# BIOTECHNOLOGICAL STRATEGIES FOR SUSTAINABLE PRODUCTION OF CAMPTOTHECIN FROM *OPHIORRHIZA SP.*

Thesis Submitted for the Award of the Degree of

**DOCTOR OF PHILOSOPHY**

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**By**

**Merinashwari**

**Konjengbam**

**(11919644)**

**Under the Supervision**
**of**

Dr. Devendra Kumar Pandey

Professor, Department of Botany

**LOVELY PROFESSIONAL UNIVERSITY, PUNJAB**
**FEBRUARY, 2024**

## DECLARATION

I, hereby declared that the presented work in the thesis entitled **"Biotechnological Strategies for Sustainable Production of Camptothecin from *Ophiorrhiza sp.*"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)**, Botany, Department of Botany, Lovely Professional University is outcome of research work carried out by me under the supervision of Dr. Devendra Kumar Pandey, working as Professor, in the Botany Department, School of Bioengineering and Biosciences, of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

Merinashwari K.
20/02/2024

**Merinashwari Konjengbam**
**Regd. No. 11919644**
School of Biosciences and Bio-Engineering
Lovely Professional University
Jalandhar, Delhi G.T.Road (NH-1)
Phagwara, Punjab-144411, India
Date: 20/02/2024

## CERTIFICATE

This is to certify that the thesis entitled **"Biotechnological Strategies for Sustainable production of Camptothecin from *Ophiorrhiza sp.*"**, which is being submitted by **Ms. Merinashwari Konjengbam** for the award of the degree of Doctor of Philosophy in Botany from the Faculty of Botany, 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.

Dr. Devendra Kumar Pandey
Professor,

Department of Botany,
School of Bioengineering and Biosciences,
Lovely Professional University
Phagwara, Punjab-144411, India
Date: 20/02/2024

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**Merinashwari Konjengbam**

## Abbreviations

| Abbreviations | Full form |
|---|---|
| **2, 4- D** | 2, 4-Dichlorophenoxyacetic Acid |
| **2iP** | 2-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 |
| **ASE** | Accelerated Solvent Extraction |
| **Asn** | L-Asparagine Monohydrate |
| **BA** | $N^6$-Benzyladenine |
| **BAP** | 6-Benzylaminopurine |
| **BBD** | Box-Behnken Design |
| **BM** | Basal Medium |
| **βME** | β-Mercapto ethanol |
| **bp** | Base Pair |
| **$CaCl_2$** | Calcium Chloride |
| **CC** | Column chromatography |
| **CS** | Chitosan |
| **CM** | Cold Maceration |
| **CTAB** | Cetyl Trimethylammonium Bromide |

| | |
|---|---|
| **DDW** | Double distilled water |
| **DMRT** | Duncan‘s Multiple Range Test |
| **DNA** | Deoxyribose Nucleic Acid |
| **dNTPs** | Deoxynucleotide triphosphates |
| **GC** | Gas Chromatography |
| **GC-MS** | Gas Chromatography- Mass Spectrometry |
| **Gln** | L-Glutamine |
| **HCl** | Hydrochloric Acid |
| **HIV** | Human Immune Deficiency Virus |
| **$HgCl_2$** | 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 |
| **IL-UMASDE** | IL-based ultrasonic/microwave-assisted simultaneous distillation and extraction |
| **ISSR** | Inter Simple Sequence Repeats |
| **JA** | Jasmonic Acid |

| | |
|---|---|
| **KN** | Kinetin |
| **$KNO_3$** | Potassium Nitrate |
| **LAF** | Laminar Air Flow |
| **LC-MS** | Liquid chromatography-mass spectrometry |
| **LOD** | Limit of Detection |
| **LOQ** | Limit of Quantification |
| **Lys** | Lysine |
| **MAE** | Microwave-Assisted Extraction |
| **MeJA** | Methyl Jasmonate |
| **$MgCl_2$** | Magnesium Chloride |
| **MEL** | Melatonin |
| **MEP** | Methylerythritol phosphate |
| **MIP-MSPD** | Molecularly imprinted polymer matrix solid-phase dispersion |
| **mT** | Metatopolin |
| **MP** | Mother Plant |
| **MS** | Murashige and Skoog |
| **MVA** | Mevalonate |
| **NAA** | α-Naphthalene Acetic Acid |
| **NaOCl** | Sodium hypochlorite |

| | |
|---|---|
| **NaOH** | Sodium hydroxide |
| **$NH_4NO_3$** | Ammonium Nitrate |
| **OD** | Oven Drying |
| **Orn** | Ornithine |
| **PBD** | Plackett-Burman Design |
| **PCR** | Polymerase Chain Reaction |
| **PEG** | Polyethylene Glycol |
| **PGRs** | Plant Growth Regulators |
| **Phe** | Phenylalanine |
| **PLE** | Pressurized Liquid Extraction |
| **PP** | Photoperiod |
| **PVP** | Polyvinyl Pyrrolidine |
| **QTL** | Quantitative Trait Locus |
| **$R^2$** | Determination Coefficient |
| **RAPD** | Random Amplified Polymorphic DNA |
| **RFLP** | Restriction Fragment Length Polymorphism |
| **RSM** | Response Surface Methodology |
| **RT** | Revised Tobacco |
| **SA** | Salicylic Acid |

| | |
|---|---|
| **SCoT** | Start codon targeted |
| **SD** | Standard Deviation |
| **SSR** | Simple Sequence Repeat |
| **TLC** | Thin layer chromatography |
| **TDZ** | Thidiazuron |
| **TIAs** | Terpene indole alkaloids |
| **Trp** | Tryptophan |
| **UAE** | Ultrasound-Assisted Extraction |
| **UARSE** | Ultrasonic Assisted Reflux Synergistic Extraction |
| **UFLC** | Ultra-fast liquid chromatography |
| **UHPLC-MS** | Ultra-high-performance liquid chromatography tandem mass spectrometry |
| **UPLC-DAD-MS** | Ultra-performance Liquid Chromatography- Diode Array Detection-Mass Spectrometry |
| **WPM** | Woody Plant Medium |
| **v/v** | Volume/volume |

## Abstract

India is one of the world's largest biologically diverse nation. India therefore plays a significant role in the development of herbal medication. The only resource that can provide a sustained supply of less expensive medications for the world's expanding population is medicinal plants. Throughout the beginning of time, people have utilized plants for medical uses. Almost 80% of people in poor nations depend on traditional medicine for their basic medical requirements. Westerners are rapidly moving to pharmaceuticals that are derived from plants as a result of the negative adverse response to man-made medications. As a result, herbal medicines are now on high demand in the market. The WHO claims that development of many medications will use medicinal plants as their primary raw material. Because of this, it is very important to carry out study on medicinal plants in order to make advantage of the beneficial features that they possess.

The genus *Ophiorrhiza* holds a significant place in the world of commerce. The main reason for choosing *Ophiorrhiza* spp. is because it ranks second in the distribution of Camptothecin which is a highest selling anticancer compound. Several pharmacological activities such as anti-cancer, antifungal, anti-malarial, anti-viral, etc., are reported in *Ophiorrhiza spp*. Apart from these it also helps in wound healing, snakebite, ulcers, stomatitis, it has analgesic, and antioxidant properties. The extract from root bark have curing effect on leprosy, gastropathy, and amenorrhea. Commonly *O. mungos* is called as mongoose plant or snakeroot for its use as a remedy for snakebites. *Ophiorrhiza* contains flavonoids, alkaloids, and triterpenes, camptothecin, according to a phytochemical investigation. Plant derived camptothecin is basically used in the preparation of anti-cancer drugs.

The plant *O.mungos* 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 *O.mungos* and the potential of well-known biotechnological techniques in view, a comprehensive investigation was carried out regarding the micropropagation and secondary metabolite synthesis on a massive scale by elicitation as well as precursor feeding. Further investigations were also performed to screen the elite population of *Ophiorrhiza spp.*, optimization of plant parts, extraction methods, solvents and other extraction parameters by using response surface methodology (RSM).

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 10 accessions of *Ophiorrhiza spp.* which were collected from Kerala, Western Ghats region of India. Bioactive compounds camptothecin was identified in the accessions and based on their content elite species were screened. The HPTLC results revealed that the accessions of *O.mungos* was screened as the elite species of *Ophiorrhiza spp.* as it contains the highest content of bioactive compound. Moreover, the HPTLC technique was shown to be easy to use, with high specificity and sensitivity, making it a viable tool for drug quality control and standardization.

- Optimization of extraction parameters was carried out by using RSM modeling and it was found that solvent composition, solid: solvent ratio, and extraction time were significant parameters for extraction of diosgenin. Furthermore, the optimal conditions for camptothecin extraction were found as solvent composition, 50%; solid: solvent ratio, 7:5; extraction time, 15 min.

  Moreover, conventional and non-conventional extraction methods and different solvents were compared and observed that UAE was efficient extraction method than

others and 60% methanol as better solvents.

- Biotechnological strategies like elicitation and precursor feeding were carried out for enhanced production of camptothecin. MeJa, JA, SA and CS as elicitors and tryptophan and geraniol as precursors were added in different concentrations to the ½ MS medium at different exposure times. The camptothecin 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 150µM each of MeJA, JA, SA and CS were found more significant combination of elicitors in terms of camptothecin production as potent precursor for the production of highest camptothecin content.

- For the purpose of micropropagation of an elite species of *O*.mungos, a new and enhanced regeneration technique was designed. For the explants surface sterilization, labolene (3-4drops) for 15min, Bavistin (1%) for 30min 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 use of ½ MS were optimized for large scale production of *O*.mungos. Simultaneous shoots and roots were developed by direct organogenesis in ½ MS medium fortified with various BA and IBA combinations. Shoot elongation was obtained in different concentrations of GA3 however 20µM containing media with 8% sucrose showed maximum elongation of shoots. Genetic and biochemical fidelity was performed by RAPD and HPTLC analysis and the in vitro progeny were a genetically same to their mother plant.

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# CHAPTER 1

# *INTRODUCTION*

## 1.1 Introduction

The only way to ensure a constant, low-cost supply of medications for the world's expanding population is to cultivate medicinal plants, which are renewable. Throughout the beginning of time, people have utilized plants for medicine since they are thought of as nature's gift for safe and effective treatment (Aras et al., 2018; Aras et al., 2021; Bursal et al., 2019). Almost eighty percent of people in poor nations depend on traditional medicine for their basic healthcare condition (Abdulkhaleq et al., 2018; Dey et al., 2021; Dutta et al., 2021). Western populations have been switching to pharmaceuticals made from plants as a result of the negative side effects of synthetic drugs. As a result, the market has seen a significant rise in demand for herbal medicines. The WHO has announced that therapeutic plants would be used as the primary ingredient in the synthesis of many different medications. For this reason, it is absolutely necessary to carry out study on medicinal herbs in in order to take advantage of the beneficial properties they contain (Igbe et al. 2018). Cunningham et al. (2018) state that in spite of plant being a significant source of medicines, there are a few challenges and factors that must be overcome before a plant can be used to produce a medicine, including sluggish development, sparse populations, restricted ranges, and a potentially devastating harvest, all of these parameters represent a threat to the survival of several species. The government of India has established facilities for on-site and off-site conservation to preserve the genetic diversity of diverse medicinal plants.

## 1.2 Medicinal Plants in India

India is one of the world's leading producers and exporters of pharmaceuticals derived from natural sources, along with Singapore, China, Brazil, South Korea, Japan, the United States of America, and Egypt. Despite widespread use of natural supplements and the existence of several therapeutic plants, around forty percent of the planet's known species are found in China and India. As a result, India is considered to be one of the world's most mega-

diverse countries. As a consequence, India can contribute significantly to the manufacture of herbal medicines. To achieve this goal, we need to learn more about the potent compounds found in therapeutic plants and the qualities of plant material that allow for efficient, reproducible, and specific extraction. India has the highest level of biodiversity in the world thanks to its 16 different agro-climatic regions and 10 different vegetative areas. Two "biodiversity hotspots" exist, adding to India's natural wealth. According to studies of Sharma et al. (2008), out of 45,000 plant species, 15,000–20,000 are recognized for their pharmacological evaluation. India's varied topography and climate are to the country's advantage. The Himalayas are hilly, while the south is tropical and wet, Rajasthan is dry, and the north-eastern regions include a wide diversity of topography, including plateaus, mountains, plains, and valleys. The Indian subcontinent is home to a rich and diversified flora due to the optimal climatic conditions (soil, temperature, precipitation, etc.).

There has been a lot of research done on medicinal plants, however the overexploitation of the species has resulted from the desire for these plants products. Therefore, there arises a need for search of alternative plants with similar medicinal properties. For e.g., there are several plants with anti-cancer properties.

**Table 1.1** List of plants exhibiting anticancer properties.

| Scientific name | Family | Anti-cancer compound | References |
|---|---|---|---|
| ***Solanum trilobatum*** | Solanaceae | Usnic acid and monoacetate | Abilasha et al., 2022 |
| ***Uvaria narum*** | Annonaceae | Quercitin | Ajaykumar et al., 2023 |
| ***Gracilaria corticata*** | Gracilariaceae | Camptothecin, Quercitin | Ashwini et al., 2017 |
| ***Taxus baccata*** | Taxaceae | Paclitaxel(taxol) | Asif et al., 2016 |
| ***Cissampelos pareira*** | Asteraceae | Tetrandrine and Berberine | Bafna et al., 2010 |
| ***Dioscorea spp.*** | Dioscoreaceae | Gracillin | Bhuia et al., 2023 |
| ***Nothapodytes foetida*** | Icacinaceae | Camptothecin | Chu et al., 2014 |
| ***Tylophora indica*** | Asclepiadaceae | Tylophorine | Cragg et al., 2005 |
| ***Ervatamia heyneana*** | Apocynaceae | Camptothecin | Dighe et al.,2012; Pu et al.,2019 |
| ***Nigella sativa*** | Ranunculaceae | Thymoquinone | Gali-Muhtasib et al., 2008 |
| ***Maytenus*** | Celastraceae | Maytansine | Huang et al., |

| | | | |
|---|---|---|---|
| ***serrata*** | | | 2021 |
| ***Merriliodendron megacarpum*** | Icacinaceae | Camptothecin | Kai et al.,2015; Pu et al.,2019 |
| ***Vinca rosea*** | Apocyanaceae | Vincristine, Vinblastine | Keglevich et al., 2012 |
| ***Ailanthus excelsa*** | Simaroubaceae | Ailanthione and glaucarubinone | Kumar et al., 2010a |
| ***Piper betle*** | Piperaceae | Hydroxychavicol | Kumar et al., 2010b |
| ***Podophyllum hexandrum*** | Berberidaceae | Podophyllotoxin | Kumar et al., 2022 |
| ***Zingiber officinale*** | Zingiberaceae | Phenolic and terpene | Lee,2016 |
| ***Colchicum autumnale*** | Colchicaceae | Colchicine | Liantinioti et al.,2018 |
| ***Psoralea corylifolia*** | Leguminosae | Aryl coumarin | Limper et al., 2013 |
| ***Taxus chinensis*** | Taxaceae | Paclitaxel(taxol) | Liu et al.,2015 |
| ***Ophiorrhiza spp.*** | Rubiaceae | Camptothecin | Mahendran et al., 2021 |
| ***Solanum dulcamarra*** | Solanaceae | Usnic acid and monoacetate | Mutlu et al., 2012 |
| ***Camptotheca acuminata*** | Nyssaceae | Camptothecin | Nacheva et al., 2020 |
| ***Solanum nigrum*** | Solanaceae | Solaoiacid, uttroside B, degalactotigonin, solanine A | Nkwe et al., 2021 |
| ***Psidium guajava*** | Myrtaceae | Apigenin | Priam et al., 2021 |
| ***Mostuea brunonis*** | Logaiaceae | Camptothecin | Pu et al.,2019 |
| ***Pyrenacantha volubilis*** | Icacinaceae | Camptothecin | Ramachandran et al., 2021 |
| ***Mappia foetida*** | Icacinaceae | Camptothecin | Ramesha et al., 2020 |
| ***Xanthium strumarium*** | Asteraceae | Xanthatin and xanthinosin | Ramírez-Erosa et al., 2007 |
| ***Spermacoce hispida*** | Rubiaceae | Flavonoids, anthocyanins, carotenoids, dietary glutathione, vitamins | Rathi et al., 2011 |
| ***Borreria hispida*** | Rubiaceae | Protein fraction F3 | Rupachandra & Sarada, 2014 |
| ***Curcuma longa*** | Zingiberaceae | Curcumin, demethoxycurcumin, bisdemethoxycurcumin, germacrone, furanodienone,zederone, andar-turmerone | Shehzad et al., 2013 |
| ***Belamcanda chinensis*** | Iridaceae | belamcanosides A and B | Song et al., 2018 |
| ***Acronychia baueri*** | Rutaceae | Acronycine | Tillequin, 2002 |
| ***Plumbago*** | Plumbaginaceae | Plumbagin | Yan et al., 2015 |

| | | | |
|---|---|---|---|
| ***zeylanica*** | | | |
| ***Uncaria tomentosa*** | Rubiaceae | Quinovic acid glycosides, oxindole alkaloids | Zari et al., 2021 |

### 1.3 Secondary metabolites from medicinal plants

Plant secondary metabolites have a crucial part in human life since they are used as food and therapeutic agents. The assurance of the effectiveness, safety, and quality of herbal plants and medicines has now become a critical issue in both developing and industrialised nations. Herbal medicines have been used for thousands of years to treat, prevent, and cure illnesses. Throughout the ages, human groups have shared knowledge of these plants' healing powers. Secondary metabolites, which are products of secondary metabolism and are employed for a variety of reasons worldwide, including the treatment of infectious diseases, are what give plants their biological characteristics. These pharmaceutically significant bioactive chemicals are separated and extracted from therapeutic plants that are used in the medication production process.

Cardoso et al. (2019) asserts that the continuous use of therapeutic plants will be extended and enhanced by significant advancements in secondary metabolite generation from plant cell culture. As a means of self-defence from herbivores or under stress, plants develop secondary metabolites, which are active photochemicals with therapeutic benefits. Several biosynthetic processes, including the shikimate, terpenoid, and polyketide pathways, allow plants to create secondary metabolites. Traditional and metabolic engineering methods of genetic modification produce a significant quantity of secondary metabolites and can sustain a consistent level of synthesis of desired substances without further assistance.

Sasidharan et al. (2011) asserts that the process of sample preparation significantly affects the qualitative as well as quantitative characteristics of the bioactive molecule. Before proceeding with extraction, thorough homogenization of the plant tissue increases the extraction rate as it increases accessibility between the solvent and the plant cell matrix. Alkaloids, terpenoids, saponins, and other chemicals are present in the crude extract of plants, thus it is necessary to choose an extraction method and analytical methodology that will best achieve the extraction, isolation, and purification of the desired bioactive molecule. The evidence shows that while secondary metabolites analysis is now easier to perform than it was in the past thanks to improvements in modern spectrometric and chromatographic techniques, the success of this effort depends on specific extraction methods, plant part characteristics, and other factors (Poole et al.,1990). Extraction

techniques are divided into two types: conventional and non-conventional techniques. The selection of a suitable extraction technique is crucial for achieving maximum yield. Therefore, in the selection of extraction techniques, one should take into consideration of factors such as type of plant sample(hard/soft), time of sample collection and its part, drying, grinding and powdering method, particle size, solvent selection for specific compounds, extraction cycles, temperature, extractor type and design. Recent advancements include MAE of anthraquinones (Suktham et al., 2021), phenols (Baltacioglu et al., 2021), essential oils (Boudraa et al., 2021), UAE of phenolic compounds (Tzima et al., 2021), flavonoid (Nguyen et al., 2021), TLC and CC are effective for the initial screening of secondary metabolites. Advanced techniques, such as HPLC, HPTLC, UPLC, UFLC, LC-MS, and GC, are effective for the subsequent analysis for identification and determination.

Tasheva and Kosturkova, (2012) suggest that rapid cloning, genotype preservation, and genetic modification of natural components in huge quantities or with improved qualities are all made possible by biotechnological approaches. The field of biotechnology has developed into a hub of interest for preservation and a reliable source of herbal plants due to its multiple advantages in various industries, including food, agricultural, forestry, pharmaceutical, and others. All of which represent a threat to the survival of several species. The government of India has established on-site and off-site conservation facilities to preserve the genetic diversity of diverse medicinal plants.

### 1.4 *In vitro* biotechnological methods

The most promising biotechnological method for the preservation and sustained cultivation of commercially valuable and critically endangered medicinal plants is in vitro culture, also known as plant tissue culture. The mass propagation of decorative, horticultural, medicinal, and disease-free plant species, cryopreservation, plant enhancement, plant breeding, creation of strategically essential secondary metabolites, and genetic modification have all seen significant increases in their significance (Debnath et al., 2006; Altpeter et al., 2016). Furthermore, in vitro culture is currently a well-established technique for cultivating and researching the biological processes of isolated plant cells, organs, tissues, cell organelles, and protoplasts under carefully regulated chemical and physical conditions. The foundation of all biotechnological research is micropropagation since nearly all applications of plant biotechnology eventually require the efficient culture of plant cells, organs, and tissues.

Compared to traditional or conventional vegetative propagation through cutting, air-

layering, grafting, and seed, this approach has a number of benefits, including the rapid growth of a rapid generation of virus-free plant stock with high quality consistency (Garcia-Gonzales et al., 2010). Considered to be among the most valuable plants for therapeutic purposes, *Coryodalis yanhusuo*, was introduced via somatic embryogenesis from the callus of the tuber to produce tubers free of pathogens (Sagare et al., 2000).

Numerous factors, including the culture medium, its makeup, the environment in which it is grown, the plant growth hormones used, and the genotype, all affect how successful micropropagation is. The development of methods for quick in vitro clonal plant production could have significant economic effects on the sector. Due to the likelihood of soma clonal inconsistency (George, 1993), certain callus cultures create clones with inheritable traits different from those of the mother plants, which promotes the development of commercially valuable improved varieties. By reducing the number of subcultures and cultivating shoot tips or axillary buds, the in vitro regenerants should have the fewest somatic changes from a conservation standpoint. In callus formations or cell suspension cultures, soma clonal differences are common and reduce the commercial relevance of the in vitro regenerants (Borse et al., 2011). The utilization of in vitro techniques for the manufacture of phytochemicals is severely hampered by the presence of soma clonal variations during micropropagation, the development of secondary metabolites, and genetically modified plants, which might result in significant commercial problems (Bhattacharya et al., 2016). Determining the genetic make-up and consistency of the in vitro regenerants is therefore necessary in order to check for soma clonal inconsistency in a cell culture. That method's procedures entail measuring potential differences at different levels using a variety of methodologies (Bhattacharyya et al., 2015, 2017a; Bhattacharyya and Van Staden, 2016; Bose et al., 2016). Numerous DNA-based molecular markers, including ISSR, RAPD, AFLP, RFLP, ScoT polymorphism, and microsatellites, have successfully been applied towards the assessment of variations in chromosome number and ploidy (Ahuja et al., 2002; Bhattacharyya et al., 2015; Bose et al., 2016; Narula et al., 2007; Thankappan and Morawalla-Patell, 2011).

Thus, micropropagation has evolved into a profitable endeavor that provides obvious advantages over traditional agricultural proliferation methods by enabling the production of enormous numbers of identical plants over a long time span, the creation of pathogen-free plants, and a notable increase in multiplication rates (Debnath et al., 2006). For example, *Stevia rebaudiana* (Alvarado-Orea et al., 2020), *Dendrobium crepidatum* (Bhattacharya et al.,2015), *Cynara scolymus* (Comino et al., 2019), *Nothapodytes foetida*

(Fulzele et al., 2001), *Camptotheca acuminata* (Nacheva et al., 2020), *Moringa oleifera* (Ridzuan et al., 2020) and *Agave salmiana* (Silos-Espino et al., 2007) are among the commercially significant plants for which a large number of micropropagation protocols have been developed. However, the inability to practice in vitro methods at a level that is viable is due to the high costs of in vitro methods relative to traditional methods and the impulsiveness of market requirements (Debnath et al., 2006; Mehrotra et al., 2007).

### 1.1.2 *In vitro* strategies for production of secondary metabolites

A well-researched technology for the manufacture of natural products from plants is provided by the in vitro cultivation of plant cells, tissues, and organs under aseptic conditions. Typically, plant material capable of producing bioactive chemicals can be obtained via micropropagating plant organs, such as roots or calluses (Atanasov et al., 2015). Plant cell culture today represents a practical way to manufacture a wide range of valuable natural bioactive chemicals (Fischer et al., 2015). In vitro culture relies on a number of variables, mostly nutrients for plant growth, to produce bioactive chemicals. The accumulation of bioactive compounds and explant development depend significantly on the nutrient concentration. The most notable example of the use of cell suspension culture for the generation of secondary metabolites is the manufacture of paclitaxel from *Taxus spp.* (Atanasov et al., 2015). However, by making certain changes to the culture media, such as accumulating elicitors and precursors, or by changing environmental factors like light, temperature, or humidity, it is possible to increase the synthesis of secondary metabolites.

Bioactive chemical production is sparked by elicitors, which are plant defense molecules. Cellulose and Pectin found in cell walls of plant, glucan and chitin found in microorganisms, and plant-resistant signaling chemicals (MeJa, SA and Ja) are among the various elicitors that can increase the production of bioactive substances. In *Azadirachta indica*, several fungal filtrates were applied to root cultures to increase the synthesis of azadirachtin in biotic elicitors (Srivastava and Srivastava, 2014). Krishnan and Siril, (2018) reported Pectin as most effective in eliciting the formation of anthraquinones in *Oldenlandia umbellate* cultures, followed by yeast extracts and xylan. The type of culture being used and the secondary metabolite being produced are the elements that influence the choice of an appropriate elicitor.

The inclusion of precursors that are intermediates in the metabolic route of the desired molecule is another tactic. In several instances, including the development of flavonoids (Masoumian et al., 2011), withanolides (Sivanandhan et al., 2013), phenolic compounds, and

triterpenoids, through the use of precursors added to the culture media improved the synthesis of the desired chemical (Rao & Ravishankar et al., 2002). Viewing the entire biosynthetic pathway of the target chemical is required before choosing the right precursor.

For the improved production of commercially valuable bioactive chemicals, other alternative biotechnology technologies include metabolic engineering, genetic transformation, hairy root cultures, bioreactor systems, endophytes, and immobilization techniques.

### 1.1 Aims and objectives of the study

*Ophiorrhiza mungos* is a medicinal plant with considerable economic value that is near threatened. However, a lot of research is still required, including phytochemical screening for elite varieties and manufacture of plants and bioactive substances using biotechnological methods. In order to develop alternative methods using biotechnological techniques for micropropagation, the production of clonally identical in vitro regenerants, the sustainable production of camptothecin by elicitation and precursor feeding, and the generation of molecular and chromatographic fingerprinting methods, the current investigations were started.

Taxonomic classification of the herb:

**Kingdom:** Plantae
**Division:** Tracheophyta
**Class:** Magnoliopsida
**Order:** Gentianales
**Family:** Rubiaceae
**Genus:** *Ophiorrhiza*

Conservation status of *Ophiorrhiza spp*.- Not evaluated (NE) (Rao, 2019)

**Objectives of the study**

- Collection of germplasm of selected *Ophiorrhiza* spp. from different indigenous sources and identify high camptothecin yielding lines.
- Study the genetic diversity of the selected *Ophiorrhiza spp*. by using phytochemicals and DNA based molecular markers.
- Standardize protocol for in vitro production of camptothecin utilizing tissue culture techniques.
- Standardize technique for mass multiplication of elite *Ophiorrhiza spp*.

# CHAPTER 2

# *Review of literature*

## 2.1: Botanical description

There is a genus of plants known as *Ophiorrhiza*, which is classified under the family Rubiaceae. The *Ophiorrhiza* species have a variety of growth patterns, from creeping plants to tiny shrubs up to 3 metres tall that grow in humid climates at various altitudes. Most of them are standing upright and have a persistent woody lower stem, while others have branches lying on the ground or forming new habits. Elliptic, lanceolate, or oval leaves are observed. Cymose inflorescences at the end of stems or along axillary branches; white flowers with funnel-shaped corollas (Jaleel and Velraj 2019; Krishnan et al. 2018a; Sibi et al. 2015). Fruits have tiny, rhomboid-shaped capsular seeds that are laterally compressed (Taher et al. 2020). There are annual and perennial species in the genus.

## 2.2: Geographical distribution

The distribution of *Ophiorrhiza* in India is presented in Table 2.1.

**Table 2.1:** *Ophiorrhiza* species distribution across India.

| Sl. no. | Species | Distribution | References |
|---|---|---|---|
| 1. | *Ophiorrhiza barberi* | Idukki, Kerala | (Rajan et al., 2013;Sibi et al., 2015; Sibi, 2017) |
| 2. | *O. caudata* | Idukki, Kerala | |
| 3. | *O. caudipetala* | Meghalaya | |
| 4. | *O. chandrasekharanii* | Andra Pradesh | |
| 5. | *O. codyensis* | Karnataka | |
| 6. | *O. eriantha* | Thiruvananthapuram, Tamil Nadu | |
| 7. | *O. fasiculata* | Sikkim, West Bengal, Orrisa, Western Himalayas | |
| 8. | *O. filistipula* | Western Himalayas, Sikkim, West Bengal, Odhisa | |
| 9. | *O. grandiflora* | Idukki, Kerala | |
| 10. | *O. heterostyla* | West Bengal, Arunachal Pradesh | |
| 11. | *O. hirsutula* | Kerala, Karnataka, Tamil Nadu, Andhra Pradesh | |
| 12. | *O. hispida* | Assam, Meghalaya | |
| 13. | *O. incarnata* | Kerala | |
| 14. | *O. lurida* | Sikkim, Manipur | |
| 15. | *O. mungos* | Kollam, Thiruvananthapuram and Idukki- Kerala | |
| 16. | *O. munnarensis* | Southern Western Ghats of Kerala | |
| 17. | *O. mungos var. angustifolia* | Ernakulam and Pathanamthitta, Kerala | |
| 18. | *O. nairii* | Thiruvananthapuram and Pathanamthitta, Kerala | |
| 19. | *O. ochroleuca* | West Bengal, Sikkim | |
| 20. | *O. oppositiflora* | Throughout eastern India | |
| 21. | *O. pectinata* | Pathanamthitta, Kerala | |
| 22. | *O. pauciflora* | Arunachal Pradesh, Meghalaya | |

| | | |
|---|---|---|
| 23. | *O. pykarensis* | Tamil Nadu |
| 24. | *O. radicans* | Kerala |
| 25. | *O. repens* | Assam, Meghalaya, Arunachal Pradesh |
| 26. | *O. rosea* | West Bengal, Sikkim |
| 27. | *O. roxburghiana* | Kerala, Tamil Nadu |
| 28. | *O. rugosa* | Kollam and Thiruvananthapuram, Kerala, Assam |
| 29. | *O. shendurunii* | Kollam and Ernakulam, Kerala |
| 30. | *O. subcapitata* | Meghalaya |
| 31. | *O. succirubra* | West Bengal, Sikkim, Meghalaya, Assam and all other states of Eastern India |
| 32. | *O. thomsonii* | West Bengal, Manipur |
| 33. | *O. tingens* | Assam, Meghalaya, Nagaland |
| 34. | *O. tirunelvelica* | Tamil Nadu |
| 35. | *O. treutleri* | West Bengal, Sikkim |
| 36. | *O. trichocarpon* | Western ghats |
| 37. | *O. villosa* | Tripura, Andaman and Nicobar Islands |
| 38. | *O. wallichii* | Meghalaya, Nagaland, Arunachal Pradesh |
| 39. | *O. wattii* | Meghalaya, Nagaland, Manipur, Arunachal Pradesh |

## 2.3 Genetic diversity assessment

Genetic diversity is described as the degree to which members of a population or species differ genetically, as well as the ability of a genotype to produce new genotypes and adapt to constantly changing ecological conditions (Brown-Guedira et al., 2000). Researchers have created a number of methods to identify somaclonal variations in plants in order to prevent them. These methods include morphological markers, biochemical markers and molecular markers. Wide germplasm characterization therefore includes visual description as well as the assessment of biochemical and molecular markers for genetic assessment (Mignouna et al., 2003).

**Table 2.2** Diversity assessment of *Ophiorrhiza spp.*

| Tools | Plant species | Results/Response | References |
|---|---|---|---|
| **Morphological markers** | | | |
| Vegetative Morphology & Floral Morphology | *Ophiorrhiza mungos, O. rugosa, O. eriantha, O. trichocarpon and O. shenduruni* | Differences in color and shape found in floral morphology, leaf shape and stem within same species & different species | Jaleel et al., 2019 |
| Floral morphology - Style length | *O. japonica* | Homostylous and distylous observed in the flower of same species. | Nakamura et al., 2007 |
| **Molecular markers** | | | |
| RAPD | *O. mungos* | Check genetic fidelity of regenerated plants | Kaushik et |

| | | | |
|---|---|---|---|
| | | propagated through somatic embryogenesis. | al., 2015 |
| Plastid markers, ETS and ITS primers | *Ophiorrhiza spp*. | ITS-ndhF-rpS16, ndhF-rps16-trnT-F data used for assessment of tribal in genetic limits and relationship of Rubiaceae | Razafimandimbison & Rydin, 2019 |
| Gene specific primer used | *O. fucosa, O. harrisiana, O. pedunculata, O. plumbea, O. pseudofasciculata, O. ridleyana, O. trichocarpon* | Phylogenetic analysis of the sequences of the nuclear TopI gene and the chloroplast matK gene revealed that primary clade of *Ophiorrhiza* taxa connected with production of CPT and its derivatives. | Viraporn et al., 2011 |
| **Phytochemical markers** | | | |
| Camptothecin, 9-methoxycamptothecin | *O. fucosa, O. harrisiana, O. pedunculata, O. plumbea, O. pseudofasciculata, O. ridleyana, O. trichocarpon* | Variations observed in the amount of Camptothecin, 9-methoxycamptothecin reported in leaves and roots. | Viraporn et al., 2011 |
| Camptothecin | *O. mungos, O. pectinata, O. eriantha, O. caudata, O. grandiflora, O. nairii, O. trichocarpon, O. barberi, O. shendurunii & and O. mungos* var. *angustifolia, O. rugosa* var. *decumbens, O. rugosa Wall.* var. *prostrata* | Camptothecin was detected in eight species of *Ophiorrhiza* collected from South Western Ghats in India. | Rajan et al., 2013 |

## 2.4 Phytochemistry & Pharmacology of *Ophiorrhiza:*

According to reports, the genus *Ophiorriza* have various medicinal attributes. Pharmacological effects reported in *Ophiorrhiza spp.* along with its active compounds are presented in Table 2.3.

