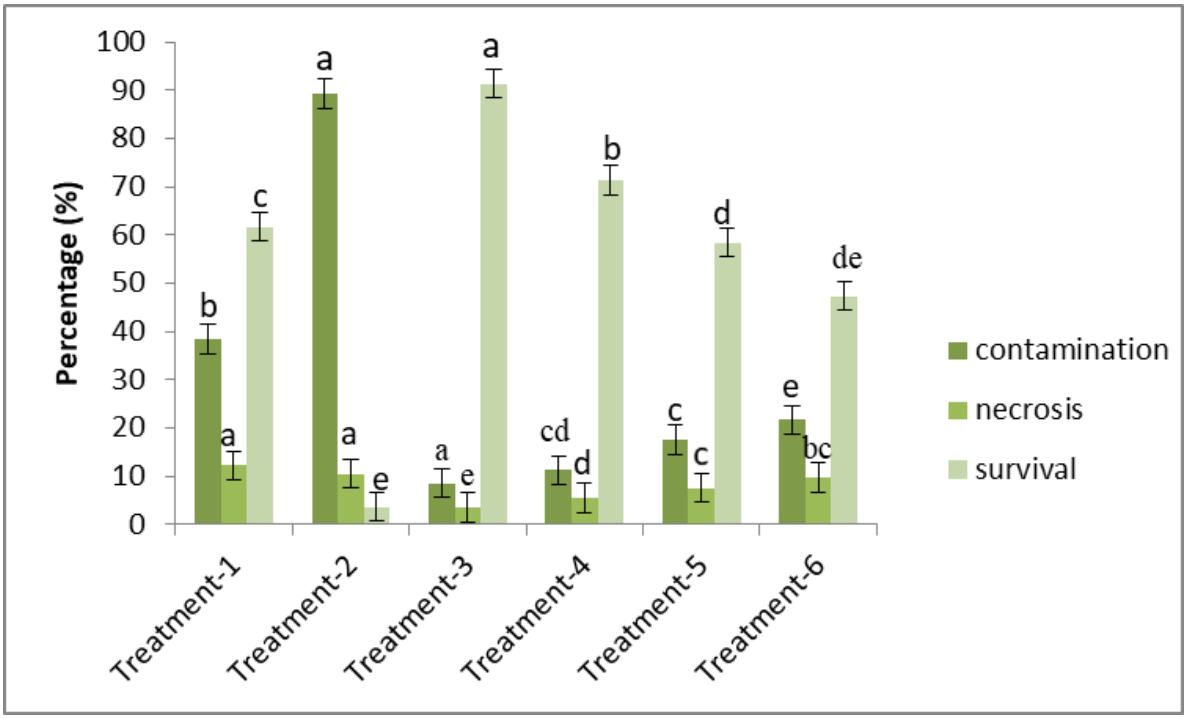


Figure 5.1: *Effect of surface sterilization processes*

5.3.1.2 Callus induction

The tuber explants excised from mother plant transplanted in CSIR-IIIM green house were inoculated on MS media fortified with 2, 4- D, BAP, IBA, IAA and NAA alone or in combinations in a concentration range of 0.5 – 2.0 mg L^{-l} individually. Among all explants used only tuber explants showed callus production. Only plant growth hormones 2, 4- D and NAA induced callus production. Morphogenic responses such as response of callus culture

percentage, nature of callus, and amount of callus were significantly influenced by both PGRs. When the concentration of 2, 4- D and NAA increased, response and callus amount also increased and reached maximum with 70% culture response in less number of days (24) when 2, 4- D was added at a 1.5 mg L^{-1} concentration (Table 5.7). Likewise 60% culture response in 29 days was obtained when NAA was added at 1.5 mg L^{-1} concentration. Further increase in concentration of both PGRs showed decline in the culture response and callus amount. Callus was also obtained at 1 mg L^{-1}, 2 mg L^{-1} concentration of 2, 4- D and NAA but in more days and with less culture response, however no callus initiation was perceived at 0.5 mg L^{-1} concentration of NAA and 2, 4- D. The callus produced by both PGRs was fragile in nature and yellow in colour (Plate 5.1).

Our outcomes are in contract with that of Kumar et al. (2017) who obtained yellow fragile callus initiation from nodal segments of *D. deltoidea* on MS media fortified with 2, 4- D. In another study led by Chen et al. (2003), NAA along with BA induced callus within 30 days in *D. zingiberensis*. Shu et al. (2005) induced callus on MS media augmented with BA and NAA though observed best callus induction in MS fortified with BA and 2, 4- D from stem explants of *D. zingiberensis*. Similar results were also obtained from hypocotyl of *D. deltoidea* by Grewal and Atal (1976) but instead of MS media, they used RT media.

Table 5.7: *Effect of NAA and 2, 4- D on callus production from tuber explant*

MS + PGR (mg L^{-1})	Response of Callus culture (%)	Days required for callus induction	Amount of callus	Nature of callus
2, 4- D				
0.5	0	-	-	-
1.0	40	32	+	Yellow green, Fragile
1.5	70	24	+++	Yellow green, Fragile
2.0	50	29	++	Yellow green, Fragile
NAA				
0.5	0	-	-	-
1.0	20	37	+	Creamy, Fragile

1.5	60	29	++	Creamy, Fragile
2.0	40	31	++	Creamy, Fragile

*- no callus: + low callus: ++ moderate callus: +++ high callus

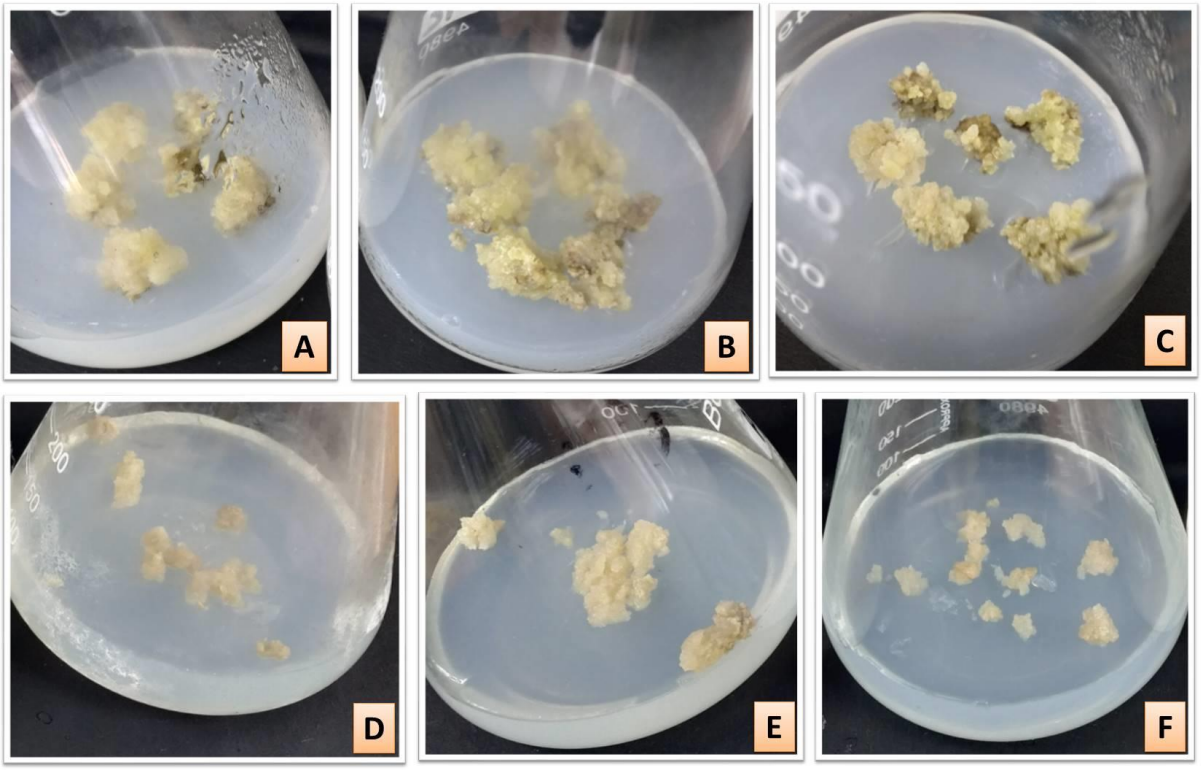


Plate 5.1: *Callus production from tuber explant on MS media comprising:*

A. 2, 4- D (1.0 mg L^{-1}) **B.** 2, 4- D (1.5 mg L^{-1}) **C.** 2, 4- D (2.0 mg L^{-1})

D. NAA (1.0 mg L^{-1}) **E.** NAA (1.5 mg L^{-1}) **F.** NAA (2.0 mg L^{-1})

5.3.1.3 Direct organogenesis

The nodal segments collected from healthy plant of *D. deltoidea* were surface sterilized and resized and then inoculated onto MS media augmented with various concentrations of BA either individually or in combinations with IBA, NAA and TDZ in order to initiate the shoots directly (Plate-5.2). The cultures were maintained in the culture room after inoculation and data were recorded with reference to percentage of culture response, number of shoots/explant and average shoot length.

5.3.1.3.1 Shoot initiation and regeneration

The culture media fortified with various concentrations of BA, IBA, NAA and TDZ individually or in combinations were studied in order to attain initiation of shoots, and all the PGRs have initiated shoots, but with varied shoot numbers ranging from 1.10 ± 0.55 to

3.86 ± 0.75 with an average length of shoots ranging from 2.13 ± 0.70 to 4.96 ± 0.70 cm (Table 5.8). Shoot initiation was found to be greatly influenced by the addition of BA, IBA and TDZ in various concentrations to the MS media. However MS media fortified with NAA in combination with BA showed poor shoot initiation. MS media devoid of any hormone (control) failed to initiate any shoot induction in the explants even after the cultures were maintained beyond the normal observation period (Figure 5.2).

MS media augmented with BA individually at different concentrations showed varied culture response percentage ranging from 46.43 ± 0.20 to 93.23 ± 0.15% with number of shoots ranging from 1.10 ± 0.55 to 3.20 ± 0.55 having average shoot length ranging from 5.20 ± 0.65 to 2.20 ± 0.70 cm. BA at 2.0 mg L^{-1} was effectual in producing 3.20 ± 0.55 shoots in number with 5.20 ± 0.65 cm shoot length in 93.23% cultures. The combination of BA and IBA in the MS media have produced 1.30 ± 0.55 to 3.86 ± 0.75 number of shoots with 2.13 ± 0.70 to 4.96 ± 0.70 cm shoot length respectively (plate 5.2). When media was augmented with BA at concentration of 2.0 mg L^{-1} and IBA at 1.0 mg L^{-1}, 3.86 ± 0.75 mean number of shoots with 4.96 ± 0.70 cm average shoot length was regenerated in 91.33% cultures, whereas 1.30 ± 0.55 mean number of shoots with 2.23 ± 0.70 cm average length of shoots was produced in 45.23% cultures on MS media enriched with BA (1.0 mg L^{-1}) and IBA (2.0 mg L^{-1}). Media augmented with different concentrations of NAA showed poor shoot induction with 1.12 ± 0.01 to 1.77 ± 0.01 mean number of shoots having 2.13 ± 0.01 to 2.96 ± 0.01 mean shoot length in 32.50% to 43.30% cultures. Same concentration of BA and NAA (2 mg L^{-1}) regenerated less shoots numbers 1.10 ± 0.02 with shoot length 2.13 ± 0.01 cm in only 32.50% of cultures (Plate 5.2: X). When TDZ was used in the media in different concentrations, shoot induction was observed at all concentrations (Table 5.8). 3.13 ± 0.02 mean number of shoots were regenerated on media containing 2.0 mg L^{-1} BA and 1.0 mg L^{-1} TDZ with 4.23 ± 0.02 cm of average shoot length in 84.33% cultures, whereas, at concentration 2.0 mg L^{-1} BA and TDZ only 1.46 ± 0.01 number of shoots were produced in 54.26% cultures with 3.13 ± 0.02 cm average shoot length.

In all combinations or treatments of PGRs adventitious shoots were regenerated directly from the nodal explants deprived of callus initiation, as in callus tissues more chances of somaclonal variations can occur. This protocol for direct regeneration of shoots moreover accounts for the fast regeneration of plants. In many therapeutic plants direct regeneration has been carried out by various researchers (Pandey et al., 2020; Savitikadi et al., 2020). Our results are in accordance with Kadota and Niimi (2004) and Tyub et al. (2007) that attained

shoot formation from *D. japonica* shoot tips and *Lavandula officinalis* on media augmented with BAP. Safdari et al. (2010) and Babu et al. (2018) too obtained direct shoot regeneration in *'Portulaca grandiflora'*L. and finger millet on MS media amended with BAP. In various earlier reports BAP along with NAA has been used commonly for direct shoot initiation (Košir et al., 2004; Sattar et al., 2016; Enyew and Feyissa, 2019). BAP in combination with TDZ was also used in many medicinal plants to obtain direct regeneration of shoots. Bhusare et al. (2018) used BAP, KN and TDZ for direct shoot regeneration from leaf and petiole explants of *Digitalis lanata* Ehrh. and found best shoot regeneration on media amended with TDZ. Kumar et al. (2017) achieved shoot regeneration on media fortified with BAP in combination with IBA, NAA and TDZ from nodal segments of *D. deltoidea* and combination of BAP and IBA induced maximum regeneration. Hesami et al. (2019) studied direct organogenesis in *Ficus religiosa* and obtained highest shoot initiation in media augmented with BAP at 1.5 mg L^{-1} of concentration and 0.15 mg L^{-1} IBA with 6.26 and 10.13 mean number of shoots/explant. Mokhtari et al. (2016) achieved maximum direct shoot regeneration on MS media containing BAP (3 or 4 mg L^{-1}) combined with IBA (0.5 mg L^{-1}) from crown explants of *Viola odorata*. Maherswari et al. (2012) used BAP, NAA, KN and IBA for direct shoot initiation in *D. oppositifolia* L. and obtained maximum shoot initiation in media augmented with BAP and IBA.

Table 5.8: *Effect of plant growth hormones on in vitro shoot formation from nodal segments of D. deltoidea after 4 weeks of culture*

MS + growth regulators (mg L^{-1})				% culture response	Shoots/explant	Shoot length (cm)
BA	**IBA**	**NAA**	**TDZ**			
0	0	0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.0	0	-	-	46.43 ± 0.20^{i}	1.10 ± 0.55^{l}	2.20 ± 0.70^{j,k}
1.0	0.5	-	-	49.70 ± 0.20^{h}	1.40 ± 0.55^{h}	2.46 ± 0.75^{g,h}
1.0	1.0	-	-	52.36 ± 0.15^{g}	2.23 ± 0.55^{c}	3.30 ± 0.60^{c,d}
1.0	1.5	-	-	48.53 ± 0.25^{h}	1.73 ± 0.60^{f,g}	3.00 ± 0.75^{e}
1.0	2.0	-	-	45.23 ± 0.15^{i}	1.30 ± 0.55^{i,j}	2.23 ± 0.70^{i,j}
1.5	0	-	-	67.26 ± 0.20^{f}	2.06 ± 0.55^{d}	2.96 ± 0.70^{e,f}
1.5	0.5	-	-	51.43 ± 0.20^{g}	2.76 ± 0.35^{b}	2.76 ± 0.70^{f}

1.5	1.0	-	-	58.46 ± 0.20^{fg}	$1.86 \pm 1.16^{e,f}$	3.90 ± 0.75^{b}
1.5	1.5	-	-	47.26 ± 0.15^{hi}	$2.43 \pm 0.50^{b,c}$	$3.76 \pm 0.61^{b,c}$
1.5	2.0	-	-	44.60 ± 0.20^{ij}	1.60 ± 0.50^{g}	$2.23 \pm 0.55^{i,j}$
2.0	0	-	-	93.23 ± 0.15^{a}	$3.20 \pm 0.55^{a,b}$	5.20 ± 0.65^{a}
2.0	0.5	-	-	88.30 ± 0.26^{b}	3.80 ± 0.55^{a}	$4.40 \pm 0.55^{a,b}$
2.0	1.0	-	-	91.33 ± 0.15^{a}	3.86 ± 0.75^{a}	4.96 ± 0.70^{a}
2.0	1.5	-	-	86.40 ± 0.10^{bc}	2.76 ± 0.65^{b}	$4.30 \pm 0.65^{a,b}$
2.0	2.0	-	-	71.46 ± 0.25^{ef}	1.90 ± 1.08^{e}	3.63 ± 0.55^{c}
2.0	-	0.5	-	43.30 ± 0.20^{ij}	$1.37 \pm 0.01^{h,i}$	2.27 ± 0.02^{i}
2.0	-	1.0	-	42.33 ± 0.15^{k}	$1.77 \pm 0.01^{f,g}$	$2.96 \pm 0.01^{e,f}$
2.0	-	1.5	-	39.60 ± 0.20^{kl}	$1.19 \pm 0.01^{j,k}$	2.73 ± 0.01^{f}
2.0	-	2.0	-	32.50 ± 0.20^{l}	1.10 ± 0.02^{l}	$2.13 \pm 0.01^{k,l}$
2.0	-	-	0.5	77.56 ± 0.20^{d}	2.53 ± 0.01^{b}	3.67 ± 0.01^{c}
2.0	-	-	1.0	84.33 ± 0.15^{cd}	$3.13 \pm 0.02^{a,b}$	$4.23 \pm 0.02^{a,b}$
2.0	-	-	1.5	70.60 ± 0.20^{e}	$2.45 \pm 0.01^{b,c}$	3.92 ± 0.01^{b}
2.0	-	-	2.0	54.26 ± 0.15^{g}	1.46 ± 0.01^{h}	3.13 ± 0.02^{d}
2.5	0	-	-	61.46 ± 0.20^{fg}	$2.16 \pm 0.50^{c,d}$	2.66 ± 0.45^{g}
2.5	0.5	-	-	71.33 ± 0.05^{e}	2.30 ± 0.72^{c}	$3.30 \pm 0.55^{c,d}$
2.5	1.0	-	-	67.10 ± 0.10^{f}	2.10 ± 0.65^{d}	$2.80 \pm 0.60^{e,f}$
2.5	1.5	-	-	53.33 ± 0.25^{gh}	1.83 ± 0.75^{f}	2.40 ± 0.61^{h}
2.5	2.0	-	-	48.73 ± 0.15^{h}	$1.36 \pm 0.55^{h,i}$	$2.13 \pm 0.70^{k.l}$