**Table 2.3** Reports on the medicinal importance of various *Ophiorrhiza spp*.

| **Pharmacological effects** | **Plant species** | **Active compounds** | **References** |
|---|---|---|---|
| Antioxidant and antibacterial | *O. pectinata* | Camptothecin | Baiju et al., 2017; Rajan et al., 2013. |
| Antiviral activities, anti-inflammatory, antimicrobial. | *O. mungos* | Camptothecin, hydroxyl camptothecin, 10-methoxy camptothecin, 9-methoxy camptothecin, luteolin-7-*O*-Glucoside, 5α-ergosterol-8(14) - ene-3β-ol, 5α-ergosterol-7-ene-3β-ol. | Baskar et al., 2011; Napagoda et al., 2020 |
| Anticancer, antifungal | *O. trichocarpon* | Ophiorrhiside E, Ophiorrhiside F, dolichantoside , 5-carboxystrictosidine , lyaloside , 3,4,5,6-Tetrahydrodolichantoside, camptothecin | Kitajima et al., 2013 |
| Angular stomatitis | *O. fistipula* | 7-Methoxy camptothecin, camptothecin | Ohba et al., 2000 |
| Anticancer | *O. eriantha* | Camptothecin | Rajan et al., 2013 |
| Anticancer | *O. grandifolia* | Camptothecin | Rajan et al., 2013 |
| Anticancer, antifungal | *O. shendurunii* | Pentacylic triterpenoid fatty acid ester, lupan-20-ol-3($^{\beta}$)-yl acetate, Olean-18-en-3($^{\beta}$)-yl, hexa decanote, dotriacontanoic, stigmasterol, rubiadin, non adecanoic acid, palmitic acid and Camptothecin | Rajan et al., 2013; Rajan et al., 2016 |
| Anticancer, antifungal | *O. rugosa* var *decumbens* | Camptothecin | Roja 2008 |
| Anticancer, antiviral | *O. wattii* | camptothecin | Sibi et al., 2015 |

## 2.5 Camptothecin

Camptothecin (CPT) [IUPAC name: (19S)-19-ethyl-19-hydroxy-17-oxa-3,13-diazapentacyclo [11.8.0.02,11.04,9.015,20] henicosa-1(21),2,4,6,8,10,15(20)-heptaene-14,18 dione] (Fig. 2.1) is Ophiorrhiza's primary active constituent. CPT is a plant-derived antitumor alkaloid. After cardiovascular disease, cancer is the leading cause of mortality worldwide (Dagenais et al., 2020). In terms of its chemical composition, it is classified as a monoterpenoid indole alkaloid with a quinolene moiety that is just slightly basic and a lactone ring as its terminal group (Pu et al., 2019). Because of its intricate structure, CPT cannot be produced artificially; instead, it must be extracted from plants only (Wink et al., 2005). By blocking the activity of type 1 DNA topoisomerase, CPT works as an effective anticancer drug (Ghanbari-Movahed et al., 2021). DNA topoisomerase I is the enzyme that is responsible for catalyzing the change in DNA topology that involves cleaving and religating one strand of DNA (Sirikantaramas et al., 2015). Colorectal, uterine, cervical, lungs, breast, and prostate cancer, AIDS, and falciparum malaria are among of the primary diseases that CPT is used to treat (Galatage et al., 2021; Partridge et al., 2021). Activity of DNA topoisomerase I reduced by CPT. Two CPT analogues, irinotecan and topotecan, are authorized for the treatments of ovarian, lung, colorectal malignancies, and cervix as well as HIV (Pizzolato and Saltz, 2003; Shaanker et al., 2008; Sirikantaramas et al., 2013).

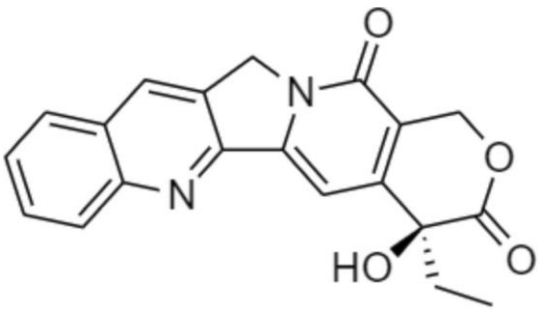



**Fig. 2.1**: Camptothecin

## 2.6 Biosynthetic pathway

According to Silvestrini et al. (2002) the process of biosynthesis of camptothecin was not much fully exposed. Several steps in the biosynthesis of CPT and the regulatory mechanism that controls this process are still poorly understood (Sirikantaramas et al., 2007; Asano et al., 2013). Reports on several cell cultures revealed that camptothecin biosynthesis and accumulation are dependent on cell development and environmental factors. Camptothecin is a nitrogen-containing, pentacyclic compound. Amino acids, such as Phe, Tyr, Lys, and Orn were considered as the precursor of different types of alkaloids. All terpene indole-

alkaloids including camptothecin are synthesized from strictosidine, a condensation product of indole tryptamine and the terpenoid secologanin, catalyzed by the enzyme strictosidine synthase. Possible biosynthetic pathways for camptothecin were reviewed by Xu *et al*. (2020). CPT is formed via a sequence of cyclization processes between tryptamine, which is derived from the shikimate pathway, and secologanin orginating from geranyl diphosphate, which is derived from the MEP and MVA pathways (Yamazaki et al., 2004). However, strictosidine to CPT metabolic pathway and the catalytic enzymes involved are not yet fully understood (Lorence and Nessler, 2004). It is believed that the flexible cytochrome P450monooxygenase (P450s) catalyzes some unidentified chemical steps, possibly including hydroxylation, oxidation, and dehydrogenation (Kai et al., 2015; Patten et al., 2010) (Fig.2.2).

## 2.7 Critical analysis of extraction and determination of camptothecin

The isolation of active secondary metabolites from medicinal plants is a critical first step in the process, and the two primary steps in sample preparation are extraction and quantification.

### 2.7.1 Extraction

Extraction is the first step for conducting qualitative and quantitative analysis of any bioactive compounds from plants. Through extraction, complex plant structures are broken down into simpler forms suitable for easy recovery and analysis of compounds of interest. Extraction techniques are divided into two types: conventional and non-conventional techniques. The selection of a suitable extraction technique is crucial for achieving maximum yield. Therefore, in the selection of extraction techniques, one should take into consideration of factors such as type of plant sample(hard/soft), time of sample collection and part used, drying, grinding and powdering method, particle size, solvent selection for specific compounds, extraction cycles, temperature, extractor type and design. From the available literature conventional techniques for extraction of camptothecin from plants include Stirring (Fulzele & Satdive, 2005b; Patil & Akamanchi, 2017a; Patil & Akamanchi, 2017b; Shen et al., 2011), Soxhlet (Ashwini et al., 2017; Palode, 2016; Rajan et al., 2013; Rani et al., 2010), Maceration (Fulzele & Satdive, 2005b; Karanje et al., 2021; Puri et al., 2005), vortex mixer (Karanje et al., 2021), cold maceration (Kulkarni et al., 2010). The main agenda of the conventional technique depends on the solubility of the solute from plant material into the solvent. Therefore, these techniques have major drawbacks of involving a large amount of solvent and sample, long extraction period with low yield. These drawbacks

are overcome by non-conventional techniques. According to Khoddami et al. (2013) non-conventional techniques on contrary to conventional use less amount of solvents and sample, shorter extraction time and produces a higher yield, thus has been categorized under 'Green Extraction Techniques'.

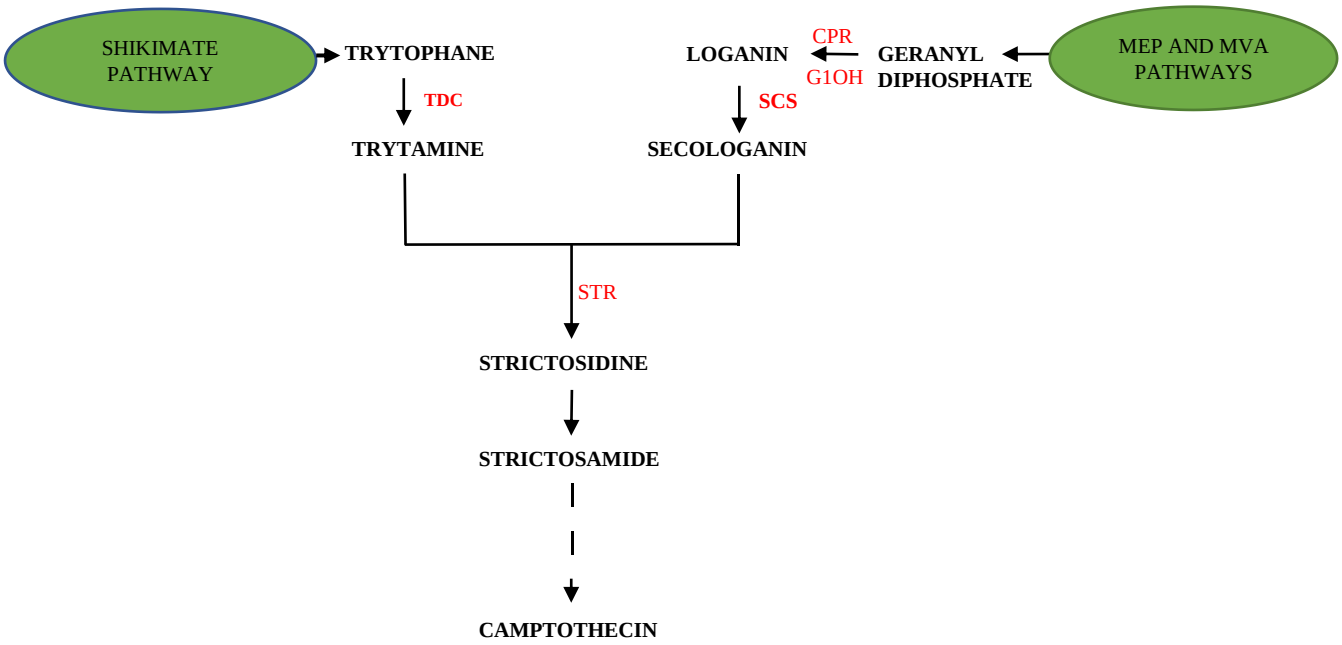



Fig 2.2: Camptothecin biosynthetic pathway; CPR-Cytochrome reductase, G1OH-Geraniol-10-hydroxylase, TDC- Tryptophan decarboxylase, SCS-Secologanin synthase, STR- Strictosidine synthase, MEP-Methyl erythritol phosphate, MVA-Mevalonate;dotted lines indicates multiple unknown steps.

Green extraction techniques can be defined as those which use an alternative solvent, maximize extract quality while minimizing energy use (Chemat et al., 2012). Several researchers have opted for green extraction techniques including UAE (Fulzele & Satdive, 2005a; Karanje et al., 2021; Li et al., 2017; Namdeo et al., 2010b), MAE (Chu et al., 2014; Jin et al., 2019; Karanje et al., 2021; Patil & Akamanchi, 2017b). Other methods such as Ionic aqueous solution ultrasonic-assisted extraction (Ma et al., 2012), UARSE (Li et al., 2017), Superficial fluid extraction (Zhang et al., 2012; Chu et al., 2014), IL-UMASDE (Zhao et al., 2016), Bilayer solid phase extraction (Chen et al., 2017), MIP-MSPD (Liu et al., 2015b) have also been reported. One common factor influencing all extraction techniques remains to be the solvent type and strength. Although factors such as agitation speed, solvent to solute ratio, temperature, and others are also taken into consideration for maximum yield (Azwanida, 2015). The most common green methods opted for extraction of CPT are UAE

and MAE. Table 2.4., shows several methods of CPT extraction from plants.

**Table 2.4** Extraction of camptothecin from different plant parts using different methods.

| **Species** | **Part used** | **Extraction** | **Solvent** | **References** |
|---|---|---|---|---|
| *Nothapodytes. nimmoniana* | Leaf, stem | Micro-extraction method | Methanol | Ankad et al., 2015 |
| *Gracilaria corticata* | Seaweeds | Soxhlet extraction | Chloroform, acetone, methanol, ethanol, water | Ashwini et al., 2017 |
| *Camtotheca acuminata* | Leaves | Ultrasonication | - | Bowen et al., 2008 |
| *N. nimmoniana* | Root | Sonication | Methanol | Chang et al., 2014 |
| *C. acuminata* | Fruit | Bilayer solid-phase extraction | Methanol | Chen et al., 2017 |
| *Nothapodytes foetida* | Stem | a) Superficial fluid extraction<br>b) reflux extraction<br>c)ultrasonication<br>d) MAE | Methanol/ water | Chu et al., 2014 |
| *C. acuminata* | Seeds | Homogenate method | Ethanol | Chunjian et al., 2009 |
| *C. acuminata* | Samaras | Ionic liquid solution | Methanol | Cui et al., 2018 |
| *N. nimmoniana* | Leaf, bark, stem, root | Sonication | Methanol | Degambada et al., 2016 |
| *Ervatamia heyneana* | Stem | Sonication | Methanol | Dighe et al., 2012 |
| *N. foetida* | Callus cultures, somatic embryos, regenerated plantlets, and 2-yr-old plants | Sonication | Methanol | Fulzele & Satdive, 2003 |
| *N. foetida* | Plant | a) Stirring<br>b) Sonication<br>c) Soxhlet<br>d) MAE | a) methanol, ethanol<br>b) methanol<br>c) methanol<br>d) methanol | Fulzele & Satdive, 2005b |
| *N. foetida* | Callus cell | Sonication | Methanol | Fulzele et al., 2001 |
| *C. acuminata* | Seed | UAE | Methanol | Jing et al., 2011 |
| *Tabernaemontana alternifolia, T. divaricata, T. citrifolia* | Leaves and stem | Maceration, Ultrasonication, Vortex mixer, Soxhlet extraction, | Methanol | Karanje et al., 2021 |

| | | | | |
|---|---|---|---|---|
| | | MAE | | |
| *N. nimmoniana* | callus cells and plant parts, viz. leaf, fruit, and stem | Sonication | Methanol | Karwasara et al., 2012 |
| *N. nimmoniana* | leaf, stem, stem bark, and root bark | MAE | Ethanol | Kavitha et al., 2010 |
| *Chonemorpha grandiflora* | callus and the stem with bark | Cold extraction | Ethanol | Kulkarni et al., 2010 |
| *C. acuminata* | Fruits | a) UARSE<br>b) HRE<br>c)UAE | Methanol | Li et al., 2017 |
| *C. acuminata* | Fruit, bark and leaf | MIP-MSPD | Methanol, acetic acid | Liu et al., 2015b |
| *C. acuminata* | First four leaves | Sonication | Methanol | Liu et al., 2015c |
| *C. acuminta* | Dried samaras | Ionic aqueous solution ultrasonic-assisted extraction | Methanol | Ma et al., 2012 |
| *N. nimmoniana* | bark, roots, leaves, and stems | Ultrasonication | Methanol | Mingzhang et al., 2011 |
| *N. nimmoniana* | Roots, stem, leaves, fruits | Sonication | Methanol | Namdeo et al., 2010a |
| *N. foetida* | roots, stems, leaves, and fruits) and tissue culture grown callus | Sonication | Methanol | Namdeo et al., 2010b |
| *C. acuminata* | Leave punches | a) Dichloromethane extraction,<br>b) chloroform/methanol extraction | chloroform/methanol | Nolte et al., 2001 |
| *O. trichocarpos* | Nodal segment | soxhlet apparatus | Methanol | Palode, 2016 |
| *N. nimmoniana* | Fresh leaves, dried leaves, bark | Solvent extraction | Methanol | Patil et al., 2016 |
| *N. nimmoniana* | Plant bark | Solvent extraction | Methanol | Patil et al., |

| | | | | |
|---|---|---|---|---|
| | | | | 2014 |
| *N. nimmoniana* | Stem | a) Stirring extraction<br>b) Ultrasonic assisted rapid extraction | a) methanol<br>b) methanol, ethanol, chloroform, and acetone | Patil & Akamanchi, 2017a |
| *N. nimmoniana* | Stem | a) Stirring extraction<br>b) MAE | a) methanol and ethanol<br>b) Methanol, ethanol, chloroform, and acetone | Patil & Akamanchi, 2017b |
| *N. foetida* | Aerial portion of plant material | Soxhlet extraction | Methanol | Puri et al., 2005 |
| *O. mungos, O. pectinata, O. eriantha, O. caudata, O. grandiflora, O. nairii, O. trichocarpon, O. barberi, O. shendurunii* and three varieties *viz., O. mungos L. var. angustifolia, O. rugosa var. decumbens, O. rugosa var. prostrata* | whole plants | Soxhlet apparatus | Methanol | Rajan et al., 2013 |
| *N. nimmoniana* | stem inner bark, root inner bark | Sonication | Methanol | Ramesha et al., 2008 |
| *Ophiorrhiza eriantha* | Tender leaves | Soxhlet | Methanol | Rani et al., 2010 |
| *a) N. foetida, b) O. mungos, c) O. rugosa* | a) juvenile plant root, stem, leaves. mature plant-roots, stem, leaves, bark, seeds,<br>b) roots, shoots<br>c) roots, | Methanol extraction | Methanol | Roja, 2006 |

| | | | | |
|---|---|---|---|---|
| | shoots | | | |
| *Ophiorrhiza rugosa* var. *decumbens* | shoot cultures | Solvent extraction | Methanol, chloroform | Roja, 2008 |
| *C. acuminata* | Freshly harvested shoots, leave, calli, and somatic embryos | Sonication | Ethanol | Sankar-Thomas & Lieberei, 2011 |
| *Ixora coccinia* | Leaves | Solvent extraction | Methanol | Saravanan & Boopalan, 2011 |
| *N. nimmoniana* | Fruits | 60% ethanol extraction | Ethanol | Sarika et al., 2019 |
| *N. pittosporoides* | Roots | SHIM-PACK VP-ODS C18 column (separation) | - | Shang-rao, 2010 |
| *C. acuminata* | - | Stirring extraction, homogenate extraction, ultrasonic extraction, MAE | - | Shen et al., 2011 |
| *C. acuminata* | Leaves | Ultrasonic bath, reflux, shaking water bath, homogenate extraction | Ethanol | Shi et al., 2009 |
| *Ophiorrhiza pumila* | In vitro hairy roots | Solvent extraction | Methanol | Sudo et al., 2002 |
| *N. nimmoniana* | Stem | (ASE) | Methanol | Upadhya et al., 2014 |
| *C. acuminata* | Leaf | Ultrasonication | Ethanol | Yan et al., 2003 |
| *C. acuminata* | Calli | Mixture of $CHCl_3$ and MetOH(4:1) | Methanol | Yang et al.,2017 |
| *Camtotheca acuminata* | Leaves, seeds | Solvent extraction | Methanol (MeOH), dichloromethane (CH2Cl2), and acetone (Me2CO) | Zhang et al., 2007 |
| *C. acuminata* | Fruit | a) conventional HRE<br>b) UAE<br>c)conventional HDE<br>d) IL-UMASDE<br>e) MASDE | Ethanol | Zhang et al., 2012 |
| *C. acuminata* | Leaf | Low-frequency Ultrasonic technology | Ethanol | Zhao et al., 2016 |

### 2.7.2 Analysis

Quantitative determination of camptothecin is generally accomplished with high-performance liquid chromatography (HPLC) or planar chromatography using HPTLC or TLC (Table 2.5). There are other less widely used methods for detecting camptothecin, namely HPLC-LTQ-Orbitrap-MS/MS and HPLC-TSQ-MS (Jin et al., 2019), RP-UPLC-PDA (Ankad et al., 2015), RP-UFLC-PDA (Upadhya et al., 2014), spectrofluorimetric method (Karwasara et al., 2012), RP-HPLC (Saravanan & Boopalan, 2011), liquid chromatography mass spectrometry (Ramesha et al., 2008), semipreparative HPLC (Puri et al., 2005), RP-HPLC/DAD/ESI/MS, RP-HPLC/DAD/ESI/MS (Yamazaki et al., 2003b) and GC-MS (Upadhya et al., 2014).

Usually, methanol is used as the solvent to extract camptothecin from plant matrix, either pure methanol or methanol blended with water in various proportions. Besides, water (Ashwini et al., 2017; Chu et al., 2014), acetonitrile (Jin et al., 2019) and dichloromethane (Zhang et al., 2007) are other less commonly used solvents.

HPLC generally uses a reversed-phase C-18 column, and both isocratic and gradient HPLC systems are discussed here (Table 2.5). Column temperatures are frequently controlled, ranging from 25$^{o}$C to 35$^{o}$C. Both isocratic and gradient modes contain a high proportion of water in the mobile phase. There are flow rates varying from 0.2ml $min^{-1}$ at a wavelength detection range of 250-370 nm ($\lambda_{max}$ for camptothecin is 254 nm) to 3 ml $min^{-1}$ at a wavelength detection range of 272 nm (Puri et al., 2005). Using a 150 mm x 4.6 mm analytical column containing acetonitrile: 5-mM di-ammonium hydrogen orthophosphate (anhydrous) (45: 55) as the isocratic solvent, a flow rate of 1.0 ml $min^{-1}$ and UV-detection at 366nm and 330 nm respectively, a HPLC-system was developed for the simultaneous quantification of camptothecin and quercetin in ethanolic extracts of *Gracilaria corticata* (Ashwini et al., 2017). Using HPTLC the detection of camptothecin and other analytes in plant extracts was conducted with silica gel coated plates (Table 2.5). The most common solvent is $CHCl_3$: MeOH: EtOAc in the ratio 4.5:0.5:5 v/v, but there are other mobile phases, such as toluene: acetonitrile: ethyl acetate: formic acid (6.0, 3.0, 1,0, and 0.1 volume ratio) (Dighe et al., 2012), a ratio of 7:3 ethyl acetate: toluene and a ratio of 1:1 chloroform: ethyl acetate (Kulkarni et al., 2010) has also been reported.

**Table 2.5** Camptothecin and related compounds: Analytical methods.

| Species | Analytical methods | Compound | References |
|---|---|---|---|
| *Nothapodytes nimmoniana* | RP-Ultra performance liquid chromatography photo-diode assay | CPT | Ankad et al., 2015 |
| *Gracilaria corticata* | TLC, HPLC | CPT, Quercitin | Ashwini et al., 2017 |
| *N. nimmoniana* | HPLC | CPT | Chang et al., 2014; Degambada et al., 2016; Kavitha et al., 2010; Mingzhang et al., 2011; Namdeo et al. 2010a; Patil & Akamanchi, 2017b |
| *Camtotheca acuminata* | UHPLC-MS | Strictosidinic acid group -Strictosidinic acid 6'-O-β-D-glucopyranoside (CA-4), strictosidinic acid (CA-18), and 3-epi-strictosidinic acid (CA-19). Vincosamide group -Vincosamide 11,6'-di-O-β-D glucopyranoside (CA-6), vincosamide (CA-15), and strictosamide (CA-16). Pumiloside group -Pumiloside (CA-20) and 3-epi-pumiloside (CA-21). CPT- Camptothecin (CA-7) and 10-methoxycamptothecin (CA-9). | Chen et al., 2017 |
| *N. foetida* | HPLC | CPT | Chu et al., 2014 |
| *Camtotheca acuminata* | HPLC | CPT, HCPT | Cui et al. 2018; Sankar-Thomas & Lieberei, 2011; Zu et al., 2003 |
| *Ervatamia heyneana* | HPTLC | CPT | Dighe et al., 2012 |
| *N. foetida* | TLC, HPLC | CPT, 9-MCPT | Fulzele et al., 2003; Fulzele et al., 2001 |
| *N. foetida* | HPLC | CPT, 9-Me-CPT | Fulzele et al., 2005b |
| *C. acuminata* | HPLC-LTQ- | CPT | Jin et al., 2019 |

| | | | |
|---|---|---|---|
| | Orbitrap-MS/MS and HPLC-TSQ-MS | | |
| *Tabernaemontana alternifolia, T. divaricata, T. citrifolia* | HPTLC | CPT | Karanje et al., 2021 |
| *N. nimmoniana* | Spectrofluorimetric method | CPT | Karwasara et al., 2012 |
| *Chonemorpha grandiflora* | TLC, HPTLC, HPLC | CPT | Kulkarni et al., 2010 |
| *C. acuminata* | HPLC | camptothecin (CPT) and betulinic acid (BA) | Li et al., 2017 |
| *C. acuminata* | HPLC | CPT | Liu et al., 2015b; Yan et al., 2003 |
| *N. foetida* | HPTLC, TLC | CPT | Namdeo et al., 2010b |
| *C. acuminata* | TLC, HPLC | CPT | Nolte et al.,2001 |
| *O. trichocarpos* | HPTLC | CPT | Palode, 2016 |
| *N. nimmoniana* | TLC, HPLC | CPT | Patil et al., 2016 |
| *N. foetida* | Semipreparative HPLC | CPT, 9-MCPT | Puri et al., 2005 |
| *Pyrenacantha volubilis* | HPLC | CPT | Ramachandran et al., 2021 |
| *N. nimmoniana* | HPLC, Liquid chromatography mass spectrometry | CPT | Ramesha et al., 2008 |
| *Ophiorrhiza eriantha* | TLC, HPLC | CPT | Rani et al., 2010 |
| *a) N. foetida, b) O. mungos, c) O. rugosa* | TLC, HPLC | CPT, 9-MCPT | Roja, 2006 |
| *Ophiorrhiza rugosa* var. *decumbens* | HPLC | CPT | Roja, 2008 |
| *Ixora coccinia* | RP-HPLC | CPT | Saravanan & Boopalan, 2011 |
| *N. nimmoniana* | HPLC | Camptothecin and methoxy camptothecin | Sarika et al., 2019 |
| *Ophiorrhiza pumila* | HPLC | CPT | Sudo et al., 2002 |
| *N. nimmoniana* | RP-UFLC-PDA | CPT | Upadhya et al. 2014 |
| *C. acuminata* | HPLC | CPT, hydroxycamptothecin (HCPT) and methoxycamptothecin | Zhang et al., 2007 |
| *C. acuminata* | HPLC | CPT, 10-HCPT, VCS-LT (vincoside-lactam), essential oils. | Zhao et al., 2016 |

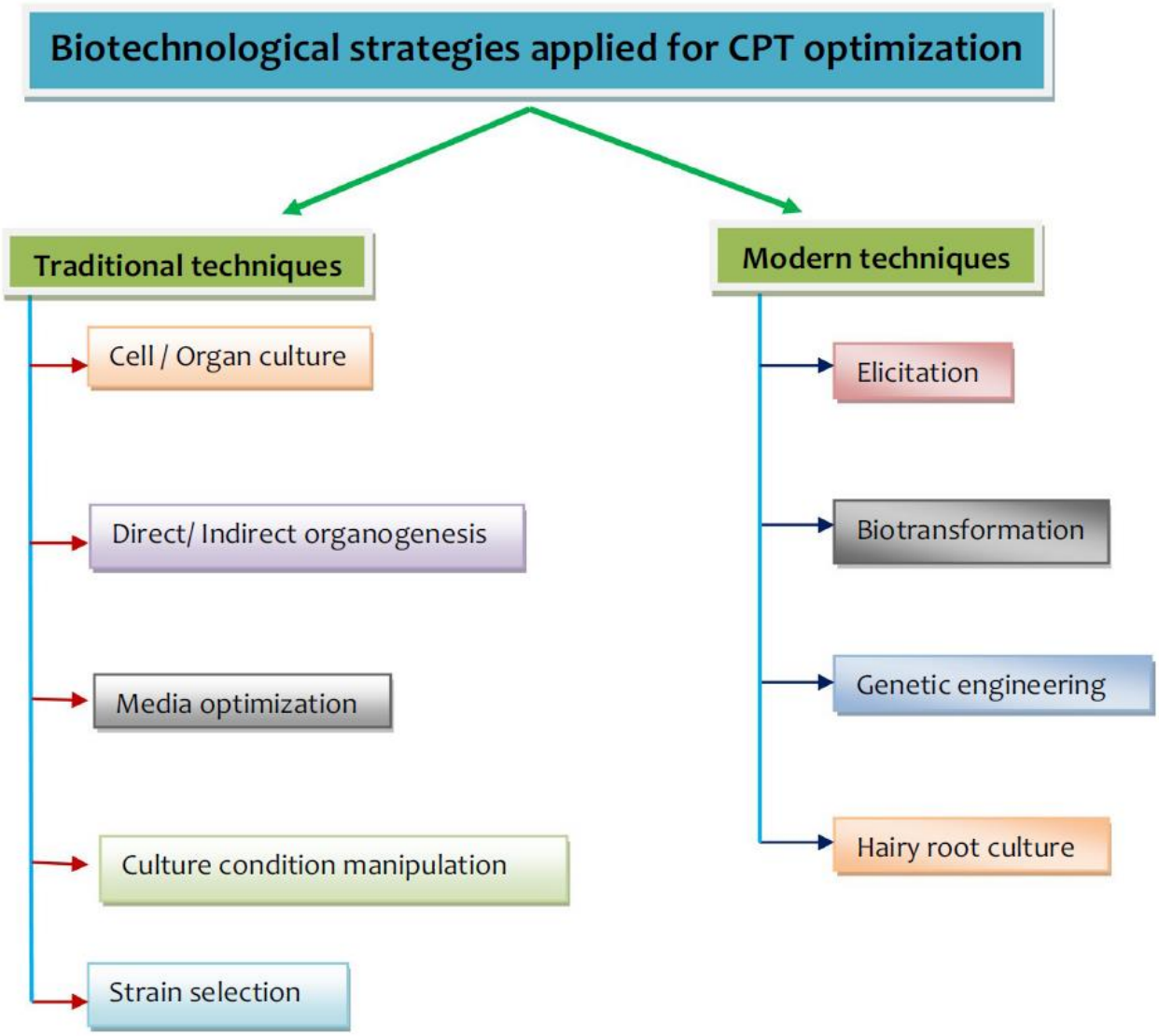



**Fig 2.3:** Different biotechnological strategies for enhancing secondary metabolite production.

### 2.8 Biotechnological strategies for *Ophiorrhiza*

*Ophiorrhiza* may be produced naturally and conventionally, however due to characteristics including slow-rate growth, low population densities, restricted geographic ranges, and destructive harvest, numerous species in threat, and it is unable to satisfy the plant's industrial needs (Applequist et al., 2020; Van Wyk & Prinsloo, 2018). Efferth (2019) state that modern technological advancements have made it possible to quickly produce key therapeutic plants in large quantities using tissue culture. Plant tissue culture provides fresh approaches to improving production, economical, durability, and sustainability (Eibl et al., 2018; Yancheva & Kondakova, 2018). Expert biotechnology approaches, such as those in tissue culture, cell and molecular biology, genetic engineering, particularly transformation technology, have opened up novel opportunities for high pharmaceutical output, nutraceuticals, and other

valuable components to address these issues. The manufacture of desired molecules and the preservation of important plant resources were both made possible in the past through biotechnological strategies (Rao & Ravishankar, 2002; Sujatha et al., 2008; Wilson et al., 2014). It also covers additional metabolic engineering procedures such as elicitor and precursor feeding, high yielding cell suspension, tissue or organ cultures, and bioreactors. Establishment of aseptic conditions, culture media and growth substances.

### 2.8.1 *In vitro* propagation in *Ophiorrhiza spp*.

Typically, meristematic plant parts like shoot tips or axillary buds are used for in vitro propagation. For the start of in vitro cultivation in *Ophiorrhiza spp*., vegetative components of the plants, such as the leaf and stem, have also been obtained as explants. Direct multiplication of shoot and induction of organs are the most effective techniques for clonal multiplication, according to Namdeo et al. (2012). For plant regeneration, direct organogenesis and somatic embryogenesis are also used since they speed up the multiplication process. According to the findings those are available, *Ophiorrhiza* has successfully been multiplied in vitro using seedlings, proximal leaf end, shoot, and nodal cultures.

#### 2.8.1.1 Establishment of *in vitro* cultures by direct organogenesis

##### 2.8.1.1.1Multiple shoot formation

Explants' ability to produce numerous shoots is influenced by several elements, including basal media concentrations, growth regulators, subcultures, and photoperiod. Basal media that has been treated with cytokinin or a mixture of auxin and cytokinin is essential for the growth of the shoot. Jose and Satheeshkumar (2004) revealed that BAP was effective in initiating numerous shoots in prior trials. Shoot elongation was attained at BA (2.22 µM) and GA3 (1.44 µM). Another study indicated that BA alone was effective at triggering many shoots (Jaimsha et al., 2010). According to Kai et al. (2008), modest concentrations of BA (2.0 mg $dm^{-3}$) and NAA (0.2 mg $dm^{-3}$) promoted the development of multiple shoots. A different study revealed that BA (8.87 µM) and IBA (2.46 µM) responded well to shoot culture (Shahanaz Beegum et al., 2007). When picloram, TDZ, and GA were added to basal medium in a 1:2:1 ratio, maximum shoot multiplication and growth occurred (Namdeo et al., 2012). Using ½ MS medium fortified with KIN and NAA, shoot multiplication was effectively observed (Ya-ut et al., 2011). BA, according to the findings, was discovered to be the best cytokinin for generating shoots from plant cuttings.

#### 2.8.1.1.2 Root initiation

The plantlet won't make it without a strong root system. The plant hormone auxin triggers root development in vitro. It has been shown that sub-culturing explants on ½ MS media without growth hormones will cause the explants to root (Roja, 2008; Ya-ut et al., 2011). Several auxin concentrations in the growth medium have been tested. Positive responses in root initiation were seen when IBA and NAA were added to ½ MS media (Kai et al., 2008), while another investigation found that the same supplementation was effective when added to full-strength MS medium (Jose & Satheeshkumar, 2004). In ½ MS media, Shahanaz Beegum et al. (2007) found that adding NAA (10.7 µM) and BA (2.32 µM) significantly improved rooting. Maximum rooting per shoot was achieved with the use of IBA in the rooting media (Jaimsha et al., 2010). According to the data we have, the most suitable strategy is to use ½ MS without a growth regulator.

#### 2.8.1.1.3 Acclimatization

Once shoots have established roots in vitro, the plantlets must be hardened and acclimatized before they can be transplanted into the field. This stage is very important since it will decide the probability that the micro-propagated plants will survive in the field. Several approaches have been used to maintain the environmental elements throughout the acclimatization process. The roots are carefully washed under running water to get rid of any remaining medium or agar before transplanting the plantlets. The in vitro raised plants were placed in a small pot (8 cm in diameter) filled with a 1:1 v/v combination of sand and soil. The pots were maintained in a darkened environment and bagged with clear polyethylene bags so as to keep in moisture. During a 3-week period, watering was performed twice. The plastic bags were taken off after three weeks, and they received regular watering (Krishnan et al., 2018b). Plantlets were transferred into paper cups containing autoclaved soil by Roja (2008), and then they were cultivated under fluorescent light for a period of ten days. It was then moved to bigger pots and cultivated inside a greenhouse. In a different experiment, the plantlets were covered with glass beakers (Kai et al., 2008). Plantlets without roots were placed in containers filled with sand and dirt. While ex vitro rooting offers several benefits, such as a decrease in cost, labour, and time, as well as the usage of auxins, their results revealed that it had a lower survival rate than in vitro rooting (Shahanaz Beegum et al., 2007). *Ophiorrhiza spp.* has a variable survival rate in natural field circumstances, with reports of 50% in rootless shoots (Shahanaz Beegum et al., 2007), 72% (Kai et al., 2008), 95% (Krishnan et al., 2018b; Kaushik et al., 2015), and up to 100% (Shahanaz Beegum et al., 2007; Roja, 2008).

**Table 2.6** In vitro studies on *Ophiorrhiza spp.*

| **Species** | **Explants** | **Disinfection procedure** | **Culture medium** | | **Shoot** | | **Root** | | **Carbohydrate, Additives, Gelling agent** | **Culture condition** | **Acclimatisation** | **Outcomes** | **References** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **shoot** | **Root** | **Auxin** | **Cytokinin** | **Auxin** | **Cytokinin** | | | | | |
| 1. *O. prostrata* | capsules | 0.5%(v/v) teepol(10-15mins), running TW, 0.5-1%(v/v) sodium hypochlorite(7-10mins), 0.005-0.1% $HgCl_2$(3-7min) | ½ MS | ½ MS | NA | 1.5 mg/ L BAP | 1mg/L NAA | NA | 3% Sucrose + agar | pH-5.8, 26±2ºC, 16/8h day/ night PP imposed using white fluorescent tube light | 3 weeks | seedlings | Gopalakrishnan et al., 2018 |
| 2. *O. rugosa* var *decumbens* | Axillary meristem | NA | MS | NA | 0.05 mg/L NAA | 4mg/L BA | NA | NA | 30g/L sucrose (w/v), 0.8% agar(w/v) | 25±2ºC, fluorescent light for 35days | NA | Mutiple shoot culture | Gopalakrishnan & Shankar, 2014 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3. *O. erianth a* | Youn g leaf | 0.1% Tween20, DW(5-6 rinses), 0.1% $HgCl_2$(3min s), SDW | MS | ½ MS | 4mg/ L NAA | 0.5mg/ L 1BA | 1-3 mg/L IBA, 1-4 mg/L NAA, 1-4mg/ L IAA | 5mg/L BA | 3% (w/v) sucrose, 0.75% agar | pH-5.7, 25±2ºC, 16h PP in cool white light 25µmol $^{-2}s^{-1}$ | NA | IBA was more responsi ve than NAA and IAA | Jaimsha et al., 2010 |
| 4. *O. mungos* | Seedli ngs | Teepol (3times), 0.1% (w/v) $HgCl_2$ (1min), SDW(3-4rinses) | MS | MS | NA | 2.22 µM BAP | 1.23 µM IBA + 1.07 µM NAA | NA | 3% Sucrose + 0.6% (w/v) agar | pH-5.8, 12h white fluoresc ent light (30-35µEm$^2$ sec$^{-1}$) | 2weeks | BAP alone was able to induce adventiti ous shoots, 100% SR | Jose & Satheesku mar, 2004 |
| 5. *O. japonic a* | Youn g leaf segme nts | 70% EtOH (30sec), 0.1% $HgCl_2$(12mi n), DDW (3 rinses) | MS | ½ MS | 0.2mg dm$^{-3}$ NAA | 2.0mg dm$^{-3}$ BA | IBA+N AA | NA | 7g dm$^{-3}$agar and 30g dm$^{-3}$ sucrose | pH-5.7, 25±2ºC, 12h PP with an irradian ce of 50µmol m$^{-2}s^{-1}$ provide d by cool white fluoresc ent lamps | 14days | Multiple shoot develop ment, 72% SR | Kai et al., 2008 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6. *O. mungos* | Axillary and terminal buds | Aseptic | MS | MS | NA | 0.25mg/ L BA + 0.25 mg/L KIN | NA | NA | 2% Sucrose + 0.8% agar 100mg/L activated charcoal | pH-5.8, 25 ± 2ºC, 16h light and 8h dark period with a light intensity of 3000lux provided by cool white fluorescent tubes | 20 days | Seedlings, 95% SR | Kaushik et al., 2015 |
| 7. *O. mungos var angustifolia* | Node and shoot apex | 1% (v/v) labolene (15min), running TW(45 min), 0.1% (w/v) $HgCl_2$(3min), SDW(4 rinses) | MS | ½ MS | NA | NA | 1.14-4.56 µM IAA, 0.98-3.92 µM IBA, 1.07-4.28 µM NAA | NA | 3% Sucrose + 0.8% (w/v) agar | pH-5.8, 25 ± 2ºC, 16h PP (35-50 $\mu Em^2/s$) provided with fluorescent tubes. | 30 days | Shoot buds, 95% SR | Krishnan et al., 2018a |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 8. *O. rugosa* var. *decumbens* | Axillary meristem | NA | MS | ½ MS | 0.05 mg/L NAA | 4mg/L BA | No PGRs | NA | 3% sucrose + 0.25% phytagel | Incubation on a gyratory shaker set at 90rpm at 23ºC for 6weeks under continuo s flourescent light | 6weeks | Shoot induction/ 100% SR | Roja, 2008 |
| 9. *O. prostrata* | Young leaves and internode | 5%(v/v) soln. Of Extran (5min), 0.01% $HgCl_2$(7-9min/ 10-12min), SDW (4 rinses) | MS | a)½ MS b)½ MS c)½ MS | 2.46 µM IBA | 8.87 µM BA | a) No PGRs b) NAA c) 10.74 µM NAA | a) – b) KIN c) 2.32 µM KIN | 0.8% (w/v) agar | pH-5.8, 25±2ºC with 16h light (at a irradiance of 25µmol $m^2s^{-1}$)/ 8h dark cycle under fluorescent tubes | 45days | Combination of IBA (2.46 µM) and BA (8.87 µM) shows positive response toward shoot culture, 100% SR | Shahanaz Beegum et al., 2007 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10. *O. alata* | seeds | 70%(w/v) EtOH (1min), Sodium hpochlorite with three drops of Tween80(15min), SDW (3rinses) | ½ MS | ½ MS liquid medium | 0, 0.54,1.08 µM NAA | 0, 4.165, 9.30 µM KIN | No PGRs | NA | 1%(w/v) sucrose + 0.5% Gelrite | pH-5.7, 25±2ºC, 16/8h light/dark cycle | NA | Hairy roots | Ya-ut et al., 2011. |

#### 2.8.1.2 Callus-mediated indirect organogenesis and somatic embryogenesis

Indirect organogenesis refers to a process in which organs are developed from a cell suspension or callus tissue that was originally grown from explants in vitro. Organogenesis induced by calluses is often performed for research into genetic alteration and for mass propagation. Both Shahanaz Beegum et al. (2007) and Martin et al. (2007) reported on *Ophiorrhiza prostrata* for the first time that shoots might regenerate from a callus. The most effective methods for stimulating callus development were obtained in MS medium fortified with 5.37 µM NAA and 2.22 µM BA. A mixture of 8.87 µM BA and 5.37 µM IBA in MS media was used to induce shoot development from calli. In ½ MS media containing 10.74 µM NAA and 2.32 µM KIN, roots were shown to develop. According to Martin et al. (2007), secondary metabolites like camptothecin tend to accumulate as the colour of the calli changes from golden yellow to crimson red. They discovered that the best MS medium for somatic embryo development was half-strength, combined with 2, 4-D (0.4-2.26 µM) alone, or with BA or KIN. Kai et al. (2008) have established a very effective procedure for indirect organogenesis of *Ophiorrhiza japonica*. They found that when MS medium was treated with NAA, growth rates increased to between 90.4% and 100%. The callus developed to develop new shoots when BA was supplemented at a greater concentration than NAA in MS media. On the other hand, the calli formed roots in MS media when NAA concentration was greater than BA concentration. The application of PVP improved the shoot regeneration process by preventing callus browning. The induction of calli in MS medium has been tested using several phytohormone combinations in recent papers. Healthy green friable calluses and brown friable calluses reported by Namdeo et al. (2012) in different hormonal combinations (Table 2.7).

**Table 2.7** Callus-mediated somatic embryogenesis.

| Species | Explants | Result/response | Medium + plant growth hormone | References |
|---|---|---|---|---|
| *O. eriantha* | Leaf | Callus | MS+NAA 4 mg/L+ BA 0.5 mg/L | Jaimsha et al., 2010 |
| *O. japonica* | Leaves | Callus | MS+BA (2.0 $mgdm^{-3}$) + NAA (0.2 $mgdm^{-3}$) | Kai et al., 2008 |
| *O. prostrata* | Leaf and internode | Callus | MS+ 5.37 µM NAA or BA or KIN; MS+ 2,4-D or in combination with N6-BA or KIN | Martin et al., 2007; Shahnaz Beegum et al., 2007 |
| *O. mungos* | Young fruits | Callus | MS + Picloram +BAP +IBA, MS+IBA+BAP+GA (2:2:1), Picloram + BAP+ GA (1:3:1), IBA+BAP+GA (2:2:2), IBA+BAP+GA (2:1:2) and IBA+ BAP+GA (5:1:1) | Namdeo et al., 2012 |

## 2.9 Role of biotic and abiotic factors

The results of an in vitro propagation or regeneration experiments is highly reliant on a number of different abiotic and biotic variables, regardless of the plant material that was utilized or the purpose of the experiment. These elements are essential to achieving a positive conclusion of in vitro proliferation and regenerative techniques. The significant impact of biotic and abiotic factors on *Ophiorrhiza spp.* micropropagation is discussed in detail in the sections and subsections that follow.

### 2.9.1 Biotic factors

#### 2.9.1.1 Source of explants and their outcomes

A successful in vitro cultivation depends on selecting the right explants. Many explants, including nodal explants (Ya-ut et al., 2011), seedlings (Jose & Satheeshkumar, 2004), proximal ends of leaves (Shahanaz Beegum et al., 2007), and axillary meristems, have been employed in the micropropagation of *Ophiorrhiza spp.* (Kaushik et al., 2015; Gopalakrishnan & Shankar, 2014; Roja, 2008). A methodology for mass plant multiplication using seedlings

(shoots) of *Ophiorrhiza mungos* was created by Jose and Satheeshkumar (2004). After two subcultures separated by four weeks, an estimated 1650 (0.60±0.53 cm) new juvenile shoots can be produced from a single explant.

### 2.9.2 Abiotic factors

#### 2.9.2.1 Basal media and composition

For the development of plantlets from explants in plant tissue culture, basal media serves as the growing medium. Basal media come in a variety of forms, including Driver and Kuniyuki Woody Plant Media (DKW), White's medium, Nitsch medium, Murashige and Skoog (MS) medium, and Gamborg's B5 Woody Plant Medium (GWPM). The most used base media for plant tissue culture is MS medium. All the nutrients needed for plantlet development are present in the basal media. Vitamins, macronutrients, micronutrients, and minerals are all present in sufficient amounts in basal medium. PGRs are introduced into the base medium in accordance with the experiment's objective. Multiple shoots, callus, rhizogenesis, and somatic embryogenesis were induced using MS media (Jaimsha et al., 2010; Jose and Satheeshkumar, 2004; Kai et al., 2008; Kaushik et al., 2015), half-strength MS medium was used for the same purposes (Gopalakrishnan et al., 2018; Krishnan et al., 2018b; Kaushik et al., 2015; Shahanaz Beegum et al., 2007; Ya-ut et al., 2011).

#### 2.9.2.2 Plant growth regulators

Induction of shoot, elongation of shoot, and induction of roots are all necessary phases on the path to the desired plant. In contrast, the first stage of indirect organogenesis is callus development, which, depending on the kind of PGR utilized, can differentiate into shoot, root, or somatic embryos. As a result, the explant's final destination is directly tied to the PGR preference. High cytokinin and low auxin, for instance, cause shoot induction, while low cytokinin and high auxin cause rhizogenesis. It has been shown that the optimal medium for bud break is MS basal medium supplemented with BA (0.5mg/L). Kai et al. (2008) found that a mixture of BA (2.0 $mgdm^{-3}$) and (0.2 $mgdm^{-3}$) NAA was optimum for shoot regeneration, whereas Kaushik et al. (2015) found that a mixture of BA (0.25 mg/L) and (0.25mg/L) KIN produced an 81% shoot regeneration response. The optimal concentrations of BA for inducing shoot growth were 1.97 μM and 8.88 μM, respectively, when coupled with the PGRs IAA, IBA, NAA, 2iP, ZN, and KIN. It has been claimed that administering BAP alone is enough to trigger shoot development (Krishnan et al., 2018b). However, as BAP (2.22 μM) concentration was increased, the percentage of shoot regeneration decreased (Gopalakrishnan et al., 2018; Jose & Satheeshkumar, 2004). The concentration of BAP used

must be closely managed, since too much of it can reduce the percentage of regenerated shoots. Similarly, 28% shoot regeneration was seen at 1.50 mg/L of BAP, but at 8.88 µM of BAP, not a single shoot bud could be seen (Jose & Satheeshkumar, 2004). In addition, it has been found that KIN (4.65 µM) (Ya-ut et al., 2011) and BA (5 mg/L) (Jaimsha et al., 2010) both induce complete shoot regeneration. A number of studies have found that NAA is the most potent auxin for rhizogenesis (Gopalakrishnan et al., 2013; Krishnan et al., 2018b; Shahanaz Beegum et al., 2007), although IAA has been found to be the most effective (Kaushik et al., 2015). A positive correlation between IBA levels and rhizogenesis was found in experimental conditions (Jaimsha et al., 2010).

#### 2.9.2.3 Additives

To raise the supply of nutrients, boost the percentage of regeneration, improve the availability of micro salt to the explants, and regulate the pH by acting as a buffer system or chelators are all examples of the types of compounds that can be classified as "additives" (Gantait et al., 2018). There have been reports of coconut water being used in place of synthetic hormones while inoculating *Ophiorrhiza mungos* explants (Namdeo et al., 2012). In addition, activated charcoal was included in the medium used for culture. ½ MS media supplemented with 100mg/L activated charcoal resulted in 92.13 % rooting frequency in *O. mungos*. Kai et al. (2008) reported the inclusion of PVP (poly-vinylpyrrolidone). The proportion of shoot regeneration is higher when PVP is present compared to when it is not.

#### 2.9.2.4 Carbohydrate sources

What goes into the culture media is crucial to the in vitro development and growth of plants. Carbohydrates play a crucial role as a carbon source in the culture medium. Carbohydrates are widely used, and many different kinds are available. Carbohydrate is an essential carbon supply and osmotic regulator for cells grown in in vitro conditions, where photosynthesis cannot occur. Because of its beneficial effect on development and low cost, sucrose has become the most popular form of carbohydrate (Muslihatin & Ratnadewi, 2012). Most *Ophiorrhiza spp*. plant tissue culture reports have used a 3% sucrose concentration for the carbohydrate supply (Jaimsha et al., 2010; Krishnan et al., 2018b; Roja, 2008). *O. japonica* and *O. decumbens* (Kai et al., 2008) were both able to regenerate several shoots when grown on MS medium supplemented with 3% sucrose. Nevertheless, *O. mungos* seedlings were formed when the sucrose content was lowered to 2% (Kaushik et al., 2015). *O. alata* hairy roots can be induced by a 1% sucrose solution in ½ MS liquid medium, despite the fact that a larger concentration of sucrose (2%-3%) is required for shoot induction (Ya-

ut et al., 2011).

### 2.10 Culture conditions

Tissue culture requires the provision of carefully managed conditions for plant growth and development. Explants, shoots, or callus tissue are kept in sterile condition with the right amount of light, temperature, and relative humidity. Photosynthesis, photomorphogenesis, and photoperiodic response will all be controlled by these physical parameters. Several scientists have found that a pH of 5.8, a temperature of 25±2°C, and a photoperiod of 16 hours of light and 8 hours of dark produce the best results (Gopalakrishnan et al., 2018; Gopalkrishnan & Shankar, 2014; Kaushik et al., 2015; Krishnan et al., 2018a; Shahanaz Beegum et al., 2007; Ya-ut et al., 2011). Light with a spectral photon flux density (SPFD) of 25 to 50 Em2/s is used to supply plants with the proton flux necessary for photosynthesis (Jaimsha et al., 2010; Jose & Satheeshkumar, 2004; Kai et al., 2008; Krishnan et al., 2018a; Shahanaz Beegum et al., 2007). The light levels, light density, and photoperiods are all consistent across experiments. Kai et al. (2008) recommended a continuous 14-day 12-h photoperiod for multiple shoot development in *O. japonica*. Jose and Satheeshkumar (2004) also suggested a photoperiod of 12 hours for regenerating adventitious shoots in *O. mungos*.

### 2.11 Surface sterilization

Many reports have indicated that mercuric chloride is widely utilized for surface sterilization, though the concentration and length of treatment time vary widely between research. Generally, the explants were cleaned with tap water and teepol before being surface sterilized with 0.1% w/v mercuric chloride (Jose & Satheeshkumar, 2004; Martin et al., 2007; Namdeo et al., 2012; Krishnan et al., 2018a; Shahanaz Beegum et al., 2007) and then 1% sodium hypochlorite with a drop of tween 20 (Ya-ut et al., 2011). To improve explant survival, surface sterilizing eliminates pathogens and other contaminants (Table 2.6).

### 2.12 Biotechnological production of camptothecin and related compounds

Plant tissue culture and other forms of biotechnology have enabled the large-scale production and collection of secondary metabolites. Improved strains, high-yielding cell line selection, and media optimization can all boost secondary metabolite production, but these methods are not foolproof. Biosynthetic pathways and the mechanisms involved in the generation of secondary metabolites are poorly known, which is the fundamental reason why these tactics fail in cell suspension culture (Karuppusamy, 2009). Table 2.8 details some of the methods tested to improve CPT and related chemical production. Modern techniques, such as the use of elicitors, hairy root culture, biotransformation, and genetic engineering, offer a viable route

for greater production of a specific secondary metabolite, as opposed to the inconsistent results obtained with traditional methods.

### 2.12.1 In vitro culture strategy

To maximise yield, it's crucial to choose the right combination of growth medium, plant h ormone, and dosage. Together including auxins and cytokinins, MS is the culture medium that is frequently used in the process of micropropagation of *Ophiorrhiza spp*. *Ophiorrhiza spp*. cultures may be started on basal media along with plant growth regulators, such as NAA and BA (Gopalakrishnan & Shankar, 2014; Roja, 2008); BAP and KIN (Kaushik et al., 2015); IAA, BAP and GA3 (Namdeo et al., 2012); BA and IAA (Shahanaz Beegum et al., 2007); NAA and KIN (Ya-ut et al., 2011). *O. pumila* callus culture was the subject of the first reported in vitro technique study (Kitajima et al., 1997a; Kitajima et al., 1997b). Midhu et al. (2019) established a successful technique for somatic embryogenesis-based plant regeneration from callus cultures. According to their research, ½ MS liquid media treated with 3 mg/L IAA was shown to be the best medium for embryo development. Researchers have used callus culture the least to produce CPT in *Ophiorrhiza.* Several research have clarified CPT production by organ culture employing leaf, shoot, and roots (Roja, 2008; Kamble et al., 2011; Sibi et al., 2016; Shahanaz Beegum et al., 2007; Vineesh et al., 2007). After carefully examining numerous culture-related factors, Deepthi and Satheeshkumar (2017b) discovered that the optimal conditions for boosting *O. eriantha* biomass and CPT production were ½ MS medium with 50% sucrose (w/v), 0.5 mg/L IBA, 0.1 mg/L GA3, 0.25 mg/L NAA, and an inoculum size of 2 g/L. Micropropagated *O. rugosa* plants derived from shoot culture had much greater alkaloid content than their wild-grown counterparts, as determined by chemical analysis (Roja, 2008). For the effective in vitro synthesis of CPT, *O. rugosa* var. *decumbens* was cultured multiple times from both its shoots and its roots (Vineesh et al., 2007). Sibi et al. (2016) showed that subculturing in medium of varying strengths may increase CPT production in *O. trichocarpon*. According to the literature, *Ophiorrhiza* hairy roots are where CPT was found in the greatest concentration.

### 2.12.2 Hairy root culture

The most effective approach for producing CPT from *Ophiorrhiza* over the last couple of decades has been hairy root culture converted by *A. rhizogenes* (Table 2.8). Generally, secondary metabolites are generated in larger amounts by hairy roots than by typical plants. The effective integration of *A. rhizogenes* strains including LBA 9402 (Kamble et al., 2011), C58C19 (Shi et al., 2020), 15834 (Saito et al., 2001), and TISTR 1450 (Ya-ut et al.,

2011) has produced hairy roots with higher CPT synthesis. Since then, *O. pumila* hairy root culture has been established by Xu et al. (2020). They discovered that OpWRKY1 targets OpCPR, a gene involved in CPT biosynthesis, and inhibits its transcription by downregulating it in *O. pumila*. Production of CPT has been associated with OpWRKY2 expression, which codes for the WRKY transcription factor in *O. pumila*. OpWRKY2 overexpression led to a threefold increase in CPT (Hao et al., 2021). By transfer of a rol B gene from T-DNA of *A. rhizogenes*. The transformation of hairy roots by *A. rhizogenes* strain 15834 was explained by Saito et al. (2001), who also observed that within 5 weeks in liquid culture, growth increased upto 16 times and major alkaloid CPT was generated. One recent research suggested that *O. pumila* chromosome-level genome assembly revealed on the development of CPT biosynthesis (Rai et al., 2021).

### 2.12.3 Elicitation strategy

Elicitation is a technique through which plants increase the production of secondary metabolites in order to defend themselves against a variety of stresses. Elicitors are the things that generate the stress that results in this reaction. These stimuli might be microbiological, physical, or chemical (Namdeo, 2007). Plants often develop secondary metabolites to defend themselves against diseases or herbivory. Numerous researchers have taken advantage of this process by boosting the production of secondary metabolites in medicinally significant plants by applying artificial stress in the form of elicitors. In the past, a few research have been carried out on the biosynthesis and biological methods for the formation of CPT in the genus *Ophiorrhiza* (Mahendran & Rahman, 2021; Wetterauer et al., 2018). It has been found by Deepthi and Satheeshkumar (2017b) that the ratio of ammonium to nitrate and the size of the inoculum influence CPT generation in *O. mungos*. Using a 50:10 mM ammonium: nitrate ratio and a 2 g/L inoculum size, they found that CPT synthesis was enhanced. The formation of CPT was unaffected by growth regulator. In a different experiment, they paired *O. mungos* cell line 3 (OMC3), a high producing cell line, with JA, CS, and SA. JA has been found to be effective for elicitation. This combination of JA and OMC3 was observed to improve biomass output and CPT production by 18.66 times (Deepthi & Satheeshkumar, 2017a). MeJa was added to culture medium, according to Asano et al. (2004), which marginally boosted CPT production. A continuous generation and increased concentration of CPT were also produced when in vitro callus of *O. mungos* was immobilized (in entrapped alginate beads) (Devasia et al., 2021). Electrical stimulation and preharvest MeJa elicitation both increased CPT in the tissue culture derived *O. ridleyana* (Pisitpaibool et al., 2021).

**Table 2.8** In vitro biotechnological strategies reported on *Ophiorrhiza spp.*

| Sl.no. | Species | Techniques | Induced gene or chemical | Types of CPT and related compounds | References |
|---|---|---|---|---|---|
| 1. | *O. liukiuensis*, *O. kuroiwai* | Hairy root culture | Methyl jasmonic acid | CPT | Asano et al., 2004 |
| 2. | *O. mungos* | Cell suspension culture;<br>Hairy root culture;<br>Direct organogenesis;<br>Seedling shoot culture | Jasmonic acid;<br>Major nutrients;<br>Media composition;<br>Concentration of ammonium to nitrate | CPT | Deepthi & Satheesh, 2017a; Deepthi &Satheesh, 2017b; Kaushik et al., 2015; Wink et al., 2005 |
| 3. | *O. eriantha* | Leaf explant culture | Media composition | CPT | Jaimsha et al., 2010 |
| 4. | *O. pumila* | Callus culture;<br><br>Hairy root cultures;<br><br><br>Investigation of optimal hydroponic root-zone environment for growth;<br><br>Hairy root cultures;<br><br>Hairy root cultures;<br>Hairy root cultures;<br>Hairy root cultures;<br><br>Hairy root cultures;<br>Hairy root cultures;<br><br>Hairy root cultures | Media composition;<br>transformation by *A.rhizogenes* (pRi15834; pGSGluc1);<br>Concentration and temperature effect on media;<br>infection by *A.rhizogenes* strain 15834;<br>OpG1OH& OpSLS;<br>brefeldin A treatment;<br>*A.rhizogenes* infection;<br><br>OpWRKY1;<br>expression of OpSTR & OpTDC;<br>transcriptome and metabolome data sets analysis | 9-β-glucosyloxycamptothecin;<br>CPT;<br><br><br><br><br><br><br><br><br><br><br><br><br>CPT and anthraquinones | Kitajima et al., 1997b;<br><br>Watase et al., 2004;<br><br><br>Yamazaki et al., 2003a;<br><br><br>Yamazaki et al., 2003b;<br><br><br>Kitajima et al., 2002;<br><br><br>Sudo et al., 2002;<br>Saito et al., 2001;<br>Xu et al., 2020;<br>Sirikantaramas et al., 2007; |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | Yamazaki et al., 2013;<br>Shi et al., 2020;<br>Lee et al., 2020;<br>Wink et al., 2005 |
| 5. | *O. rugosa var decumbens* | Multiple shoot culture;<br><br>Hairy roots and transformed shoots;<br><br>root culture;<br>Albino multiple shoots | Media composition;<br><br>*Agrobacterium rhizogenes* strain LBA9402;<br>growth hormones;<br>media composition | CPT | Roja, 2008;<br><br>Gopalakrishnan & Shankar, 2014;<br><br>Kamble et al., 2011;<br>Vineesh et al., 2007 |
| 6. | *O. mungos var angustifolia* | Regeneration of adventitious shoots from in vitro leaf of explants | Media composition | CPT | Krishnan et al., 2018 |
| 7. | *O. prostrata* | Indirect somatic embryogenesis;<br><br>shoot organogenesis | Subculture passages in media of alternating strength;<br>growth regulators | CPT | Martin et al., 2007;<br><br>Shahanaz Beegum et al., 2007 |
| 8. | *O. pectinata* | Callus tissue culture through direct embryo genesis | Diclorophenoxy acetic acid | Bioactive compounds | Midhu et al., 2019 |
| 9. | *O. rugosa* | Shoot culture;<br>Overexpression of Nfstr | Media composition; (Nfstr) cloned using homology based approach | CPT | Roja, 2008; Singh et al., 2020 |
| 10. | *O. trichocarpon* | Shoot culture | Subculture passages in media of alternating strength | CPT | Sibi et al., 2016 |
| 11. | *O. alata* | Hairy root culture | *Agrobacterium rhizogenes* strain TISTR 1450 | CPT | Ya-ut et al., 2011 |

# CHAPTER 3

*Collection of plants from different geographical locations of Western Ghats (Kerala) and screening of elite species of Ophiorrhiza on the basis of camptothecin by HPTLC method.*

## 3.1 Introduction

*Ophiorrhiza* spp. are undershrubs with a height of 20cm to 3m. *Ophiorrhiza* is a member of the Rubiaceae family, with 47 species and nine variants identified in India (Deb & Mondal, 2001). The majority of *Ophiorrhiza* spp. are found in abundance in India's South Western Ghats area (Sasidharan, 2004). Traditionally, it was applied to treat snake bites. wound healing, ulcers, and stomatitis, among other things. Because of the presence of the powerful anticancer chemical CPT, *Ophiorrhiza* has been a prominent focus in study. CPT was discovered in *Camptotheca acuminata* for the first time. It is also detected in *Nothapodytes foetida* in a promising proportion. However, because of the strong demand for CPT in the industrial sector, this major source plant has been meticulously collected. As a result, an alternate plant source with a potential CPT content is in considerable demand. *Ophiorrhiza sp*. has a role in this regard. To locate the high producing camptothecin lines, many researchers performed trials on several species of *Ophiorrhiza*. Rajan et al. (2013) conducted research on several *Ophiorrhiza* species and discovered that *O. mungos* var *angustifolia* has the highest CPT concentration. CPT is an indole monoterpenoid alkaloid with a lactone ring at the end of a mildly basic quinoline moiety (Pu et al., 2019). Artificial synthesis is not feasible due to its intricate structure, thus it must rely on plants as a natural supply. The presence of CPT varies depending on the species. *Ophiorrhiza* spp. are small cultivable plants with a promising CPT content, making them a viable CPT supply option.

Qualitative as well as quantitative results are strongly impacted by the method of sample preparation. properties of the bioactive compound. Before proceeding with extraction, thorough homogenization of the plant tissue increases the extraction rate (Sasidharan et al.,

2011) as it increases accessibility between the solvent and the plant cell matrix. Several techniques for the extraction of CPT from plants have been employed. Some of the reported ones include Stirring, Soxhlet, Maceration, vortex mixer, cold maceration, HRE, ASE, Solvent extraction, Ultrasonication, MAE, UARSE, Superficial fluid extraction, IL-UMASDE (Table 2.4)

Based on the available literature, numerous analytical methods on quantification of CPT were made including reverse phase HPLC/DAD/ESI/MS, TLC, HPTLC, HPLC, RP-HPLC, LC-MS, UHPLC-MS (Table 2.5). Recently, Karanje et al. (2021) determined CPT in *Tabernaemontana* sp. (Apocynaceae) using HPTLC, while simultaneous analysis of CPT and Quercitin in ethanolic extracts of *Gracilaria corticate* (Gracilariaceae) was carried out by HPLC (Ashwini et al., 2017). CPT contents in *O. mungos* varied from 170.11 (µg/g,dr.wt) to 503.15 (µg/g,dr.wt) (Lorence & Nessler, 2004), 127.86(µg/g,dr.wt) to 476.89 (µg/g,dr.wt) (Rajan et al., 2013). Previous reports on detection of CPT on *O. mungos*, *O. eriantha*, *O. grandifolia*, *O. pectinata*, *O. shendurunii*, *O. trichocarpon*, *O. rugosa* var. *prostrata*, *O. mungos* var. *angustifolia* were made (Gharpure et al.,2010; Lorence & Nessler, 2004; Martin et al., 2007; Rani et al., 2010; Rajan et al., 2013; Roja, 2008). According to reports, HPTLC is a superior option to HPLC for determining phytoconstituents since it is more affordable, quick, and time-efficient, and it can detect numerous samples at once on a plate (Pandey & Kaur, 2018). Previous studies on medicinal plants have used HPTLC for detection of marker compound such as diosgenin from *Dioscorea deltoidei* (Nazir et al., 2021), mangiferin from *Swertia* spp. (Pandey & Kaur, 2018), from *Stevia rebaudiana* leaves secondary metabolites stevioside (Stev), steviol glycosides, and rebaudioside A (Reb A) (Nawaz et al., 2022), stigmasterol from *Rauvolfia serpentina* (Dey & Pandey, 2014).

## 3.2 Materials and methods

### 3.2.1 Chemicals and marker compound

Marker compound camptothecin (>90% purity) was procured from Sigma-Aldrich Company, USA and stored at -20 ℃. Methanol, ethyl acetate and chloroform - HPLC grade purchased from Himedia, India.

### 3.2.2 Collection of plant material

The plant material of *Ophiorrhiza hirsutula* Wight ex Hook.f., *Ophiorrhiza rugosa* var *prostrata* (D.Don) Deb& Mondal from Palode, Thiruvananthapuram *and Ophiorrhiza mungos* L. from Kottakkal, Malappuram (Kerala), were collected during the period of 2021-2022. All the samples were collected at the maturity stage in the winter season (December-February). The collection sites along with morphological descriptions and time of collection

are presented in Table 3.1.

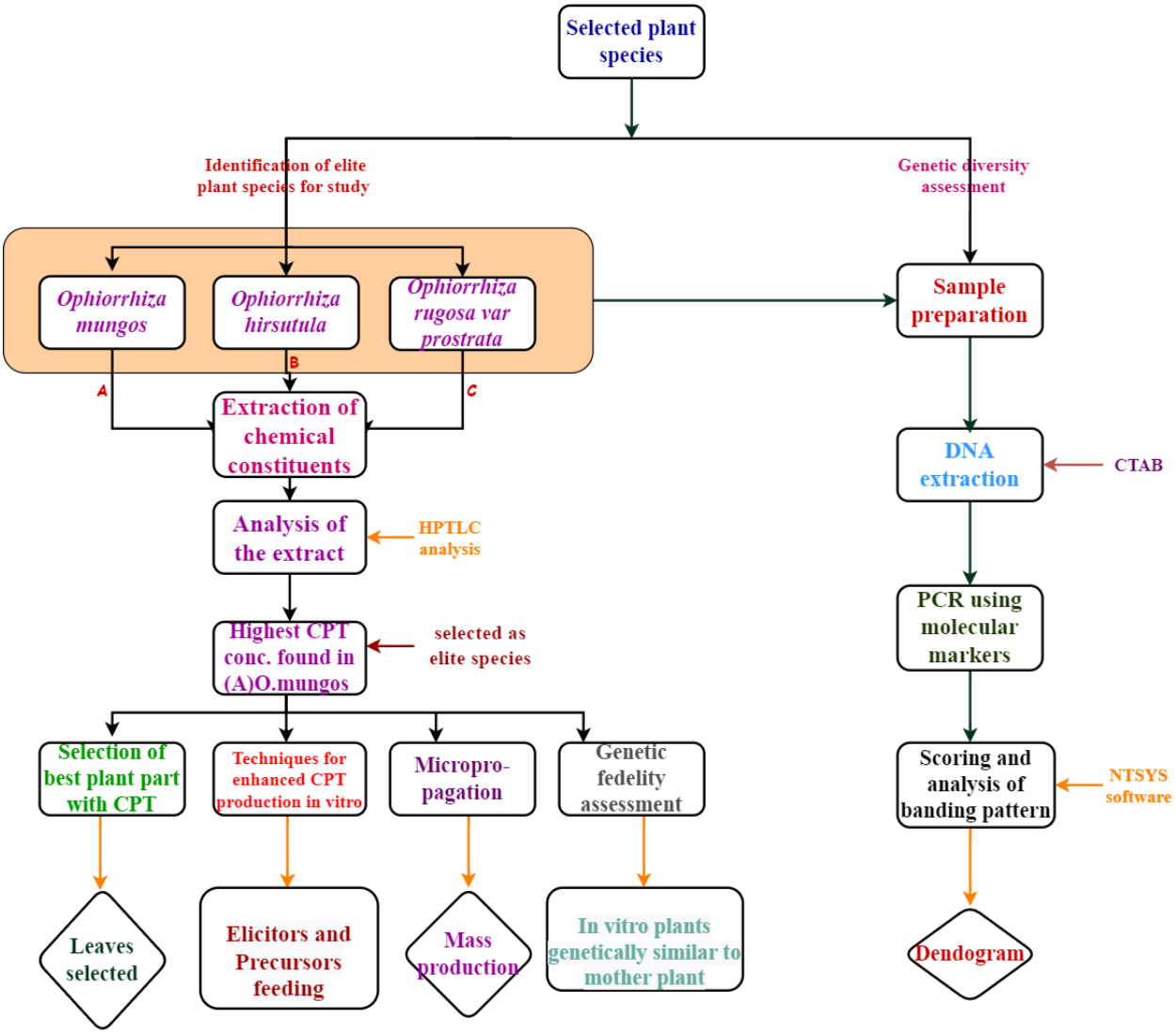



**Fig.3.1:** Graphical abstract – Selection of elite *Ophiorrhiza spp*. and in vitro strategies for increased camptothecin production.

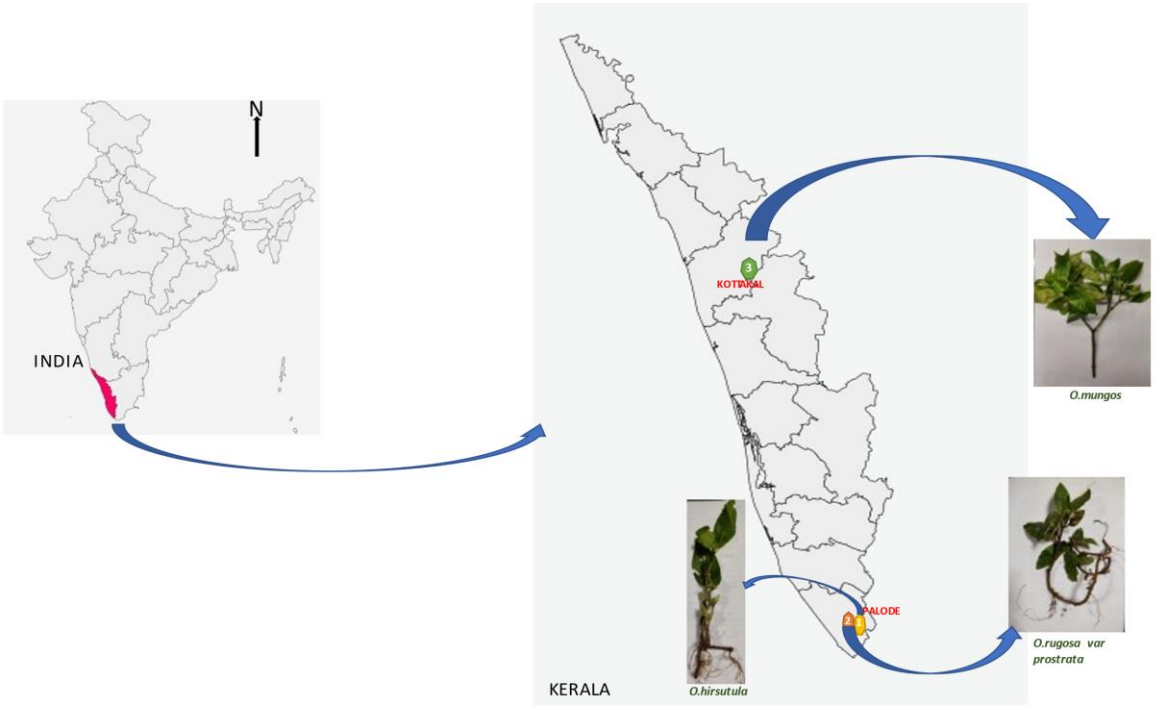



**Fig 3.2:** Location for sample collection on India map.


**Plate 3.1:** Collected samples (1) *Ophiorrhiza hirsutula* (2) *O. rugosa* var *prostrata* (3) *O. mungos* (Scale bar— 5 cm).