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

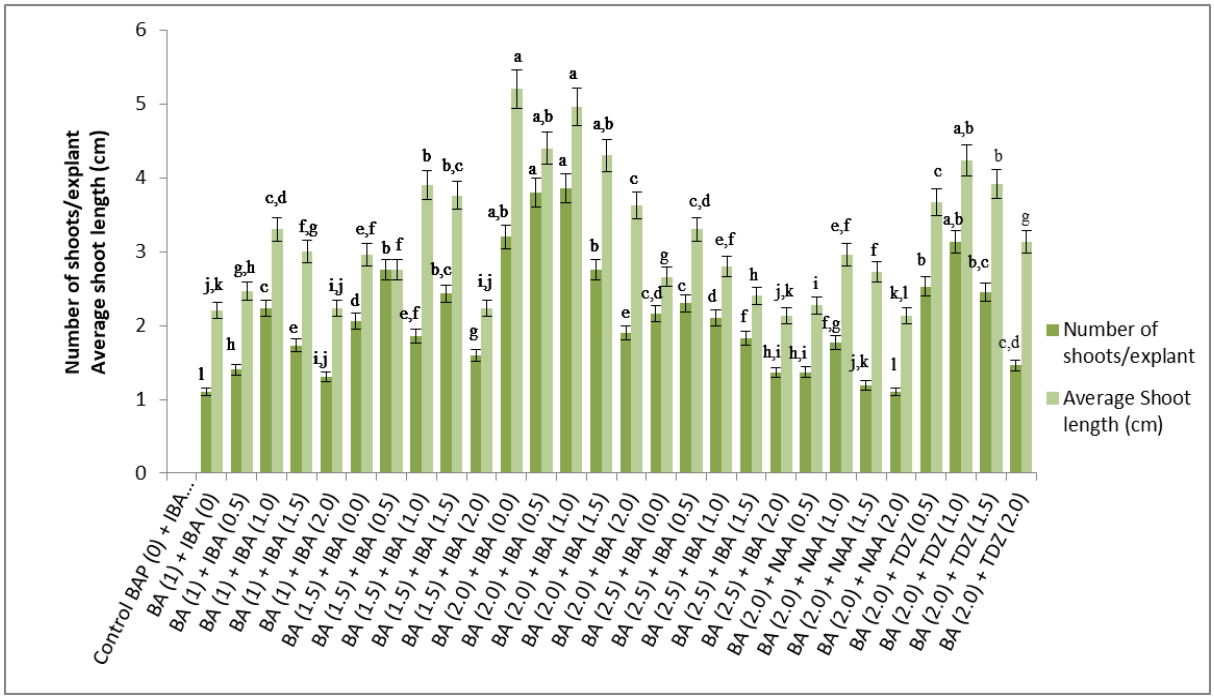
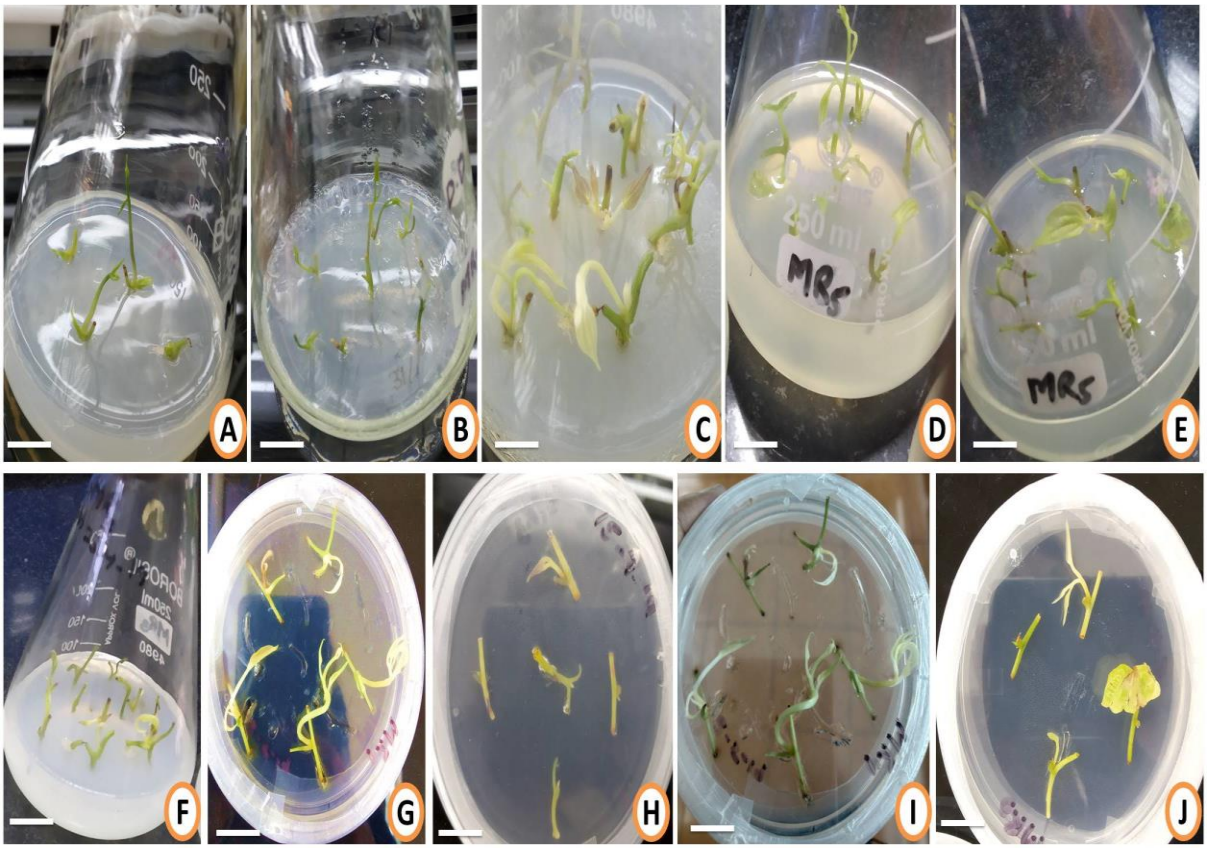


Figure 5.2: *Effect of different growth regulators on shoot induction*



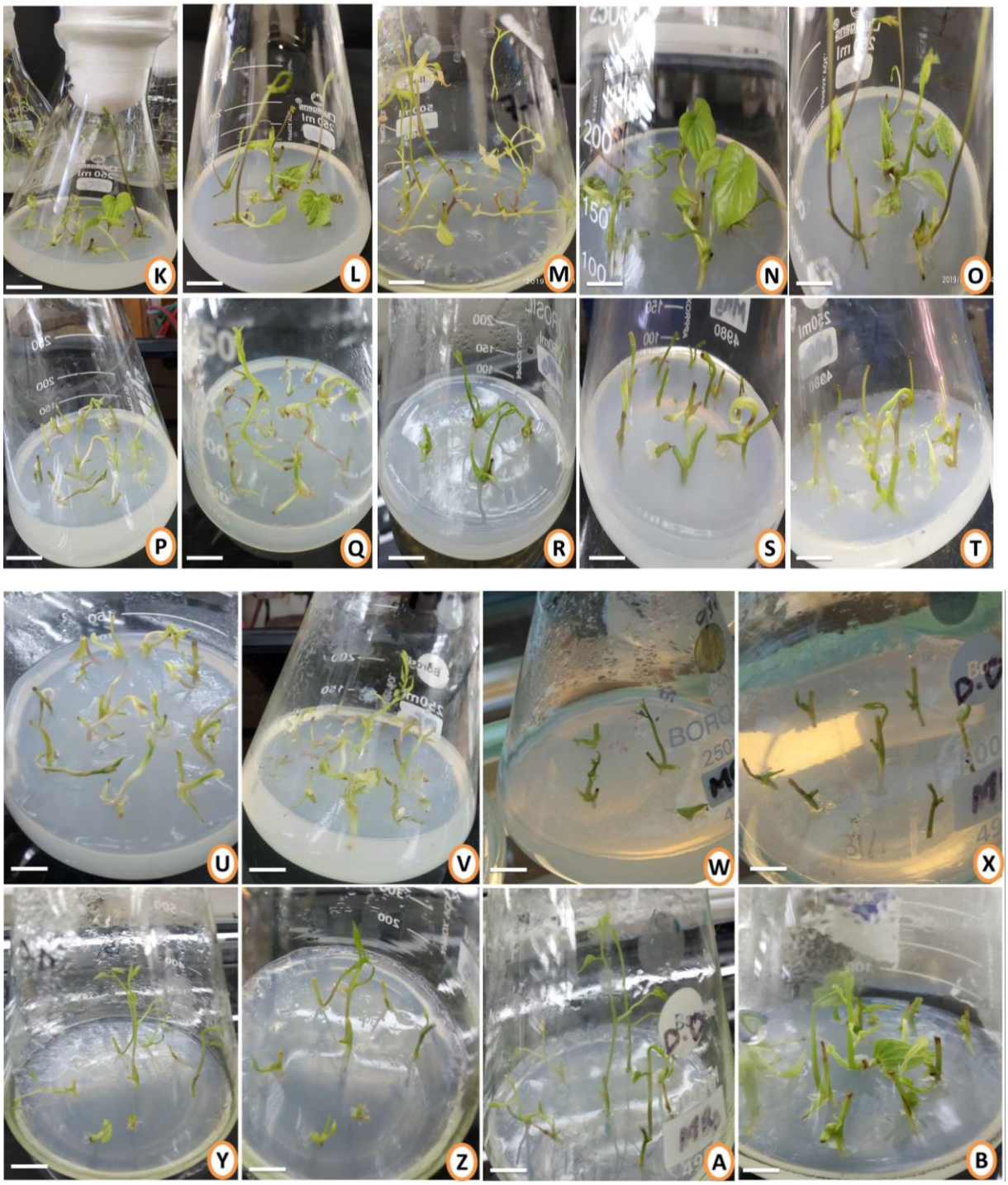


Plate 5.2: *Effect of different growth regulators on shoot induction*

(A) *Media fortified with BA 1.0 mg* L^{-1}

(B-E) *Media fortified with BA 1.0 mg* L^{-1} *+IBA 0.5 – 2.0 mg* L^{-1}

(F) *Media fortified with BA 1.5 mg* L^{-1}

(G-J) *Media fortified with BA 1.5 mg* L^{-1} *+IBA 0.5- 2.0 mg* L^{-1}

(K) *Media fortified with BA 2.0 mg* L^{-1}

(L-O) *Media fortified with BA 2.0 mg* L^{-1} *+IBA 0.5 mg* L^{-1}

(P) *Media fortified with BA 2.5 mg L^{-1}*

(Q-T) *Media fortified with BA 2.5 mg L^{-1}+IBA 0.5- 2.0 mg L^{-1}*

(U-X) *Media fortified with BA 2.0 mg L^{-1}+NAA 0.5- 2.0 mg L^{-1}*

(Y-B) *Media fortified with BA 2.0 mg L^{-1}+TDZ 0.5 – 2.0 mg L^{-1}*

5.3.1.3.2 Shoot multiplication

The initiated shoots after initiation stage were transferred on the different media consisting of solid MS media (with agar) and liquid MS media (without agar) augmented with various BA and IBA concentrations for further multiplication. After subculture in multiplication media, data was recorded in respect of percentage of multiplication response, number of shoots/explant and the average shoot length after 4 weeks of culture (Table 5.8 and 5.9).

5.3.1.3.2.1 Effect of BA and IBA on shoot multiplication

Solid MS media was augmented with the combination of BA in a concentration range of 1.0 – 2.5 mg L^{-1} and IBA in the range of (0.5 – 2.0 mg L^{-1}) (Plate 5.3). At all BA and IBA combinations shoot multiplication was observed and the percentage of response culture ranges from 32.60 ± 0.20 to 100.26 ± 0.15 and the shoot numbers ranges from 4.04 ± 0.01 to 11.86 ± 0.01 with an average length of shoot 2.94 ± 0.01 to 5.96 ± 0.02 cm (Table 5.9; Figure 5.3). The MS media fortified with 1.0 mg L^{-1} BA and IBA (0.5 – 2.0 mg L^{-1}) regenerated 4.04 ± 0.01 to 6.75 ± 0.02 number of shoots/explant with 2.94 ± 0.01 to 4.44 ± 0.02 shoot length cm. BA at 1.0 mg L^{-1} and IBA at 2.0 mg L^{-1} showed poor shoot multiplication. The percentage of shoot multiplication 48.30 ± 0.20 to 88.73 ± 0.15 was noticed on media augmented with 1.5 mg L^{-1} BA and IBA (0.5 – 2.0 mg L^{-1}) and produced 4.13 ± 0.02 to 7.76 ± 0.01 mean number of shoots/explant with 3.25 ± 0.02 to 4.76 ± 0.01 cm average shoot length (Figure 5.3). MS media amended with 2 mg L^{-1} BA and 1.0 mg L^{-1} IBA showed maximum shoot multiplication response 100.26 ± 0.15% and produced maximum mean number of shoots/explant 11.86 ± 0.01 with 5.96 ± 0.02 cm average shoot length (Plate 5.2: J). 9.83 ± 0.02 mean number of shoots with 5.43 ± 0.01 cm shoot length was observed on media comprising 2.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA. BA at 2.0 mg L^{-1} showed good response as compared to other concentrations. MS media fortified with BA (2.5 mg L^{-1}) and IBA (0.5 – 2.0 mg L^{-1}) produced 5.37 ± 0.01 to 6.44 ± 0.01 mean number of shoots with 3.13 ± 0.02 to 4.82 ± 0.01 cm average shoot length and the percentage of response was 36.60 ± 0.10 to 53.66 ± 0.15% (Plate 5.3: M). Among different BA and IBA

combinations, BA (2.0 mg L^{-1}) and low concentrations of IBA (0.5 mg L^{-1} and 1.0 mg L^{-1}) showed good shoot multiplication.

Liquid MS media was fortified with different combinations of BA (1.0 – 2.5 mg L^{-1}) and IBA (0.5 – 2.0 mg L^{-1}). Shoot multiplication was observed in all the BA and IBA combinations and the percentage of response culture ranges from 42.46 ± 0.02 to 100.33 ± 0.02% and the shoot numbers ranges from 4.30 ± 0.20 to 13.50 ± 0.17 with an average length of 3.33 ± 0.25 to 7.53 ± 0.15 cm (Table 5.10). When BA was 1.0 mg L^{-1} in combination with IBA (0.5 – 2.0 mg L^{-1}) on the MS media, 54.25 ± 0.02 to 65.38 ± 0.02% of cultures were responded with 5.43 ± 0.35 to 6.73 ± 0.20 mean number of shoots/explant were produced having average shoot length 3.33 ± 0.25 to 4.46 ± 0.23 cm. MS media augmented with combination of BA 1.5 mg L^{-1} and IBA (0.5 – 2.0 mg L^{-1}) produced 4.30 ± 0.20 to 7.53 ± 0.25 mean number of shoots/explant with 3.50 ± 0.20 to 6.63 ± 0.15 cm average shoot length (Figure 5.4). The response percentage of shoot multiplication in this media was in the range of 48.30 ± 0.20 to 89.42 ± 0.01%. MS media fortified with BA 2.0 mg L^{-1} in combination with various IBA concentrations regenerated 4.50 ± 0.36 to 13.50 ± 0.17 mean number of shoots with 4.26 ± 0.20 to 7.53 ± 0.15 cm average shoot length (Plate 5.4). The best percentage of culture responses 100.33 ± 0.02% were recorded in media containing BA 2.0 mg L^{-1} and IBA 1.0 mg L^{-1} and produced highest number of shoots/explant 13.50 ± 0.17 with 7.53 ± 0.15 cm shoot length (Plate 5.4: J). This combination was considered the optimum PGR combination for shoot multiplication in *D. deltoidea* among all treatments. When concentration of BA was increased to 2.5 mg L^{-1} and used in combination with IBA there was decline in the number of shoots and percentage of response. The response percentage was recorded as 43.76 ± 0.03 to 52.35 ± 0.03% and shoot numbers per explant as in the range of 5.52 ± 0.30 to 6.62 ± 0.36 with 3.36 ± 0.25 to 4.72 ± 0.20 cm average shoot length.

In earlier studies, role of auxins in combination with cytokinins has been reported in several species of plants (Xu et al., 2008; Khan et al., 2011; Singh et al., 2014; Revathi et al., 2020). Our outcomes accord with the results of Kumar et al. (2017) who recorded 17.33 ± 1.45 mean number of shoots/explant with 5.50 ± 0.29 mean shoot length on the solid MS media amended with BAP (1.5 mg L^{-1}) and IBA (0.5 mg L^{-1}). In another study, Sen and Sharma (1991) recorded maximum numbers of multiple shoots per explant on media fortified with 4.4 μM BA and 2.5 μM IBA. Furmanowa et al. (1985) obtained 120 number of shoots/explant on the Nitsch and Nitsch medium (Nitsch and Nitsch 1969) augmented with combination of BA and IBA. Piatczak et al. (2005) used liquid MS media for the shoot

multiplication of *Centaurium erythraea* and obtained 60 microshoots within 4 weeks but IAA was used instead of IBA. Mazri (2015) cultured shoot tip explants of *Phoenix dactylifera* L. in both liquid and semi-solid half MS media amended with different plant hormones and observed best shoot multiplication in semi-solid half MS media fortified with 3 µM IBA and 3 µM BAP. Loc et al. (2005) attained 5.6 shoots per explant of *Curcuma zedoaria* in multiplication media fortified with 13.31 µM L^{-1} BAP and 2.85 µM L^{-1} IBA. Brassard et al. (1996) obtained shoot induction and multiplication of *Larix x eurolepis* Henry on the Schenk and Hildebrandt (SH) medium amended with 5 µM BA and 1 µM IBA. In *in vitro* shoot multiplication of *Ficus anastasia*, a mean number of 20 lateral branches/stem was obtained in multiplication media augmented with 8 mg L^{-1} of BA and 1 mg L^{-1} of IBA (Al Malki and Elmeer 2010).