**Table 3.1** Morphological description of plant samples collected from Western ghats, Kerala.

| Sample | Morphological description | Location | Time of collection |
|---|---|---|---|
| *Ophiorrhiza hirsutula* Wight ex Hook. f. | Undershrub, (23cm) hairy stem, rooting at lower nodes, (6.2cm x3.1cm) leaves opposite, reticulate venation, ovate lanceolate, acute apex, cymose terminal inflorescence. | Palode, Thiruvananthapuram | 30th December 2021-3rd January 2022 |
| *Ophiorrhiza rugosa var angustifolia* (D. Don) Deb&Mondal | Herb, (16.3cm) runner stem, rooting at lower nodes, (5.4cmx2.6cm) opposite ovate, reticulate venation, elliptic lanceolate, acute apex, cymose inflorescence. | Palode, Thiruvananthapuram | 30th December 2021 -3rd January 2022 |
| *Ophiorrhiza mungos* L. | Erect herb(27.8cm), rooting at lower nodes, (7.9x3.4cm) leaves opposite, reticulate venation, elliptic lanceolate, acuminate, terminal scorpiod cymose inflorescence. | Kottakal, Malappuram | 17th February 2022 |

### 3.2.3 Authentication of plant material

Three species of *Ophiorrhiza* viz *O. mungos* (Voucher number: KUBH 1822), *O. hirsutula* (Voucher number: KUBH 1823) and *O. rugosa* var *prostrata* (Voucher number: KUBH 1824) were collected from Kottakkal and Palode (Western Ghats region, Kerala), India from December-February 2021-22. The curator of the botany department at the Kerala University in India identified the species, and herbarium sheet was added to the department division of herbarium.

### 3.2.4 Sample preparation

Each plant sample was separated on the basis of inflorescence, leaves, stem, roots and oven dried at 50°C for 16h and powdered using mortar and pestle. For screening of elite species powdered material 100mg were microwave extracted at 100W, 90sec in 10ml 60%methanol (Saravanan & Boopalan, 2011). Extraction was carried out thrice. After extraction combined solvents concentrated using a rotary evaporator (Buchi, Switzerland) and residue dissolved in 1ml methanol. Centrifuged at 10000rpm for 10min. Supernatant filtered using 45µM nylon membrane and stored at 4°C for analysis.

#### 3.2.4.1 Extraction techniques

For selection of extraction technique, powdered material 100mg was extracted by different methods in10ml methanol. Each extraction was carried out thrice. After extraction combined solvents concentrated using a rotary evaporator (Buchi, Switzerland) and residue dissolved in 1ml methanol. Centrifuged at 10000rpm for 10min. Supernatant filtered using 45µM nylon membrane and stored at 4°C for analysis.

##### 3.2.4.1.1 Maceration

Powdered sample macerated for 24hrs in different concentrations of methanol (100%,60%,0%) with intermittent shaking in between (Zhang et al., 2007).

##### 3.2.4.1.2 Hot water bath

Powdered sample extracted with methanol (100%,60%,0%) at 60°C for 90mins in shaking water bath (Sarika et al., 2019).

##### 3.2.4.1.3 Microwave extraction

Powdered sample extracted with methanol (100%,60%,0%) at 100W, 2mins. Microwave irradiation stopped at intervals, flask taken out, cooled under running tap water for few secs to avoid superboiling. Total time taken for one complete cycle was 10mins (Fulzele & Satdive, 2005b).

##### 3.2.4.1.4 Ultrasonic bath extraction

Powdered sample extracted with methanol (100%,60%,0%) at 40KHz,10mins at room temperature (Sankar-Thomas & Lieberei, 2011; Zu et al., 2003).

##### 3.2.4.1.5 Optimization of extraction parameters by RSM model

Box Behnken design (BBD) was applied for the optimization of extraction parameters. Three independent variables solvent composition, solid: solvent ratio and ultrasonication time were chosen as the independent variables and the coded levels are shown in Table 3.5. The optimal level and possible collaboration among the relevant parameters were analysed using BBD. Minitab software was used to create the experimental layout.

**Table 3.2** Treatment variables for optimization of camptothecin extraction from *O. mungos* using BBD.

| Variables | Coded levels | | | |
|---|---|---|---|---|
| | Codes | + 1 | 0 | -1 |
| **Solvent composition** | $X_1$ | 70 | 50 | 30 |
| **Solid: Solvent ratio (g/ml)** | $X_2$ | 10:60 | 7.5:40 | 5:20 |
| **Ultrasonication time (min)** | $X_3$ | 20 | 15 | 10 |

This model depends on first-order model:

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

According to the equation given below, the coding of variables was done (Maran & Manikandan, 2012):

$$x_i = X_i - X_0 / \Delta X_i \qquad i = 1, 2, 3..., k \quad (2)$$

The ideal point was predicted using a second-order polynomial equation, which allowed us to evaluate the correlation between the independent variables and the CPT material. Below is the equation with four independent variables:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (3)$$

Where Y represents predicted response; $\beta_0$ shows modal constant; $X_1$, $X_2$ and $X_3$ are significant factors; $\beta_1, \beta_2,$ *and* $\beta_3$ are linear coefficients; $\beta_{11}, \beta_{22}$ and $\beta_{33}$ are quadratic coefficients and $\beta_{12}, \beta_{13},$ *and* $\beta_{23}$ are the interactive coefficients. The regression coefficient and analysis of variance have been used to analyse the CPT content.

### 3.2.5 Phytochemical analysis

#### 3.2.5.1 Marker compound preparation

Marker compounds camptothecin (1mg) was dissolved in DMSO: methanol (1:4v/v) (1 mL) to prepare stock solution (1 mg/ mL) and working concentration 0.02 mg/ mL was achieved by dissolving 20 µl of stock solution in 880µl of methanol.

#### 3.2.5.2 Calibration curve preparation (Linearity)

The linearity range of 40-200 ng/spot was achieved by generating a camptothecin

calibration curve using varying amounts of standard solution (2, 4, 6, 8, 10 µl). Peak area versus concentration at 366 nm wavelength was used to generate the calibration graph. Camptothecin levels were calculated across samples by using a regression equation and a standard peak area.

### 3.2.5.3 HPTLC Instrumentation

The contents of CPT in the sample were screened utilizing an HPTLC system (CAMAG Muttenz, Switzerland) equipped with a Linomat-5 automated sample applicator and a CAMAG TLC scanner-3 ("Scanner 180710" S/N 180710 (2.01.02)) that was controlled by WinCATS software (version: 1.4.6.2002). 10 µl of various *Ophiorrhiza* extracts (10 µg/ µl) were put on pre-coated silica gel 60 F254 TLC plates (20 x10 cm, E. Merck, Darmstadt, Germany) automatically. Each sample was applied to the plates as 6 mm wide bands with 13 mm between tracks using a Linomat-5 automatic sample applicator with a 100µl Hamilton syringe in $N_2$ flow (application rate 150 nL/s, space between two bands-11mm, slit dimension-6mm x 0.45mm, scanning speed 20 mm/step, and data resolution 100 m/step). The HPTLC was carried out at a temperature of 24±2 °C and a relative humidity of 45 percent. In the CAMAG twin trough glass chamber (20 cm x 10 cm) saturated prior for 10 minutes with mobile phase vapour and run period 24 minutes, HPTLC plates were developed up to 8cm in 20ml of EtOAc: $CHCl_3$: MeOH(5:4.5:0.5v/v) mobile phase.

### 3.2.5.4 Quantification of camptothecin

The HPTLC method for the simultaneous quantification of camptothecin was developed and validated. Different mobile phases were tried to give clear separation and resolution of camptothecin. Each sample (10 µL) was applied on 20 × 10 cm precoated silica gel 60 $F_{254}$ TLC plate. Among different solvent systems used for mobile phase, ethyl acetate: chloroform: methanol (4.5:5:0.5 v/v) gave well resolute spots of compound from crude samples of *Ophiorrhiza spp.* After development, plates were dried using hair dryer (Nova, India) for 5 min and then scanned at 254 nm and 366nm.

### 3.2.5.5 Method validation

According to the guidelines of ICH (International Council for Harmonisation, 2005), method validation was carried out on the basis of sensitivity, precision, specificity, accuracy, robustness and recovery presented in Table 3.3.

#### 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). LOD and LOQ values were calculated using the following formula: LOD = 3.1 (SD/S) and LOQ 10.1(SD/S) based on the standard deviation (SD) of the response and the slope (S) of the calibration curve of camptothecin.

#### 3.2.5.5.2 Specificity

Peak purity and densitograms were used to assess specificity. The starting, maximum, and ending peak purity of the reference compound were aligned with those of the sample, and the overlay spectra of the sample were aligned with the reference chemical. The reference chemical band and samples band were examined using the $R_f$ value.

#### 3.2.5.5.3 Accuracy and Precision

Instrument accuracy was evaluated by measuring the same amount of CPT (250 ng/spot) in five separate spots. Three times on the same day, as well as after three days, different concentrations of the reference drug were used to measure repeatability and reproducibility. These examinations were carried out five times, and the mean and %RSD show the results.

## 3.3 Result and Discussion

### 3.3.1 Method development

The mobile phase ratio for HPTLC was optimized to achieve accurate and well-resolved peaks for camptothecin. The mobile phase ethyl acetate: chloroform: methanol (4.5:5:0.5 v/v) showed good separation and symmetrical peaks at $R_f$ value of 0.54 for camptothecin (Figure 3.3).

### 3.3.2 Method validation

The linearity plot of peak area against amount of camptothecin was found linear in the range of 40-200 ng/spot (Figure 3.4). Good linear relationship for the plot was confirmed by linear regression data (Table 3.3). 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 10 and 30 for camptothecin (Table 3.3).

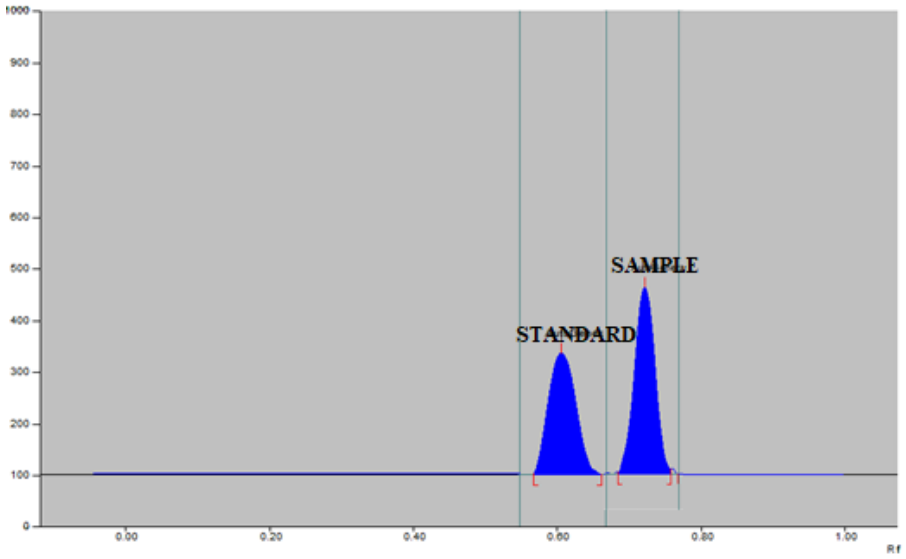



**Fig 3.3:** HPTLC densitogram of standard camptothecin and *Ophiorrhiza mungos* sample.

**Table 3.3** Method validation for camptothecin quantification.

| S. No | Parameters | Camptothecin |
|---|---|---|
| 1 | Linearity range (ng/spot) | 40-200 |
| 2 | Correlation coefficient ($r^2$) | 0.997 |
| 3 | Regression equation | Y = 13.113X + 149.42 |
| 4 | [b]Limit of detection (LOD) (ng) [3×SD/S] | 10 ng |
| 5 | [b]Limit of quantification (LOQ) (ng) [10×SD/S] | 30 ng |
| 6 | $R_f$ | 0.54 |
| **Precision and accuracy** | | |
| 7 | Intra-day RSD (%), n = 5 | 0.21 |
| 8 | Inter-day RSD (%), n = 5 | 0.27 |
| **Recovery** | | |

| | | |
|---|---|---|
| 9 | Amount of standard in plant samples (µg $mg^{-1}$) having maximum bioactive compounds | 9.6 |
| 10 | Amount of standards added in plant samples (µg $mg^{-1}$) | 4.0, 8.0, 12.0 |
| 11 | Amount of standard found (µg $mg^{-1}$) | 13.56, 17.57, 21.63 |
| 12 | Recovery (%) | 100.52,100.12, 100.6 |
| 13 | Mean Recovery (%) | 100.41 |

[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

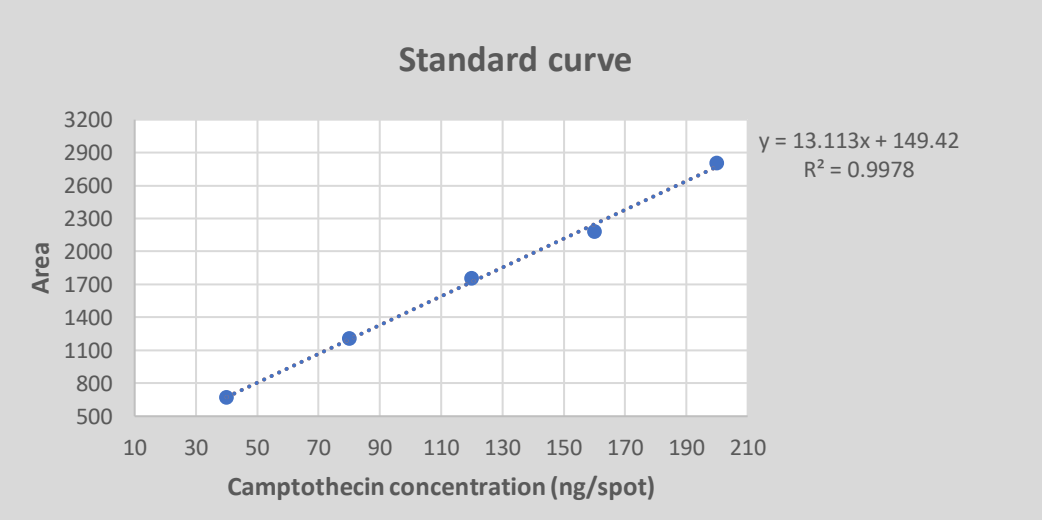



**Fig 3.4:** The linearity graph of the standard camptothecin.

### 3.3.3 Quantification of camptothecin

#### 3.3.3.1 Screening of elite species of *Ophiorrhiza* and camptothecin content in different plant parts of *Ophiorrhiza spp*.

The camptothecin content of the various components (inflorescence, leaf, stem, and roots) of 10 different accessions i.e., 4 accessions of *O. mungos* (OM-I: *O. mungos* inflorescence, OM-S: *O. mungos* stem, OM-L: *O. mungos* leaves and OM-R: *O. mungos* roots), 3 accessions of *O. hirsutula* (OH-S: *O. hirsutula* stem, OH-L: *O. hirsutula* leaves, OH-R: *O. hirsutula* roots) and 3 accessions of *O. rugosa* var *prostrata* (OR-S:*O. rugosa* var *prostrata* stem, OR-L: *O. rugosa* var *prostrata* leaves and OR-R: *O. rugosa* var *prostrata* roots) which were collected from Kottakkal and Palode (Western Ghats of Kerala), was screened by using HPTLC. Camptothecin peak from the extracts of *Ophiorrhiza* samples were confirmed by matching their single spot at $R_f$ =0.54 with the peaks of standard (Figure 3.3). The quantity of camptothecin in plant samples of *Ophiorrhiza* was evaluated by applying the linear regression equation and the content are displayed in Table 3.4. In all accessions camptothecin was found, however, their content was dependent on which sample was analyzed. The accessions of *O. mungos* showed higher content of camptothecin in leaves (0.37%) followed by inflorescence (0.16%), roots (0.16%) and lowest value in stem (0.14%). HPTLC analysis (Fig.3.5) confirms camptothecin in 10 different accessions belonging to three different species of *Ophiorrhiza*. *Ophiorrhiza* extracts were analyzed in different mobile phases and highly defined CPT peaks were obtained in the extracts run in $CHCl_3$: MeOH: EtOAc (5:0.5:4.5v/v) (Fig.3.3). Compared to *O. rugosa* var *prostrata* and *O. hirsutula*, *O. mungos* showed highest CPT content and is screened as the elite species suitable for traditional cultivation and tissue culture based propagation. This is the first report on use of different parts of plant samples for analysis and also of CPT detection on *O. hirsutula*. Previous reports on detection of CPT on *O. eriantha*, *O. grandifolia*, *O. pectinata*, *O. shendurunii*, *O. trichocarpon*, *O. rugosa* var. *prostrata*, *O. mungos*, *O. mungos* var. *angustifolia* were made (Gharpure et al., 2010; Lorence & Nessler, 2004; Martin et al., 2007; Rani et al., 2010; Rajan et al., 2013; Roja, 2008). Table 3.5, displays camptothecin concentration in different parts *O. mungos* extracted using the optimal solvent and extraction method (60% methanol and UAE). *Ophiorrhiza* extracts were analyzed in different mobile phases and $CHCl_3$: MeOH: EtOAc (5:0.5:4.5v/v) gave highly defined CPT peaks in the extracts (Fig.3.5).

Compared to *O. rugosa* var *prostrata*(0.001%) and *O. hirsutula*(0.11%), *O. mungos* (0.37%) showed highest CPT content and screened as the elite species suitable for traditional

cultivation and tissue culture based propagation. *O. hirsutula* (0.11%) showed low content of CPT while negligible content below detection level by TLC scanner was observed in *O. rugosa* var. *prostrata*(0.001%). Table 3.5, shows different parts of the same plant showing different contents of CPT. Thus, selecting the most productive *Ophiorrhiza* species and also to the particular plant part is crucial for tissue culture based large scale production and industrial purposes. Of the three species *O. rugosa* var *prostrata* and *O. hirsutula* leaf showed CPT below detection level. This is the first report on use of different parts of plant samples for analysis and also of CPT detection on *O. hirsutula*. It was revealed that through UAE the leaves (0.96%) contain highest CPT followed by inflorescence (0.64%), root (0.51%) and stem (0.49%). Camptothecin is present in the inflorescence, stem, leaf, and roots, according to HPTLC fingerprinting (Fig. 3A,3C). The interspecific variation in camptothecin content may result from genetic differences, different habitats or microclimates, environmental factors such as temperature, humidity, soil composition, and light availability, as well as from evolutionary history that resulted in the development of various chemical profiles.

#### 3.3.3.2 Effects of various extraction methods and solvent systems on camptothecin production

The study's goals were to (a) compare two widely used conventional extraction methods (hot water bath and maceration) with two modern, environmentally friendly methods (MAE and UAE using methanol as a solvent), (b) optimize the conditions for the most effective method, and (c) suggest a method for the efficient, large-scale extraction of bioactive compounds from leaves. The extraction technique i.e., UAE, MAE, Heat reflux extraction (HRE) and maceration were used in the present investigation. The plant matter was oven dried. Under different circumstances, the dried material was employed for extraction.

Four extraction techniques were conducted on different parts of *O. mungos* using methanol and water (100%,60%,0%) as solvent. Leaf sample UAE extracted in 60% methanol showed the highest content of CPT (0.96%). According to published research, a variety of solvents were used to extract camptothecin from *Ophiorrhiza* species. Our research on various plant sections showed that 60% methanol was the most effective solvent for extracting camptothecin. The physicochemical characteristics of methanol may be used to explain why it is so easy to extract bioactive chemicals from it. Earlier Kaur et al. (2019) reported similar results while extracting secoiridoids and xanthones from *Swertia spp*. This could also be because the plant material contains polar compounds that are soluble in solvents with high polarity such as methanol.

### 3.3.3.3 Screening of significant extraction parameters by BBD(RSM)

The statistical methods and their interplay in the framework of the inquiry quantify the effects of changes to the test variables. BBD is a widely used and highly effective design (Ekren & Ekren, 2008). Coded levels for different variables used are shown in Table 3.2.

According to the experimental findings, the total %CPT content varied from 0.67% in 19 run (15min extraction time, 7.5:40 solid: solvent ratio and 50% aqueous ethanol) to 0.098% in run 2 (10min extraction time, 10:60 solid: solvent ratio and 30% aqueous ethanol).

The model was assessed in accordance with the significance of the regression coefficients. In order to enhance the number of significant variables, the level of significance was set at 0.1 in accordance with earlier research. The significant variables on the response variable of CPT were the intercept ($X_0$) ($p$ =0.655220), the linear effect of solvent composition ($X_1$) ($p$ = -0.104749) and its quadratic effect ($X_{11}$) ($p$ = -0.065996), the linear effect of solid: solvent ratio ($X_2$) ($p$ =0.092261) and its quadratic effect ($X_{22}$) ($p$ =-0.080492), the linear effect of ultrasonication time ($X_3$) ($p$ =0.046440) and its quadratic effect ($X_{33}$) ($p$ =0.105064), the interaction between solvent composition and solid: solvent ratio ($X_{12}$) ($p$ =0.068125), the interaction between solvent composition and ultrasonication time ($X_{13}$) ($p$ =0.006625), and the interaction between solid: solvent ratio and ultrasonication time ($X_{23}$) ($p$ =0.004375).
Multi-regression analysis of experimental data yields a second-order polynomial that mathematically reflects the relationship between the independent factors and the response (% CPT content: coded and uncoded value).

CPT (% dry weight) (coded unit)

$$Y = 0.655220 - 0.104749X_1 + 0.092261X_2 + 0.046440X_3 - 0.065996X_1^2 - 0.080492X_2^2 - 0.105064X_3^2 + 0.068125X_1X_2 + 0.006625X_1X_3 + 0.004375X_2X_3 \quad (4)$$

CPT (% dry weight) (uncoded unit)

$$Y = -0.981482 + 4.90538E\text{-}05X_1 + 0.156709X_2 + 0.129427X_3 - 1.64990E\text{-}04X_1^2 - 0.0128787X_2^2 - 0.00420254X_3^2 + 0.00136250X_1X_2 + 6.62500E\text{-}05X_1X_3 + 0.000350000X_2X_3 \quad (5)$$

### 3.3.3.4 Method validation

#### 3.3.3.4.1 Linearity

The aspects of method validation for camptothecin quantification are provided in Table 3.3. With regard to peak area, a good linearity-range of $r^2$ (coefficient of determination) was found

to be 40–200 ng/spot for camptothecin, as illustrated in Fig. 3.4. Table 3.3 displays the predicted LOD and LOQ of camptothecin used to determine the method's correct sensitivity.

**3.3.3.4.2 Precision**

Utilizing 50% of the camptothecin present in the plant extract allowed researchers to test the method's accuracy. To calculate the repeatability and reproducibility of the data, coefficient variation (% RSD) was examined for intra-day and inter-day precision (n= 5). Table 3.3 displays the data gathered from average recoveries and intra-day and inter-day precision.

**3.3.3.4.3 Specificity**

Peak purity and densitograms were used to carefully examine specificity. Camptothecin peak purity was decided upon based on $r^2$ value. $R_f$ values were used to compare the test samples to the reference compound, and it was discovered that both samples fit the criteria for superimposable spectra. Testing the developed method's specificity revealed no contaminants.

## 3.4 Conclusions:

In this study comparison between conventional (maceration and hot water bath) and non-conventional (MAE and UAE) extraction techniques, various plant parts of three different species of *Ophiorrhiza* and different solvent concentrations were evaluated. Different parts of the same plant showed variations in their CPT contents (Table 3.4). Thus, choosing the highly productive *Ophiorrhiza* species and the particular plant part is crucial for tissue culture based large scale production and industrial purposes. When compared to other sections of the plant extract, leaves of *O. mungos* (3731 µg/g, dr. wt or 0.37%) had the greatest amount of CPT. Lorence and Nessler, (2004), reported the amount of CPT ranging from 503.15(µg/g, dr. wt) to 170.11(µg/g, dr. wt) and also Rajan et al. (2013), reported CPT concentration was found from 127.86 (µg/g, dr. wt) to 476.89 (µg/g, dr. wt) when extracted from whole plant. In comparison to other extraction methods, UAE with 60% methanol as solvent showed the maximum concentration of marker compound in minimal extraction time (10min). The presence of CPT in *O. hirsutula* was also established in this investigation. This is the first report on the use of different parts of plant samples and comparison of different extraction techniques for analysis and also of CPT detection on *O. hirsutula*. This experiment also demonstrated the need of selecting *Ophiorhiza* species with high CPT yields for industrial uses. The presence of considerable CPT content in *O. hirsutula* and *O. rugosa* var. *prostrata*, which were examined in this experiment, is ruled out.

HPTLC fingerprinting has emerged in recent years as a necessary and effective technology

for the identification and authenticity of pharmaceutical plants as well as the standardization of herbal medications. The HPTLC method created and validated in this study for the estimation of camptothecin in crude samples of *Ophiorrhiza spp*. was found to be straightforward, exact, and sensitive. It can be used for quality control and standardization of drugs that contain camptothecin as their main component, as well as by laboratories for the analysis of products that contain camptothecin. In order to filter and distinguish the *Ophiorrhiza mungos* from other species of the genus *Ophiorrhiza*, the profiles will also be helpful. The findings of our study can also be applied to the screening and identification of elite populations whose propagation, whether for commercial or in vitro usage, may significantly contribute to the expansion of global commerce. The results of our analysis, which included 10 accessions of *Ophiorrhiza* spp., led to the discovery of an elite species with a high camptothecin content. Future mass cultivation techniques might be applied to such promising populations.

**Table 3.4** Quantity of camptothecin in plant accessions collected from Western Ghats (Kerala).

| **Accession no.** | **Plant part (dry powder)** | **Species** | **CPT%** |
|---|---|---|---|
| 1. OM-I | Inflorescence | *O. mungos* | 0.16 |
| 2. OM-S | Stem | *O. mungos* | 0.14 |
| 3. OM-L | **Leaves** | ***O. mungos*** | **0.37** |
| 4. OM-R | Roots | *O. mungos* | 0.16 |
| 5. OH-S | Stem | *O. hirsutula* | 0.03 |
| 6. OH-L | Leaves | *O. hirsutula* | 0.02 |
| 7. OH-R | Roots | *O. hirsutula* | 0.11 |
| 8. OR-S | Stem | *O. rugosa* var *prostrata* | 0.001 |
| 9. OR-L | Leaves | *O. rugosa* var *prostrata* | 0.003 |
| 10. OR-R | Roots | *O. rugosa* var *prostrata* | 0.002 |

**(OM-I: *O. mungos* inflorescence, OM-S: *O. mungos* stem, OM-L: *O. mungos* leaves and OM-R: *O. mungos* roots; OH-S: *O. hirsutula* stem, OH-L: *O. hirsutula* leaves, OH-R: *O. hirsutula* roots; OR-S:*O. rugosa* var *prostrata* stem, OR-L: *O. rugosa* var *prostrata* leaves and OR-R: *O. rugosa* var *prostrata* roots)

**Table 3.5** HPTLC analysis of different parts of *Ophiorrhiza mungos.*

| **Extraction** | **Parts of plant (dry powder)** | **CT% (100% methanol)** | **CT% (60% methanol)** | **CT% (0% methanol/aqueous soln.)** |
|---|---|---|---|---|
| UAE | Inflorescence | 0.48 | 0.64 | 0.27 |
| | Leaves | **0.75** | **0.96** | **0.47** |
| | Stem | 0.27 | 0.49 | 0.11 |
| | Root | 0.31 | 0.51 | 0.20 |
| MAE | Inflorescence | 0.06 | 0.21 | 0.04 |
| | Leaves | 0.05 | 0.11 | 0.02 |
| | Stem | 0.03 | 0.08 | 0.01 |
| | Root | 0.04 | 0.09 | 0.01 |
| MACERATION | Inflorescence | 0.27 | 0.51 | 0.16 |
| | Leaves | 0.23 | 0.49 | 0.09 |
| | Stem | 0.18 | 0.36 | 0.09 |
| | Root | 0.17 | 0.26 | 0.08 |
| HOT WATER BATH | Inflorescence | 0.17 | 0.38 | 0.09 |
| | Leaves | 0.25 | 0.42 | 0.10 |
| | Stem | 0.08 | 0.19 | 0.05 |
| | Root | 0.17 | 0.31 | 0.07 |

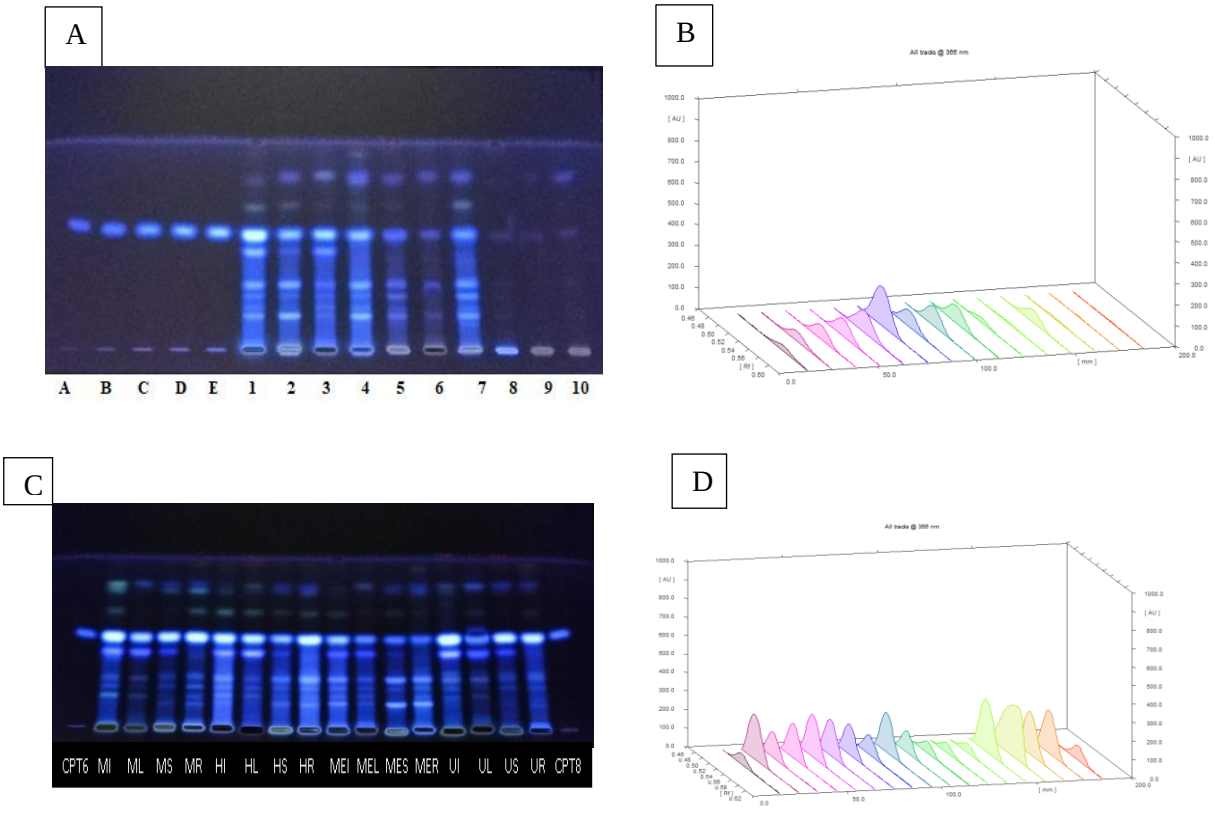



**Fig 3.5:** (A) -HPTLC fingerprinting of different accessions compared with standards at 366nm.(A-E standard CPT);(1-10 Table 3.3)
**(**B)- HPTLC densitogram of *O. mungos, O. hirsutula* and *O. rugosa* showing camptothecin peaks.
(C)-HPTLC fingerprinting of different accessions compared with standards ; A-366nm,

- **CPT6-** 6µl of camptothecin standard stock solution (20µg/ml).,**MI-** Macerated inflorescence**, ML-** Macerated leaves**, MS-**Macerated stem**, MR-**Macerated root**, HI-** Hot water bath Inflorescence**, HL-** Hot water bath leaves**, HS-** Hot water bath stem**, HR-** Hot water bath roots**, MEI-**Microwave extracted Inflorescence**, MEL-** Microwave extracted leaves**, MES-** Microwave extracted stem**, MER-** Microwave extracted roots**, UI-** Ultrasonicated Inflorescence**, UL-** Ultrasonicated leaves**, US-**Ultrasonicated stem**, UR-** Ultrasonicated root**, CPT8-** 8µl of camptothethecin standard stock solution (20µg/ml).

(D)- HPTLC densitogram of different parts of *O. mungos* extracted by maceration, hot water bath, microwave and ultrasonication.
STD-standard CPT.