Table 5.9: *Effect of solid MS media containing BA and IBA on in vitro shoot multiplication from nodal segments of D.deltoidea after 4 weeks of culture*

Solid MS + growth regulators (mg L^{-1})		**% response**	**Shoots/explant**	**Shoot length (cm)**
BA	**IBA**			
0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.0	0.5	51.30 ± 0.20^{d,e}	5.43 ± 0.02^{f}	4.44 ± 0.02^{c}
1.0	1.0	55.46 ± 0.15^{d}	6.75 ± 0.02^{c}	3.37 ± 0.02^{f}
1.0	1.5	49.40 ± 0.20^{e}	4.32 ± 0.01^{g}	3.07 ± 0.01^{g,h}
1.0	2.0	44.66 ± 0.15^{f}	4.04 ± 0.01^{h}	2.94 ± 0.01^{h}
1.5	0.5	79.43 ± 0.15^{b,c}	7.76 ± 0.01^{b}	4.76 ± 0.01^{b}
1.5	1.0	88.73 ± 0.15^{b}	6.87 ± 0.01^{c}	3.92 ± 0.02^{d}
1.5	1.5	75.26 ± 0.15^{c}	4.43 ± 0.02^{g}	3.66 ± 0.02^{e}
1.5	2.0	48.30 ± 0.20^{e}	4.13 ± 0.02^{g,h}	3.25 ± 0.02^{f,g}
2.0	0.5	95.40 ± 0.20^{a}	9.83 ± 0.02^{a,b}	5.43 ± 0.01^{a}
2.0	1.0	100.26 ± 0.15^{a}	11.86 ± 0.01^{a}	5.96 ± 0.02^{a}
2.0	1.5	82.50 ± 0.20^{b,c}	5.75 ± 0.01^{e}	4.67 ± 0.01^{b,c}
2.0	2.0	32.60 ± 0.20^{g,h}	5.07 ± 0.02^{f}	3.67 ± 0.02^{e}

2.5	0.5	$46.53 \pm 0.25^{e,f}$	6.27 ± 0.02^{d}	$4.37 \pm 0.01^{c,d}$
2.5	1.0	53.66 ± 0.15^{d}	$6.44 \pm 0.01^{c,d}$	4.82 ± 0.01^{b}
2.5	1.5	48.30 ± 0.20^{e}	5.85 ± 0.02^{e}	3.45 ± 0.02^{f}
2.5	2.0	36.60 ± 0.10^{g}	5.37 ± 0.01^{f}	3.13 ± 0.02^{g}

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

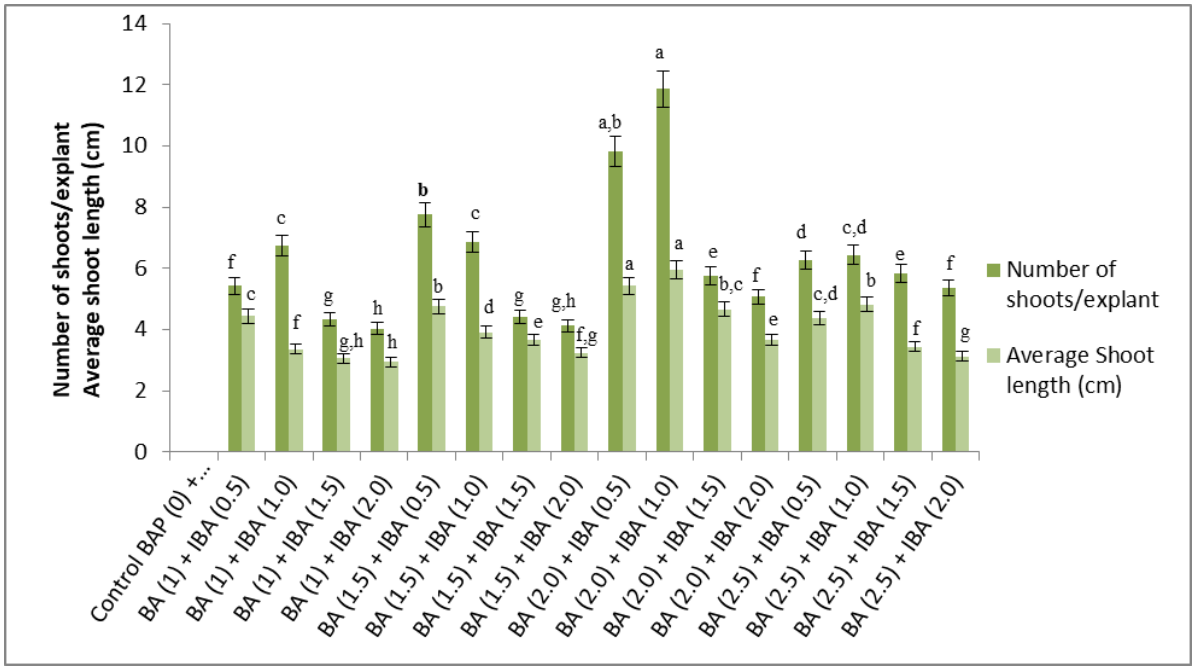


Figure 5.3: *Effect of different plant regulators on shoot multiplication*

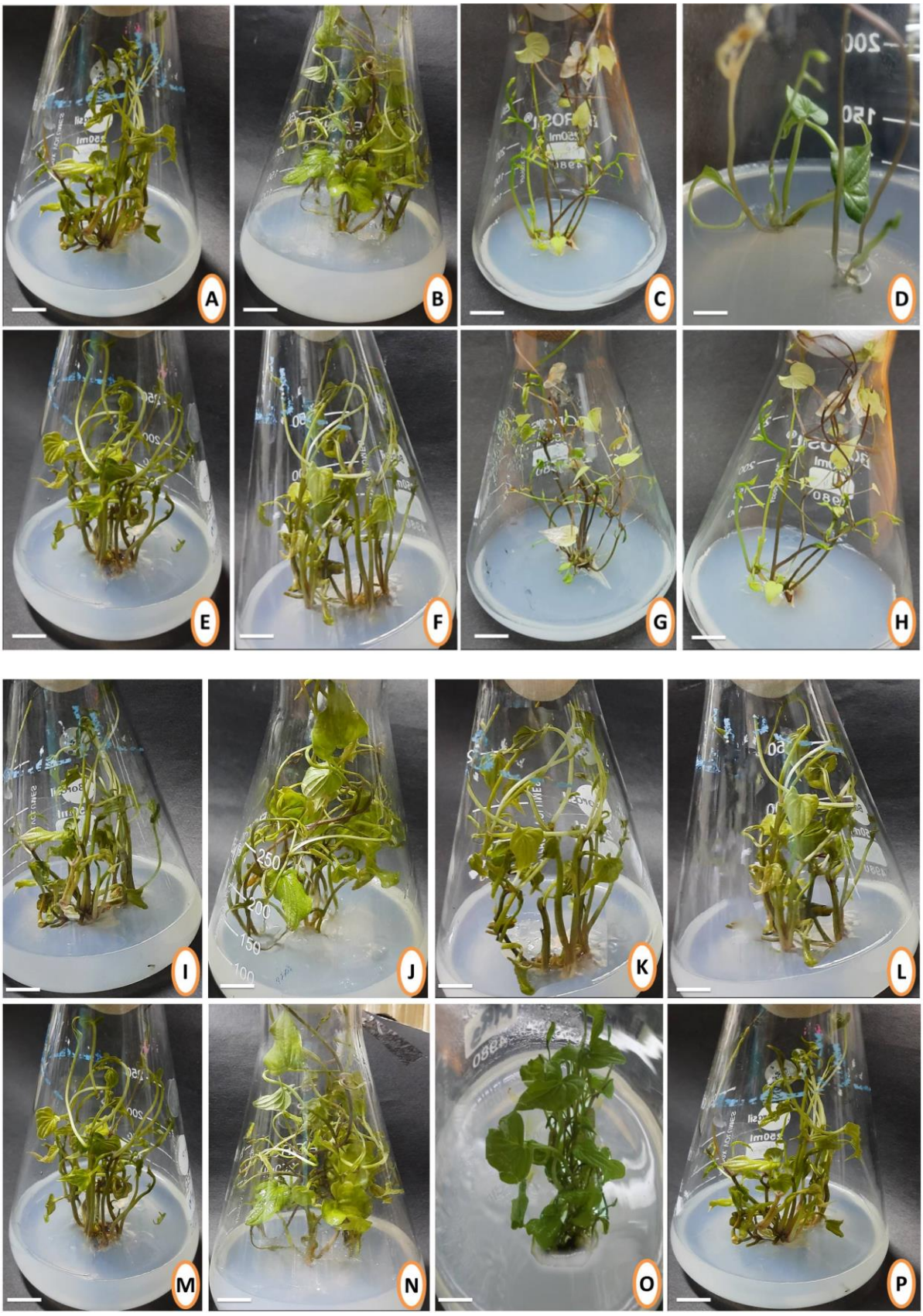


Plate 5.3: *Shoot multiplication on solid MS medium fortified with different combinations of BA and IBA*

(A-D) *Media fortified with BA 1.0 mg L^{-1}+IBA 0.5 – 2.0 mg L^{-1}*

(E-H) *Media fortified with BA 1.5 mg L^{-1}+IBA 0.5 -2.0 mg L^{-1}*
(I-L) *Media fortified with BA 2.0 mg L^{-1}+IBA 0.5- 2.0 mg L^{-1}*
(M-P) *BA 2.5 mg L^{-1}+IBA 0.5- 2.0 mg L^{-1}*

Table 5.10: *Effect of liquid MS media containing BA and IBA on in vitro shoot multiplication from nodal segments of D. deltoidea after 4 weeks of culture*

Liquid MS + growth regulators (mg L^{-1})		% response	Shoots/explant	Shoot length (cm)
BA	**IBA**			
0	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1.0	0.5	61.75 ± 0.03^{d}	6.73 ± 0.20^{d}	4.46 ± 0.23^{d,e}
1.0	1.0	65.38 ± 0.02^{c,d}	6.56 ± 0.20^{e}	3.40 ± 0.10^{g}
1.0	1.5	59.55 ± 0.03^{d,e}	5.66 ± 0.20^{f,g}	3.43 ± 0.35^{f,g}
1.0	2.0	54.25 ± 0.02^{e}	5.43 ± 0.35^{g}	3.33 ± 0.25^{g,h}
1.5	0.5	89.42 ± 0.01^{b}	7.53 ± 0.25^{c}	6.63 ± 0.15^{b}
1.5	1.0	86.46 ± 0.02^{b}	6.66 ± 0.25^{d,e}	5.50 ± 0.10^{c}
1.5	1.5	71.25 ± 0.03^{c}	4.40 ± 0.30^{g,h}	3.66 ± 0.25^{f}
1.5	2.0	58.64 ± 0.02^{d,e}	4.30 ± 0.20^{h}	3.50 ± 0.20^{f}
2.0	0.5	98.33 ± 0.02^{a}	10.43 ± 0.35^{a,b}	7.43 ± 0.15^{a}
2.0	1.0	100.33 ± 0.02^{a}	13.50 ± 0.17^{a}	7.53 ± 0.15^{a}
2.0	1.5	96.43 ± 0.02^{a}	9.63 ± 0.25^{b}	5.53 ± 0.32^{c}
2.0	2.0	42.46 ± 0.02^{h}	4.50 ± 0.36^{g,h}	4.26 ± 0.20^{e}
2.5	0.5	48.34 ± 0.01^{f}	6.50 ± 0.26^{e}	4.53 ± 0.25^{d}
2.5	1.0	49.14 ± 0.03^{f}	6.62 ± 0.36^{d,e}	4.72 ± 0.20^{d}
2.5	1.5	52.35 ± 0.03^{e}	5.89 ± 0.20^{f}	3.75 ± 0.20^{f}
2.5	2.0	43.76 ± 0.03^{g}	5.52 ± 0.30^{g}	3.36 ± 0.25^{h}

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

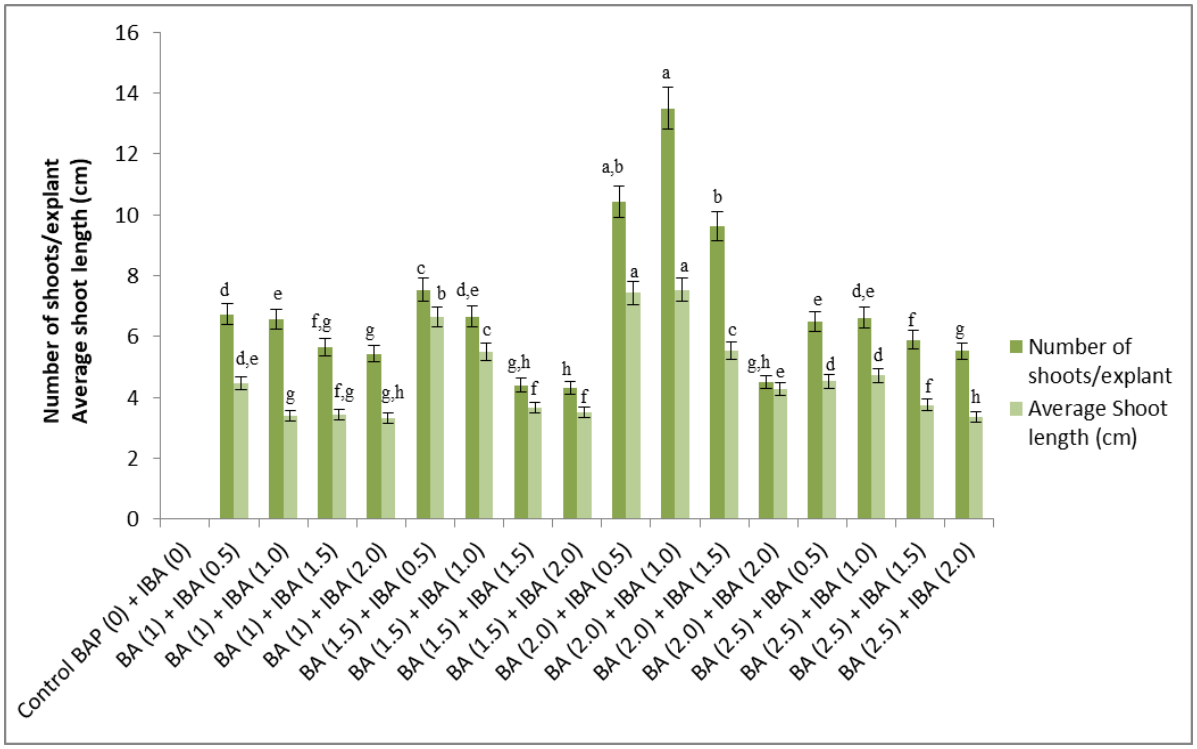


Figure 5.4: *Effect of liquid MS media containing BA and IBA on in vitro shoot multiplication*

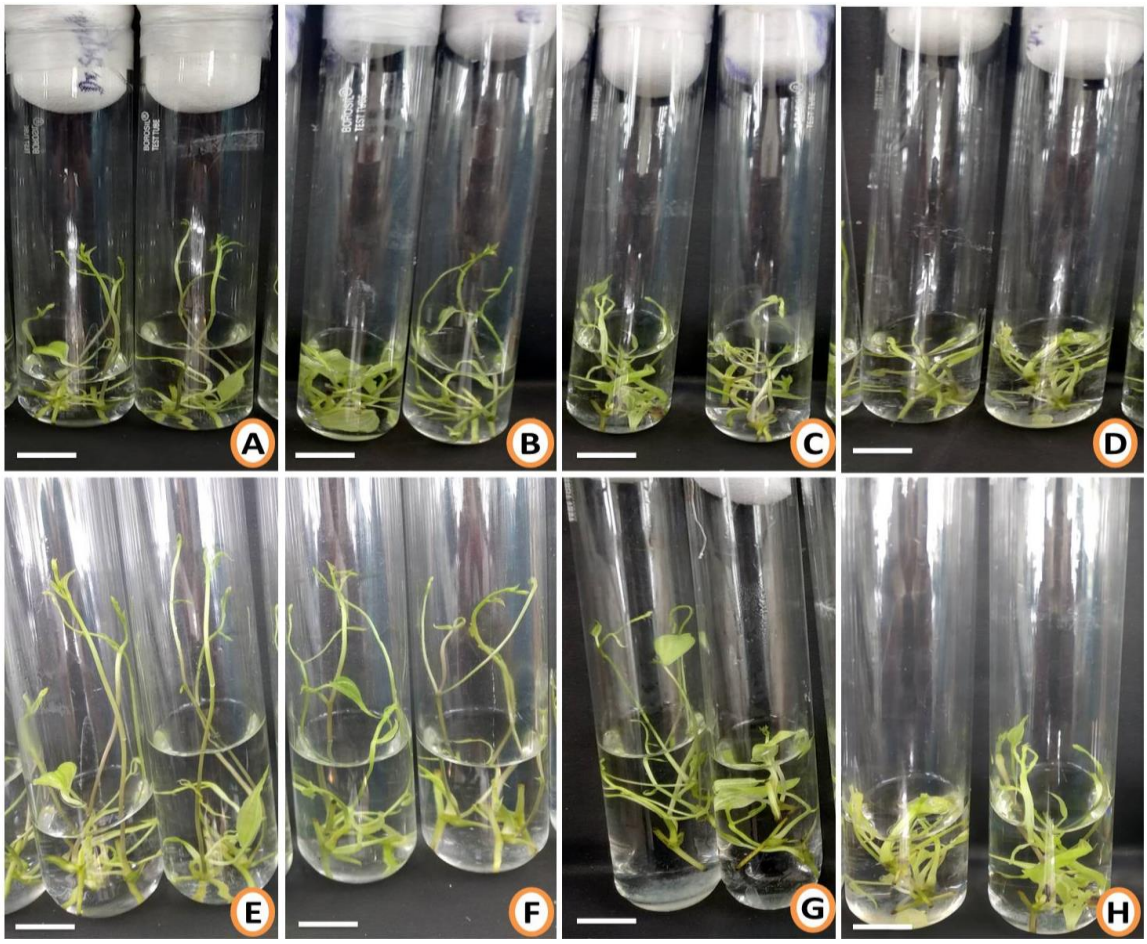


Plate 5.4: *Shoot multiplication on Liquid MS medium.*

(A-D) *Media augmented with BA 1.0 mg L^{-1}+IBA 0.5 – 2.0 mg L^{-1}*

(E-H) *Media augmented with BA 1.5 mg L^{-1}+IBA 0.5 – 2.0 mg L^{-1}*

(I-L) *Media augmented with BA 2.0 mg L^{-1}+IBA 0.5- 2.0 mg L^{-1}*

(M-P) *Media augmented with BA 2.5 mg L^{-1}+IBA 0.5- 2.0 mg L^{-1}*

5.3.1.3.3 Root formation

In Micropropagation, rooting is one of the important stage in terms of root induction for the *in vitro* developed shoots. The well-regenerated shoots were required to be transferred to rooting media for proper development of roots. The potential of plant tissues towards the initiation of adventitious roots relies on the interaction of different endogenous and exogenous factors. The role of auxins (IBA and NAA) and cytokinins (BAP) in root induction is well established and has been studied by (Scout, 1972; Gasper et al, 1996; Gaba, 2005). Rooting of *in vitro* shoots is considered as an important step and also influences the survival rate of plants during acclimatization process (Goncalves et al., 1998). In this study, rooting was simultaneous in liquid shoot multiplication medium containing BA and IBA in combinations. The spontaneous rooting in the multiplication media was due to the effect of IBA. BA alone in media was not able to induce the rooting. High

concentration of BA and low concentration of IBA showed good response of rooting in liquid MS media. Besides that, another MS media fortified with different concentrations of NAA was also used as rooting media. The influence of different rooting media on culture response, number of roots/explant and length of roots is presented in Table 5.11 and Table 5.12. Root induction was detected within 2 weeks of subculture in all combinations tested except PGR free media (Table 5.11; 5.12). Liquid MS media fortified with BA and IBA showed best rooting (Table 5.12; Plate 5.6). Maximum response 99.43% with 7.10 ± 0.20 roots/explant having average root length of 5.33 ± 0.20 cm was observed in liquid MS media amended with BA (2.0 mg L^{-1}) + IBA (1.0 mg L^{-1}). MS medium augmented with BA (1.5 mg L^{-1}) + IBA (0.5 mg L^{-1}) also showed good culture response (88.66%) with 6.70 ± 0.20 mean number of roots having 4.10 ± 0.20 cm average root length (Plate 5.5: E). Moderate culture response (69.30%) was observed in media fortified with 1.5 mg L^{-1} of BA and IBA at 1.0 mg L^{-1} producing 4.30 ± 0.20 mean number of roots having 3.80 ± 0.10 cm average root length (Figure 5.6). Culture response (75.23%) was obtained in media augmented with BA 1.0 mg $L^{-1}$and IBA 0.5 mg L^{-1} producing 4.30 ± 0.20 of roots/explant with 2.66 ± 0.25 cm root length. Minimum culture response 17.56% was observed in media fortified with BA 1.0 mg L^{-1} and IBA 2.0 mg L^{-1} producing 2.23 ± 0.15 mean number of roots with 2.33 ± 0.25 cm root length.

MS media augmented with higher concentration of NAA (2.0 mg L^{-1}) mean number of roots/explant 5.4 ± 0.70 with 4.8 ± 0.70 cm root length was regenerated in 92% cultures within 32 days. NAA at 1.5 mg L^{-1} showed 3.2 ± 0.60 mean number of roots with 3.2 ± 0.70 cm average root length (Figure 5.5). MS media augmented with NAA 1.0 mg L^{-1} showed moderate culture response (57.3%) produced 1.9 ± 0.40 roots/explant having root length 2.9 ± 0.80 cm (Plate 5.5: B). However, NAA at 0.5 mg L^{-1} revealed minimum culture response (38.6%) with 0.5 ± 0.30 mean number of roots/explant having 1.9 ± 0.40 cm root length cm.

In the literature, NAA was used for rooting of *in vitro* plants of many *Dioscorea* spp. by various researchers (Bahera et al., 2008; Chen et al., 2003; Kumar et al., 2017). Bahera et al. (2008, 2009) attained maximum rooting in ½ MS media adjuvanted with 2.0 mg L^{-1}NAA in *D. Oppositifolia* and *D. alata.* Poornima and Ravishankar, (2007) reported, the successful rooting was perceived on the media adjuvanted with 2.67 µM NAA in *D. pentaphylla* after 30 days of culture. In earlier reports, simultaneous production of roots was also observed in *Curcuma aromatic* by Nayak (2000); *Curcuma longa* by Prathanturaug et al. (2005); *Zingiber petiolatum* by Prathanturarug et al. (2004); *Boesenbergia rotunda* by Yusuf et al. (2011).

Table 5.11: *Effect of solid MS with PGRs on in vitro rooting of D. deltoidea*

Solid MS + NAA (mg L^{-1})	% culture response	Roots/explant	Root length (cm)
0	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
0.5	38.6 ± 6.50^{d}	0.5 ± 0.30^{d}	1.9 ± 0.40^{d}
1.0	57.3 ± 6.50^{c}	1.9 ± 0.40^{c}	2.9 ± 0.80^{c}
1.5	78.3 ± 6.50^{b}	3.2 ± 0.60^{b}	3.2 ± 0.70^{b}
2.0	92.0 ± 6.50^{a}	5.4 ± 0.70^{a}	4.8 ± 0.70^{a}

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

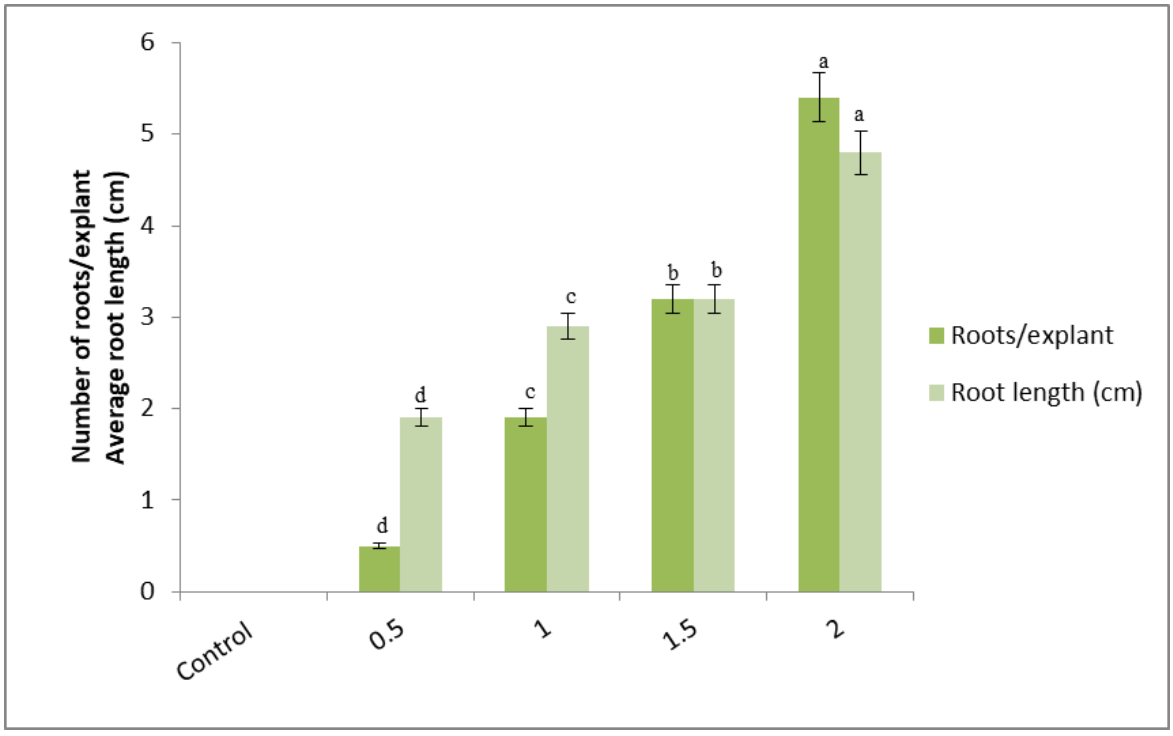


Figure 5.5: *Effect of solid MS with NAA on in vitro rooting of D. deltoidea*

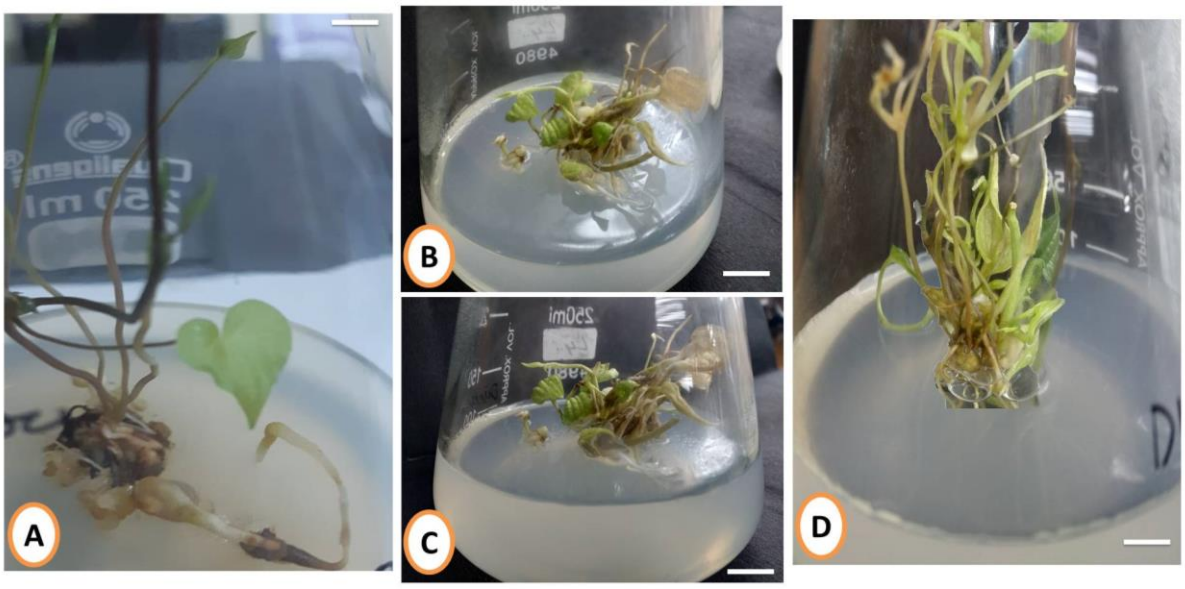


Plate 5.5: *Root induction from multiple shoot on solid MS medium*

(A) *NAA 0.5 mg L^{-1}* ***(B)*** *NAA 1.0 mg $L^{-1\ 1}$* ***(C)*** *NAA 1.5 mg L^{-1}* ***(D)*** *NAA 2.0 mg L^{-1}*

Table 5.12: *Effect of liquid MS with PGRs on in vitro rooting of D. deltoidea*

Liquid MS + PGR (mg L^{-1})		% response	Roots/explant	Root length (cm)
BA	**IBA**			
1.0	0.5	75.23 ± 0.15^{b}	$4.30 \pm 0.20^{c,d}$	2.66 ± 0.25^{e}
1.0	1.0	39.73 ± 0.15^{e}	$3.70 \pm 0.10^{e,f}$	2.50 ± 0.20^{g}
1.0	1.5	$23.46 \pm 0.15^{f,g}$	2.40 ± 0.10^{h}	2.63 ± 0.25^{f}
1.0	2.0	17.56 ± 0.15^{h}	2.23 ± 0.15^{g}	2.33 ± 0.25^{h}
1.5	0.5	$88.66 \pm 0.15^{a,b}$	$6.70 \pm 0.20^{a,b}$	$4.10 \pm 0.20^{b,c}$
1.5	1.0	69.30 ± 0.20^{d}	$4.30 \pm 0.20^{c,d}$	3.80 ± 0.10^{c}
1.5	1.5	$29.20 \pm 0.10^{e,f}$	3.73 ± 0.15^{e}	3.56 ± 0.15^{d}
1.5	2.0	19.60 ± 0.10^{g}	2.56 ± 0.11^{f}	$3.26 \pm 0.20^{d,e}$
2.0	0.5	71.26 ± 0.15^{c}	5.23 ± 0.15^{b}	4.73 ± 0.15^{b}
2.0	1.0	99.43 ± 0.20^{a}	7.10 ± 0.20^{a}	5.33 ± 0.20^{a}
2.0	1.5	74.40 ± 0.10^{b}	4.66 ± 0.15^{c}	5.20 ± 0.10^{a}
2.0	2.0	27.43 ± 0.15^{f}	3.80 ± 0.10^{d}	4.50 ± 0.20^{b}

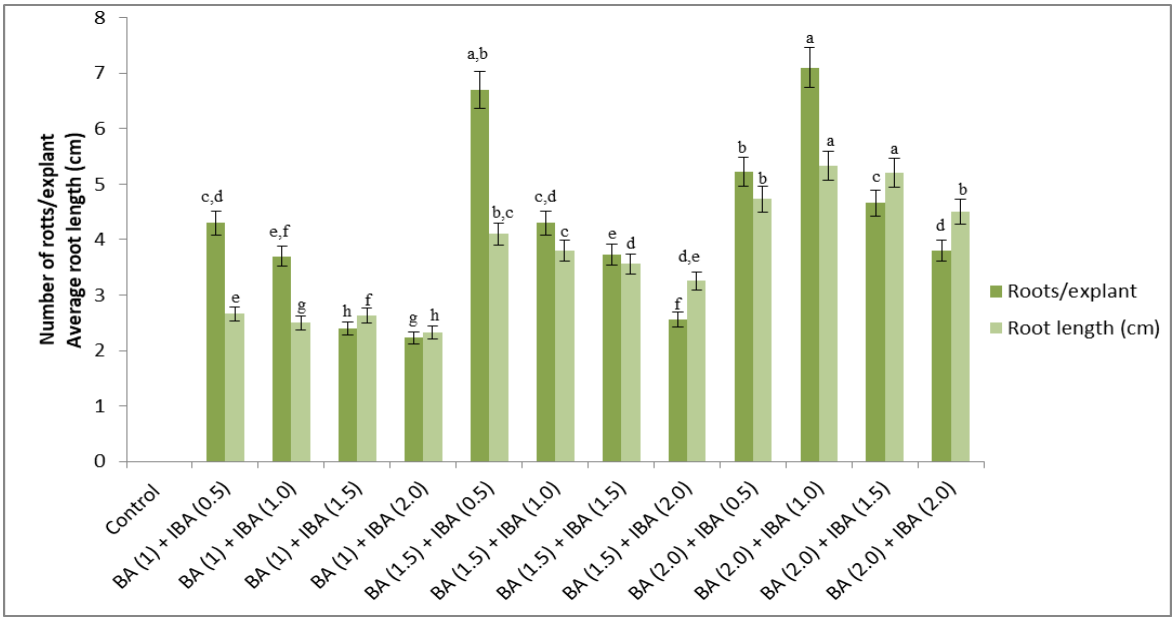


Figure 5.6: *Effect of liquid MS with PGRs on in vitro rooting of D. deltoidea*

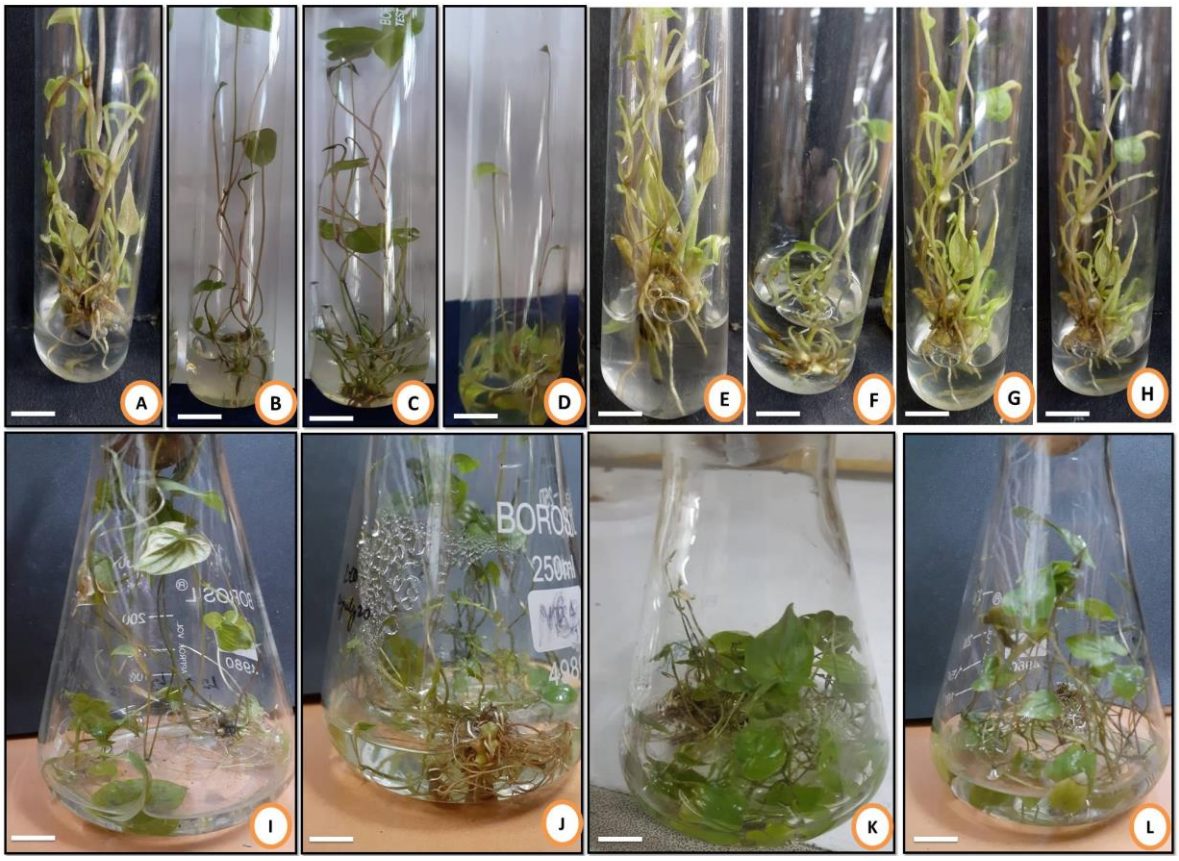


Plate 5.6: *Root induction from multiple shoot on Liquid MS medium*

(A-D) *MS medium fortified with BA 1.0 mg L^{-1} + IBA 0.5 – 2.0 mg L^{-1}*

(E-H) *MS medium fortified with BA 1.5 mg L^{-1} + IBA 0.5- 2.0 mg L^{-1}*

(I-L) *MS medium fortified with BA 2.0 mg L^{-1} + IBA 0.5 mg L^{-1}*

5.3.1.3.4 Tuber formation

Tubers of *D. deltoidea* are the main sources of diosgenin and many researchers have reported tuber formation in different species of *Dioscorea* (Ondo et al., 2007; Ng, 1988; Chu et al., 2002; Jean and Cappadocia, 1992). In this work, different combination of BAP, NAA and KN and different concentration of sucrose were tested on tuberization of *D. deltoidea*, however only three media containing BAP alone in the range of 0.5 ml L^{-1}, 0.9 ml L^{-1} and 1.5 ml L^{-1} and in combinations only one combination i.e. BAP 0.15 mg L^{-1} + 0.1 mg L^{-1} NAA + 0.25 mg L^{-1} KN resulted in the formation of tubers. Tuberization process was accompanied in liquid MS media as in our study liquid media showed good results in shoot multiplication and rooting process as compared to solid MS media and also many researchers have recommended liquid MS media (Mehrotra et al., 2007; Jo et al., 2008; Sivanandhan et al., 2013). Media augmented with BAP 0.15 mg L^{-1} + 1.0 mg L^{-1} NAA + 0.25 mg L^{-1} KN at sucrose showed 2.3 ± 0.20 mean number of tubers in 42 days, while as 3.4 ± 0.17 number of tubers were obtained in 42 days of culture in the media augmented with BAP 1.5 mg L^{-1}. MS media fortified with BAP at concentration of 0.9 mg L^{-1} produced 5.8 ± 0.19 mean number of tubers.