**Table 3.6** BBD criteria of extraction variables with corresponding experimental and predicted value.

| Run Order | Solvent composition | Solid: solvent | Ultrasonication Time | Experimental CPT (%) | Predicted CPT (%) |
|---|---|---|---|---|---|
| **1** | 30 | 5 | 10 | 0.457 | 0.448842 |
| **2** | 70 | 5 | 10 | 0.098 | 0.089844 |
| **3** | 30 | 10 | 10 | 0.501 | 0.488363 |
| **4** | 70 | 10 | 10 | 0.419 | 0.401866 |
| **5** | 30 | 5 | 20 | 0.526 | 0.519721 |
| **6** | 70 | 5 | 20 | 0.198 | 0.187224 |
| **7** | 30 | 10 | 20 | 0.592 | 0.576743 |
| **8** | 70 | 10 | 20 | 0.539 | 0.516746 |
| **9** | 16.36414 | 7.5 | 15 | 0.635 | 0.644721 |
| **10** | 83.63586 | 7.5 | 15 | 0.269 | 0.29239 |
| **11** | 50 | 3.295518 | 15 | 0.268 | 0.272392 |
| **12** | 50 | 11.70448 | 15 | 0.554 | 0.582719 |
| **13** | 50 | 7.5 | 6.591036 | 0.268 | 0.279953 |
| **14** | 50 | 7.5 | 23.40896 | 0.415 | 0.436158 |
| **15** | 50 | 7.5 | 15 | 0.67 | 0.65522 |
| **16** | 50 | 7.5 | 15 | 0.63 | 0.65522 |
| **17** | 50 | 7.5 | 15 | 0.68 | 0.65522 |
| **18** | 50 | 7.5 | 15 | 0.67 | 0.65522 |
| **19** | 50 | 7.5 | 15 | 0.637 | 0.65522 |
| **20** | 50 | 7.5 | 15 | 0.65 | 0.65522 |

**Table 3.7** Estimated Regression Coefficients for Camptothecin (%) using data in coded units.

| **Model parameters** | **Regression coefficient** | **S.E. Cofficient** | ***T*** | ***P*** |
|---|---|---|---|---|
| **Constant** | 0.655220 | 0.009588 | 68.335 | 0.000 |
| **Solvent composition** | -0.104749 | 0.006362 | -16.466 | 0.000 |
| **Solid: Solvent** | 0.092261 | 0.006362 | 14.503 | 0.000 |
| **Ultrasonication Time** | 0.046440 | 0.006362 | 7.300 | 0.000 |
| **Solvent composition$^2$** | -0.065996 | 0.006193 | - 10.657 | 0.000 |
| **Solid: Solvent$^2$** | -0.080492 | 0.006193 | -12.997 | 0.000 |
| **Ultrasonication Time$^2$** | -0.105064 | 0.006193 | -16.965 | 0.000 |
| **Solvent composition × Solid: Solvent** | 0.068125 | 0.008312 | 8.196 | 0.000 |
| **Solvent composition × Ultrasonication Time** | 0.006625 | 0.008312 | 0.797 | 0.444 |
| **Solid:Solvent × Ultrasonication Time** | 0.004375 | 0.008312 | 0.526 | 0.610 |

**Table 3.8** Estimated Regression Coefficients for Camptothecin (%) using data in uncoded units

| Model parameters | Regression coefficient |
|---|---|
| **Constant** | -0.981482 |
| **Solvent composition** | 4.90538E-05 |
| **Solid: Solvent** | 0.156709 |
| **Ultrasonication Time** | 0.129427 |
| **Solvent composition$^2$** | -1.64990E-04 |
| **Solid: Solvent$^2$** | -0.0128787 |
| **Ultrasonication Time$^2$** | -0.00420254 |
| **Solvent composition × Solid: Solvent** | 0.00136250 |
| **Solvent composition × Ultrasonication Time** | 6.62500E-05 |
| **Solid:Solvent × Ultrasonication Time** | 0.000350000 |

**Table 3.9** Analysis of variance for Camptothecin (%) by using BBD criterion.

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| **Regression** | 9 | 0.599270 | 0.599270 | 0.066586 | 120.47 | 0.000 |
| **Linear** | 3 | 0.295548 | 0.295548 | 0.098516 | 178.24 | 0.000 |
| **Square** | 3 | 0.266090 | 0.266090 | 0.088697 | 160.48 | 0.000 |
| **Interaction** | 3 | 0.037632 | 0.037632 | 0.012544 | 22.70 | 0.000 |
| **Residual Error** | 10 | 0.005527 | 0.005527 | 0.000553 | | |
| **Lack-of-Fit** | 5 | 0.003486 | 0.003486 | 0.000697 | 1.71 | 0.286 |
| **Pure Error** | 5 | 0.002041 | 0.002041 | 0.000408 | | |
| **Total** | 19 | 0.604797 | | | | |

R-Sq = 99.09% R-Sq(pred) = 95.16% R-Sq(adj) = 98.26%

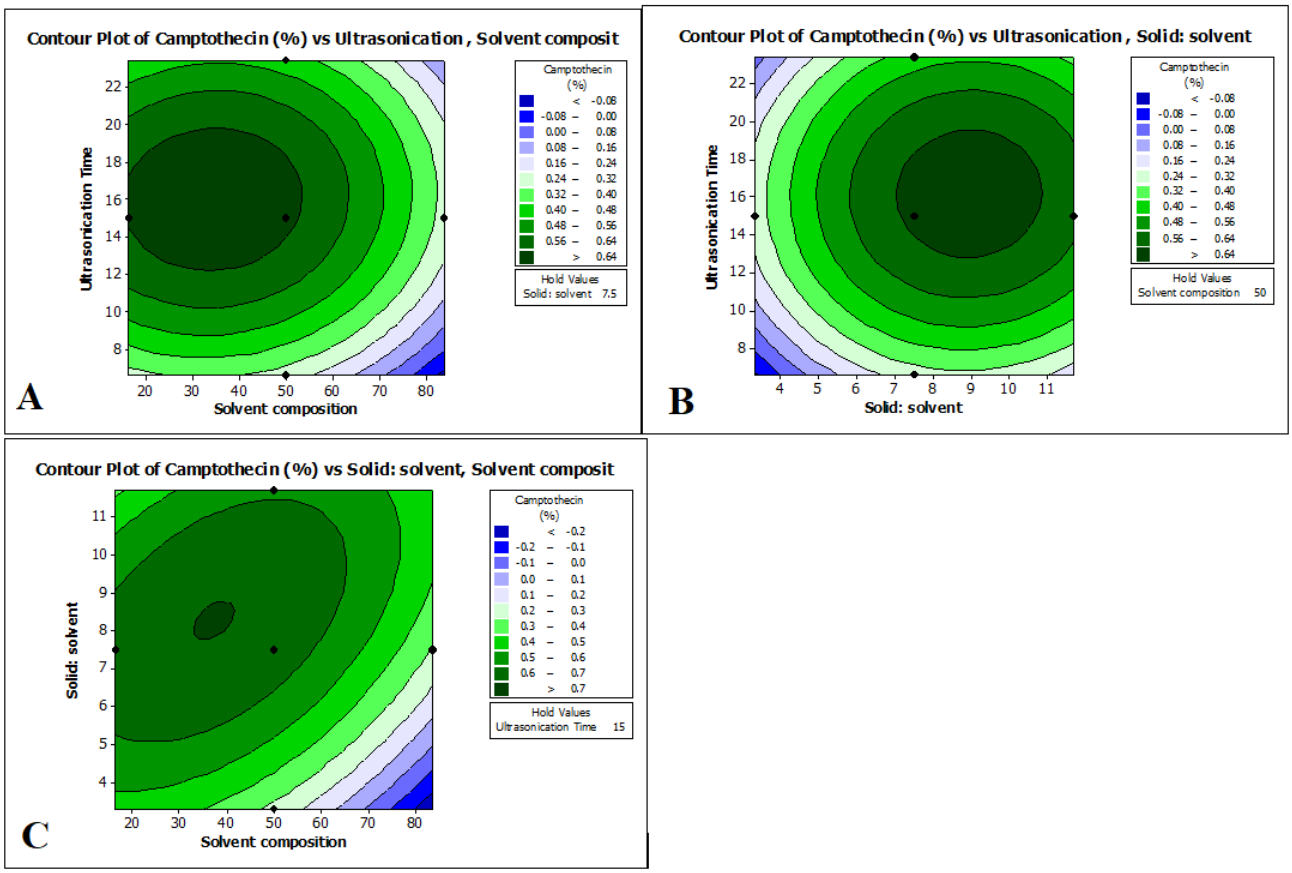



**Fig 3.6:** Contour Plot of CPT (%) vs (A) Ultrasonication, Solvent Composition(B) Ultrasonication, Solid: Solvent (C) vs Solid: Solvent, Solvent Concentration.

# CHAPTER 4

*Study the genetic diversity of the selected Ophiorrhiza spp. by using phytochemicals and DNA based molecular markers.*

## 4.1 Introduction

Plant genetic diversity (PGD) is crucial to the survival of plant and animal species because it increases their chances of surviving in a dynamic world. PGD facilitates the emergence of novel alleles, sub-species, and species in a population by providing a platform for natural selection to drive reproductive isolation (Qian et al., 2001). The glimpse of evolution in action can be seen in the way a species genetic makeup shifts throughout time. Understanding natural genetic variation is crucial for determining the degree of endangerment of uncommon and/or medicinally important species, which is necessary for developing effective conservation efforts to protect them. The plants under this genus may either be annual or perennial. There are nine variations and 47 species of the genus that are found in India. The southern Western Ghats area has documented sixteen species and three variants. Others have been recorded from Sikkim, Meghalaya, Arunachal Pradesh, and all other eastern Indian states. Several popular multiherbal products have *Ophiorrhiza* as a key ingredient. Its medicinal potential is mainly because it contains several bio-active chemicals, including camptothecin, 10-hydroxy-camptothecin, and 9-methoxy-camptothecin. *O. mungos* is the most prestigious and well-known species of *Ophiorrhiza*. Yet, research shows that camptothecin has been found in non-endemic species as well. Additional significant species, were detected with camptothecin viz. *O. eriantha*, *O. grandifolia*, *O. pectinata*, *O. shendurunii*, *O. trichocarpon*, *O. rugosa* var. *prostrata*, *O. mungos*, *O. mungos* var. *angustifolia* (Gharpure et al., 2010; Lorence & Nessler, 2004; Martin et al., 2007; Rani et al., 2010; Rajan et al., 2013; Roja, 2008). Because of the strong demand for camptothecin in the industrial sector, the major source plants have been meticulously collected. As a result, an alternate plant source with a potential camptothecin content is in considerable demand.

*Ophiorrhiza sp*. has a role in this regard. The genetic diversity of important *Ophiorrhiza* species/populations is poorly understood despite the plant's broad recognition as a powerful therapeutic plant. The main processes in the plant breeding programme are the identification, characterisation, and evaluation of the germplasm. *Ophiorrhiza*, a genus with a great deal of misconceptions and a difficult categorization, is incredibly polymorphic. Important *Ophiorrhiza* species collected from various geographic regions have not yet undergone thorough molecular study. For the characterisation of plant germplasm and the evaluation of plant genetic diversity, the using DNA based genetic markers may be a reliable method. There are significant differences between plant accessions gathered from various sites in the makeup of their biomarker metabolites (Kaur et al., 2019). Phytochemicals diversity is attributable to a number of climatic conditions as well as to the genotypes of the plant genes (Kumar & Roy, 2018). Understanding the relationships between DNA based genetic markers and phytochemical markers, which is regarded as the greatest method to assess, preserve, and improve the group of superior genotypes, is the best way to identify plant genetic diversity (Hennicke et al., 2016; Kaur et al., 2019). They have an extremely long shelf life and are specific to the plant in question. Until now, there have been no reports on the use of molecular markers to evaluate the genetic diversity of *Ophiorrhiza spp*. Recently, the use of ISSR and RAPD markers to analyse the genetic diversity of many industrially significant plants has proven to be an efficient method (Baruah et al., 2017). Despite the fact that ISSR markers have been widely used, and rely upon employing microsatellites to detect simple-sequence repeats and reveal additional polymorphism DNA segments, RAPD markers are relatively affordable and are widely recognised for spanning the whole genome (Baruah et al., 2017). Characterizing the genetic diversity of *Ophiorrhiza* species as well as populations in India's Western Himalayas using molecular and phytochemical markers has not yet been documented. Utilizing molecular (RAPD) and phytochemical marker (camptothecin), the current study sought to examine three different species of wild *Ophiorrhiza* namely *O. mungos*, *O. hirsutula*, and *O. rugosa* var *prostrata* for evidence of genetic variety and population genetic variability. The main objectives of this study were (i)to assess the genetic diversity within and between *Ophiorrhiza* species, (ii)the utility of RAPD primers in assessing population genetics and the study of genetic variation, and (iii) intrapopulation variation in phytochemical composition of 30 *O. mungos* plants based on camptothecin content. At the industrial scale, knowing the genetic relationships across *Ophiorrhiza* species/populations based on molecular and phytochemical markers will be useful for identifying the superior genotypes.

## 4.2 Materials and methods

### 4.2.1 Sample collection

Chapter 3(3.2.2).

**Table 4.1** Locations where plant samples were collected in the Western Ghats (Kerala), India.

| Plant accessions | Plant species | Location | Elevation (m) | Coordinates |
|---|---|---|---|---|
| S1 | *Ophiorrhiza mungos* | Kottakkal | 225m | 11.2980118, 75.9420137 |
| S2 | | Kottakkal | 227m | 10.9854136, 76.1508147 |
| S3 | | Kottakkal | 225m | 10.8451753, 76.0628985 |
| S4 | | Kottakkal | 230m | 10.8343850, 75.9310242 |
| S5 | | Kottakkal | 254m | 10.7842771, 75.9859718 |
| S6 | *Ophiorrhiza hirsutula* | Thiruvananthapuram | 304m | 8.4817700, 76.9301022 |
| S7 | | Thiruvananthapuram | 324m | 8.5193667, 76.9246075 |
| S8 | | Thiruvananthapuram | 536m | 8.4922030, 76.9850498 |
| S9 | | Thiruvananthapuram | 552m | 8.4894865, 77.0207658 |
| S10 | | Thiruvananthapuram | 565m | 8.4731873, 77.0180184 |
| S11 | *Ophiorrhiza rugosa* var *prostrata* | Thiruvananthapuram | 458m | 8.4759039, 77.0482396 |
| S12 | | Thiruvananthapuram | 402m | 8.5166505, 77.0015341 |
| S13 | | Thiruvananthapuram | 468m | 8.4731876, 76.9218601 |
| S14 | | Thiruvananthapuram | 478m | 8.4731873, 76.9850498 |
| S15 | | Thiruvananthapuram | 488m | 8.4759039, 76.9493339 |

### 4.2.2 Morphological features

Chapter 3 Table 3.1; Plate 3.1.

## 4.3 Assessment of genetic diversity among *Ophiorrhiza* species by RAPD analysis:

Leafy shoots of 15 different accessions were gathered in the zippered polybags containing ice bags from various places (Fig. 3.1) in the Indian Western Himalayas (Kerala). These included 5 accessions of *O. mungos*, 5 accessions of *O. hirsutula*, and 5 accessions of *O. rugosa* var *prostrata*.

### 4.3.1 DNA isolation

The CTAB method (Doyle and Doyle, 1990) was used to isolate genomic DNA, which was then amplified through polymerase chain reaction (PCR) with RAPD primers (Sigma-Aldrich, USA). The procedure is discussed below:

I. 500mg of fresh leaves along with liquid nitrogen was crushed into powder using chilled mortar and pestle.

II. 1ml of preheated (60°C) CTAB extraction buffer added to the crushed sample and the slurry was transferred to a clean micro-centrifuge tube (MCT). The MCTs were incubated

in water bath for 1 hour at 65 ℃ and mixed by inversion after every 10 minutes.

III. The MCTs were centrifuged at 6000rpm for 10mins.

IV. Equal volume of Chloroform: Isoamyl alcohol (C: I, 24:1 v/v) was added and the MCTs were inverted for 5mins for proper mixing in rotatory spin.

V. The MCTs were centrifuged at 12,000rpm for 10mins.

VI. The supernatant was transferred in fresh tubes and steps IV, and V were repeated.

VII. Clear supernatant transferred to fresh tubes.

VIII. Ice cold isopropanol was added to the supernatant and mixing in rotatory spin for 10mins.

IX. MCTs were incubated for 1hr at -80°C to precipitate the DNA.

X. Centrifuged at 12,000rpm for 10mins at 10°C for pelleting the DNA.

XI. Supernatant was decanted and 500µl ethanol (70%) was added.

XII. The MCTs were inverted for 5mins in rotatory spin.

XIII. 70% ethanol drained carefully in order to retain the pellet in MCT and pellet was dried in laminar air flow.

XIV. The pellet was dissolved in 50 µl TE buffer.

**Table 4.2** CTAB Extraction buffer 100ml.

| **Chemicals** | **Required concentration** | **Working concentration (100 ml)** |
|---|---|---|
| Tris base (pH 8.0) | 1 M | 10.0 ml |
| EDTA | 0.5 M | 4.0 ml |
| NaCl | 5 M | 28.0 ml |
| CTAB | 10 % | 2.0 g |
| $H_2O$ | Distilled water | 40.0 ml |
| PVP 40 | Mw 40,000 | 1 g |

*All chemicals added, adjusted to pH 5.0 with HCl and total volume make up to 100ml with $H_2O$.

### 4.3.2 Purity and quantification of isolated DNA

The purity or quality of isolated genomic DNA was tested in 0.8g/100ml agarose gel by electrophoresis and Gel Doc (BioRad) revealed the presence of DNA bands. The quantity of DNA was determined by using UV- spectrophotometer.

### 4.3.3 PCR amplification by RAPD primers

Initially ten RAPD primers were screened (Sigma-Aldrich) for RAPD analysis, of which only six showed consistent and unique amplification using PCR (Table 4.3). The PCR reaction mixture contained 5.0 µL of DNA template (50 ng/l), 3.75 µL 5x Reaction buffer (Promega, USA), 0.85 µL of 10 µM primer, 0.09 µL of 2.5 U/l GO G2 Taq DNA Polymerase (Promega, USA), and 6.75 µL of sterile water (in a total volume of 20 µL). Biorad Thermo cycler was used for the amplification. Initial denaturation stage at 94 °C for 2 minutes, followed by 35 cycles of denaturation for 1 minute at 94 °C, annealing at 37 °C for 1 minute, extension at 72 °C for 2 minutes, and final extension at 72 °C for 5 minutes. Electrophoresis of the amplified products was performed on a 1.4% agarose gel in 1 TAE buffer (pH 8.0), with 0.01 µL/ml of EtBr for staining. Gel Documentation System was utilized to visualize and imaging of the gels (Bio-rad, USA). Size of amplicons was determined by comparing them to a 100 bp DNA ladder (GeneRuler Thermo Scientific, USA). The intensity of bands was ignored in favour of their ability to be reproduced and categorized as homologous only if they had the same migration.

### 4.3.4 Data scoring and statistical analysis

First, we used a molecular ladder (1000 bp) in a gel documentary system to determine the molecular size of all the amplified bands for each RAPD marker. Next, we assembled a binary data matrix for all plant accessions in which a score of 1 indicated the presence of a band and a score of 0 indicated its absence (Matching of bands were produced with Molecular weight analysis by using Image LabTM –version 6.0.0 program). In order to create a cluster analysis, we used the NTSYSpc (2.2 version) software package and relied on the Jaccard's coefficient of similarity, UPGMA (unweighted pair group method with arithmetic average), and the neighbor-joining tree. The Minitab programme was used to compute a number of measures of dissimilarity between the units of interest, including distance/metric, Euclidean distance, Furnas portraiture, and factorial analysis.

## 4.4 Assessment of phytochemical diversity among *O.mungos* population by HPTLC analysis:

### 4.4.1 Chemicals and reference compounds

Chapter 3(3.2.1)

### 4.4.2 Plant material

Collection of 30 accessions (KE1-KE30) of *O. mungos* for phytochemical diversity assessment was done from six different locations in Kerala (Table 4.4).

### 4.4.3 Sample preparation

The plant samples were washed in running tap water and oven dried, 50°C, 16hr. The oven dried sample was finely crushed using a mortar and pestle. Each sample (100 mg) was subjected to UAE, 40Khz, 180W for15min, based on the most effective extraction method selected for the extraction of camptothecin from *Ophiorrhiza mungos* (Table 3.4).

#### 4.4.4.1 Preparation of standard stock solution

Chapter 3(3.2.5.1).

#### 4.4.4.2 Preparation of standard curve

Chapter 3(3.2.5.2).

### 4.4.5 HPTLC instrumentation and conditions

Chapter 3(3.2.5.3).

#### 4.4.5.1 Quantification of camptothecin from different accessions of *O.mungos*

Chapter 3(3.2.5.4).

## 4.5 Results and Discussions

### 4.5.1 Genetic diversity analysis

Even though morphological analysis can be used as a starting point for identifying medicinal plants, it is important to remember that many different plants can have very similar appearances; this can lead to confusion and even adulteration of medicinal plant material among pharmaceutical corporations. As a result, therapeutic drug efficacy declines and potentially hazardous compounds are generated that can lead to a wide range of health problems. There is a great deal of genetic variation across samples of plants grown in various environments (Kaur et al., 2019). The expression of various genes in medicinal plants, results in a wide range of chemotypes, which is influenced by both environmental and genetic variables. Therefore, screening the key genotypes and conserving their gene pool in industrially important plants necessitates molecular characterization and evaluation of genetic diversity at intraspecific and interspecific level (Qian et al., 2001). The first stage of any plant breeding effort is the collection, characterization, and evaluation of germplasm (Kaur et al., 2019). Molecular marker technology is a reliable method for identifying plant genetic material and studying variations among crops. The molecular markers or DNA-based identifiers we use are extremely reliable and specific to the target plant. These markers have the potential to serve as a reliable method of identifying germplasm, measuring genetic variability, and keeping standards high. The validity and genetic variety of medicinal plants may now be verified using a range of classical molecular markers established over the past

few decades. These markers include RAPD, RFLP, AFLP, SSR, ISSR, and SNPs. In the recent past, ISSR and RAPD markers have proven useful for assessing genetic diversity in a wide range of economically significant plants (Baruah et al., 2017; Kaur et al., 2019; Kumar & Roy, 2018).

Molecular data serves as a valuable tool for the construction of phylogenetic trees, enabling researchers to elucidate the evolutionary lineage and interrelationships among species. This analytical approach proves especially advantageous when morphological characteristics provide limited or potentially misleading information. Furthermore, molecular analysis permits the identification of cryptic species within the plant kingdom, instances where species exhibit close morphological resemblance but possess distinct genetic profiles.

*Ophiorrhiza* as a genus is highly polymorphic nevertheless, this is often misunderstood due to the complexities of classifying the various species within the genus. The current investigation evaluates the genetic diversity of three Western Ghats (Kerala) *Ophiorrhiza* species, including *O. mungos*, *O. hirsutula*, and *O. rugosa* var *prostrata*, gathered from a various locations(Table 4.1). Genetic variability in the wild genotypes of *Ophiorrhiza spp*., was explored by one single primer-based amplification reactions (SPAR) technique, viz. markers. A total of 6 SPAR primers were used to check the intra-specific and inter-specific variability among 15 accessions of *Ophiorrhiza spp*.

### 4.5.2 RAPD analysis

The PCR-RAPD results were used to gain information about polymorphisms both within and between species. For this study, we used 10 RAPD primers to evaluate genetic diversity among *Ophiorrhiza* species/populations collected from several sites in the Western Ghats (Kerala). Six of these primers produced bands on the agarose gel that were clearly visible, easily reproducible, and brilliant (Table 4.3). Amplified bands were found in all of the markers that were examined, ranged from seven to eighteen with molecular size 750 bp (OPD-20) to 2000 bp (OPA-11). Six selected RAPD markers used detected 69 polymorphic bands out of a total of 77 amplified bands. The average level of polymorphism was 89.5%, with the percentage ranging from 83.0% (OPA-11) to 94% (OPD-18).

**Table 4.3** Estimated RAPD polymorphism percentage.

| Sl.no | Primer code | Primer sequence(5′-3′) | GC content % | Total bands | PB | MB | %polymorphism |
|---|---|---|---|---|---|---|---|
| 1. | OPD 20 | ACCCGGTCAC | 70 | 11 | 10 | 1 | 91 |
| 2. | OPD 18 | GAGAGCCAAC | 60 | 17 | 16 | 1 | 94 |
| 3. | OPA 11 | CAATCGCCGT | 60 | 18 | 15 | 3 | 83 |
| 4. | OPA 18 | AGGTGACCGT | 60 | 14 | 13 | 1 | 93 |
| 5. | OPA 20 | GTTGCGATCC | 60 | 7 | 6 | 1 | 86 |
| 6. | OPC 02 | GTGAGGCGTC | 70 | 10 | 9 | 1 | 90 |
| | | Total bands | | 77 | 69 | 8 | 89.5 |

* PB= Polymorphic bands, MB= Monomorphic bands

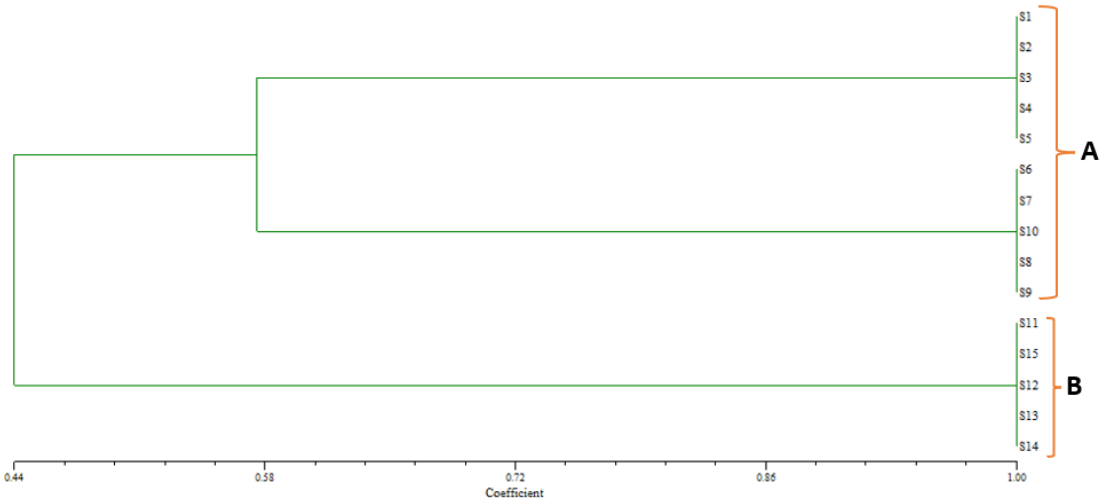



**Fig 4.1:** UPGMA dendogram constructed using RAPD data. The abbreviation on the right are *Ophiorrhiza spp*. accessions {*O. mungos*(S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa var prostrata* (S11-S15)}.

**Table 4.4** CPT quantification in *Ophiorrhiza mungos* from Kerala, India.

| Accession no. | Collection site | CPT±SE(µg/g,dr.wt.)* |
|---|---|---|
| KE1 | Idukki | 276.00±3.49$^{j}$ |
| KE2 | Idukki | 319.80±10.74$^{gh}$ |
| KE3 | Idukki | 300.20±1.16$^{hi}$ |
| KE4 | Idukki | 278.00±4.01$^{j}$ |
| KE5 | Idukki | 345.00±2.64$^{f}$ |
| KE6 | Thiruvananthapuram | 317.20±2.08$^{gh}$ |
| KE7 | Thiruvananthapuram | 393.80±5.15$^{e}$ |
| KE8 | Thiruvananthapuram | 383.20±5.83$^{e}$ |
| KE9 | Thiruvananthapuram | 291.40±4.71$^{ij}$ |
| KE10 | Thiruvananthapuram | 343.00±6.16$^{f}$ |
| KE11 | Kollam | 218.20±2.43$^{l}$ |
| KE12 | Kollam | 273.60±4.53$^{j}$ |
| KE13 | Kollam | 336.00±3.27$^{fg}$ |
| KE14 | Kollam | 307.00±1.92$^{hi}$ |
| KE15 | Kollam | 418.60±3.82$^{d}$ |
| KE16 | Kottakal | 511.20±4.65$^{a}$ |
| KE17 | Kottakal | 480.80±8.36$^{b}$c |
| KE18 | Kottakal | 491.40±6.70$^{ab}$ |
| KE19 | Kottakal | 497.80±5.33$^{ab}$ |
| KE20 | Kottakal | 464.40±12.81$^{c}$ |
| KE21 | Pathanamthitta | 429.00±8.16$^{d}$ |
| KE22 | Pathanamthitta | 383.40±6.86$^{e}$ |
| KE23 | Pathanamthitta | 171.20±7.82$^{mn}$ |
| KE24 | Pathanamthitta | 166.80±0.58$^{n}$ |
| KE25 | Pathanamthitta | 215.00±14.51$^{l}$ |
| KE26 | Ernakulam | 124.80±4.56$^{o}$ |
| KE27 | Ernakulam | 151.20±9.22$^{n}$ |
| KE28 | Ernakulam | 188.00±3.29$^{m}$ |
| KE29 | Ernakulam | 243.00±8.86$^{k}$ |
| KE30 | Ernakulam | 246.00±12.94$^{k}$ |

* Each CPT±SE data is an average of five values

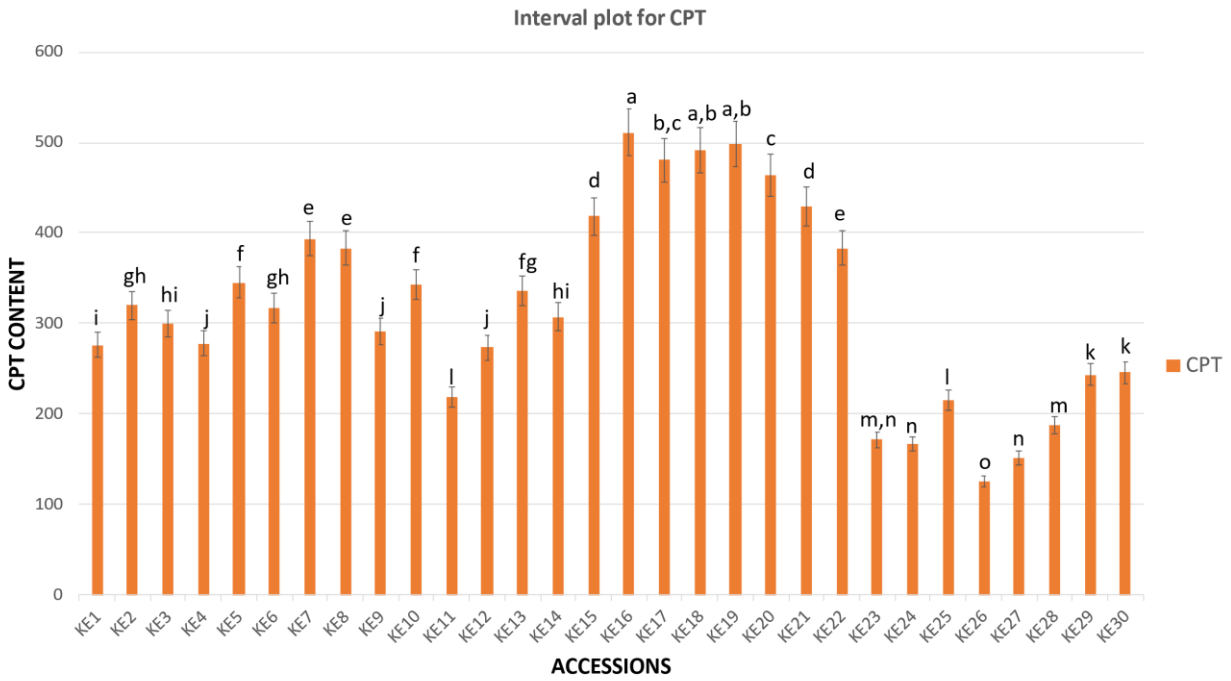



**Fig.4.2:** Graphical presentation of CPT (%) in different accessions of *O. mungos*

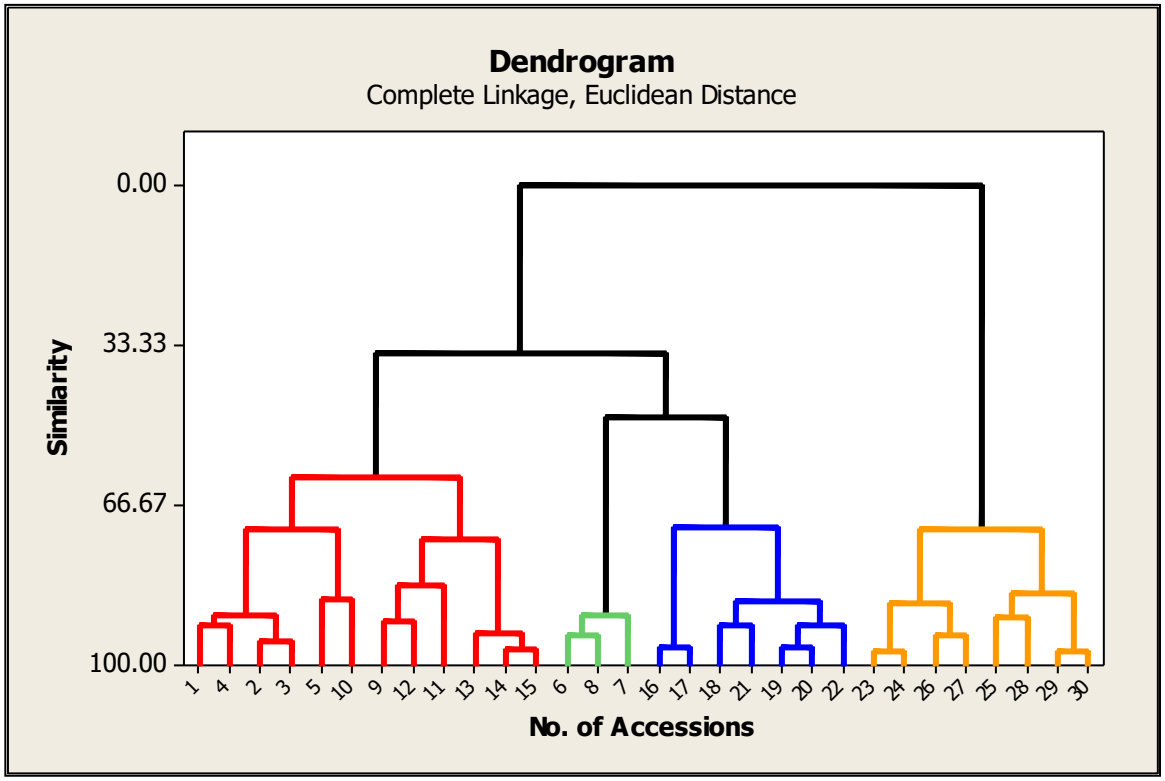



**Fig.4.3:** Dendrogram based on the CPT content of collected *O. mungos* accessions (KE1-KE30) from different geographical locations in Kerala with three major clusters having two subclusters in each major cluster.

### 4.5.3 Cluster analysis:

The three *Ophiorrhiza* species may be easily distinguished from one another owing to the use of 6 SPAR primers (RAPD) to collect binary data, the results were then tabulated and evaluated to form a UPGMA (unweighted pair group method with arithmetic average) tree (Fig. 4.1). Nei's genetic diversity was used as a proxy for the overall molecular diversity among all the accessions, both within and between species. Remarkably, the dendrograms produced by both approaches revealed two clearly differentiated clusters labelled A and B. Using RAPD markers, it would be possible to distinguish between *O. mungos, O. hirsutula,* and *O. rugosa* var *prostrata,* all of which belong to the genus *Ophiorrhiza*. Cluster A and Cluster B, determined from RAPD markers, easily differentiate the two types viz. *O. mungos* (S1-S5) and *O. hirsutula* (S6-S10) accessions from *O. rugosa* var *prostrata*, whereas Cluster A further sub-divided into two sub-clusters, includes five *O. mungos* accessions (S1-S5), and *O. hirsutula* (S6-S10) accessions (Fig. 4.1). The lowest levels of genetic diversity were seen between the *O. mungos* and *O. hirsutula* species. *Ophiorrhiza spp*. accessions lacked in intra-specific genetic diversity. The population of *O. rugosa* var *prostrata* found in Thiruvananthapuram (Kerala) regions had the highest level of genetic diversity (0.44 similarity co efficient), followed by the populations of *O. mungos* and *O. hirsutula* (0.58 similarity co efficient). Our research may be useful in assessing the present distribution of various *Ophiorrhiza* species. Our research shows that the two closely related species *O. mungos* and *O. hirsutula* have a very low level of genetic diversity and gene flow, which poses a serious threat to the survival of these valuable species. Although *O. mungos* is the most elite species in its genus, it is presently considered to be in a near threatened species state. Because of this, *O. mungos* species require urgent conservation efforts. The evolution of genetic characteristics for adaption to changing environmental situations leads to a high amount of intra-specific genetic variation. The genetic similarity of the accessions from the closely adjacent regions was indicated by their clustering. Diverse population differentiation is also caused via gene flow and genetic drift. RAPD markers are well-known for their coverage of the complete genome at a low cost. The loss of genetic diversity in several medicinally important plant genera, including the *Ophiorrhiza* genus, poses a severe threat to future sustainable development and genetic improvement efforts. Molecular markers have a long shelf life and can only be used on the targeted plant. There have been no previous reports on the diversity evaluation of *Ophiorrhiza* spp. This research reveals molecular variation across germplasms of *O. rugosa* var *prostrata* grown in a range of environments. According to a review of the relevant literature, the camptothecin content of *Ophiorrhiza* specimens from various localities exhibits a wide range of variance (Rajan et al., 2013). By using DNA-based

molecular markers, unique herbal medicines can be identified and authenticated for their safety, efficacy, and validity. The current research might be useful in identifying and developing novel DNA alleles that may reveal the important genetic features in *Ophiorrhiza spp*. The ability of a species to maintain its genetic makeup in the face of environmental change makes plant genomic diversity (PGD) crucial. PGD allows for the formation of novel alleles, sub-species, or taxa in a population by providing a setting in which natural selection can promote reproductive isolation (Qian et al., 2001). Observing how a species genetic makeup is shifting throughout time might provide a look into the species' dynamic process. To evaluate the likelihood of extinction of threatened or rare medicinal plant and to design appropriate conservation strategies, an understanding of natural genetic diversity and population structure is crucial.