Effect of sucrose concentration on tuberization was also checked and it was found that 60 g L^{-1} sucrose produced maximum number of tuber (7.5 ± 0.11) with 10.2 mm length. Sucrose at 60 g L^{-1} produced 5.9 ± 0.15 numbers of tubers having weight 3.1 ± 0.19 and 8.9 ± 0.22 mm length. When sucrose concentration was increased to 80 g L^{-1} number of tubers was decreased to 4.7 ± 0.19 having 7.7 ± 0.25 mm length and 2.4 ± 0.17 g weight (Table 5.14).

Various researchers have found sucrose significant for tuber formation. Our results are in contradictory to Jasik and Mantell. (2000) and Mantell et al., 1978 that reported 20 g L^{-1} sucrose was better than 40 g L^{-1} in terms of number and size of microtubers. The outcomes of our study are in accord to the results of Kohmura et al. (1995), who reported 60 g L^{-1} sucrose more effective than 30 g L^{-1} in the production of tubers. Ng. (1988) reported that was decrease in microtubers production in *D. rotundata* by the addition of 8 or 10% sucrose and 2.5 µM KN in MS media whereas Mantell et al., 1978 found 2% sucrose produced highest microtubers in *D. alata* and *D. opposite* nodal culture. Chen et al., 2007 stated that sucrose helps in shoot and micro tuber induction by providing carbon source and energy. Polyamines effect was also studied by Onvono et al. (2010) on tuber formation and found that putrescine in low concentration (10^{-5}, 10^{-6} M) causes early tubers in *D. cayenensis – D.*

rotundata complex. Alizadeh et al. (1998) reported that NAA and IBA promoted *in vitro* tuberization and growth of *D. composite* and also 80 – 100 g/L sucrose induced tuber development.

Table 5.13: *Effect of plant hormones on tuber formation*

PGR (mg L^{-1})			Mean number of tubers	Tuber length (mm)	Tuber weight (g)
BAP	**NAA**	**KN**			
0.15	0	0	1.8 ± 0.22^{d}	2.9 ± 0.18^{d}	1.6 ± 0.18^{d}
0.15	1.0	0.25	2.3 ± 0.20^{c}	4.1 ± 0.17^{c}	2.1 ± 0.22^{c}
0.9	0	0	5.8 ± 0.19^{a}	9.3 ± 0.21^{a}	3.4 ± 0.11^{a}
1.5	0	0	3.4 ± 0.17^{b}	5.6 ± 0.25^{b}	2.8 ± 0.13^{b}

Table 5.14: *Effect of sucrose concentration on tuber formation*

BAP (mg L^{-1})	Sucrose (g L^{-1})	Mean number of tubers	Tuber length (mm)	Tuber weight (g)
0.9	40	5.9 ± 0.15^{b}	8.9 ± 0.22^{b}	3.1 ± 0.19^{b}
0.9	60	7.5 ± 0.11^{a}	10.2 ± 0.18^{a}	3.9 ± 0.13^{a}
0.9	80	4.7 ± 0.19^{c}	7.7 ± 0.25^{c}	2.4 ± 0.17^{c}

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

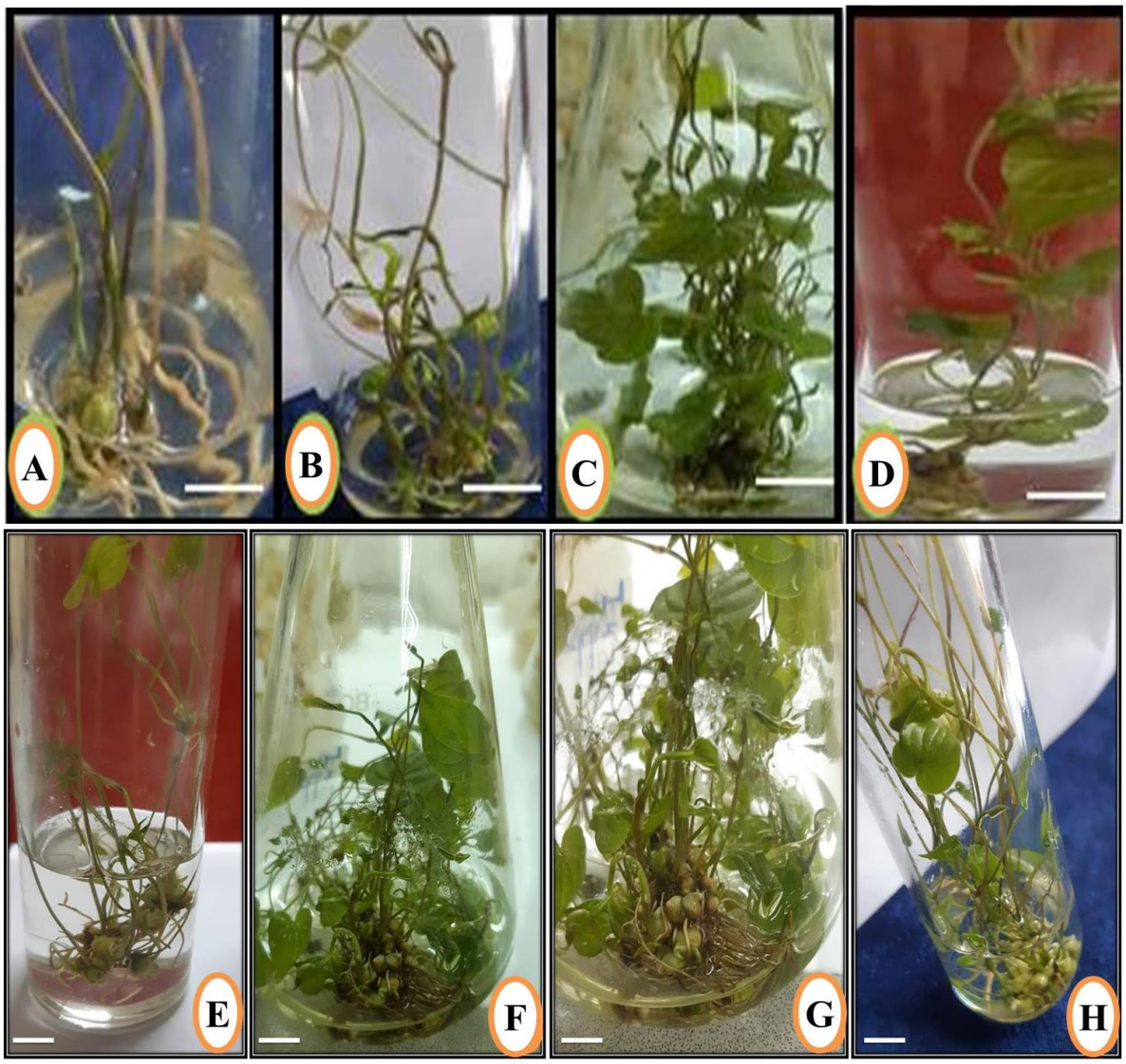


Plate 5.7: *Tuber formation in D. deltoidea through nodal segments*

(A) *Media fortified with BAP 2.0 mg L^{-1} + 1.0 mg L^{-1} NAA + 0.25 mg L^{-1} KN*

(B, C) *Media fortified with BAP 0.15 mg L^{-1} and BAP 0.5 mg L^{-1}*

(D) *Media devoid of any hormone (control)*

(E) *Media fortified with BAP 0.9 mg L^{-1}*

(F) *Media fortified with 4%, 6% and 8% sucrose.*

5.3.1.3.5 Hardening

The *in vitro* grown plantlets were further taken to hardening unit in a phased routine. In this study, 96% survival rate was recorded (Plate 5.7). Behera et al. (2008, 2009) also used soil: sand: manure (1:1:1) for acclimatization of micropropagated plants of *D. hispida* and *D. oppositifolia* and reported 90% survival rate. Jova et al. (2005) attained successful

acclimatization by using soil rite which contains equivalent percentage of decomposed coir and peat moss. Kadota and Niimi (2004) recorded 80% acclimatization rate of *D. japonica* in vermiculite and soil mixture (1:1). Bazabakana et al. (1999) used sand: soil: mould (1:1:2) for the acclimatization of rooted plantlets of *D. alata*.

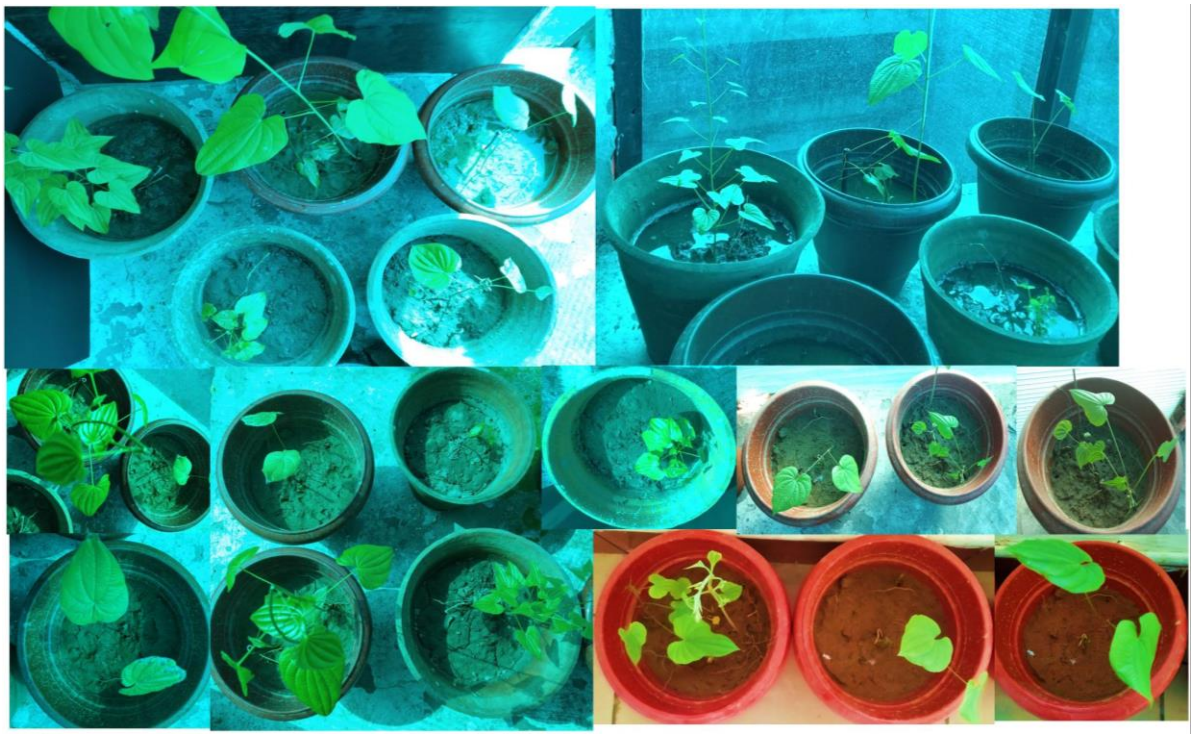


Plate 5.8: *Hardening of in vitro grown plants in green house*

5.3.2 Genetic fidelity study

Somaclonal variation is the main problem associated with the *in vitro* cultures and there are many ways to detect these variations. The detection of variation and confirmation of genetic fidelity in *in vitro* regenerants have been done by several isozyme, cytological and molecular markers, however PCR based molecular markers for instance ISSR, RAPD, AFLP, SSR etc. are the most used techniques. ISSR markers are considered appropriate in variation detection among *in vitro* regenerated plantlets (Leory et al., 2001; Joshi and Dhawan, 2007).

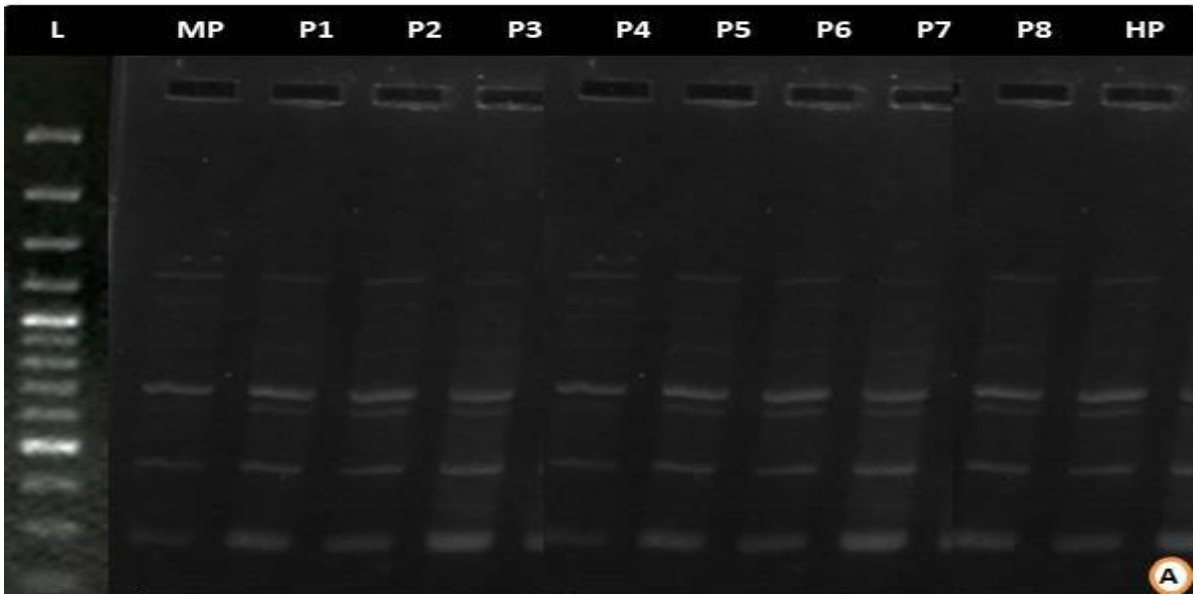
In the current study, ISSR a cost effective and simple PCR based molecular marker was used to evaluate the genetic fidelity of *in vitro* plantlets of *D. deltoidea.* A total of seven primers were tried and only five primers (Table 5.5) were selected for their good resolution and reproducibility. The amplification products range from 100-1400 and the number of bands regenerated varied from 4 in ISSR 13 primer to 11 in ISSR 10 primer (Table 5.13).

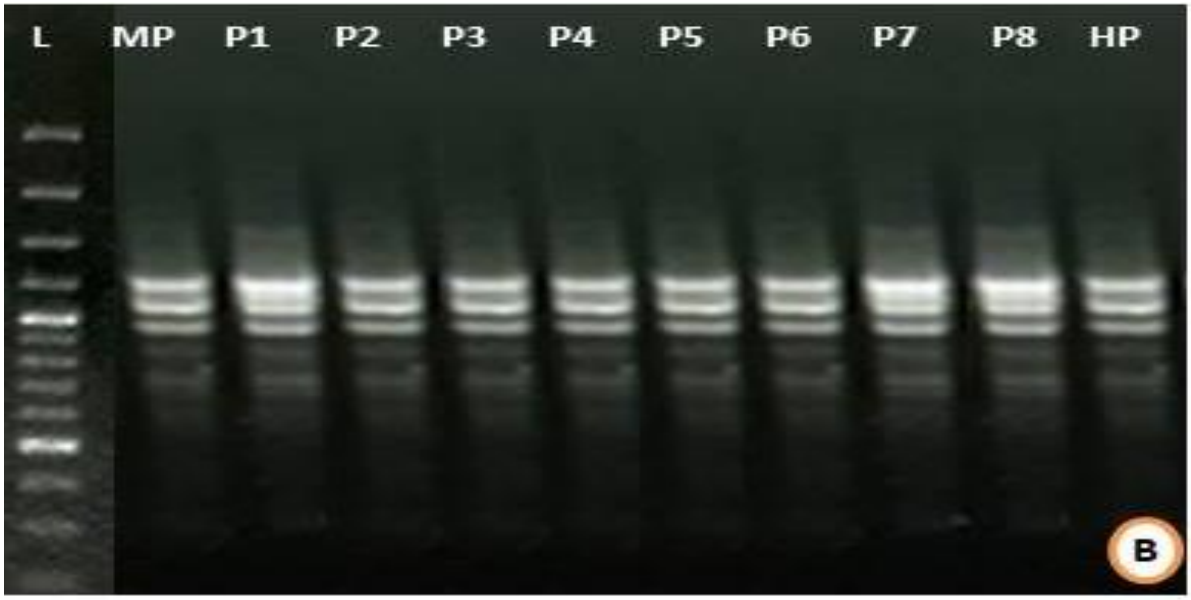
Only monomorphic banding without any polymorphism was reproduced by all the primers in *ex vitro* and *in vitro* plantlets of *D. deltoidea* (Plate 5.8).

Our outcomes are in contract with the results of many authors in the monomorphism study of micropropagated plants analysed with ISSR markers. Genetic fidelity analysis of *in vitro* regenerants of Garcinia and *Calliandra tweedii* were assessed by ISSR markers and were found monomorphic (Mohan et al., 2012; Heikrijam, 2014). Joshi and Dhawan (2007) multiplied *in vitro* plants of *Swertia chirayita* via axillary multiplication and used 16 ISSR primers generated total 102 amplicons and homogenous amplification profile was observed. The genetic constancy conformation of *in vitro* plantlets of *Moringa peregrina* by ISSR markers showed pure genetic stability in all *in vitro* regenerants (Khateeb et al., 2013). The genetic homogeneity test has been effectively evaluated in various *Dioscorea* species by various authors (Nascimento et al., 2013; Wu et al., 2014; Nudinet al., 2017). However, in various studies genetic and phenotypic variations have been informed to occur through *in vitro* regeneration producing somaclonal variations in several species of plants (Larkin and Scowcroft, 1981; Kaeppler et al., 2000). The present outcomes of ISSR analysis can be used to evaluate the genetic uniformity of *in vitro* conserved regenerants of *D. deltoidea* on large scale, thus easing the crop enhancement program in *Dioscorea* species and can be used for commercially by breeders.

Table 5.15: *ISSR analysis banding pattern of in vitro, hardened and mother plant*

Primer code	Primer sequence (5′-3′)	Number of bands	Range of amplified bands (bp)
ISSR 10	TCTCTCTCTCTCTCTCG	11	200 – 1400
ISSR 12	ACACACACACACACACC	4-5	700 – 1700
ISSR 13	ACACACACACACACACG	4	100 – 800
ISSR 17	GGAGAGGAGAGGAGA	8	100 – 500
ISSR 20	AGAGAGAGAGAGAGAGCT	5	100 – 400


L
MP
P1
P2
P3
P4
P5
P6
P7
P8
HP
A


L
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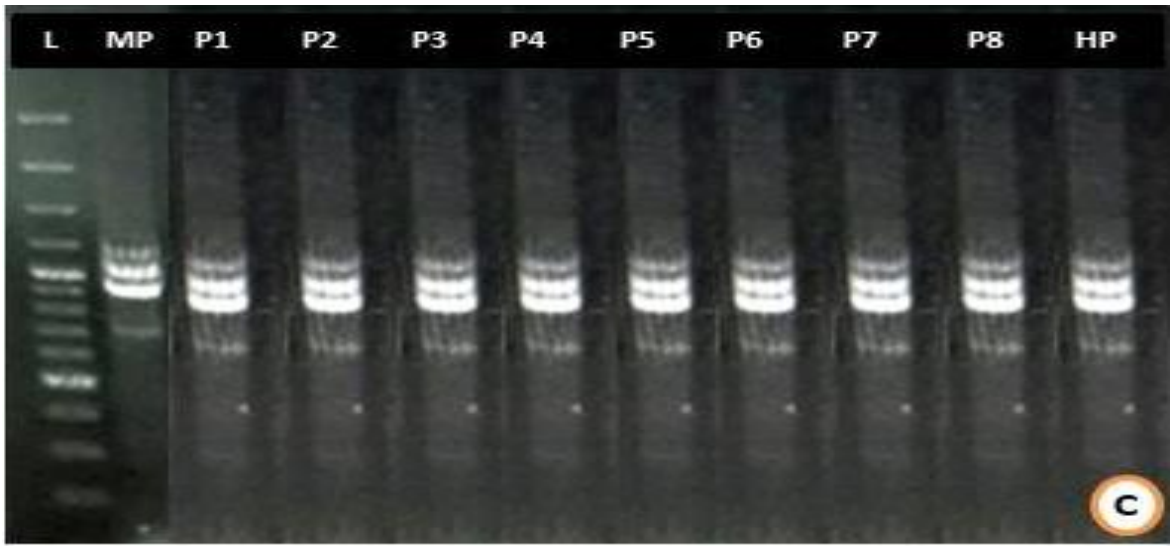
L
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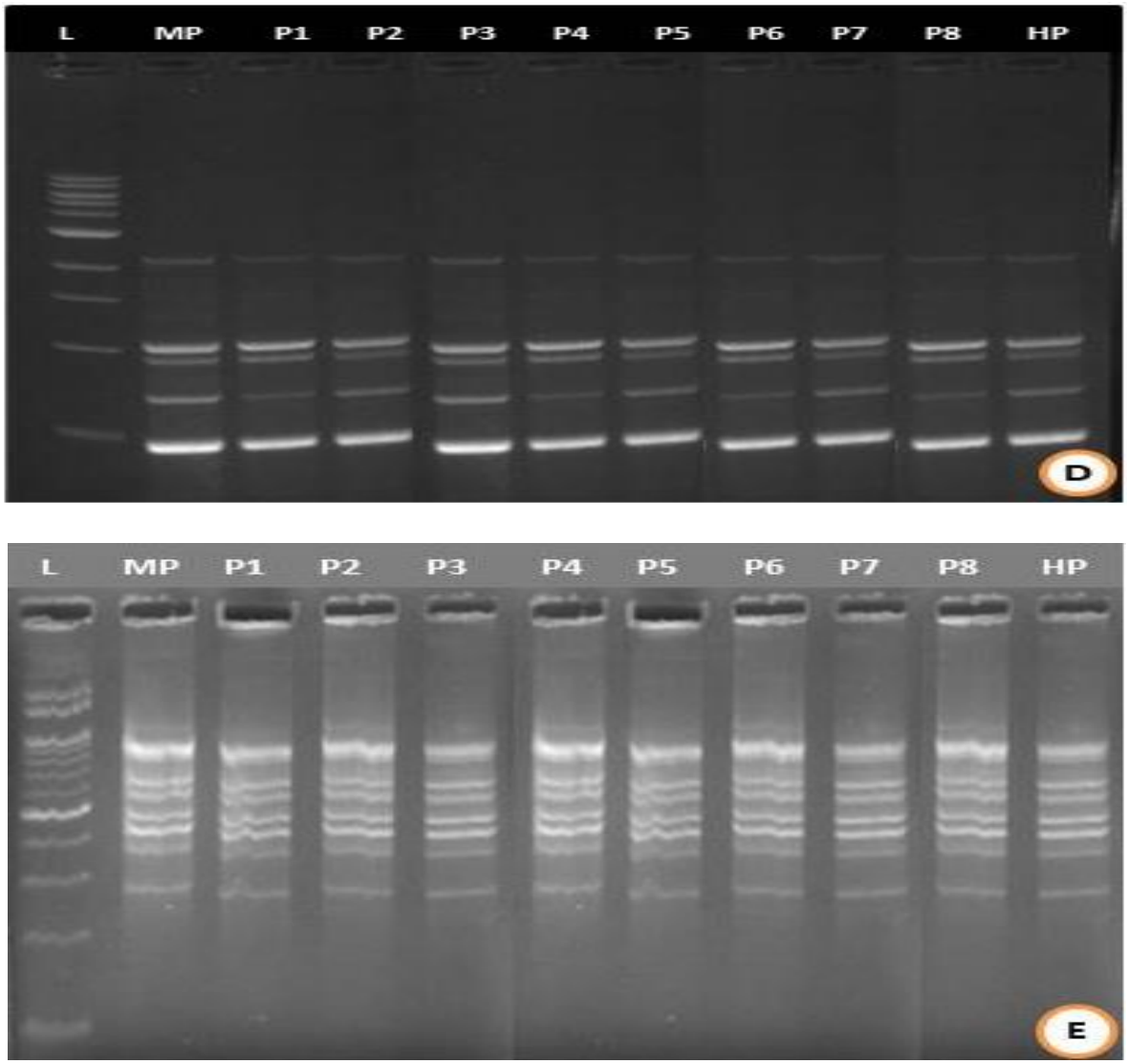


Plate 5.9: *Gel picture of amplicons amplified using five ISSR primers* ***A:*** *ISSR 10* ***B:*** *12* ***C:*** *13* ***D:*** *17* ***E:*** *20 where M represents ladder (100 bp) and P1-P8 are the randomly selected regenerated plants and HD are hardened plants*

5.3.3 Biochemical fidelity study

The assessment of biochemical fidelity among *in vivo* (mother plant) and *in vitro* plantlets are imperious for the efficiency of any proposed protocol of tissue culture. The plants possess many bioactive compounds that are responsible for their therapeutic properties. In this species *D. deltoidea*, diosgenin is the main bioactive compound possessing various medicinal properties. So, in this present study diosgenin content in different extracts of ex vitro and *in vitro* regenerants were quantified by HPTLC analysis and compared on the basis of peak area. The results had confirmed that all extracts of leaf, stem and root of *in vitro* and *ex vitro* plants of *D. deltoidea* contain diosgenin. According to HPTLC, *in vitro* regenerants showed good content of diosgenin than *ex vitro* plants. The percentage of diosgenin in *in*

vitro leaf is 0.055% and in *ex vitro* is 0.054%, in *in vitro* stem is 0.063% and in *ex vitro* diosgenin is 0.058%, in *in vitro* root diosgenin is 0.492% and in *ex vitro* 0.428% as shown in Table 5.15 and Figure 5.7. Comparable studies were carried out by Sasidharan et al. (2017) and Behera et al. (2018) and determined biochemical fidelity by HPTLC analysis in various medicinal plants. Coronarin D in *ex vitro* and *in vitro* plants of *Hedychium coronarium* was analyzed and compared by HPTLC analysis and revealed that coronarin D was higher in *in vitro* plants than *ex vitro* mother plant (Behera et al., 2019). In this study, the developed micropropagation protocol does not reveal any contrary effect on diosgenin production.

Table 5.16: *Diosgenin content in in vivo and in vitro raised plants of D. deltoidea*

Plant part	*In vivo* Diosgenin (%)	*In vitro* Diosgenin (%)
Leaf	0.0547 ± 0.008	0.055 ± 0.006
Stem	0.0580 ± 0.009	0.063 ± 0.005
Root	0.428 ± 0.006	0.492 ± 0.006

*All the values are expressed as mean ± SD (n = 3); SD = standard deviation.

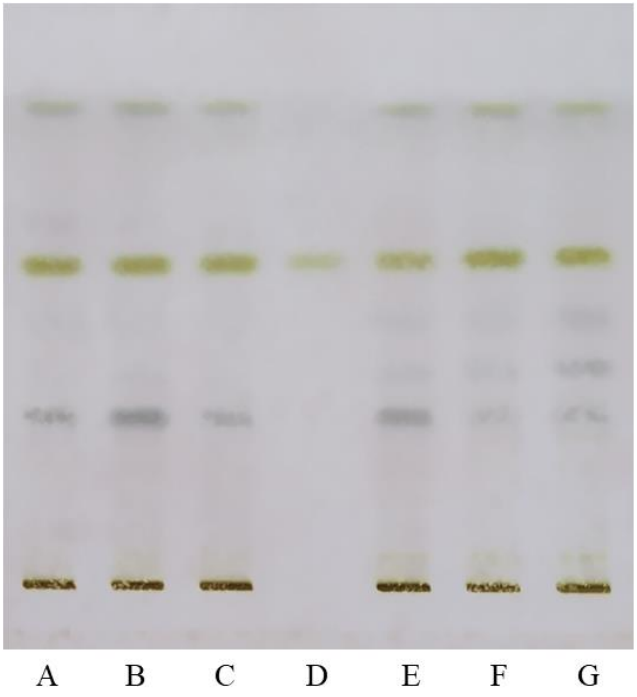


Figure 5.7: *HPTLC fingerprinting of diosgenin **A** = in vitro Leaf, **B** = in vitro stem, **C** = in vitro root, **D** = standard (diosgenin), **E** = in vivo leaf, **F** = in vivo stem, **G** = in vivo root.*

5.4 Conclusion

The results described in this chapter explain the development of highly efficient and improved plant regeneration protocol for *D. deltoidea*. In this study, nodal segments inoculated into MS medium augmented with different PGRs successfully showed callus initiation along with direct shoot and root induction. Moreover, simultaneous production of shoots and roots i.e. one-step plant regeneration was achieved in shoot multiplication medium augmented with various combinations of BA and IBA but without agar. By using this protocol a large number of plantlets can be produced yearly from a single nodal segment. Most immensely these *in vitro* plantlets were found genetically and biochemically similar to their mother plant as determined by ISSR and HPTLC analysis respectively. Being an economically significant but endangered plant, the current protocol for Micropropagation of *D. deltoidea* might prove useful for mass production of clonal plants to be utilized for *ex-situ* germplasm conservation, re-establishment in wild habitats as well as commercial use in pharmaceutical industries.

CHAPTER 6

Application of elicitors and precursors for the enhancement of bioactive compound diosgenin in the plants

6.1 Introduction

An ample number of bioactive compounds were produced by plants which are having tremendous applications in biopesticides, food, cosmetics and pharmaceutical companies and around one-fourth of recommended medicines are obtained from plants. Currently, the utilization of these compounds in huge amounts caused extinction of their population and genetic diversity has eroded. Furthermore, the wild plants suffer more from climatic changes and ecological conditions which cause variations in their biochemical profile. From past few centuries, there has been a rehabilitated curiosity in the utilization of pharmaceutical plants and their drugs as a substitute to synthetically prepared medicines for the cure of ailments, which caused development of pharmaceutical industries every year.

Plant cell or organ cultures as an auspicious technology for the secondary metabolites production provide opportunities over bioactive compounds extraction from plants or by chemical synthesis (Rao and Ravishankar, 2002). However, application of plant cell cultures commercially have been met with limited success and the reasons are low yield of products, instability of biosynthesis and challenges to scale-up (Bourgaud et al., 2001). Out of them, low productivity of the bioactive compounds is the universal phenomenon (Wiedenfeld et al., 1997). In order to solve this issue, great work and efforts have been made on it in recent years and many strategies such as elicitation, precursor feeding, optimization of the medium, and so on (Qu et al., 2011). Out of these strategies, elicitation is the most efficient process that stimulates the production of bioactive compounds (Qu et al., 2011; Largia et al., 2015; Chauhan et al., 2018; Jirakiattikul et al., 2020). Elicitors may be biotic or abiotic based on their origin are external stimuli molecules capable of stimulation defense or stress-induced responses in plants. In *in vitro* cultures the exogenous use of elicitors are useful to improve the biosynthesis of desired secondary metabolites and to study responses of plants against pathogen attacks. According to Largia et al. (2015) low concentration of elicitors in

the medium improve the quantity of bioactive compounds. Cultures elicited with chemical substances has found more consistent for the production of elevated amounts of bioactive (Radman et al., 2003).

Methyl Jasmonate (MeJa) and salicylic acid (SA) out of these chemical elicitors have been well known for the enhanced production of various bioactive compounds in different cultures (Qu et al., 2011; Sivanandhan et al., 2013; Largia et al., 2015). Being a volatile methyl ester of jasmonic acid MeJa has been known in abiotic and biotic stresses as a signaling molecule (Creelman and Mullet, 1995). Yu et al. (2002) stated that MeJa helps in the defense compounds formation by involving in the pathway of signal transduction that makes specific enzymes to generate those particles. SA one more stress signaling molecule is widely used for its role in inducing resistance towards pathogens in plants (Rao et al., 2000). According to Kang et al (2004), SA appeared as key-signaling molecule in the last decades that is responsible in the stimulation of certain defense responses in plants. Both the elicitors have also been reported to improve the quantity of bioactive compounds in wide range of plant methods for instance shoot cultures of *Digitalis purpurea* (Patil et al., 2013), whole plant cultures of *Centella asiatica* (Kim et al., 2004), cell cultures of *Ginkgo biloba* (Kang et al., 2006), hairy root cultures of *Gentiana dinarica* (Krstić-Milošević et al., 2017) and *Daucus carota* callus cultures (Sudha and Ravishankar, 2003). The quantity of triterpenoid saponins was enhanced by the elicitation of MeJa and SA in hairy root cultures of *Centella asiatica* (Kim et al., 2007) and in whole plant cultures of *Glycyrrhiza glabra* (Shabani et al., 2009).

In addition to elicitation, precursor feeding is considered another effectual process to enhance the production of bioactive compounds in different cultures such as shoot cultures of *Digitalis purpurea* (Patil et al., 2013), cell suspension cultures of *Vitis vinifera* (Qu et al., 2011), hairy root cultures of *Psoralea corylifolia* (Shinde et al., 2009) and whole plant cultures of *Rauwolfia serpentine* (Panwar and Guru, 2015). With precursor feeding, the quantity of digitoxin and digoxin in *Digitalis purpurea* shoot cultures was increased to 9.1- and 11.9-folds.

In order to fulfil the demand of diosgenin, application of elicitors and precursors are the need of the hour. Therefore, in this study elicitation and precursor feeding has been attempted on various parameters such as exposure time, type and concentration of elicitors and precursors. At this time we precisely target to develop an effective elicitation and precursor feeding approach for enhanced biomass production and improved accumulation of diosgenin upon using two different elicitors which are MeJa and SA individually and three

different precursors which are squalene, β-sitosterol and cholesterol. Till date, as per our knowledge is concerned, this is the first study on the elicitation and precursor feeding on diosgenin production and biomass accumulation in *in vitro* cultures of *D. deltoidea.*

6.2 Materials and methods

6.2.1 Chemicals and reagents

Squalene, methyl Jasmonate, salicylic acid, β-sitosterol, cholesterol, 6-Benzylaminopurine (BAP), Indole-3-butyric acid (IBA) were purchased from Sigma-Aldrich, USA. Reference compound and solvents already described in Chapter 3 (Section 3.2.1).

6.2.2 Plant material

In vitro cultures of *D. deltoidea* established in MS media augmented with BA (2.0 mg L^{-1}) and IBA (1.5 mg L^{-1}) for direct organogenesis were used for precursor and elicitor treatment. *In vitro* culture methodology already described in Chapter 5.

6.2.3 Elicitors and precursors preparation and treatment

In this study, SA and MeJa as elicitors and squalene, β-sitosterol and cholesterol were used as precursors in the concentration of (100 and 200 µM). The stock solutions were prepared separately by dissolving elicitors in 50% ethanol and precursors in 99% ethanol. After preparation, all stock solutions were filter sterilized through 0.22 µM syringe filter. The *in vitro* regenerants having maximum biomass 5 g fresh weight on 5^{th} week of culture so were treated with elicitors on the 5^{th} week of culture at different exposure times (4, 8, 16 h). The multiple shoots with roots were transferred immediately after the given exposure time of the elicitor treatment to the liquid MS medium enriched with 2.0 mg L^{-1} BA and 1.5 mg L^{-1} IBA aseptically. After elicitor treatment, the *in vitro* plantlets were harvested on 7^{th} week for the production of biomass and diosgenin. Similarly, precursor treatments were given *to in vitro* plants on the 5th day of culture and were harvested on 5^{th} and 10^{th} day after the precursor treatment. All the treatments were carried out in triplicates and control cultures were added with 50% ethanol for each trial.

6.2.4 Quantification Analysis

6.2.4.1 Biomass quantification

Biomass was quantified by weighing the fresh weight of shoots and roots of both control and treated cultures after harvesting. The harvested *in vitro* shoots and roots were freeze-dried and lyophilized and dry weight measurement was obtained.

6.2.4.2 Diosgenin quantification

6.2.4.2.1 Sample preparation

Sample preparation has been described in Chapter 3 (Section 3.2.4).

6.2.4.2.2 GC-MS instrument

An Agilent 7890A Gas chromatography fitted to a 5875C mass spectrometer detector (XL MSD) with triple axis and mass hunter work station software (USA) was used for the diosgenin quantification. Chromatography was executed on DB-5: 30 m x 0.25 mm i.d. x 0.25 µM film thickness column. Helium as a gas carrier works at a flow rate of 0.5 mL/min. The oven temperature of Gas chromatography was elevated from 200 °C for 2 min to 280 °C for 20 min at a heating rate of 10 °C/min. 5 µl was used as injection volume in the split ratio of 1:1. The Mass spectra were noted in electron impact mode with ionization energy of 70 eV and scan range of 50-600 amu with scan rate of 0.5 s/scan. Inlet and transfer line temperature were set 2500 C. Wiley, NIST libraries were used for identification of compounds and also confirmed by peak enrichment on co-injection with existing authentic standards. Peak area percentages were attained electronically from the TIC response deprived of the use of correction factors. GC-MS chromatogram of diosgenin is shown in Figure 6.3.

6.3 Statistical analysis

All the values obtained are represented in triplicates as mean ± standard deviation. Analysis of variance (ANOVA) with Tukey's test was implemented for the statistical analysis by using the software Minitab 15 English ($p < 0.05$).

6.4 Result and discussion

6.4.1 Effect of elicitors on biomass accumulation and diosgenin production

Use of liquid culture for elicitation has been suggested by many scholars. Mehrotra et al. (2007) confirmed an increase in the bioactive compound production as well as in shoot growth by liquid culture system in lesser culture period. The reason can be the adjacent interaction of the tissue with the medium stimulating the uptake of nutrients causing enhanced growth. Another important activity of liquid culture system is the reduction of apical dominance activity because of constant shaking state of the tissues in the medium comprising the initiation and multiplication of various axillary buds. Jo et al. (2008) and Sivanandhan et al (2013) reported same responses. Prasad et al. (2013) recently used liquid culture system for the elicitation of *Centella asiatica* shoot cultures. Therefore, in this study the elicitation process was accomplished in liquid MS medium augmented with 2.0 mg L^{-1}BA and 1.5 mg L^{-1} IBA as it was noticed to be more eminent for plant harvest also.