### 4.5.4 Phytochemical analysis

Trends in medicine today favor herbal remedies over synthetic ones, and there is solid proof that ancient civilizations used herbal remedies. Newman & Cragg (2012) report that in the last 30 years, over half of the licensed medications for treating cancer have been derived from plants. More pharmacophores need to be documented to increase the therapeutic and preventative options available from herbal medicine. Pharmaceutical companies use the chemicals found in medicinal plants to manufacture biomedicines due to the strong therapeutic potential of these compounds. Secondary metabolites in medicinal herbs have been shown to be affected by morphological, genetic, and environmental characteristics, all of which have implications for medicine quality. Despite their obvious advantages, the health benefits of herbal pharmaceuticals are sometimes overshadowed by issues of quality that can be resolved by the application of reliable methods of standardizing herbal materials. Medicinal plant chemo-profiling has been accomplished through the use of a number of different analytical methods. In order to extract natural compounds from even the most complicated matrices, High-Performance Liquid Chromatography (HPLC) is an extremely flexible chromatographic technique. HPLC is widely used for analyzing diverse natural materials since it is both fast and accurate (Ramachandran et al., 2021). High performance thin layer chromatography (HPTLC) has recently emerged as a highly effective method for the study of crucial bioactive chemicals. When compared to HPLC, HPTLC is superior for the simultaneous estimate of a large number of samples because to its rapid processing time, high reproducibility, high accuracy, and low cost.

There is variation with the content of camptothecin with the geographical location in *O.*

*mungos* analyzed by HPTLC (Rajan et al., 2013). There evidence suggests that climatic conditions influence the content of camptothecin. There is high content of camptothecin found in Kottakkal, Kerala.

Hennicke et al. (2016) found that other plants' environments significantly affect the development of secondary metabolites. When it comes to *Swertia*, fluctuations in altitude cause subtle but noticeable shifts in the plant's genetic and phytochemical make-up (Kaur et al., 2019). Similar, *Stevia rebaudiana* phytochemical changes were reported to be altitude and climate dependent (Nawaz et al., 2022). Many studies have shown that different regions with similar climates produce similar amounts of secondary metabolites in their plants. Numerous investigations of various medicinal herbs support the connection. The morphological, molecular, and phytochemical diversity in 50 *H. coronarium* accessions from different states in Eastern India was studied. The association between diversity pattern and geographical origin was clearly demonstrated using cluster analysis based on molecular and phytochemical characterization. The results showed that there are substantial geographical differences in the phytochemical and molecular composition of *H. coronarium* accessions (Ray et al., 2019). These analyses demonstrated unequivocally that environmental factors, such as latitude and humidity, have a significant impact on plant secondary metabolites. The climate and landscape of India varies widely from region to region. This means that secondary metabolites might exhibit a wide range of variability. In the current research, we compared the camptothecin levels in *O. mungos* from different geographic regions and associated them with local climate. KE16 accessions in Kottakkal, Kerala, with its highlands(254m) and wet marine environment of the Western Ghats of India, had the highest concentration (511.20 µg/g, dr.wt.). Camptothecin is produced by *O. mungos* because the climate is ideal for its growth. Pathanamthitta and other low-lying areas exhibited a moderate concentration of camptothecin (50-60m altitude). The climate in these areas is different from that of the Highlands, and this has a great impact on the formation of secondary metabolites. Ernakulam (KE26) had the least amount of content (124.80 µg/g, dr.wt.). Camptothecin is used widely in the pharmaceutical business as an anticancer ingredient, making it crucial to identify high-yielding accessions of the plant. There is a correlation between the genetic and phytochemical diversity. However, just a little amount of research has been done so far to investigate these links. We can learn more about the exceptional species of *O. mungos* and how its genetics and phytochemistry interact in the future by conducting further research.

## 4.6 Conclusion

Quantitative modulation of the biochemical marker viz. camptothecin was shown in 30 *Ophiorrhiza mungos* samples collected from 6 districts in Kerala, India, using the current quick, verified, and repeatable HPTLC method. Assessment of phytochemical diversity due to difference in climatic conditions and geographical location plays a very major effect on variance in camptothecin concentration, making it useful in selecting and screening the elite species of *O. mungos*. Phytochemical changes in *O. mungos* were detected in the present study, and these variations were found to be connected to geographic location. In this work, we found substantial intra-specific variation in camptothecin content across *O. mungos* samples and screened the elite species for commercial propagation and camptothecin production. Some of the most elite *O. mungos* have been spotted in the highlands and in the wet maritime environment of Kottakal, Kerala, India. In order to preserve and cultivate them, these can be investigated. The study found that the chemical makeup of plant accessions varies with their geographical origins. Additionally, the most elite *O. mungos* accessions can be preserved, widely disseminated, and put to good use by the industrial sector.

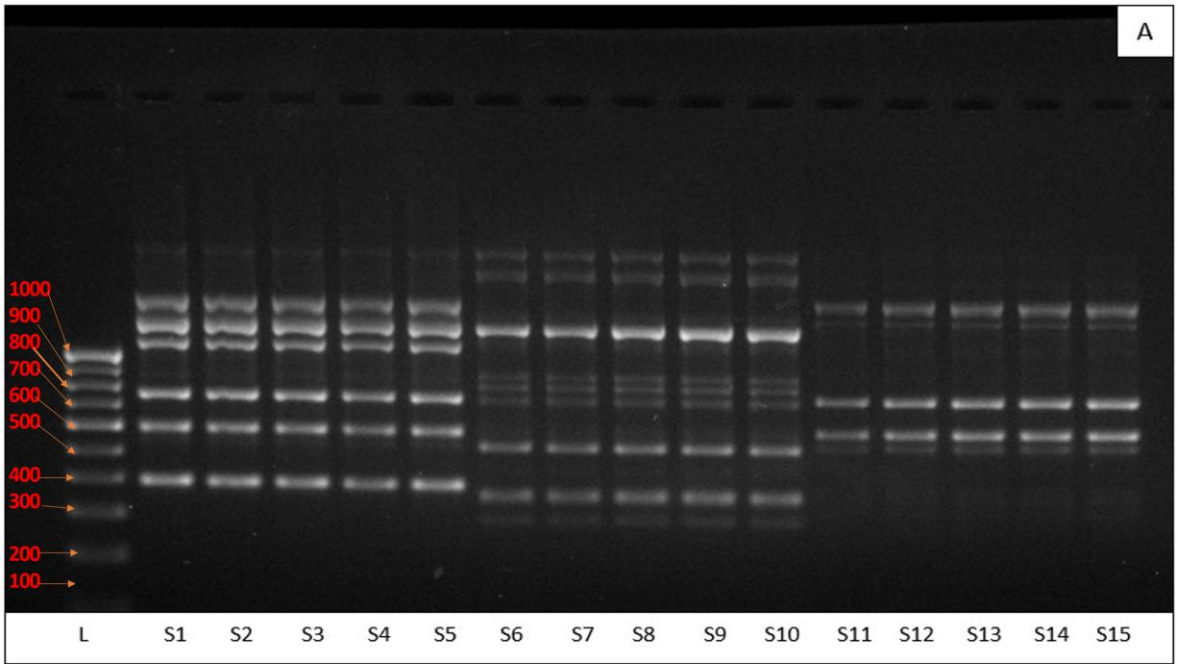
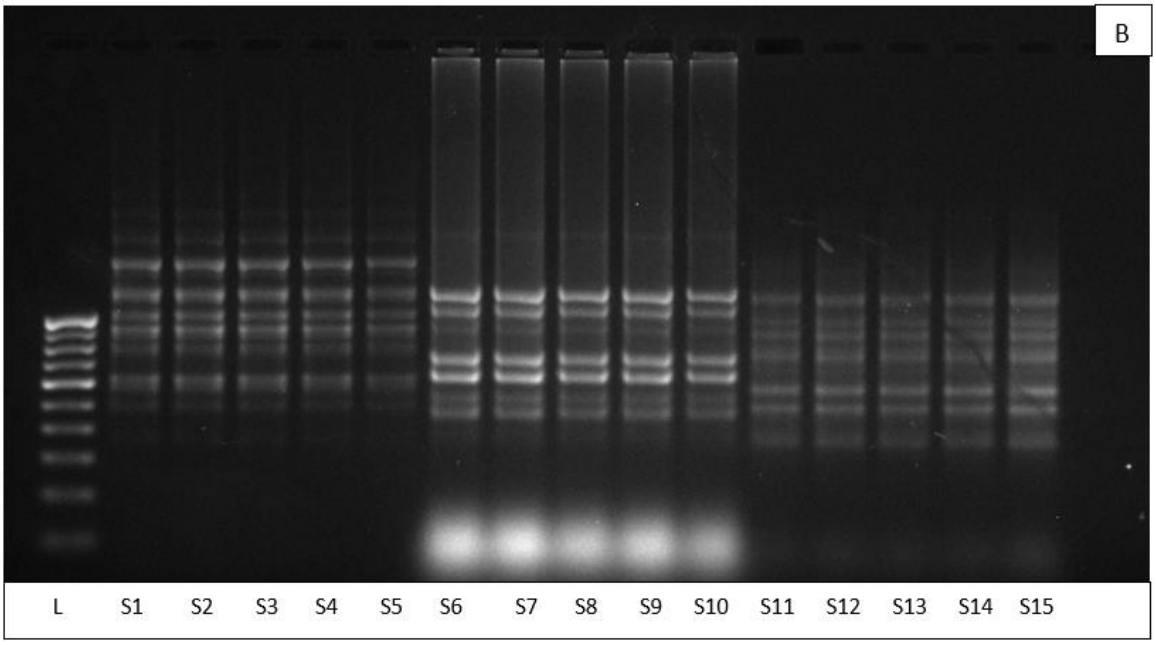
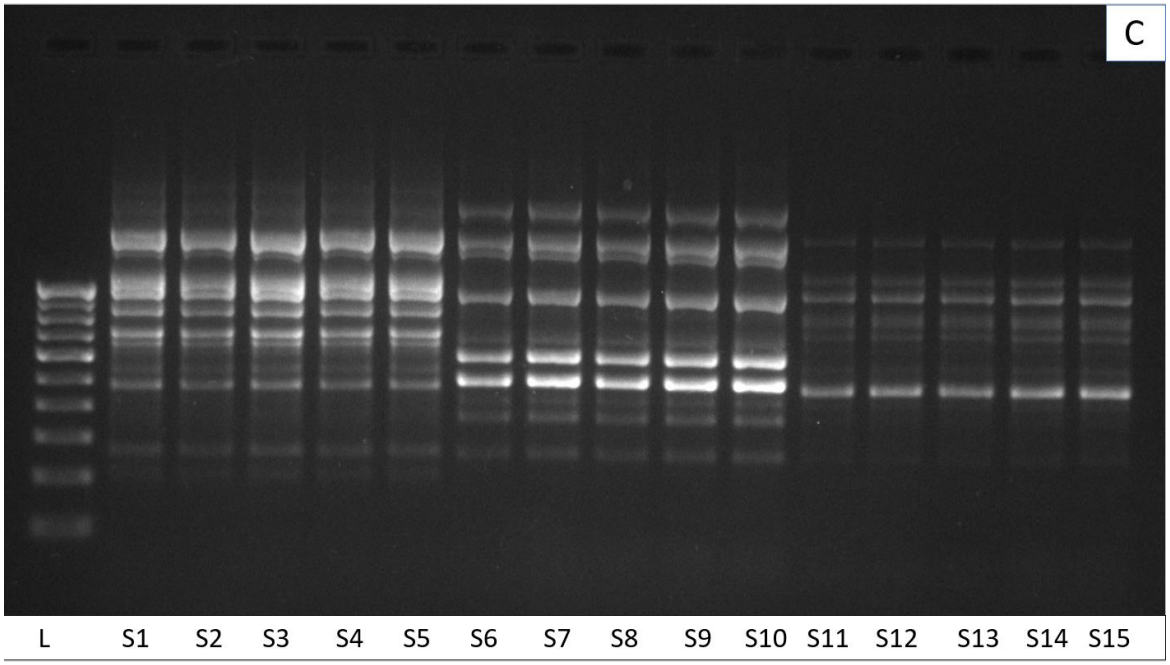


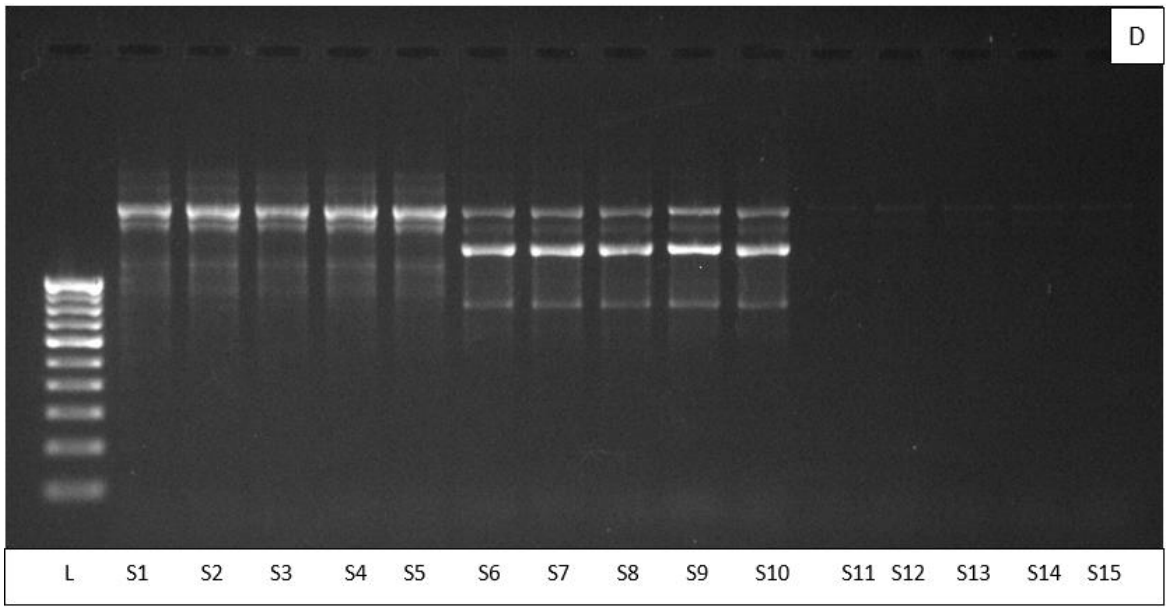

**Plate 4.1** (A) Bands produced with RAPD marker- OPA-18 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S11-S15).



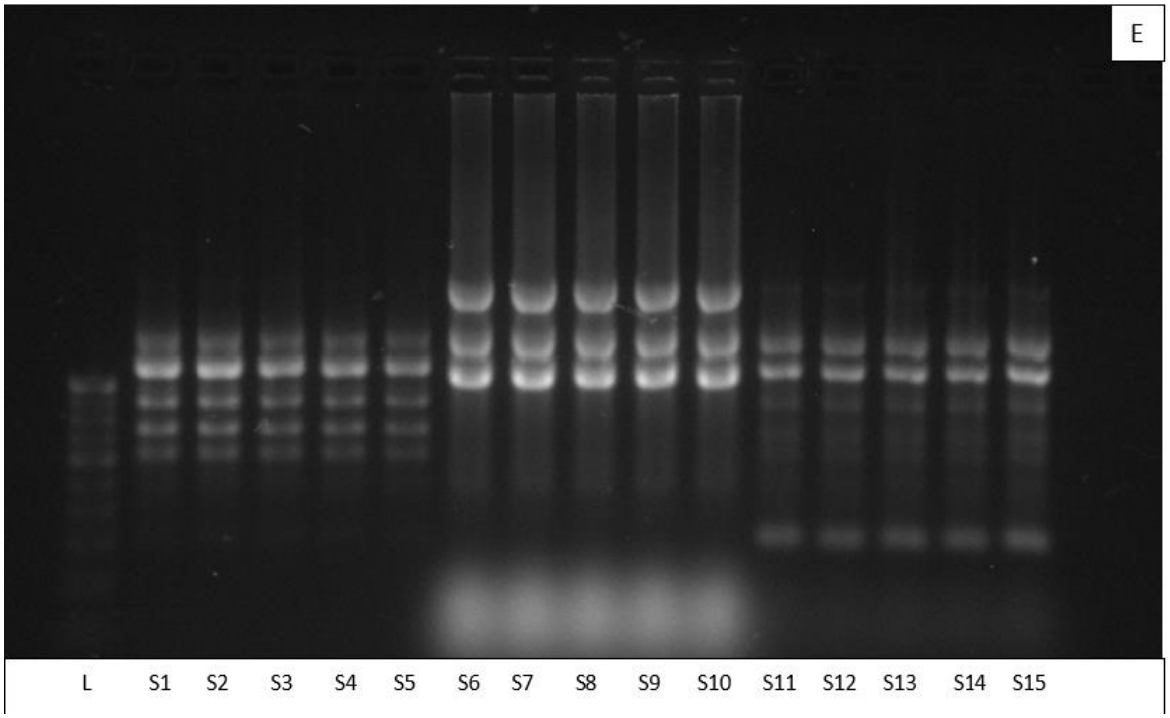

(B) Bands produced with RAPD marker- OPD-18 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S11-S15).



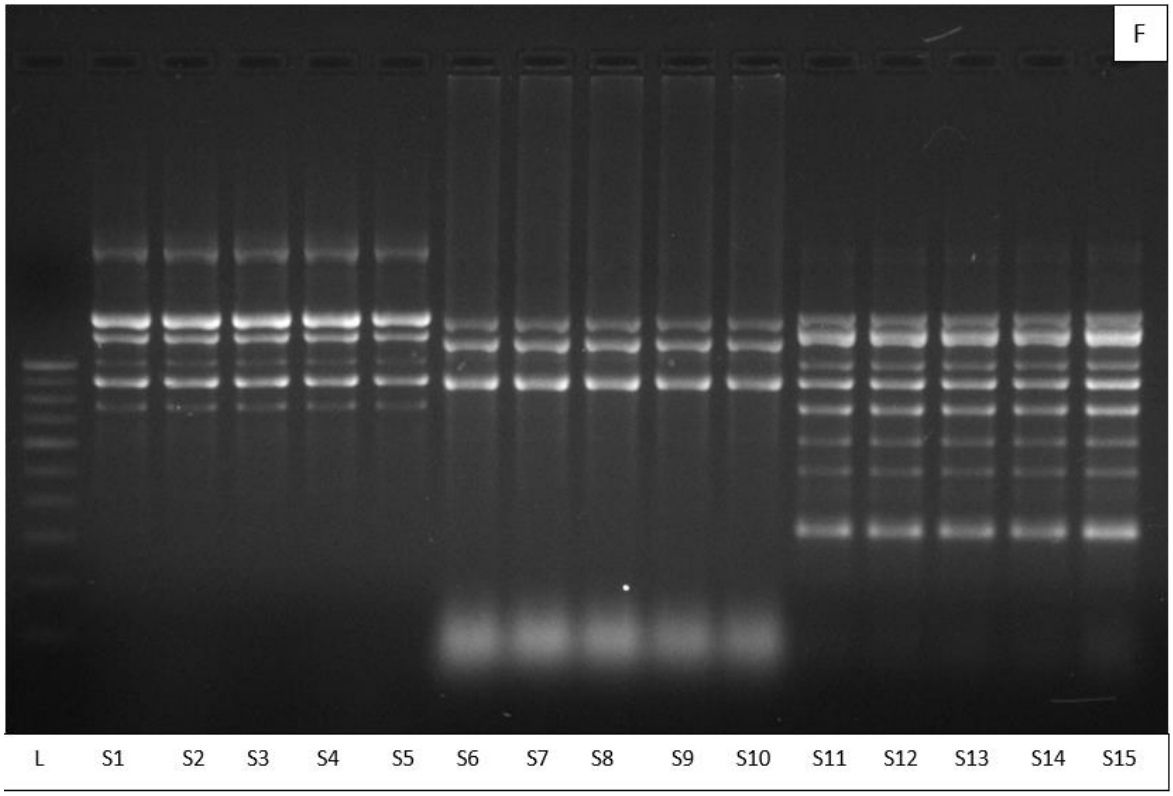

(C) Bands produced with RAPD marker- OPA-11 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S10-S15).




(D) Bands produced with RAPD marker- OPA-20 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S11-S15).




(E) Bands produced with RAPD marker- OPC-02 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S11-S15).




(F) Bands produced with RAPD marker- OPD-20 in *Ophiorrhiza* accessions; *O. mungos* (S1-S5), *O. hirsutula* (S6-S10) and *O. rugosa* var *prostrata* (S11-S15).

# CHAPTER 5

# *Application of elicitors and precursors for the enhancement of bioactive compound camptothecin in the plants*

## 5.1 Introduction

Over one-fourth of pharmaceutical drugs are derived from plants, which are the source of a large variety of bioactive chemicals with significant uses in biopesticides, food, cosmetics, and pharmaceutical industries (Neumann et al., 2020). Because of the massive quantities of these drugs generally utilized, their population are at risk of extinction, and genetic diversity has deteriorated (Pan et al., 2014). Moreover, the wild plants are more impacted by ecological and climatic changes that alter their metabolic profiles. Throughout the last several centuries, there has been a reinvigorated interest in using medicinal plants and their pharmaceuticals as an alternative to synthetically prepared medicines for the treatment of diseases, which has led to the yearly growth of the pharmaceutical industry (Dey et al., 2021; Igbe et al., 2008).

As a promising method for the generation of secondary metabolites, plant cell or organ cultures provide advantages over chemical synthesis or extracting bioactive substances from plants (Rao & Ravishankar, 2002). Nevertheless, the commercial use of plant cell cultures has had mixed results due to poor product yield, unstable biosynthesis, and scaling-up issues (Bourgaud et al., 2001). The general characteristic among them is poor bioactive compound production (Wiedenfeld et al., 1997). In recent years, several techniques, including elicitation, precursor feeding, medium optimization, and so on, have been used to address this problem (Namdeo, 2007). Elicitation is the method that promotes the creation of bioactive substances most effectively among these techniques (Deepthi & Satheeskumar, 2017a; Krishnan et al., 2018b; Largia et al., 2015; Namdeo, 2007). Elicitors, which may be either biotic or abiotic depending on where they came from, are chemicals that act as external stimuli and can stimulate a plant's stress- or defense-induced responses. Exogenous elicitors may be used in in vitro cultures to enhance the manufacture of desired secondary metabolites and to research how plants react to pathogen infections. According to Largia et al. (2015) low concentration

of elicitors in the amount of bioactive substances increases due to the medium. Chemically induced cultures have been reported to produce bioactive compounds in greater quantities (Radman et al., 2003).

Salicylic acid (SA) and methyl jasmonate (MeJa), two of these chemical elicitors, are well recognised for increasing the synthesis of a variety of bioactive chemicals in diverse cultures (Largia et al., 2015; Srivastava & Srivastava, 2014). MeJa, a volatile methyl ester of jasmonic acid, has been used as a signalling molecule under both biotic and abiotic conditions (Creelman & Mullet, 1995). According to Yu et al. (2002), MeJa participates in the process of signal transduction that creates certain enzymes to produce those particles, which aids in the synthesis of defensive chemicals. Another stress signalling molecule, SA, is often employed because of its function in promoting disease resistance in plants (Rao et al., 2000). SA, according to Kang et al. (2004), emerged as a crucial signalling molecule in recent decades that is in charge of activating certain defensive responses in plants. Both elicitors have been shown to increase the amount of bioactive compounds in a variety of plant methods, including cultures of *Digitalis purpurea* shoots (Patil et al., 2013), *Centella asiatica* whole plants (Kim et al., 2004), *Ginkgo biloba* cells (Kang et al., 2006), and *Gentiana dinarica* hairy roots (Krstić-Milońević et al., 2017). In hairy root cultures of *Centella asiatica* the amount of triterpenoid saponins was increased by the elicitation of MeJa and SA (Kim et al., 2007) and entire plant cultures of *Glycyrrhiza glabra* (Shabani et al., 2009).

Precursor feeding is thought to be an additional effective method to increase the production of bioactive compounds in a variety of cultures, including shoot cultures of *Digitalis purpurea* (Patil et al., 2013), hairy root cultures of *Psoralea corylifolia* (Shinde et al., 2009), and whole plant cultures of *Rauwolfia serpentine* (Panwar & Guru, 2015). Digitoxin and digoxin concentrations in *Digitalis purpurea* shoot cultures rose 9.1 and 11.9 fold with precursor feeding, respectively.

Using elicitors and precursors is essential in order to fulfill the need for camptothecin. As a result, elicitation and precursor feeding have been performed in this work on a variety of parameters, including exposure length, elicitor kind, and elicitor and precursor concentration. Using four distinct elicitors, Ja, MeJa, SA and CS separately, and two different precursors, tryptophan, and geraniol, we are now aiming to establish an efficient elicitation and precursor feeding method for higher biomass production and accumulation of camptothecin. Elicitation parameters such as elicitor concentrations, type of elicitor as well as the optimal concentration of precursor and type were investigated by RSM, PBD screened important factors, and BBD optimized key variables to establish target values. As far as we

are aware, this is the first investigation into the effects of precursor feeding and elicitation on camptothecin synthesis in *O. mungos* in vitro cells using RSM experimental design.

## 5.2 Materials and methods

### 5.2.1 Chemicals and reagents

Chemicals ordered from Sigma-Aldrich, USA: methyl jasmonate, jasmonic acid, salicylic acid, chitosan, tryptophan, geraniol, 6-benzylaminopurine (BAP), and indole-3-butyric acid (IBA). Compounds of reference and appropriate solvents have been detailed in detail in Chapter 3. (Section 3.2.1).

### 5.2.2 Modeling and optimization studies

For elicitors, BBD was applied for the analysis of different types and concentrations of elicitors. And for precursor research was conducted in two stages. First, the significant independent parameters are screened using a Plackett-Burman design (PBD), and then, the optimal level and possible collaborations among the relevant parameters are analyzed using a Box-Behnken design (BBD). Minitab was used to create the experimental layout.

#### 5.2.2.1 Plackett–Burman design

PBD is an efficient method for selecting key elements, and it involves a large number of variables but only a few runs (Asfaram et al., 2016). Six precursors of varying concentrations were tested and their effects were estimated using PBD. There are k+1 possible trials in PBD, where k is the number of independent variables. Each precursor has a high and low state, denoted by (+) and (1), respectively. Table 5.1 lists the encodings for each precursor and its concentration, and Table 5.4 details the experimental design that involved 12 trials screening 6precursors. All experiments were performed in triplicate, and the significant features were tested using 5% ($p < 0.05$) regression analysis.

This model depends on first-order model:

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

In this notation, Xi is the coded independent variable, Y is the predicted target function, β0 is the scaling constant, and βi is the regression coefficient.

**Table 5.1** Different precursors with coded levels employed in PBD for screening of best precursor and its optimal concentration influencing camptothecin extraction.

| Variable code | Variables | High level (+) | Low level (-) |
|---|---|---|---|
| $X_1$ | L- tryptophan | 2 | 1 |
| $X_2$ | L-leucine | 2 | 1 |
| $X_3$ | Tryptamine | 2 | 1 |
| $X_4$ | Loganin | 2 | 1 |
| $X_5$ | Secologanin | 2 | 1 |
| $X_6$ | Geraniol | 2 | 1 |

#### 5.2.2.2 Box-Behnken design (BBD)

Once the factors that had the greatest effect on camptothecin extraction had been screened by using PBD, they could be optimized using BBD. Table 5.6 displays the design of 13 separate tests in which each design variable was administered at one of three levels: -1, 0 and +1. All variables were coded using the following equation (Maran & Manikandan, 2012):

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

Where xi stands for the coded value's independent variable, Xi for the actual value's independent variable, $X_0$ for the actual value at the midpoint, and $\Delta X_i$ for the step change value's independent variable. Each experiment was repeated three times, and the average result was used for statistical analysis. The optimal levels were determined using the following second-order polynomial equation:

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 11X12 + \beta 22X22 + \beta 12X1X2 \qquad (3)$$

Predicted response is denoted by Y, the modal constant by $\beta_0$, significant factors by X1, and X2, linear coefficients by $\beta_{1,\ and}\ \beta_2$ quadratic coefficients by $\beta_{11,}$ and $\beta_{22}$, and interactive coefficients by $\beta_{12}$. The data was analyzed by regression and ANOVA using Minitab statistical software, and a quadratic polynomial model was derived.

Similarly, for optimization of elicitors and their concentrations BBD was employed. Table 5.10 displays the design of 27 separate tests in which each design variable was administered at one of three levels: -1, 0 and +1. All variables were coded using the following equation

(Maran & Manikandan, 2012):

$$xi = Xi - X0/\Delta Xi \qquad i = 1,2,3,\ldots\ldots\ldots,k \qquad (4)$$

Where xi stands for the coded value's independent variable, Xi for the actual value's independent variable, $X_0$ for the actual value at the midpoint, and $\Delta X_i$ for the step change value's independent variable. Each experiment was repeated three times, and the average result was used for statistical analysis. The optimal levels were determined using the following second-order polynomial equation:

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 4X4 + \beta 11X12 + \beta 22X22 + \beta 33X32 + \beta 44X42 + \beta 12X1X2 + \beta 13X1X3 + \beta 14X1X4 + \beta 23X2X3 + \beta 24X2X4 + \beta 34X3X4 \qquad (5)$$

Predicted response is denoted by Y, the modal constant by 0, significant factors by $X_1$, $X_2$, $X_3$, and $X_4$, linear coefficients by $\beta_1, \beta_2, \beta_3$, and $\beta_4$ quadratic coefficients by $\beta_{11}, \beta_{22}, \beta_{33}$, and $\beta_{44}$ and interactive coefficients by $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24}, \beta_{34}$. The data was analyzed by regression and ANOVA using Minitab statistical software, and a quadratic polynomial model was derived.

**Table 5.2** Significant variables(precursors) with coded levels employed in BBD.

| **Variables** | **Coded levels** | | | |
|---|---|---|---|---|
| | **Codes** | **+ 1** | **0** | **-1** |
| L-Tryptophan(mM) | $X_1$ | 2 | 1.5 | 1 |
| Geraniol(mM) | $X_2$ | 2 | 1.5 | 1 |

**Table 5.3** Significant variables(elicitors) with coded levels employed in BBD.

| **Variables** | **Coded levels** | | | |
|---|---|---|---|---|
| | **Codes** | **+ 1** | **0** | **-1** |
| MeJA(µM) | $X_1$ | 200 | 150 | 100 |
| JA(µM) | $X_2$ | 200 | 150 | 100 |
| SA(µM) | $X_3$ | 200 | 150 | 100 |
| CS(µM) | $X_4$ | 200 | 150 | 100 |

### 5.2.3 Plant material

Precursors and elicitors were applied to *O. mungos* in vitro cultures grown in ½ MS media supplemented with BA (13.37µM) and IBA (1.96µM) for direct organogenesis. The in vitro culture process is detailed in detail in Chapter 6.

### 5.2.4 Statistical analysis

Experiments were run in triplicate, and results were reported as the mean SD (SD). One-way analysis of variance (ANOVA) was performed, and then Tukey's test was performed for post-hoc comparisons. Minitab 15 was used for statistical testing, and significance levels of $p < 0.05$ were evaluated.

### 5.2.5 Elicitors and precursors preparation and treatment

In this study, SA, Ja, MeJa, and CS as elicitors, and L- tryptophan and geraniol were used as precursors in the concentration of (100, 150, and 200 µM) and (1, 1.5, and 2 mM) respectively. Stock solutions of 10 mM were prepared separately. 250µL each of Ja & MeJa were dissolved separately in 12ml ethanol: 13ml DW, 34.53mg SA was dissolved in 12ml ethanol: 13ml DW, CS in 1%acetic acid and precursors in 99% ethanol. After preparation, all stock solutions were filter sterilized through a 0.22 µM syringe filter into autoclaved amber bottles (50ml) inside the laminar airflow chamber. The *in vitro* regenerants on $9^{th}$ week of culture so were treated with elicitors on the $9^{th}$ week of culture for 4 days. After elicitor treatment, the *in vitro* plantlets were harvested for the production of biomass and camptothecin. Similarly, precursor treatments were given *to in vitro* plants on the $7^{th}$ day of culture and were harvested on $14^{th}$ day after the precursor treatment. All the treatments were carried out in triplicates.

### 5.2.6 Camptothecin quantification

#### 5.2.6.1 Sample preparation

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

##### 5.2.6.1.1 HPTLC instrumentation

Chapter 3, section 3.2.5.3

### 5.3 Statistical analysis

The results are shown as the mean standard deviation of three independent samples. Minitab 15 English was used for RSM experimental design, analysis of variance

(ANOVA) followed by a Tukey's test ($p< 0.05$).

## 5.4Result and discussion

### 5.4.1 Effect of precursors on camptothecin production

Feeding precursors at optimal concentrations and exposure times can boost bioactive chemical production in plant cell cultures. Nevertheless, feedback inhibition to the metabolite pathway may be induced by an excess concentration of precursors with an insufficient exposure duration. In order to maximize secondary metabolite formation, it is necessary to identify the optimal exposure period and precursor concentration (Liu et al., 2007). Of different precursors tried (Table 5.4) in the current study, L-Typtophan and Geraniol was found to give the best result. In order to maximize camptothecin accumulation in *O.mungos* cultures, we examined the effects of exposing the in vitro plants to L-Typtophan and Geraniol, two precursors involved in the manufacture of camptothecin, at three different concentrations (1mM, 1.5mM, and 2mM) and for 14 days. For this experiment two-month-old in vitro plants were used. Precursor treatment had a major impact on camptothecin production by *O. mungos* in liquid culture (Table 5.6). The highest content of camptothecin (1678.53-1747.63µg/g) was recorded in combination of 1.5mM each of both L-Tryptophan and Geraniol, followed by 1486.48 µg/g in 1.5mM L-Tryptophan and 2.2 mM Geraniol and least content was (768.81 µg/g) seen in 1mM each of both L-Tryptophan and Geraniol. It was observed that camptothecin content decreased with the increase in concentration of the precursors.

#### 5.4.1.1 Fitting the RSM model

The Box-Behnken experimental design was developed to improve the CPT extraction parameters and considered experimental values obtained for the variable responses, as shown in Table 5.6. According to the experimental findings, the total CPT content varied from 768.81µg/g in run 1 (1mM L-tryptophan: 1mM Geraniol) to 1747.63 µg/g in run 10 (1.5mM L-tryptophan: 1.5mM Geraniol).

The model was assessed in accordance with the significance of the regression coefficients. In order to enhance the number of significant variables, the level of significance was set at 0.1 in accordance with earlier research. The significant variables on the response variable of CPT were the intercept ($X_0$) ($p$ =1.70200), the linear effect of L-Tryptophan concentration ($X_1$) ($p$ = -0.12394) and its quadratic effect ($X_{11}$) ($p$ = -0.30475), the linear effect of Geraniol concentration ($X_2$) ($p$ = 0.16041) and its quadratic effect ($X_{22}$) ($p$ = -0.21975), and the interaction between L-Tryptophan and Geraniol ($X_{12}$) is ($p$ =-0.08500),

The second-order polynomial acquired by multi-regression analysis of experimental data

represents the mathematical relationship between independent variables and the response (CPT content: coded and uncoded value).

CPT (µg/g) (coded unit)

$$Y = 1.70200 - 0.12394X_1 + 0.16041X_2 - 0.30475X_1^2 - 0.21975X_2^2 - 0.08500X_1X_2 \quad (3.1)$$

CPT (µg/g) (uncoded unit)

$$Y = -4.63655 + 4.41489X_1 + 3.46781X_2 - 1.21900X_1^2 - 0.879000X_2^2 - 0.340000X_1X_2 \quad (3.2)$$

Individual linear, quadratic, and interaction factors' effects and regression coefficients were calculated using an analysis of variance (ANOVA) with a 95% confidence level. ANOVA showed that the models had strong coefficients of determination ($R^2$) 0.9909 between independent factors and response variables. The model fits well since the p-value for lack-of-fit was non-significant ($p > 0.05$), which was used to assess the model's suitability (Table 5.9). Furthermore, all instances had p-values that were lower than 0.05, indicating that the models were statistically acceptable.