The application of elicitors has shown significant effect on biomass accumulation of *D. deltoidea* cultures. Two different concentrations (100, 200 µM) of SA and MeJa were incorporated at three different incubation times (4 h, 8 h and 16 h) in the liquid MS medium on 5^{th} week of culture. The biomass accumulation in *D. deltoidea* cultures affected by SA and MeJa were presented in Table 6.1 and Figure 6.1. Cultures treated with MeJa exhibited biomass production in the range of 0.487 ± 0.059 g to 0.727 ± 0.057 g DW in the shoots and 0.571 ± 0.042 to 0.782 ± 0.061 g DW in the roots. However, the biomass production of control cultures was higher than treated ones. At all incubation times, there was constant reduction in the biomass accumulation in *D. deltoidea* cultures. MeJa treated cultures showed increase in the content of diosgenin at all levels. At incubation 4 h, diosgenin content in shoots was 0.348% and 0.475% in roots at 100 µM MeJa and 0.432% in shoots to 0.497% in roots at 200 µM MeJa. However, 8 h MeJa treated shoots produced 0.814% diosgenin and 0.856% content was produced in roots at 100 µM MeJa. While, 200 µM MeJa produced 0.765% diosgenin in shoots and 0.825% in roots that was less than 100 µM MeJa treated cultures. The incubation time 16 h also produced good diosgenin content at both concentrations of MeJa but higher concentration of MeJa produced less content.

Cultures elicited with SA showed biomass production in the range of 1.539 ± 0.0223 g to 1.767 ± 0.028 g DW in shoots and 1.517 ± 0.131 to 1.798 ± 0.027 g DW in the roots and diosgenin content in the range of 0.471 ± 0.016 to 0.912 ± 0.011 g DW in shoots and 0.582

± 0.012 to 0.978 ± 0.018 g DW in roots. SA (100 µM) showed least biomass reduction in both shoot and root biomass when incubation time was 4 h but showed good diosgenin content in shoots and roots. SA at 200 µM yielded maximum diosgenin content 0.912% in shoots and 0.978% in roots. At incubation time 8 h, biomass of shoots was 1.552 ± 0.330 at 100 µM SA to 1.767 ± 0.028 g DW at 200 µM SA and biomass of roots were 1.779 ± 0.210 at 100 µM SA to 1.767 ± 0.116 g DW at 200 µM SA. The diosgenin content at 8 h exposure time was in the range of 0.819% in shoots and 0.867% in roots at 100 µM SA and 0.664% in shoots and 0.775% in roots at 200 µM SA. Incubation time 16 h showed 1.656 ± 0.194 to 1.539 ± 0.022 g DW shoot biomass production and 1.643 ± 0.150 to 1.517 ± 0.131 g DW root biomass production. However, the diosgenin content at 16 h exposure time was observed in between 0.625% in shoots and 0.658% in roots at 100 µM SA and 0.549% in shoots to 0.595% in roots at 200 µM SA. From Table 6.1, it is clear that in both elicitor treatments and at all incubation times biomass production was seen less than that of control cultures and SA showed minimum reduction in biomass as compared to MeJa. As for diosgenin production SA treatment enhanced its yield at all concentrations and incubation times but content varies in all treatments.

Our results are in agreement with the outcomes of Largia et al. (2015) in which SA was noticed less toxic than MeJa. Suzuki et al. (2005) also revealed that MeJa at higher concentrations was found toxic. Comparable results were observed in the shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum* in which biomass production was significantly inhibited at higher concentrations of jasmonic acid (JA) and as relative to control cultures SA showed slight effect on biomass accumulation but 200 µM SA increased production of hypericin and pseudohypericin (Coste et al., 2011). Diwan and Malpathak. (2011) also found 200 µM SA optimal concentration for the furanocoumarin production in the *Ruta.graveolens* shoot cultures. In a recent report, it has been revealed that SA showed no effect on shoot cultures of *D. membranacea*, whereas JA showed low effect (Jirakiattikul et al., 2020). MeJa at concentration above 0.1 mM showed reduction in the growth of *Centella asiatica* cultures but increased asiaticoside production (Kim et al., 2004). Our outcomes are also in contract with the outcomes of Sivanandhan et al. (2013) who also reported that biomass production was completely inhibited at 150-250 µM MeJa however insignificant reduction was observed in contrast to control cultures when cultures were elicited with SA. Chauhan et al. (2018) reported that SA (50 µM) showed negative impact on diosgenin production in the microtubers of *Chlorophytum borivilianum*. Hari et al.

(2018) found enhancement in the psoralen production in the suspension culture of *Psoralea corylifolia* by the addition of 100 µM SA and Sirvent and Gibson (2002) obtained high content of hyperforin in the shootlet meristem cultures of *Hypericum perforatum* by the treatment of 1 mM SA.

However, in a study led by Giri and Zaheer (2016) reported adverse effects of SA on biomass production of *Andrographis paniculata*. According to Zhao et al. (2010) the efficacy of elicitors varied depending on the type of culture (shoot or root culture, suspension culture or whole plant culture), concentration, exposure or incubation time, genotypes and this theory has been revealed in our work also.

Table 6.1: *Elicitors effect on biomass accumulation and diosgenin content in shoot cultures of D.deltoidea*

Elicitor (µm)	Harvest Time (h)	Biomass (g DW)		Diosgenin (%)	
		Shoot	Root	Shoot	Root
MeJa					
0	4	$1.74 \pm 0.091^{a,b}$	$1.74 \pm 0.091^{a,b}$	$0.229 \pm 0.014^{i,j}$	$0.229 \pm 0.014^{i,j}$
100		0.727 ± 0.057^{d}	0.782 ± 0.061^{d}	0.348 ± 0.026^{g}	$0.475 \pm 0.016_{g,h}$
200		0.487 ± 0.059^{g}	0.571 ± 0.042^{f}	0.432 ± 0.013^{f}	$0.497 \pm 0.019^{e,f}$
0	8	$1.748 \pm 0.043^{a,b}$	$1.748 \pm 0.043^{a,b}$	$0.276 \pm 0.010^{i,j}$	$0.276 \pm 0.010^{i,j}$
100		$0.674 \pm 0.045^{d,e}$	0.724 ± 0.033^{d}	0.814 ± 0.013^{b}	$0.856 \pm 0.022^{a,b}$
200		$0.632 \pm 0.031^{e,f}$	$0.702 \pm 0.035^{d,e}$	0.765 ± 0.020^{c}	0.825 ± 0.024^{b}
0	16	$1.726 \pm 0.024^{a,b}$	$1.726 \pm 0.024^{a,b}$	0.255 ± 0.053^{j}	0.255 ± 0.053^{j}
100		$0.663 \pm 0.026^{d,e}$	0.763 ± 0.031^{d}	0.533 ± 0.018^{e}	0.622 ± 0.042^{e}
200		$0.523 \pm 0.024^{f,g}$	$0.691 \pm 0.024^{d,e}$	0.628 ± 0.019^{e}	$0.643 \pm 0.045^{d,e}$
SA					
0	4	1.826 ± 0.020^{a}	1.826 ± 0.020^{a}	$0.265 \pm 0.006^{i,j}$	$0.265 \pm 0.006^{i,j}$
100		$1.742 \pm 0.037^{a,b}$	$1.798 \pm 0.027^{a,b}$	0.471 ± 0.016^{f}	0.582 ± 0.012^{e}
200		$1.664 \pm 0.038^{b,c}$	$1.745 \pm 0.022^{a,b}$	$\mathbf{0.912 \pm 0.011^{a}}$	$\mathbf{0.978 \pm 0.018^{a}}$
0	8	$1.810 \pm 0.136^{a,b}$	$1.810 \pm 0.136^{a,b}$	$0.263 \pm 0.006^{i,j}$	$0.263 \pm 0.006^{i,j}$

100		1.552 ± 0.330^{b,c}	1.779 ± 0.210 ^{a,b}	0.819 ± 0.012^{b}	0.867 ± 0.022^{a,b}
200		1.767 ± 0.028^{a,b}	1.767 ± 0.116 ^{a,b}	0.664 ± 0.019^{d}	0.775 ± 0.026^{c}
0	16	1.817 ± 0.025^{a}	1.817 ± 0.025^{a}	0.267 ± 0.005^{i,j}	0.267 ± 0.005^{i,j}
100		1.656 ± 0.194^{b}	1.643 ± 0.150^{b}	0.625 ± 0.007^{d}	0.658 ± 0.011^{d}
200		1.539 ± 0.022^{c}	1.517 ± 0.131^{c}	0.549 ± 0.026^{e}	0.595 ± 0.016^{e}

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

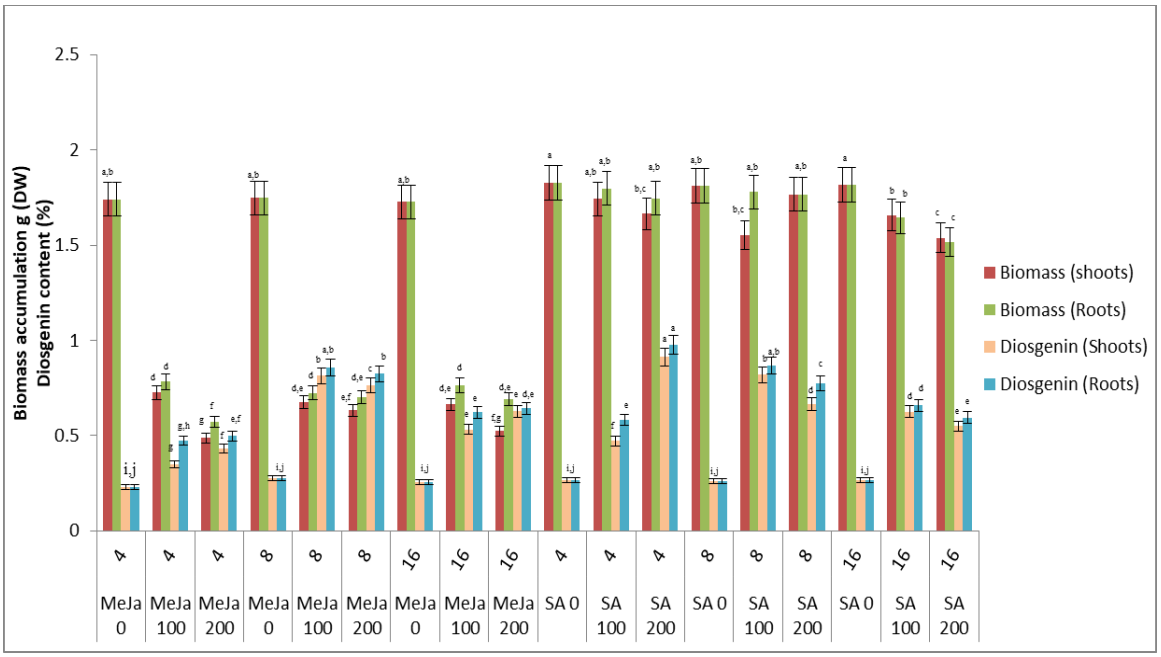


Figure 6.1: *Effect of MeJa and SA on biomass and diosgenin production*

6.4.2 Effect of precursors on biomass accumulation and diosgenin production

In the bioactive compounds biosynthesis of plant cell cultures, precursor feeding at optimum concentrations and exposure time can promote the bioactive compounds production. However, excess concentration of precursors with inappropriate exposure time might induce feedback suppression to the metabolite pathway. Determination of suitable concentration of precursor with optimum exposure time is important to attain high production of secondary metabolites (Liu et al., 2007). Therefore, in our study three precursors associated with biosynthesis of diosgenin namely squalene, cholesterol and β-sitosterol at two concentrations (100 µM and 200 µM) with two exposure times (5 days and 10 days) were optimized for the accumulation of biomass and diosgenin in the *D. deltoidea* cultures. The

biomass and diosgenin production in liquid culture of *D. deltoidea* was significantly affected by the treatment of precursor (Table 6.2). At 100 µM squalene, the biomass of shoots was 1.121 ± 0.017 g DW and 1.271 ± 0.081 g DW of roots when cultures were harvested on 5th day and diosgenin production was 0.947% in shoots and 0.973% in roots. However, when concentration of squalene was increased to 200 µM and harvesting time was same as 5th day, the biomass and diosgenin production was decreased in both shoots and roots as compared to 100 µM squalene but as compared to control ones diosgenin production increased from 0.319% to 0.636% in shoots and 0.764% in roots. When *in vitro* plantlets were harvest on 10th day after treatment with squalene the biomass production was greatly reduced in both shoots and roots but slight increase in the diosgenin production. In β-sitosterol treated cultures, at concentration 100 µM there was slight reduction in the biomass production as 1.559 ± 0.025 g DW of shoots and 1.617 ± 0.015 g DW of roots were obtained when harvested on 5th day of treatment, though diosgenin content increased in both shoots (0.782%) and in roots (0.834%). But at 200 µM β-sitosterol maximum diosgenin content 1.006% in shoots and 1.216% in roots was obtained (Figure 6.2). When cultures were harvested on 10th day, biomass production was significantly reduced at both concentrations of β-sitosterol and insignificant increase was observed in diosgenin content. Cholesterol at different concentrations reduced biomass accumulation at both exposure time but showed slight increase in diosgenin production at both exposure times. Cholesterol at 100 µM showed less biomass reduction but when its concentration increased to 200 µM significant reduction in biomass was observed. Though, diosgenin content increased more at 200 µM and yielded 0.559% diosgenin in shoots and 0.691% diosgenin in roots on 5th day and on 10th day 0.518% diosgenin in shoots and 0.541% in roots was obtained.

Of different precursors tried in the current study, β-sitosterol at 200 µM increased maximum diosgenin content in both shoots and roots of *D. deltoidea* and also exhibited much better effects than squalene and cholesterol. The outcomes of this study are in accord with the outcomes of Sivanandhan et al (2014) in which addition of squalene and cholesterol in the suspension cultures of Withania somnifera reduced biomass production, though 6 mM squalene produced maximum withanolides. In another study, squalene and cholesterol showed biomass reduction and also significantly affected production of cardiotonic glycosides in the shoot cultures of *Digitalis purpurea* (Patil et al., 2013). Many reports on precursor feeding in plant cell or organ cultures are available to enhance the production of secondary metabolites while the precursor feeding significantly reduces the growth

characteristics relies upon the plant species tested. It is also confirmed in our study that the biomass accumulation reduced, whereas diosgenin content enhanced due to precursor treatment.

Table 6.2: Precursors effect on biomass accumulation and diosgenin content in shoot cultures of *D.deltoidea*

Precursor (µm)	Harvest Time (Days)	Biomass (g DW)		Diosgenin (% DW)	
		Shoot	Root	Shoot	Root
Squalene					
0	5	$1.780 \pm 0.092^{a,b}$	$1.780 \pm 0.092^{a,b}$	$0.319 \pm 0.008^{i,j}$	$0.319 \pm 0.008^{i,j}$
100		1.121 ± 0.017^{d}	$1.271 \pm 0.081^{c,d}$	0.947 ± 0.021^{a}	0.973 ± 0.005^{a}
200		1.034 ± 0.022^{d}	1.142 ± 0.054^{d}	0.636 ± 0.048^{c}	0.764 ± 0.011^{b}
0	10	$1.737 \pm 0.191^{a,b}$	$1.737 \pm 0.191^{a,b}$	0.307 ± 0.019^{i}	0.307 ± 0.019^{i}
100		$0.736 \pm 0.019^{e,f}$	$0.861 \pm 0.116^{d,e}$	$0.485 \pm 0.012^{e,f}$	0.557 ± 0.022^{d}
200		0.638 ± 0.016^{g}	$0.782 \pm 0.119^{e,f}$	$0.412 \pm 0.010^{f,g,h}$	$0.528 \pm 0.025^{d,e}$
β-Sitosterol					
0	5	$1.721 \pm 0.024^{a,b}$	$1.721 \pm 0.024^{a,b}$	$0.315 \pm 0.014^{i,j}$	0.315 ± 0.014^{ij}
100		1.559 ± 0.025^{c}	1.617 ± 0.015^{b}	0.782 ± 0.011^{b}	$0.834 \pm 0.033^{a,b}$
200		$0.745 \pm 0.028^{e,f}$	$0.885 \pm 0.017^{d,e}$	**1.006 ± 0.001^{a}**	**1.216 ± 0.031^{a}**
0	10	1.823 ± 0.012^{a}	1.823 ± 0.012^{a}	0.301 ± 0.010^{j}	0.301 ± 0.010^{j}
100		1.121 ± 0.016^{d}	$1.221 \pm 0.113^{c,d}$	$0.432 \pm 0.009^{f,g}$	$0.538 \pm 0.019^{d,e}$
200		0.517 ± 0.011^{h}	0.598 ± 0.121^{g}	$0.486 \pm 0.011^{e,f}$	0.503 ± 0.017^{e}
Cholesterol					
0	5	$1.730 \pm 0.014^{a,b}$	$1.730 \pm 0.014^{a,b}$	$0.313 \pm 0.013^{i,j}$	$0.313 \pm 0.013^{i,j}$
100		1.032 ± 0.017^{d}	1.122 ± 0.021^{d}	0.562 ± 0.011^{d}	0.655 ± 0.034^{c}
200		0.725 ± 0.011^{f}	0.875 ± 0.027^{e}	0.635 ± 0.012^{c}	$0.691 \pm 0.032^{b,c}$
0	10	$1.777 \pm 0.019^{a,b}$	$1.777 \pm 0.019^{a,b}$	$0.414 \pm 0.007^{f,g,h}$	$0.414 \pm 0.007^{f,g,h}$
100		0.819 ± 0.017^{e}	$0.889 \pm 0.011^{d,e}$	$0.474 \pm 0.009^{e,f,g}$	$0.496 \pm 0.015^{e,f}$

200		$0.559 \pm 0.015^{g,h}$	0.691 ± 0.014^{f}	$0.518 \pm 0.009^{d,e}$	$0.541 \pm 0.013^{d,e}$

Values represent Mean ± SE (standard error) of 12 explants per treatment of three repeated treatments. Means followed by the same letter within the columns are not significantly different according to Duncan's multiple range at 95% confidence interval

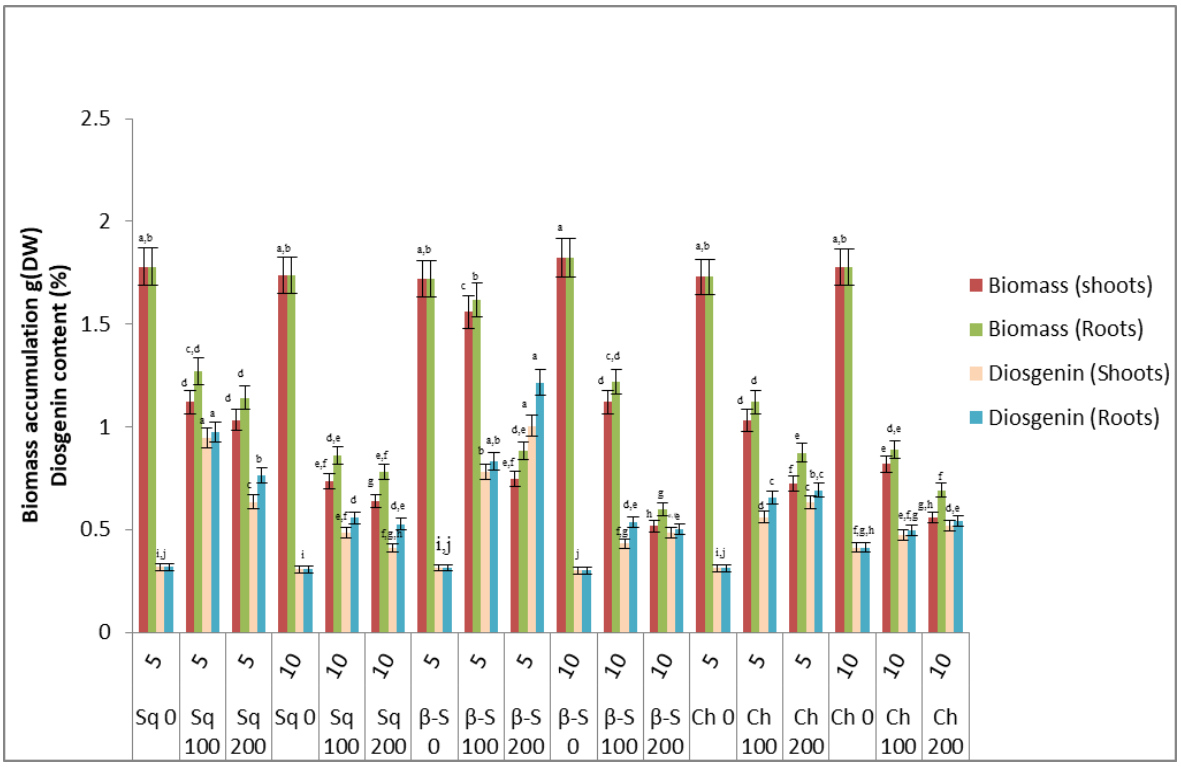
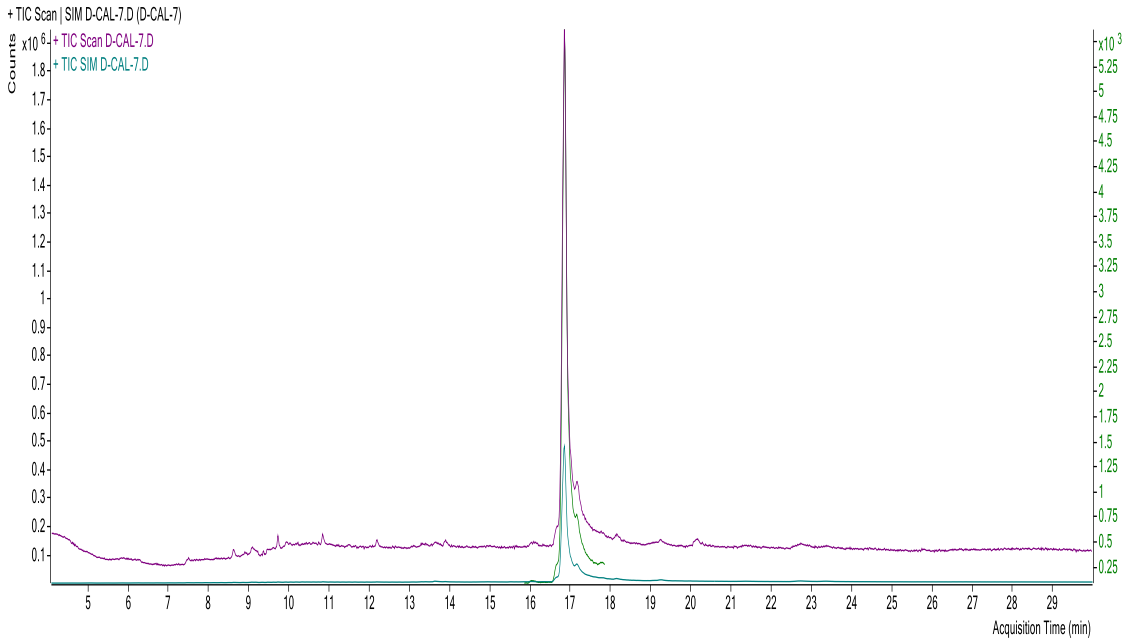


Figure 6.2: *Effect of squalene,* β-Sitosterol and cholesterol *on biomass and diosgenin production*



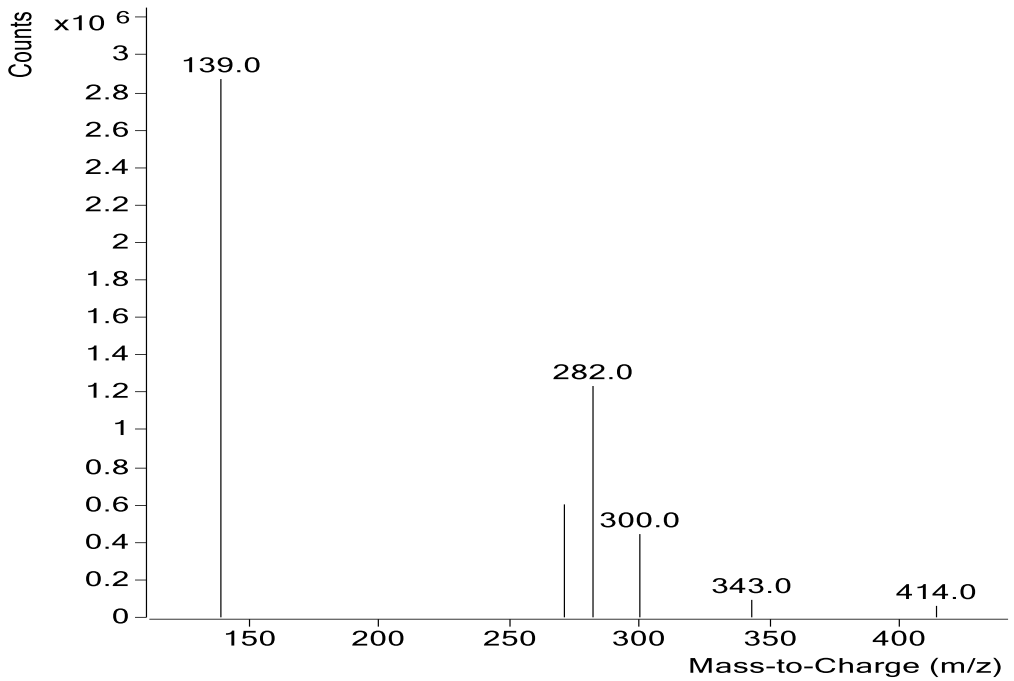


Figure 6.3: *TIC and SIM chromatograms of diosgenin*

6.5 Conclusion

This work has been accompanied with the objective of enhancing the production of diosgenin in *in vitro* cultures of *D. deltoidea* by the method of elicitation and precursor feeding. The plant cultures of *D. deltoidea* were elicited with MeJa and SA as elicitors and with precursors squalene, β-sitosterol and cholesterol separately at different concentrations. The outcomes of the present study revealed that SA was found more significant elicitor in terms of diosgenin production and β-sitosterol as potent precursor for the production of highest diosgenin content. Negative effect of elicitors and precursors were seen on biomass accumulation though positive effect on diosgenin content. The study also reveals that diosgenin content varied significantly in the *D. deltoidea* cultures depending on the type and concentration of elicitor or precursor used in relationship with exposure time. Squalene and cholesterol also promoted diosgenin production in *D. deltoidea* cultures but were less effective than β-sitosterol. The outcomes attained from this work can turn as a roadmap to promote investigations about the enhancement in diosgenin production. Furthermore, the treatment of elicitor and precursor can be used to scale-up the production of bioactive compounds and mass propagation of medicinal plants by the utility of bioreactors. Moreover, further studies are critical to design metabolic engineering methodologies that would improve the production of valued secondary metabolites in *in vitro* cultures.

CHAPTER 7

Summary and Conclusion

7.1 Summary

Dioscorea deltoidea generally famous as "Singli Mingli" is a rare high value therapeutic plant having huge economic significance. Due to its excessive and careless harvesting from its wild habitat and adulteration in its commercial trade, natural germplasm of *D. deltoidea* are vanishing promptly. Therefore, keeping these facts in view the current studies were commenced for the development of alternate strategies using biotechnological techniques for micropropagation, production of clonally identical *in vitro* regenerants, sustainable production of diosgenin by elicitation and precursor feeding and generation of molecular and chromatographic fingerprinting methods. The HPTLC and ISSR fingerprinting developed in the current study might be utilized efficiently in future as a consistent reference kit for the screening of elite variety having high potential of drug yielding and furthermore for detecting adulteration in commercial trade. The major objectives of the study were as follows:

- Collection of plants from different geographical locations and screening of elite variety of *D. deltoidea* on the basis of diosgenin and other related compounds by HPTLC method.
- Optimization of extraction methods on diosgenin yield from plants of *D. deltoidea*.
- Micropropagation of elite variety of *D. deltoidea* for mass production.
- Application of elicitors and precursors for the enhancement of diosgenin in the plants.

For the present study, plant accessions of *D. deltoidea* were collected from three different geographical zones of India namely Jammu and Kashmir, Himachal Pradesh and Uttarakhand and have been used for the screening of elite variety on the basis of bioactive compounds. An innovative and improved method of HPTLC was developed and validated for the simultaneous quantification of diosgenin and β-sitosterol. The plants collected from Gulmarg, J& K at an elevation of 2990 m was found potent sources of diosgenin and β-

sitosterol. The results revealed that altitudinal and environmental variations play important role to affect the eminence and amount of secondary metabolites. Moreover, HPTLC method was found simple, precise and sensitive and can be used in quality control and standardization of drugs.

The bioactive compounds value and quantity mainly depends on the selection of appropriate extraction technique. Basically extraction is the first phase in the study of any therapeutic plant however plays very important and critical part on the final outcome. In **Chapter 4**, extraction parameters were optimized by RSM and ANN modeling and revealed that among seven parameters, four parameters which are as solid: solvent ratio, particle size, extraction time and temperature were found significant parameters for the extraction of diosgenin. Furthermore, the optimum conditions for diosgenin extraction were found as solid: solvent ratio, 1: 45; particle size, 1.25 mm; extraction time, 45 min; temperature, 45 °C. In addition conventional and non- conventional methods such as cold maceration (CM), heat reflux extraction (HRE) and microwave-assisted extraction (MAE) methods and solvent selection (ethanol (100%), ethanol (50%) and water) has been compared to determine the proficient method for diosgenin extraction. MAE with 50% ethanol showed very good yield of diosgenin (0.985%) in less time duration (3 min) as compare to CM and HRE. Moreover, influence of seasonal variation on diosgenin content was also checked. Four seasons spring (March), summer (June), autumn (September) and winter (December) were selected and diosgenin amount was estimated three monthly by HPTLC analysis and results showed that December month is the best harvesting month that yielded highest diosgenin content (1.187%) followed by March (1.143%). Drying methods also played significant part in the quantity and quality of bioactive compounds and keeping this fact into consideration four different drying methods such as sun drying, shade drying, oven drying and microwave drying were tried. Out of these drying methods tried shade drying method was found optimum drying method as it yielded maximum diosgenin content (1.428%) than other methods.

Chapter 5 describes the micropropagation of elite variety of *D. deltoidea* by direct organogenesis. For the explants surface sterilization, NaOCl (2%) for 2 min and 0.1% $HgCl_2$ for 3 min was found to be an effectual treatment in respect of survival rate and establishment. In order to check most responsive explants, different explants such as leaf, internode, nodal segment and tuber were tried and nodal segments were found most responsive in terms of direct regeneration and tuber explants only respond to callus

formation. The *in vitro* response of regenerants was found to be significantly affected by the composition of media and also type and concentration of PGRs. Out of the growth hormones used, 2, 4- D and NAA in different concentration proved effective in regeneration of callus from tuber explant. Among different concentration of 2, 4- D and NAA, 1.5 mg L^{-1} produced maximum amount of callus with 70% culture response in 24 days and 60% culture response in 29 days. Callus generated in both the cases was yellow green and creamy fragile. For direct shoot initiation, BA either alone or in combinations with IBA, NAA and TDZ was used. Out of two auxins (IBA and NAA) in combination with BA, IBA was found more effective one as it significantly affected the shoot number and regeneration frequency. However, BA alone at concentration 2.0 mg L^{-1} showed maximum culture response (93.23%) and produced 3.80 ± 0.55 shoot numbers. For shoot multiplication both solid and liquid MS media augmented with different combinations of BA and IBA were used, however liquid MS medium was found efficient one as it produced more shoot numbers. Liquid MS medium augmented with BA 2.0 mg L^{-1} and IBA 1.0 mg L^{-1} produced maximum number of shoots/explant 13.50 ± 0.17 with 100.33% culture responses. This combination was considered the optimum PGR combination for shoot multiplication in *D. deltoidea* among all treatments. In shoot multiplication medium, simultaneous rooting was found, that is an additional advantage in the large scale micropropagation method. Besides that, another MS media augmented with different concentrations of NAA was also used as rooting media and 5.4 ± 0.70 roots were produced at 2.0 mg L^{-1} NAA with 92% culture response. The well-developed rooted plants were acclimatized successfully with 96% of survival rate and were attained on equal proportions of sand: manure (1:1:1). The micropropagated plants were screened for genetic fidelity and biochemical fidelity by using ISSR and HPTLC analysis. The randomly selected *in vitro* regenerants were found genetically similar to their mother plant as revealed by ISSR analysis and HPTLC results confirmed the presence of diosgenin in both micropropagated and mother plant which means they are biochemically identical and no change has occurred due to tissue culturing. Thus the micropropagation protocol is appropriately standardized to produce genetically and biochemically identical plants.

Due to the high medicinal value of bioactive compound diosgenin, *D. deltoidea* has attained much importance in the scientific community over the past few years. In **Chapter 6** genuine efforts have been made to enhance the yield of secondary metabolites in *in vitro* regenerants through biotechnological strategies which are as elicitation and precursor feeding. Methyl

Jasmonate and salicylic acid was used as elicitors and squalene, β-sitosterol and cholesterol as precursors at different concentrations (100 µM and 200 µM) and exposure times. Elicitor treatment was given at three exposure times (4 h, 6 h and 8 h) and precursor treated cultures were harvested after 5^{th} and 10^{th} of treatment. The outcomes of this study revealed that SA was found more significant elicitor in terms of diosgenin production and β-sitosterol as potent precursor for the production of highest diosgenin content. SA at 200 µM produced maximum diosgenin content 0.912% in shoots and 0.978% in roots and β-sitosterol at 200 µM maximum diosgenin content 1.006% in shoots and 1.216% in roots was obtained. Negative effect of elicitors and precursors were seen on biomass accumulation though positive effect on diosgenin content.

Finally, it can be concluded that through the current study a promising variety (G2, belonging to Gulmarg area) has been identified as the elite variety displaying clear superiority in terms of secondary metabolite (diosgenin) content in comparison to other investigated varieties. In the future, such promising variety may be utilised for mass cultivation. The HPTLC method developed was found to be simple, precise and sensitive and can be used in quality control and standardization of drugs and also used by the laboratories for the analysis of diosgenin and β-sitosterol containing products. The profiles will also be beneficial to screen and differentiate the *D. deltoidea* from other species of the genus Dioscorea. Moreover, ANN model was found superior than RSM in terms of predicting abilities and accuracy. Shade drying was observed as the most proficient and cost effective drying method in terms of diosgenin yield and seasons greatly affected bioactive compound quantity. Furthermore, MAE could be used as a competent and environmental friendly alternate to conventional methods to extract desired compound from plant matrices for their utilization in various industrial areas. In this study an alternative strategies have been made for mass propagation and germplasm conservation of genetically similar plants in a viable way. DNA and HPTLC fingerprinting generated in this work might be helpful in future. Furthermore, elicitor and precursor treatment can be used to scale-up the production of bioactive compounds and mass propagation of medicinal plants by the utility of bioreactors. Moreover, further studies are critical to design metabolic engineering methodologies that would improve the production of valued secondary metabolites in *in vitro* cultures. This work may be useful to researchers endeavoring to use biotechnological strategies to improve the quality and quantity of viable plants and bioactive compounds.

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1. Romaan Nazir, Suphla Gupta, Abhijit Dey, Vijay Kumar, Muzamil Yousuf,.... & D.K. Pandey (2020). *In vitro* propagation and assessment of genetic fidelity in *Dioscorea deltoidea*, a potent diosgenin yielding endangered plant. *South African Journal of Botany*. DOI https://doi.org/10.1016/j.sajb.2020.07.018
2. Romaan Nazir, Suphla Gupta, Abhijit Dey, Vijay Kumar,… & D. K. Pandey (2021). Biotechnological strategies for the sustainable production of diosgenin from *Dioscorea* spp. *Applied Microbiology and Biotechnology*. 105; 569–585. https://doi.org/10.1007/s00253-020-11055-3.
3. Romaan Nazir, Abhijit Dey, Vijay Kumar, & D. K. Pandey (2021). HPTLC quantification of diosgenin in *Dioscorea deltoidea*: evaluation of extraction efficacy, organ selection, drying method and seasonal variation. *South African Journal of Botany*. 138; 386–393. https://doi.org/10.1016/j.sajb.2020.12.027.
4. Dey Biswas, Romaan Nazir, Protha Biswas, Vijay Kumar, Samapika Nandy, Anuradha Mukherjee, ... & D. K. Pandey (2020). Endophytic sources of diosgenin, a natural steroid with multiple therapeutic values. *South African Journal of Botany*. DOI https://doi.org/10.1016/j.sajb.2020.04.009
5. Romaan Nazir, Suphla Gupta, Abhijit Dey, Vijay Kumar,… & D. K. Pandey (2021). Enhanced *in vitro* production of diosgenin in shoot cultures of *Dioscorea deltoidea* by elicitation and precursor feeding. BMC Plant Biology. Under Revision
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