Response surfaces were developed to ascertain the optimal levels of independent variables for the extraction of the CPT content from *O. mungos*. Three-dimensional surface plots were used to show each pair of variables, with the centre variable being held constant. The HPTLC plates at 366nm in Plate 6.1(A&B) depict the presence of CPT in every precursor treated samples and densitogram of CPT peaks respectively, on the amount of CPT. Plate 6.2: A&B surface plot and contour plots shows the maximum CPT content at 1.5 mM L-Tryptophan and 1.5 mM Geraniol.

This screening experiment of interactive effect of precursors on camptothecin synthesis reveals that the concentrations 1.5mM each of both L-Tryptophan and Geraniol had the best yield (1658.52-1747.63µg/g) out of the rest combinations. The outcomes of this study are in accord with the outcomes of Amna et al. (2012) in which addition of L-trytophan (1mM) in the endophytic spore suspension cultures isolated from *Nothapodytes foetida* resulted in significant increase in camptothecin content but Geraniol(1mM) had no significant effect. In another study, L-trytophan and Geraniol significantly affected production of camptothecin with injection of precursors in the stem of *C. acuminata* (Liu et al., 2015c). Precursor feeding has been shown to increase secondary metabolite production in plant cell and organ cultures, however this practice has also been shown to have a major negative impact on plant development, depending on the species. Our research also confirms that camptothecin level was increased as a result of precursor treatment.

**Table 5.4** Yield of camptothecin from *O. mungos* using different levels of extraction variables of Plackett-Burman design criteria.

| Runs | L-tryptophan (mM) $X_1$ | L-leucine (mM) $X_2$ | Trptamine (mM) $X_3$ | Loganin (mM) $X_4$ | Secologanin (mM) $X_5$ | Geraniol (mM) $X_6$ | CPT(µg/g) |
|---|---|---|---|---|---|---|---|
| 1 | 2 | 1 | 2 | 1 | 1 | 1 | 564.07 |
| 2 | 2 | 2 | 1 | 2 | 1 | 1 | 649.13 |
| 3 | 1 | 2 | 2 | 1 | 2 | 1 | 383.54 |
| 4 | 2 | 1 | 2 | 2 | 1 | 2 | 721.01 |
| 5 | 2 | 2 | 1 | 2 | 2 | 1 | 813.08 |
| 6 | 2 | 2 | 2 | 1 | 2 | 2 | 1120.01 |
| 7 | 1 | 2 | 2 | 2 | 1 | 2 | 762.24 |
| 8 | 1 | 1 | 2 | 2 | 2 | 1 | 458.91 |
| 9 | 1 | 1 | 1 | 2 | 2 | 2 | 716.03 |
| 10 | 2 | 1 | 1 | 1 | 2 | 2 | 782.45 |
| 11 | 1 | 2 | 1 | 1 | 1 | 2 | 687.32 |
| 12 | 2 | 1 | 1 | 1 | 1 | 1 | 343.21 |

**Table 5.5** Regression analysis for prediction of significant extraction parameters by PBD.

| Term | Coefficient | SE coefficient | *T* | *P* |
|---|---|---|---|---|
| Constant | 0.662500 | 0.03148 | 21.05 | 0.000 |
| L-tryptophan | 0.109167 | 0.03148 | 3.47 | 0.018 |
| L-leucine | 0.069167 | 0.03148 | 2.20 | 0.079 |
| Tryptamine | 0.002500 | 0.03148 | 0.08 | 0.940 |
| Loganin | 0.019167 | 0.03148 | 0.61 | 0.569 |
| Secologanin | 0.045833 | 0.03148 | 1.46 | 0.205 |
| Geraniol | 0.132500 | 0.03148 | 4.21 | 0.008 |

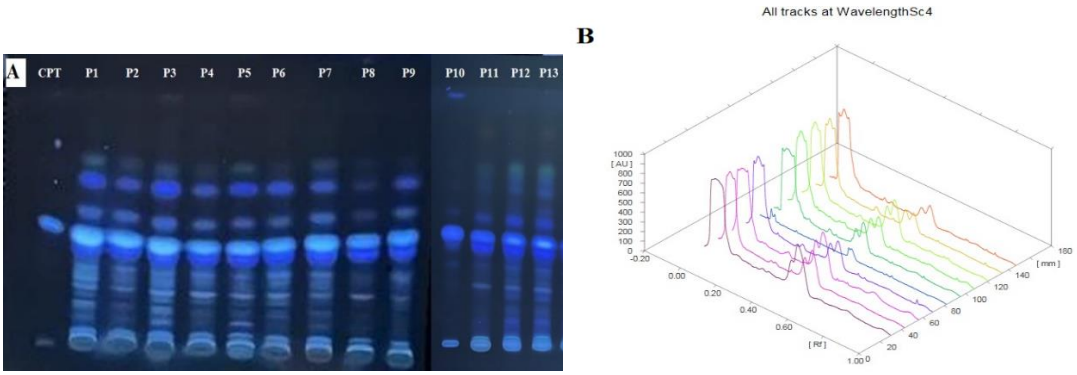



**Fig 5.1:** (A) HPTLC fingerprinting of different precursors compared with standards at 366nm.(CPT-Standard, P1-P13-Precursors treated *O. mungos* sample );(1-13 Table 4.6) **(**B)HPTLC densitogram of *O. mungos* treated with precursors showing camptothecin peaks.

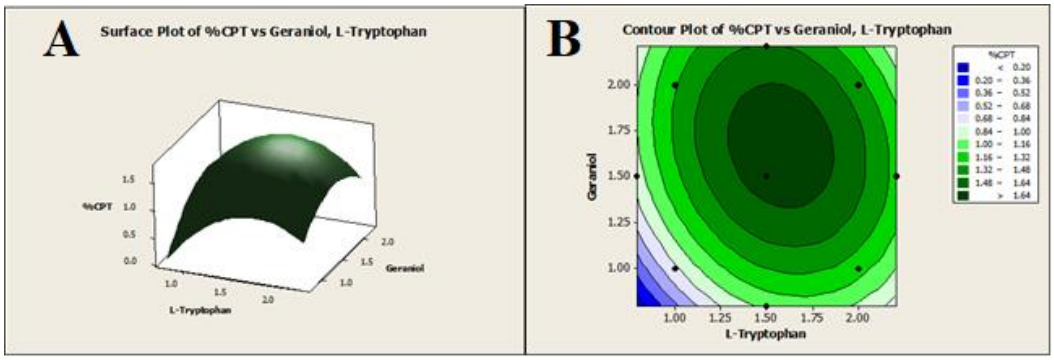



**Fig 5.2:** (A) Surface plot of %CPT vs Geraniol, L-Tryptophan (B) Contour plot of %CPT vs Geraniol, L-Tryptophan.

**Table 5.6** BBD criteria of precursors with corresponding experimental and predicted value.

| StdOrder | RunOrder | PtType | Blocks | L-Tryptophan(mM) | Geraniol(mM) | CPT(µg/g) | Predicted |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 1 | 768.81 | 808.15 |
| 2 | 2 | 1 | 1 | 2 | 1 | 1156.48 | 1226.04 |
| 3 | 3 | 1 | 1 | 1 | 2 | 1319.28 | 1298.96 |
| 4 | 4 | 1 | 1 | 2 | 2 | 1364.67 | 1376.85 |
| 5 | 5 | -1 | 1 | 0.79 | 1.5 | 935.18 | 917.22 |
| 6 | 6 | -1 | 1 | 2.20 | 1.5 | 1327.80 | 1267.78 |
| 7 | 7 | -1 | 1 | 1.5 | 0.79 | 1112.17 | 1035.65 |
| 8 | 8 | -1 | 1 | 1.5 | 2.20 | 1486.48 | 1489.35 |
| 9 | 9 | 0 | 1 | 1.5 | 1.5 | 1724.02 | 1702.00 |
| 10 | 10 | 0 | 1 | 1.5 | 1.5 | 1747.63 | 1702.00 |
| 11 | 11 | 0 | 1 | 1.5 | 1.5 | 1678.53 | 1702.00 |
| 12 | 12 | 0 | 1 | 1.5 | 1.5 | 1734.50 | 1702.00 |
| 13 | 13 | 0 | 1 | 1.5 | 1.5 | 1658.52 | 1702.00 |

**Table 5.7** Estimated Regression Coefficients for Camptothecin (µg/g) using data in coded units(precursor).

| Term | Coefficient | SE coefficient | T | P |
|---|---|---|---|---|
| Constant | 1.70200 | 0.02580 | 65.978 | 0.000 |
| L-tryptophan | 0.12394 | 0.02039 | 6.077 | 0.001 |
| Geraniol | 0.16041 | 0.02039 | 7.865 | 0.000 |
| L-tryptophan *L-tryptophan | -0.30475 | 0.02187 | -13.935 | 0.000 |
| Geraniol *Geraniol | -0.21975 | 0.02187 | -10.048 | 0.000 |
| L-tryptophan * Geraniol | -0.08500 | 0.02884 | -2.947 | 0.021 |

**Table 5.8** Estimated Regression Coefficients for Camptothecin (µg/g) using data in uncoded units.

| Term | Coefficient |
|---|---|
| Constant | -4.63655 |
| L-tryptophan | 4.41489 |
| Geraniol | 3.46781 |
| L-tryptophan *L-tryptophan | -1.21900 |
| Geraniol *Geraniol | -0.879000 |
| L-tryptophan * Geraniol | -0.340000 |

**Table 5.9** Analysis of variance for Camptothecin (µg/g) by using BBD criterion(precursor).

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| **Regression** | 5 | 1.23300 | 1.233001 | 0.246600 | 74.11 | 0.000 |
| **Linear** | 2 | 0.32874 | 0.328739 | 0.164369 | 49.40 | 0.000 |
| **Square** | 2 | 0.87536 | 0.875362 | 0.437681 | 131.54 | 0.000 |
| **Interaction** | 1 | 0.02890 | 0.028900 | 0.028900 | 8.69 | 0.021 |
| **Residual Error** | 7 | 0.02329 | 0.023291 | 0.003327 | | |
| **Lack-of-Fit** | 3 | 0.01701 | 0.017011 | 0.005670 | 3.61 | 0.123 |
| **Pure Error** | 4 | 0.00628 | 0.006280 | 0.001570 | | |
| **Total** | 12 | 1.25629 | | | | |

R-Sq = 98.15% R-Sq(pred) = 89.59% R-Sq(adj) = 96.82%

### 5.4.2 Effect of elicitors camptothecin production

Many researchers have proposed using liquid culture as a method of elicitation. Increased generation of bioactive compounds and faster shoot growth were both validated by the liquid culture system, as reported by Mehrotra et al. (2007). One possible explanation is that the proximity of the tissue to the medium enhances its ability to absorb nutrients, leading to faster growth. Due to the frequent shaking of the tissues in the medium, apical dominance activity is diminished in the liquid culture system, which is crucial for the development of many axillary buds. In their respective studies, Jo et al. (2008) and

Sivanandhan et al. (2013) found the same results. *Centella asiatica* shoot cultures were recently elicited using a liquid culture technique by Prasad et al. (2013). Thus, the elicitation procedure was carried out in liquid ½ MS medium supplemented with 13.37µM BA and 1.96 µM IBA in this investigation since it was found to be more prominent for in vitro plant multiplication as well. The application of elicitors has shown significant effect on CPT content of *O. mungos* cultures. Three different concentrations (100, 150 µM and 200 µM) of SA, Ja, MeJa and CH were incorporated with 4 days incubation period in the liquid MS medium on 2 months of culture. Similar BBD design was used for this study as well. The independent variables were MeJa, Ja, SA and CS (Table 5.3). The CPT content was analyzed in the whole plant. The plant was harvested after 4days of elicitor treatment and analyzed by HPTLC. A total of 27 experimental runs were performed as per BBD-RSM design and the experimental and predicted camptothecin yield is outlined in Table 5.10. The model validation was confirmed by ANOVA and the quadratic effect of the independent variables on the camptothecin yield was also determined. The second-order polynomial obtained by multi-regression analysis of experimental data represents the mathematical correlation between the independent variables and the response value of camptothecin in whole plant (Equation 4.1).

According to the experimental findings, the total CPT content varied from 285.480µg/g in run 18 (200 µM MeJA, 150 µM JA, 100 µM SA and 150 µM CH) to 675.830 µg/g in run 27 (150µM for all elicitors i.e., MeJA, JA, SA and CH).

The model was assessed in accordance with the significance of the regression coefficients. In order to enhance the number of significant variables, the level of significance was set at 0.1 in accordance with earlier research. The significant variables on the response variable of CPT were the intercept ($X_0$) ($p$ = 671.967), the linear effect of MeJa concentration ($X_1$) ($p$ = -31.639) and its quadratic effect ($X_{11}$) ($p$ = -207.621), the linear effect of Ja concentration ($X_2$) ($p$ =22.280) and its quadratic effect ($X_{22}$) ($p$ =-120.895), the linear effect of SA concentration ($X_3$) ($p$ = 40.298) and its quadratic effect ($X_{33}$) ($p$ = -84.157), the linear effect of CH concentration ($X_4$) ($p$ = 5.783)and its quadratic effect ($X_{44}$) ($p$ = -100.783) and the interactive effect of MeJa and Ja (X12) ($p$ = -11.133), interactive effect of MeJa and SA (X13) ($p$ = 19.752), interactive effect of MeJa and CH (X14) ($p$ = 50.372), interactive effect of Ja and SA (X23) ($p$ = -74.720), interactive effect of Ja and CH (X24) ($p$ = 35.787) and interactive effect of SA and CH (X34) ($p$ = -24.103).

Multi-regression analysis provides a second-order polynomial that models the mathematical connection between the experimental variables and the response (CPT content: coded and uncoded value).

CPT (µg/g dry weight) (coded unit)

$$Y = 671.967 - 31.639X1 + 22.280X2 + 40.298X3 + 5.783X4 - 207.621X12 - 120.895X22 - 84.157X32 - 100.783X42 - 11.133X1X2 + 19.752X1X3 + 50.372X1X4 - 74.720X2X3 + 35.787X2X4 - 24.103X3X4 \quad (5.1)$$

CPT (µg/g dry weight) (uncoded unit)

$$Y = -4095.67 + 20.7422X1 + 17.9569X2 + 15.6489X3 + 8.48620X4 - 0.0830483X12 - 0.0483578X22 - 0.0336628X32 - 0.0403133X42 - 0.00445300X1X2 + 0.00790100X1X3 + 0.0201490X1X4 - 0.0298880 + 0.0143150X2X4 - 0.00964100X3X4 \quad (5.2)$$

Individual linear, quadratic, and interaction factors effects and regression coefficients were calculated using an analysis of variance (ANOVA) with a 95% confidence level. The results of the analysis of variance for the quadratic model fit are shown in Table 5.13. The P-values were used to verify the statistical significance of each coefficient. A significant coefficient was suggested by a high F-value and a low P-value (Luo et al., 2020). ANOVA showed that the models had strong coefficients of determination ($R^2$) 0.9909 between independent factors and response variables. The model fits well since the p-value for lack-of-fit was non-significant ($p > 0.05$), which was used to assess the model's suitability (Table 5.13). Furthermore, all instances had p-values that were lower than 0.05, indicating that the models were statistically acceptable.

Response surfaces were developed to ascertain the optimal levels of independent variables for the extraction of the CPT content from *O. mungos*. Three-dimensional surface plots were used to show each pair of variables, with the centre variable being held constant. The HPTLC plates at 366nm in Fig. 5.3(A&B) depict the presence of CPT in every elicitor treated samples and densitogram of CPT peaks respectively, on the amount of CPT. Fig. 5.4: A-F contour plots shows the maximum CPT content at different combination of elicitors, (A) depicts maximum CPT content when CS and SA are both at 150µM, (B) depicts maximum CPT content when CS and JA are both at 150µM, (C) depicts maximum CPT content when CS and MeJa are both at 150µM, (D) depicts maximum CPT content when SA and JA are

both at 150µM, (E) depicts maximum CPT content when SA and MeJa are both at 150µM and (F) depicts maximum CPT content when JA and MeJa are both at 150µM. This screening experiment of interactive effect of elicitors on camptothecin synthesis reveals that the concentrations 150µM each of all elicitors viz MeJa, JA, SA and CS had the best yield (666.390-675.830µg/g) out of the rest combinations followed by 559.240 µg/g at 150 µM MeJa, 100 µM JA, 200 µM SA and 150 µM CS. Least concentration of CPT (285.480 µg/g) was recorded in concentrations 200 µM MeJa, 150 µM JA, 100 µM SA and 150 µM CS. Based on the results obtained MeJa and SA concentration play prominent role than the rest of the elicitors. Results from this study agree with those from Keshavan et al. (2022), who found that by adding 5%Jasmonic acid and 10% chitosan to a cell suspension culture of *Nothapodytes nimmoniana* yielded a considerable rise in camptothecin content. In another study, 150 µM MeJa significantly affected production of camptothecin by 7-fold in *O. mungos* var *angustifolia* (Krishnan et al., 2018b). In plant cell and organ cultures, the exogenous injection of elicitors into the culture media has been demonstrated to boost the production of secondary metabolites; however, this approach has also been proven to have a severe deleterious impact on plant development. Our research also confirms that camptothecin level was increased as a result of elicitor treatment.

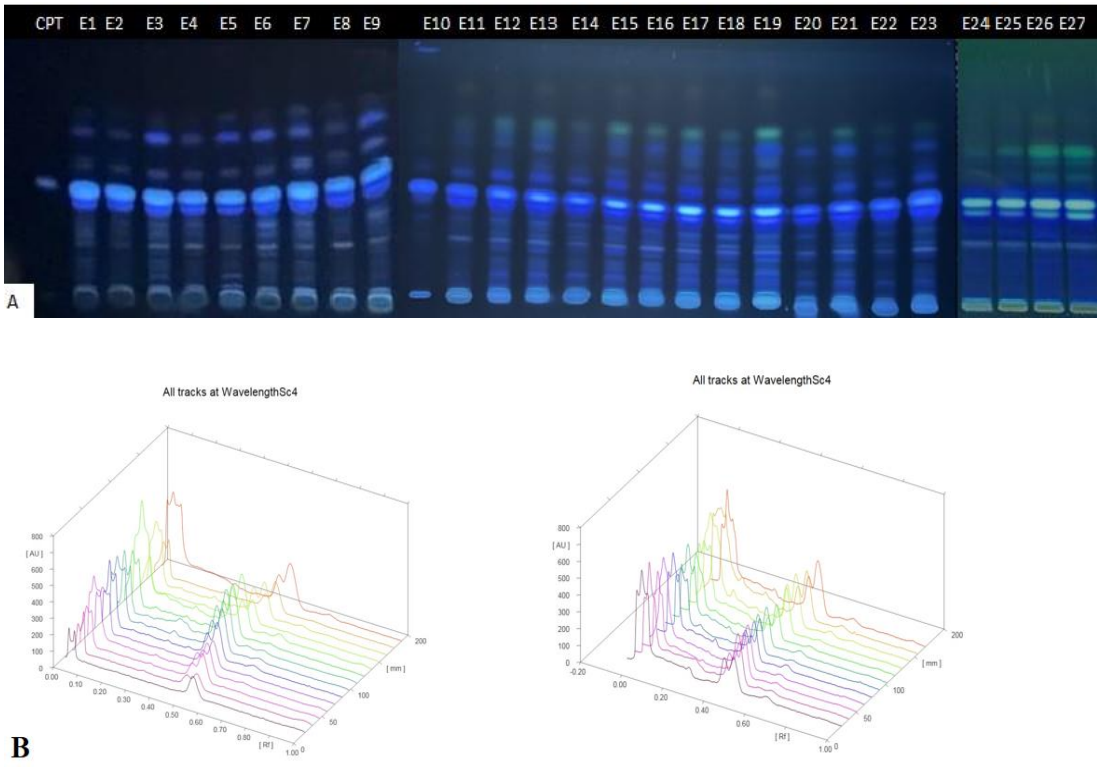



**Fig 5.3:** (A) -HPTLC fingerprinting of different elicitors compared with standards at 366nm.(CPT-Standard, E1-E27-Elicitors treated *O.mungos* sample );(1-27 Table 4.9)

**(**B)HPTLC densitogram of *O. mungos* treated with elicitors showing camptothecin peaks.

**Table 5.10** BBD criteria of elicitors with corresponding experimental and predicted value.

| Std order | MeJa(µM) X1 | JA(µM) X2 | SA(µM) X3 | CS(µM) X4 | CPT(µg/g) | Predicted |
|---|---|---|---|---|---|---|
| 1 | 100 | 100 | 150 | 150 | 347.880 | 341.678 |
| 2 | 200 | 100 | 150 | 150 | 308.040 | 300.665 |
| 3 | 100 | 200 | 150 | 150 | 402.580 | 408.503 |
| 4 | 200 | 200 | 150 | 150 | 318.210 | 322.960 |
| 5 | 150 | 150 | 100 | 100 | 415.180 | 416.848 |
| 6 | 150 | 150 | 200 | 100 | 539.620 | 545.640 |
| 7 | 150 | 150 | 100 | 200 | 484.090 | 476.618 |
| 8 | 150 | 150 | 200 | 200 | 512.120 | 509.000 |
| 9 | 100 | 150 | 150 | 100 | 445.760 | 439.792 |
| 10 | 200 | 150 | 150 | 100 | 286.060 | 275.768 |
| 11 | 100 | 150 | 150 | 200 | 343.370 | 350.612 |
| 12 | 200 | 150 | 150 | 200 | 385.160 | 388.078 |
| 13 | 150 | 100 | 100 | 150 | 320.910 | 329.622 |
| 14 | 150 | 200 | 100 | 150 | 527.080 | 523.622 |
| 15 | 150 | 100 | 200 | 150 | 559.240 | 559.648 |
| 16 | 150 | 200 | 200 | 150 | 466.530 | 454.768 |
| 17 | 100 | 150 | 100 | 150 | 393.760 | 391.287 |
| 18 | 200 | 150 | 100 | 150 | 285.480 | 288.504 |
| 19 | 100 | 150 | 200 | 150 | 430.890 | 432.369 |
| 20 | 200 | 150 | 200 | 150 | 401.620 | 408.595 |
| 21 | 150 | 100 | 150 | 100 | 453.750 | 458.014 |
| 22 | 150 | 200 | 150 | 100 | 426.690 | 430.999 |
| 23 | 150 | 100 | 150 | 200 | 397.810 | 398.004 |
| 24 | 150 | 200 | 150 | 200 | 513.900 | 514.139 |
| 25 | 150 | 150 | 150 | 150 | 666.390 | 671.967 |
| 26 | 150 | 150 | 150 | 150 | 673.680 | 671.967 |
| 27 | 150 | 150 | 150 | 150 | **675.830** | 671.967 |

**Table 5.11** Estimated Regression Coefficients for Camptothecin (µg/g) using data in coded units(elicitor).

| Term | Coefficient | SE coefficient | T | P |
|---|---|---|---|---|
| Constant | 671.967 | 4.818 | 139.456 | 0.000 |
| MeJa(µM) | -31.639 | 2.409 | -13.132 | 0.000 |
| JA(µM) | 22.280 | 2.409 | 9.248 | 0.000 |
| SA(µM) | 40.298 | 2.409 | 16.724 | 0.000 |
| CS(mg/L) | 5.783 | 2.409 | 2.400 | 0.034 |
| MeJa(µM) * MeJa(µM) | -207.621 | 3.614 | -57.451 | 0.000 |
| JA(µM)* JA(µM) | -120.895 | 3.614 | -57.451 | 0.000 |

| | | | | |
|---|---|---|---|---|
| SA(µM)* SA(µM) | -84.157 | 3.614 | -33.453 | 0.000 |
| CS(mg/L)* CS(mg/L) | -100.783 | 3.614 | -27.888 | 0.000 |
| MeJa(µM) * JA(µM) | -11.133 | 4.173 | -2.668 | 0.020 |
| MeJa(µM) * SA(µM) | 19.752 | 4.173 | 4.733 | 0.000 |
| MeJa(µM) * CS(mg/L) | 50.372 | 4.173 | 12.071 | 0.000 |
| JA(µM) * SA(µM) | -74.720 | 4.173 | -17.906 | 0.000 |
| JA(µM) * CS(mg/L) | 35.787 | 4.173 | 8.576 | 0.000 |
| SA(µM) * CS(mg/L) | -24.103 | 4.173 | -5.776 | 0.000 |

**Table 5.12** Estimated Regression Coefficients for Camptothecin (µg/g) using data in uncoded units(elicitor).

| **Term** | **Coefficient** |
|---|---|
| Constant | -4095.67 |
| MeJa(µM) | 20.7422 |
| JA(µM) | 17.9569 |
| SA(µM) | 15.6489 |
| CS (mg/L) | 8.48620 |
| MeJa(µM) * MeJa(µM) | -0.0830483 |
| JA(µM) * JA(µM) | -0.0483578 |
| SA(µM) * SA(µM) | -0.0336628 |
| CS (mg/L) * CH (mg/L) | -0.0403133 |
| MeJa(µM) * JA(µM) | -0.00445300 |
| MeJa(µM) * SA(µM) | 0.00790100 |
| MeJa(µM) * CS (mg/L) | 0.0201490 |
| JA(µM) * SA(µM) | -0.0143150 |
| JA(µM) * CS (mg/L) | 0.0143150 |
| SA(µM) * CS (mg/L) | -0.00964100 |

**Table 5.13** Analysis of variance for Camptothecin (µg/g) by using BBD criterion(elicitor).

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| **Regression** | 14 | 328015 | 328015 | 23429.6 | 336.37 | 0.000 |
| **Linear** | 4 | 37853 | 37853 | 9463.3 | 135.86 | 0.000 |
| **Square** | 4 | 248177 | 248177 | 62044.2 | 890.75 | 0.000 |
| **Interaction** | 6 | 41985 | 41985 | 6997.5 | 100.46 | 0.000 |
| **Residual Error** | 12 | 836 | 836 | 69.7 | | |
| **Lack-of-Fit** | 10 | 787 | 787 | 78.7 | 3.21 | 0.261 |
| **Pure Error** | 2 | 49 | 49 | 24.5 | | |
| **Total** | 26 | 328851 | | | | |

R-Sq = 99.75%  R-Sq(pred) = 98.59%  R-Sq(adj) = 99.45%

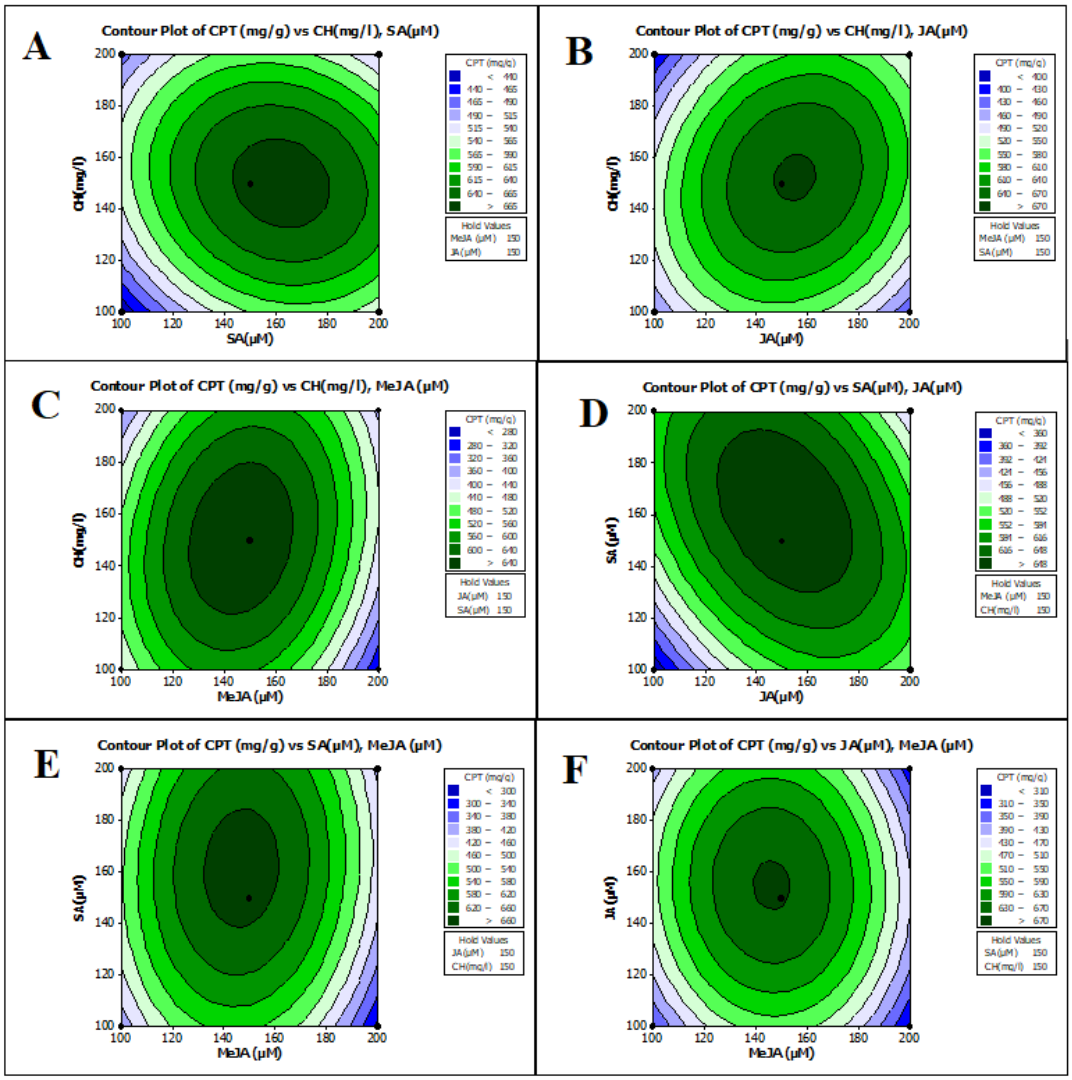



**Fig 5.4** A-F: Contour Plots of different elicitors interaction.

## 5.5 Conclusion

The goal of this work has been to increase camptothecin production in in vitro cultures of *O. mungos* by the use of elicitation and precursor feeding. MeJa, JA, SA, and CS as elicitors, and L-Tryptophan and Geraniol as precursors, were used in various concentrations to elicit the plant cultures of *O. mungos*. The results of the present study showed that a concentration of 150 mM of all elicitors viz. MeJa, JA, SA, and CS produced the highest camptothecin content, while a concentration of 1.5 µM of both L-Tryptophan and Geraniol was found to be a potent precursor concentration for the production of the highest camptothecin content. The study also shows that the amount of camtothecin in *O. mungos* cultures varies greatly with the type and concentration of elicitor or precursor employed in conjunction with the different combinations. The results of this study can serve as a road map for future research into optimizing camptothecin production. Bioreactors can be used in conjunction with elicitor and precursor treatments to dramatically increase the yield of bioactive chemicals and the number of plants available for therapeutic application. Research is also needed to develop metabolic engineering approaches that can boost in vitro culture production of valuable secondary metabolites.

# CHAPTER 6

# *Mass multiplication of elite Ophiorrhiza sp.*

## 6.1 Introduction

Tissue culture plants can be rapidly propagated and accumulate biomass and secondary metabolites from medicinal plant. When it comes to specific genotypes, clonal propagation by plant tissue culture, also known as micropropagation, is the gold standard for true-to-type propagation that may be accomplished in a relatively short duration and area. Even in the dead of winter, a single explant can be grown into hundreds or thousands of new plants in a relatively short amount of time and space. High levels of multiplication and lower demands on primary plants and space make micropropagation ideal for propagating and preserving rare and endangered plant species, however, the use of growth hormones can limit the success of this technique (Hussain et al., 2012).

Plant growth hormones establish a specific form of growth in cultured cells or tissues, which may be triggered by a chemical increase. Hormones supplied to the medium singly or in mixtures maintain specific and consistent organic and inorganic components in the developing tissue and drive cells/tissues to form callus and subsequently segregate into shoots, roots, or die (Haq & Dahot, 2007). Since auxins are primarily necessary for root formation and cytokinins for shoot creation and bud growth, these two hormones are the most important for establishing in vitro cultures (North et al., 2012). The growth and development activities of plant cultures are affected by changes in the concentration of auxins and cytokinins (Ngomuo et al., 2013). However, the success of plant regeneration depends on the correct concentration and combination of auxins and cytokinins in the culture media. The emergence and growth of the meristem are just two of the crucial morphological processes regulated by the cytokinin-auxin relationship. Shoot multiplication in in vitro culture can be triggered by administering low concentrations of auxins and high concentrations of cytokinins, as shown by Su et al. (2011).

Eventually, scientists discovered that cytokinins, a varied group of N6-a substituted purine derivative that promotes cell division in plants, are actually an essential natural hormone for the healthy growth and development of plants. Many other plant developmental processes,

such as the suppression of leaf senescence, cell expansion, chloroplast growth, nutrient mobilisation, and shoot and root branching, are influenced by cytokinins as well (Mok,1994). There is strong evidence that cytokinins support either direct or indirect shoot induction. Cytokinin accumulation triggered shoot proliferation at the level of the superficial meristem, and later on callus was used to facilitate root formation (Paterson & Rost,1981). However, auxins cause a wide variety of activities in plants and are therefore a crucial component of the culture medium used in the tissue culture and micropropagation process. Auxins play a crucial role in stimulating apical dominance, increasing shoot and root development, initiating cell division in callus or specified organs, differentiating and elongating cells or conductive tissues, and motivating apical dominance in in vitro cultures (Singh et al., 2016).

The most common auxins used in in vitro cultures are 2,4-D, which is used for both callus induction and suspension cultures, NAA, which is used for organogenesis and primarily for root formation, and IBA, which is utilized for both shoot multiplication and root creation (Hussain et al., 2012). However, BAP and KN are the most often employed cytokinins for inducing both axillary and adventitious shoot development and for establishing apical meristem dominance. But BAP is the cytokinin of choice for the vast majority of researchers (Ngomuo et al., 2013).

One significant benefit of in vitro cultivation is the generation of genetically inert plantlets. To successfully clone elite genotypes with superior traits and preserve them for future generations, in vitro regenerants must share a high degree of genetic similarity. Despite this, numerous studies have demonstrated the existence of somaclonal diversity among in vitro plantlets. These distinctions depend on the genotype and the environmental conditions in which the explant is grown (da Silva et al., 2007). Biochemical and DNA-based molecular markers, as well as morphological character analysis, can all be used to learn more about these variants. The changes found by morphological, cytological, and biochemical analyses may be influenced by developmental and environmental factors, thus, researchers rely increasingly on molecular markers (Alizadeh et al., 2015).

Evaluation of the genetic and biochemical stability of the in vitro raised plantlets is crucial following large-scale tissue culture plant propagation. Generally speaking, clonal proliferation through tissue culture must result in progeny that are identical to the parent explants, yet several studies indicated that in vitro cultures are often susceptible to genetic alterations by stress encountered during culture (Kumar et al., 2017; Kumar & Chandra, 2013). Utilizing explants with already developed meristems can improve the genetic fidelity of regenerants in clonal propagation studies. Plant growth regulators are typically added to

the medium to enhance the multiplication of shoots from explants. However, this also raises the likelihood of cellular mutations and genetic variability in regenerated plants. A powerful tool for characterizing variability is PCR-based molecular markers (DNA Markers)such as RAPD, ISSR, and SSR. Different samples of *Ophiorrhiza* have been examined for clonal fidelity and genetic stability using molecular markers such as RAPD (Kaushik et al., 2015). Since RAPD technique is being simple and cost effective, it has been widely used by many researchers to assess genetic stability in micropropagated plants (Agnihotri et al., 2009; Chalageri & Babu, 2012; Kaushik et al., 2015).

Even a cursory review of the literature on micropropagation of *Ophiorrhiza spp*. reveals a severe lack of information on either direct or indirect organogenesis. Considering the voids, genuine attempts have been made to design an effective and enhanced protocol of micropropagation utilizing explants of the elite species of *Ophiorrhiza sp. (O. mungos*), as detailed in **Chapter 3**. This investigation also makes an effort, utilising RAPD and HPTLC analysis, to assess the genetic and biochemical stability of the in vitro raised plantlets. The presence of camptothecin, the main compound of interest in *O. mungos*, was used to evaluate the biochemical accuracy.

## 6.2 Materials and Methods

### 6.2.1 *In vitro* studies

The most important part of conducting in vitro research is keeping cultures in an aseptic environment. The following outlines the sterile chambers and methods used for in vitro culture of *O. mungos*:

- ❖ Source of explant.
- ❖ Sterilization of glassware and other equipment.
- ❖ 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.

#### 6.2.1.1 Mother Plant source

All in vitro investigations and genetic fidelity experiments in this work were conducted using a mother plant source derived from the elite species of *Ophiorrhiza sp*. reported in our Chapter 3, which was gathered from the Kottakal area of Kerala. All of the plant's parts were

separated and washed to get rid of any possible traces of dirt or mud.

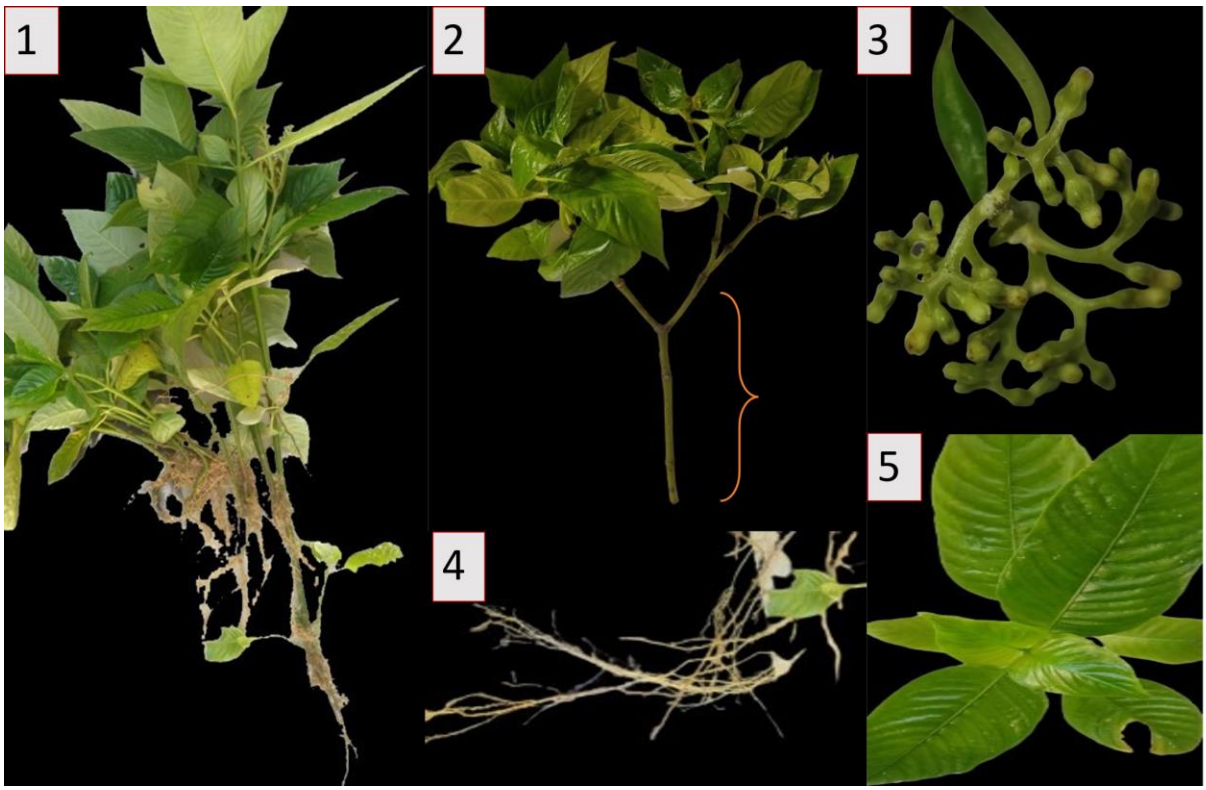



**Plate 6.1:** Different parts of *O. mungos* (1) whole plant (2) nodal parts (3) roots (4) inflorescence (5) leaves.

### 6.2.1.2 Sterilization of glassware and other equipments

Flasks, petri dishes, test tubes, beakers, culture bottles, forceps, and scalpels are just some of the glassware that gets a good scrub before each use. They were first soaked in liquid detergent, scrubbed and then washed with water to rinse away any remaining residue. After that, they were dried in a hot air oven at 60-80 °C to kill any remaining contaminants. After drying, glassware and other equipment were autoclaved for use.

### 6.2.1.3 Preparation and sterilization of nutrient media

The experiment employed Murashige and Skoog (1962) (MS) with added $CaCl_2$ and pre-packaged vitamin packets (Hi-Media). For the media, 1 liter of double-distilled water was used to dissolve 4.9 g of MS media and 30 g of sucrose (at a 3% concentration) while stirring constantly. Using stock solutions, MS media at 50% strength (1/2 MS) were produced (Table 6.1). pH was adjusted to 5.8 by adding 1N NaOH (Sodium hydroxide) or HCl (Hydrochloric acid) and heat-stable growth hormones were added to the MS media at the necessary concentrations. The media was then boiled and stirred occasionally to dissolve

the addition of agar 0.8% (8 g). The media was then transferred to clean conical flasks, petri dishes, and test tubes before being autoclaved at 121°C and 15 psi for 15-20 minutes.

#### 6.2.1.4 Preparation of stock solution of plant growth hormones

Auxin and cytokinins are the primary hormones investigated here. To stimulate the development of callus, shoots, and roots, several preparations including BA, IBA, NAA, IAA, pictogram, GA3, TDZ, metatopolin and melatonin, were combined in required combinations and poured onto ½ MS media. Due to the insolubility of PGRs in water, a relative solvent was used to dissolve them, and then DDW was added to get the desired volume. Table 6.2 detailed the soluble range for various PGRs. 100µM PGRs stock solution was made in advance. The stock solutions of BA, NAA, IBA, IAA, metatopolin, melatonin, picloram, GA3 and TDZ were prepared by dissolving measured amounts of respective hormones (according to their molecular weight) in suitable solvents (Table 6.2) and adjusting the final volume with double distilled water, and the resulting stock solutions were placed in the refrigerator at -20 °C until use.

**Table 6.1** MS media and ½ MS media 1L preparation.

| **Stock** | | **Salt component** | **Mass(mg)/1000 ml** | **Volume stock(mL/L) final medium(MS)** | **Volume stock(mL/L) final medium (1/2 MS)** |
|---|---|---|---|---|---|
| 1 | | $NH_3NO_3$ | 165 | 10 | 5 |
| 2 | | $KNO_3$ | 95 | 20 | 10 |
| 3 | | $CaC1_2.2H_2O$ | 44 | 10 | 5 |
| 4 | | $MgSO_4.7H_2O$ | 37 | 10 | 5 |
| 5 | | NaFeEDTA | 4 | 10 | 5 |
| 6 | | $KH_2PO_4$ | 17 | 10 | 5 |
| | | $H_3BO_4$ | 0.62 | 10 | 5 |
| 7a | | $ZnSO_4.7H_2O$ | 0.86 | 10 | 5 |
| | | Kl | 0.083 | 10 | 5 |
| 7b | | $MnSO_4.4H_2O$ | 2.23 | 10 | 5 |
| | | $NaMoO_4.2H_2O$ | 0.025 | 10 | 5 |

| | | | | | |
|---|---|---|---|---|---|
| 8 | | $CuSO_4.5H_2O$ | 0.0025 | 10 | 5 |
| | | $CoC1_2.6H_2O$ | 0.0025 | 10 | 5 |
| 9 | | Thiamin HCI (B1/Aneurine) | 0.01 | 10 | 5 |
| | | Niacine (Nicotinic acid) | 0.05 | 10 | 5 |
| | | Pyridoxine HCI | 0.05 | 10 | 5 |
| | | Glycine | 0.2 | 10 | 5 |

**Table 6.2** Plant growth hormones stock solution preparation.

| Plant hormone | Molar mass (g/mol) | Formula | Solubility |
|---|---|---|---|
| BA | 225.2492 | $C_{12}H_{11}N_5$ | 1N NaOH/ EtOH |
| IBA | 203.24 | $C_{12}H_{13}NO_2$ | 1N NaOH/ EtOH |
| IAA | 175.184 | $C_{10}H_9NO_2$ | 1N NaOH/ EtOH |
| NAA | 186.2066 | $C_{12}H_{10}O_2$ | 1N NaOH/ EtOH |
| KIN | 215.21 | $C_{10}H_9N_5$ | 1N NaOH/ EtOH |
| GA3 | 346.37 | $C_{19}H_{22}O_6$ | 1N NaOH/ EtOH |
| mT | 241.25 | $C_{12}H_{11}N_5O$ | 1N NaOH |
| MEL | 232.278 | $C_{13}H_{16}N_2O_2$ | EtOH |
| TDZ | 220.25 | $C_9H_8N_4OS$ | 1N KOH/ DMSO |

### 6.2.1.5 Setting up sterile conditions in a LAF

Wiping LAF with ethanol (70%) and then subjecting it to 15 minutes of UV sterilization was done to ensure that it was completely sterile prior to its use to inoculate a new batch. All exposed orifices, including mouth and nose, were protected by a mask, and hands were disinfected with 70% ethanol. Every instrument, from forceps to scalpels to scissors to surgical blades, was autoclaved, immersed in 95% ethanol and then disinfected by being heated in a flame. A cotton plug or cap of culture test-tubes/conical flasks was kept on hand

and inoculation was performed close to the flame. After inoculation, the flasks or test tubes were recapped, wrapped in parafilm, and placed in the culture room for incubation.

#### 6.2.1.6 Optimization and surface sterilization of explants

Leaf, node, internode, and shoot tip explants were chosen for in vitro regeneration of *O. mungos*. To eliminate contamination, the explants were surface sterilized. Surface sterilizing agents such as bavistin, tween-20 (Ya-ut et al., 2011), ethanol (Kai et al., 2008), mercuric chloride, and labolene (Krishnan et al., 2018a) were utilized in this investigation with varying exposure times (Table 6.3). All explants were carefully cleansed in flowing tap water before being treated in tween-20 and bavistin for 30 minutes. The tween-20 and bavistin solution was discarded, and the explants were washed three times with running tap water. The cleaned explants were then transferred to a laminar air flow chamber to continue the surface sterilization procedure, in which several sterilizing agents with variable time intervals were used to standardize the sterilization protocol, as stated below:

- **Treatment-1:** After washing, explants were subjected to liquid detergent treatment for 15mins, rinsed with tap water, treated with tween 20 (2-3drops) and Bavistin (3%) for 30mins, rinsed with distilled water, washed with 70% ethanol (40-50sec), mercuric chloride ($HgCl_2$) for 4 minutes in laminar airflow chamber (LAF) and then splashed with autoclaved distilled water 4-5times.
- **Treatment-2:** After washing, explants were subjected to tween 20 (2-3drops) treatment and Bavistin (0.1%) for 10mins, washed with regular water, then double-washed with distilled water, sterilized with mercuric chloride ($HgCl_2$) for 1-2 minutes in LAF and then splashed with autoclaved distilled water 5-6times.
- **Treatment-3:** After washing, explants were subjected to labolene (2drops) treatment for 15mins, rinsed in running tap water(20-30min), treated with tween 20(2-3drops) and Bavistin (1%) for 30mins, washed with regular water, then double-washed with distilled water, sterilized with mercuric chloride ($HgCl_2$) for 2 minutes in LAF and then splashed with autoclaved distilled water 5-6times.
- **Treatment-4:** After washing, explants were subjected to labolene (3-4drops) treatment for 15mins, rinsed in running tap water (20-30min), treated with tween 20 (2-3drops) and Bavistin (1%) for 30mins, washed with regular water, then double-washed with distilled water, washed with 70% ethanol (30-40sec), sterilized with

mercuric chloride ($HgCl_2$) for 3 minutes in LAF and then splashed with autoclaved distilled water 5-6times.

- **Treatment-5:** After washing, explants were subjected to labolene (3-4drops) treatment for 15mins, rinsed in running tap water(20-30min), treated with tween 20 (2-3drops) and Bavistin (1%) for 30mins, washed with regular water, then double-washed with distilled water, sterilized with mercuric chloride ($HgCl_2$) for 3 minutes in LAF and then splashed with autoclaved distilled water 5-6times.

**Table 6.3** Sterilization agent concentration, exposure period, and preparation.

| S. No | Sterilization agents | Concentrations (%)/ amount | Exposure period (min) | Preparation |
|---|---|---|---|---|
| **1** | Mercuric chloride ($HgCl_2$) | 0.1 | 1 | 150 mg $HgCl_2$ dissolved in 150 ml of autoclaved double distilled water |
| | | | 2 | |
| | | | 3 | |
| | | | 4 | |
| **2** | Labolene | 3-4 drops | 15 | - |
| **3** | Bavistin | 0.1 | 10 | 1g of Bavistin in 100ml of distilled water (1%) |
| | | 1 | 30 | |
| | | 3 | 30 | |
| **4** | Tween 20 | 2-3 drops | 10 | - |
| | | | 30 | |
| **5** | Ethanol | 70 | 2 | 70 ml ethanol in 30 ml distilled water |
| | | | 3 | |

### 6.2.1.7 Inoculation and maintenance of cultures

Explants were taken from leaves, nodes, internodes, and the tips of the shoots, and then trimmed to the appropriate size (1–1.5 cm) with a slanting cut at the base before being inoculated on ½ MS media. The nodes, internodes and shoot tips were grown vertically, while

the leaf segments were placed with their abaxial and adaxial sides touching the medium. Explants are sub-cultured at 4-week intervals.

The cultures were maintained clean and sterile environment equipped with fluorescent tubes emitting (40-50 mol $m^{-2}$ $s^{-1}$). From day two after inoculation, the cultures were observed and interpretations were made. Callus formation, root and shoot formation, root and shoot size, culture response, and so forth were all measured.

#### 6.2.1.8 Direct shoot initiation and multiplication

Shoot induction was achieved by inoculating *O. mungos* explants onto ½ MS media supplemented with various doses of plant hormones including BA alone and in combination with KIN (Table 6.8). Cultures were moved from shoot induction medium to shoot multiplication media containing varying concentrations of BA + IBA (Table 6.9) and mT (Table 6.11) for additional shoot proliferation once shoots were developed

#### 6.2.1.9 Shoot elongation

Well-developed shoots were inoculated in ½ MS media supplemented with various concentrations of GA3 for shoot elongation (Table 6.12).

#### 6.2.1.10 Root induction

After the shoots had achieved sufficient length, they were sub cultured in the rooting medium. Rooting was achieved using a variety of PGRs including BA, NAA, and IBA at varying concentrations in solid ½ MS. (Table 6.13).

#### 6.2.1.11 Callus formation

The explants were cultured on callus initiation media augmented with different concentration of IAA, Picloram, TDZ, BAP, GA3, and IBA.

### 6.2.2 Acclimatization or hardening of plants

Tissue culture raised plantlets possessing well-developed roots were rinsed properly for removal of all remnants of agar prior to hardening. In vitro plants with well-developed root system were put in small flower pots filled with vermiculite and placed at 28°C in greenhouse. The plantlets were watered with ½ MS every 5–6 days without sugar or vitamins and wrapped with a plastic bag to trap moisture (70–80% humidity). After 3weeks they were then moved to larger container with soil, sand, and manure (1:1:1) to help them acclimatize. The plants that had become acclimated were moved to a greenhouse with a natural photoperiod after two weeks.

### 6.2.3 Genetic fidelity assessment

#### 6.2.3.1 Plant material

The genetic fidelity of tissue cultured plants produced was evaluated using RAPD markers. To evaluate the clonal stability of in vitro cultures, we randomly sampled young leaves from both wild plants (WP) and in vitro cultures (1-4).

**Table 6.4** Different PGRs used for direct and indirect organogenesis with concentration.

| Plant growth hormone (PGR) | Concentration (µM) | Culture |
|---|---|---|
| BA | 0-30 µM | Shoot induction, multiplication, root and callus formation |
| KN | 0-22.25 µM | Shoot induction, multiplication |
| IBA | 0-30 µM | Shoot multiplication, root and callus formation |
| IAA | 0-30 µM | Callus induction |
| NAA | 0-4 µM | Root induction |
| Picloram | 0-30 µM | Callus induction |
| TDZ | 0-30 µM | Callus induction |
| GA3 | 0-30 µM | Shoot elongation and callus induction |
| metatopolin | 0-12.5µM | Shoot multiplication and development |
| melatonin | 0-12.5µM | root induction, development |

#### 6.2.3.2 Genomic DNA extraction

Chapter 4(4.3.1).

#### 6.2.3.3 Purity and quantification of isolated DNA

Chapter 4(4.3.2).

### 6.2.3.4 PCR amplification by RAPD primers

Table 6.16 displays the results of six RAPD primers (Sigma-Aldrich) which showed consistent and unique amplification using PCR out of the ten RAPD primers (Sigma-Aldrich) used. The PCR reaction mixture contained 5.0 µL of DNA template (50 ng/l), 3.75 µL 5x Reaction buffer (Promega, USA), 0.85 µL of 10 µM primer, 0.09 µL of 2.5 U/l GO G2 Taq DNA Polymerase (Promega, USA), and 6.75 µL of sterile water (in a total volume of 20 µL). Biorad Thermo cycler was used for the amplification. Initial denaturation stage at 94 °C for 2 minutes, followed by 35 cycles of denaturation for 1 minute at 94 °C, annealing at 37 °C for 1 minute, extension at 72 °C for 2 minutes, and final extension at 72 °C for 5 minutes. Electrophoresis of the amplified products was performed on a 1.4% agarose gel in 1 TAE buffer (pH 8.0), with 0.01 µL/mL of EtBr for staining. Gel Documentation System was utilised to visualize and imaging of the gels (Bio-rad, USA). Size of amplicons was determined by comparing them to a 100 bp DNA ladder (GeneRuler Thermo Scientific, USA). The intensity of bands was ignored in favour of their ability to be reproduced and categorized as homologous only if they had the same migration.

**Table 6.5** Sequence details of RAPD primers.

| Sl.no | Primer code | Primer sequence (5′-3′) | GC content % |
|---|---|---|---|
| 1. | OPD 20 | ACCCGGTCAC | 70 |
| 2. | OPD 18 | GAGAGCCAAC | 60 |
| 3. | OPA 11 | CAATCGCCGT | 60 |
| 4. | OPA 18 | AGGTGACCGT | 60 |
| 5. | OPA 20 | GTTGCGATCC | 60 |
| 6. | OPC 02 | GTGAGGCGTC | 70 |

### 6.2.3.5 Biochemical fidelity assessment

The biochemical fidelity was evaluated by measuring camptothecin concentrations in in vitro plant samples and ex vitro mother plant material.

### 6.2.3.6 Plant sample preparation, extraction and HPTLC instrumentation

All the contaminants were removed by thoroughly rinsing the stem, leaves, and roots of in vitro and ex vitro cultivated plants with running water. In Chapter 3, we discussed the process to prepare a plant sample for analysis by High Performance Liquid Chromatography (Section 3.2.4; 3.2.5.3).

### 6.2.4 Statistical analysis

The tests were conducted with a minimum of 12 explants per treatment and in duplicate. Duncan's Multiple Range Test (DMRT) was used at a 5% significance level for each trial, and data was analyzed using SPSS 16.0 software (SPSS Inc., Chicago, USA). Results are shown as means ± standard errors.

## 6.3 Result and Discussion

### 6.3.1 *In vitro* Micropropagation of *O. mungos*

Methods for the successful in vitro micropropagation of *O. mungos* were developed in this study, using a variety of explants like leaves, nodes, internodes, and shoot tips. Explants tested on ½ MS media supplemented with varying concentrations of plant hormones showed variation in response, whereas control explants grown on plant hormone-free media showed no change. Different plant hormones have the following effects on callus development, shoot emergence, shoot proliferation, and root establishment:

#### 6.3.1.1 Surface sterilization

Standardizing the surface sterilization process for micropropagation of *O. mungos* required the use of a variety of surfactants for the various explants to ensure appropriate sterilization of explants. Five distinct treatments were utilized in the surface sterilization (Treatment-1, 2, 3, 4 and 5). The percentage of infected tissue, and the total number of tissues were all noted.

In treatment-1 70% ethanol treatment for 50 sec and $HgCl_2$(0.1%) was applied to sterilize the explants for 4 min and results showed that contamination was low (18.46 ± 0.30), and survival rate (10.73 ± 0.15) was found less (Table 6.6). In Treatment-2 in which tween 20(0.1%) and bavistin (0.1%) for 10mins and $HgCl_2$(0.1%) for 1-2 min used showed maximum contamination (89.30) and survival (3.63 ± 0.25). In Treatment -3 labolene (2 drops) for 15mins, tween 20(2-3drops) and Bavistin (1%) for 30mins, and sterilized with mercuric chloride $HgCl_2$(0.1%) for 2 min showed moderate contamination (28.50 ± 0.20), and survival rate (9.40 ± 0.30). In Treatment -4 labolene (3-4drops) for 15mins, tween 20 (2-3drops) and Bavistin (1%) for 30mins, 70% ethanol (30-40sec), surface sterilization with $HgCl_2$(0.1%) for 3 min showed moderate contamination (31.30 ± 0.20), and very low survival (2.36 ± 0.30). In Treatment -5 labolene (3-4drops) for 15mins, tween 20 (2-3drops) and Bavistin (1%) for 30mins, sterilized with $HgCl_2$(0.1%) for 3 minutes showed very low contamination (1.56 ± 0.25) with high survival rate (92.43 ± 0.25). Among all the treatment,

Treatment 5 had the highest survival rate and the lowest rates of contamination compared to other techniques as the optimal concentrations, along with suitable exposure durations of respective sterilizing agents, were found to be highly efficacious in surface sterilizing plant samples prior to inoculation. The utilization of Labolene (3-4drops) for a duration of 15 minutes proved advantageous in both the removal of extraneous particulate matter and acted as an effective disinfectant. Concurrently, Tween 20 (2-3drops) facilitated enhanced distribution and penetration of the sterilizing agent. Bavistin (1%) played a pivotal role in eradicating fungal contaminants within the sample. Lastly, the inclusion of $HgCl_2$ (0.1%), a broad-spectrum surface disinfectant, demonstrated its efficacy in eradicating a diverse spectrum of microorganisms, including bacteria and fungi. However, it is noteworthy that elevated concentrations or prolonged exposure times of these disinfectants can have adverse effects on plant cells. Conversely, insufficient concentrations and shorter exposure durations may fail to yield the desired disinfectant effect on the plant sample. Table 6.6 and Figure 6.1 show the aggregated outcomes across all treatments. Treatment-5 was deemed the optimal treatment for the surface sterilization of *O. mungos* explants because it resulted in the lowest rates of contamination and the highest explant survival rates. To achieve the highest possible rate of sterile culture for a variety of species, many scientists have utilized $HgCl_2$ for surface sterilization of explants at varying time exposures. The explants were typically rinsed using regular water and teepol, then surface sterilized with 0.1% w/v $HgCl_2$, occasionally in 70% ethanol, followed by 1% NaOCl with a drop of Tween 20 (Jaimsha et al., 2010; Jose & Satheeshkumar, 2004; Kai et al., 2008; Krishnan et al., 2018a; Ya-ut et al., 2011). Surface sterilization lowers undesired material and pathogens to improve explant survival.

**Table 6.6** *Effect of surface sterilization process.*

| Sterilization process | Contamination of explants (%) | Survival of explants (%) |
|---|---|---|
| Treament-1 | $18.46 \pm 0.30^{c}$ | $10.73 \pm 0.15^{b}$ |
| Treament-2 | $89.30 \pm 0.20^{a}$ | $3.63 \pm 0.25^{d}$ |
| Treament-3 | $28.50 \pm 0.20^{bc}$ | $9.40 \pm 0.30^{c}$ |
| Treament-4 | $31.30 \pm 0.20^{b}$ | $2.36 \pm 0.30^{d}$ |
| Treament-5 | $1.56 \pm 0.25^{d}$ | $92.43 \pm 0.25^{a}$ |

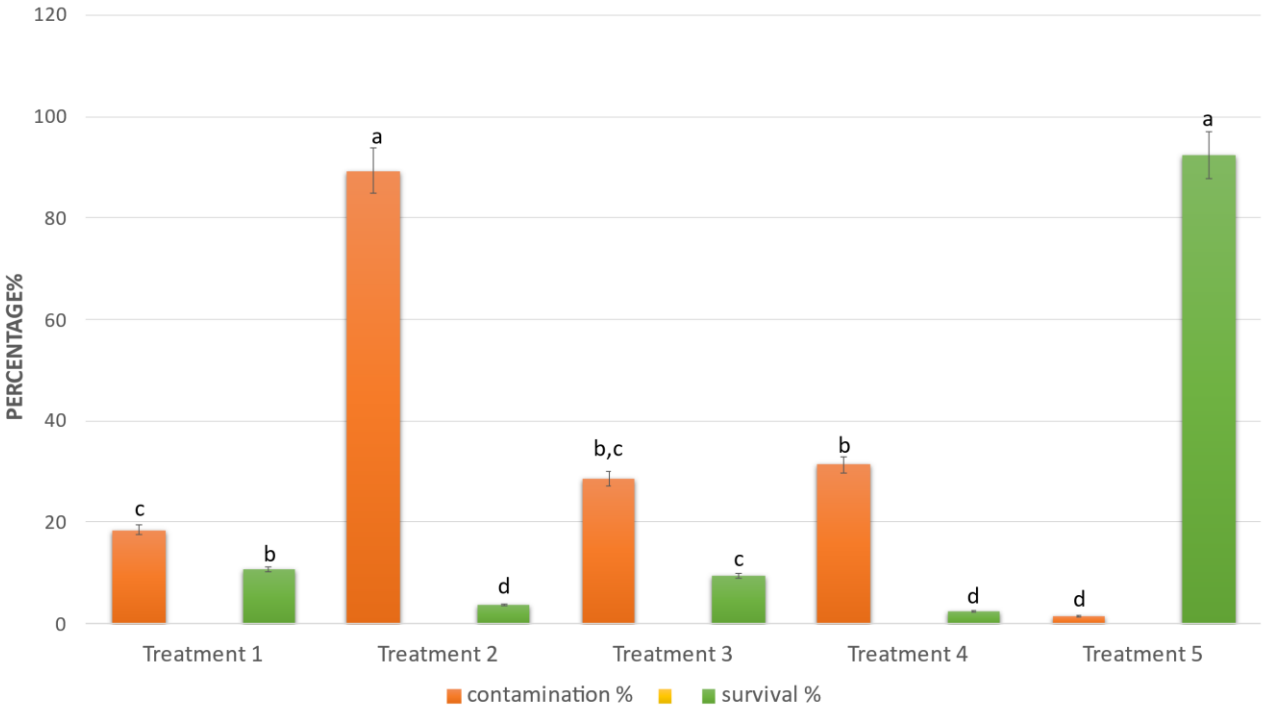



**Fig 6.1:** Effect of surface sterilization processes.

### 6.3.1.2 Callus induction

Explants from the shoot tip, node, and root were placed in ½ MS media supplemented with BAP, IBA, IAA, GA3, TDZ, and Picloram in concentrations ranging from 10-30µ M. Callus formation was observed with nodal, leaves, and root explants, but not with other explant types. The generation of callus was stimulated by the plant growth hormones BAP, IBA, GA3, TDZ, Piclo, and IAA. The percentage of callus formation, the type of callus, and the total number of calluses formed were all correlated strongly with PGR levels. When the concentration of BAP (20µM) and IAA (20µM) increased, response and callus amount also increased and reached maximum with 71% culture response in less number of days (27) in combination with GA3(10µM) (Table 6.7). Likewise, 65% culture response in 31 days was obtained when Piclo was added at 10µM concentration in combination with BAP (30µM) and GA3(10µM). Further increase in concentration of both PGRs showed decline in the culture response and callus amount. Callus was also obtained at 10:20:20 µM concentration of BAP, IAA and GA3 but in more days and with less culture response, however no callus initiation was perceived at 30:10:20 µM, 30:20:10 µM concentration of BAP: IBA: GA3 and 30:10:10 µM concentration of GA3: TDZ: Piclo. The callus produced was fragile in nature, green and brown in colour (Plate 6.2).

Our outcomes are in contract with that of Namdeo et al. (2012) who obtained green and brown fragile callus initiation from nodal segments of *O. mungos* on ½ MS and MS media fortified with BAP, IBA, GA3, TDZ, Piclo and IAA. In another study led by Kai et al. (2008), NAA alone or in combination with BA induced callus from in vitro leaf explant within 14 days in *O. japonica*. Deepthi & Satheeskumar (2015) induced callus on MS media augmented with KN, 2,4-D and NAA though observed best callus induction in MS fortified with KN(0.5mg/L), 2,4-D (1.0 mg/L) and NAA (3.0 mg/L) from leaves explants of *O. mungos*.

**Table 6.7** Effect of various PGRs on callus production from nodal explant.

| ½ MS + PGRs (µM) | | | | | | Response of Callus culture (%) | Days required for callus induction | Amount of callus | Nature of callus |
|---|---|---|---|---|---|---|---|---|---|
| **BA** | **IBA** | **GA3** | **TDZ** | **Piclo** | **IAA** | | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | - |
| 10 | 20 | 30 | 0 | 0 | 0 | 40 | 27 | ++ | *Gr* |
| 10 | 30 | 20 | 0 | 0 | 0 | 20 | 27 | + | *Gr* |
| *20* | *10* | *30* | *0* | *0* | *0* | *50* | *28* | ++ | *Gr* |
| 20 | 30 | 10 | 0 | 0 | 0 | 23 | 30 | + | *Gr* |
| 30 | 10 | 20 | 0 | 0 | 0 | 0 | - | - | - |
| 30 | 20 | 10 | 0 | 0 | 0 | 0 | - | - | - |
| 0 | 0 | 10 | 10 | 30 | 0 | 21 | 30 | + | *Br* |
| *0* | *0* | *10* | *30* | *10* | *0* | *55* | *30* | ++ | *Br* |
| 0 | 0 | 30 | 10 | 10 | 0 | 0 | - | - | - |
| 10 | 0 | 10 | 0 | 30 | 0 | 12 | 29 | + | *Gr* |
| *30* | 0 | *10* | 0 | *10* | 0 | *65* | *31* | +++ | *Gr* |
| 10 | 0 | 30 | 0 | 10 | 0 | 46 | 32 | ++ | *Gr* |
| 10 | 0 | 20 | 0 | 0 | 20 | 36 | 27 | + | *Gr* |
| *20* | *0* | *10* | *0* | *0* | *20* | *71* | *27* | +++ | *Gr* |
| 20 | 0 | 20 | 0 | 0 | 10 | 20 | 28 | ++ | *Gr* |

*- no callus: + low callus: ++ moderate callus: +++ high callus; Br: Brown friable callus; Gr: Green friable callus.

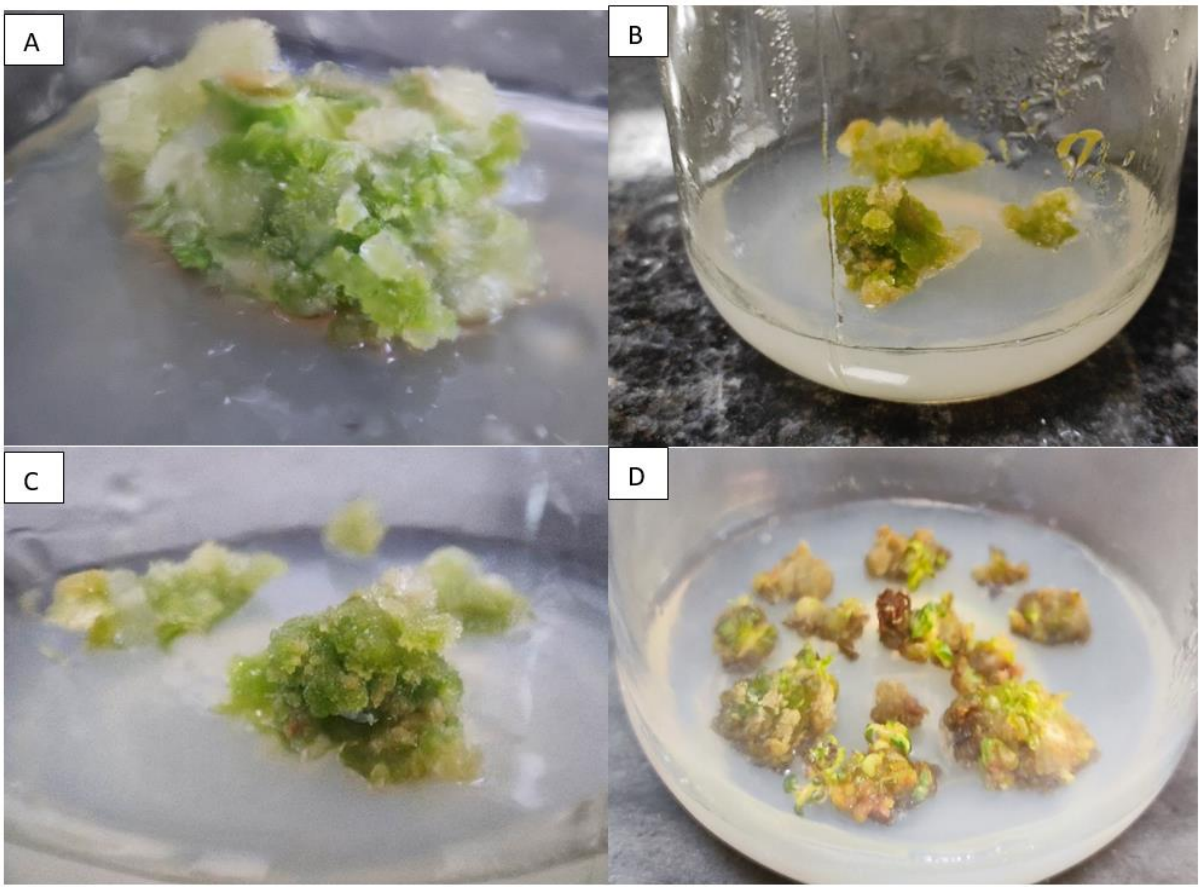



**Plate 6.2:** Callus production from nodal explant on ½ MS media comprising:
**(A)** BA: IAA:GA3 (20: 20: 10) **(B)** *BA: IBA:GA3 (20: 10: 30)* (**C)** *GA3: BAP: Piclo (10:30:10)* **(D)** *GA3: TDZ: Piclo (10: 30: 10)*

### 6.3.1.3 Direct organogenesis

Healthy *O. mungos* nodal segments were collected, surface sterilized, and resized before being inoculated onto ½ MS media supplemented with varying concentrations of BA alone or in combination with IBA, and KN to start shoot growth from the nodes (Plate-6.4). After inoculation, the cultures were kept in the culture room, where information about the percentage of culture response, the number of shoots per implant, and the average shoot length was recorded.

#### 6.3.1.3.1 Shoot initiation and regeneration

All PGRs have initiated shoots, however, the number of shoots per explant ranges from 1.66±0.03 to 4.62±0.06, and the average number of days for shoot induction ranges from 13.60±0.04 to 34.34±0.09, depending on the concentration of BAP and KIN in the culture media (Table 6.8). Multiple concentrations of BAP and KIN added to the ½ MS media were found to have a significant effect on shoot initiation. Nevertheless, ½ MS media supplemented with KIN exhibited delayed initiation and weak shoot start. Even after extending the culture time beyond what would normally be considered normal, explants grown in ½ MS media devoid of any hormone (control) did not commence shoot induction (Figure 6.2). The percentage of cultural responses varied from 46.31±0.06 to 98.59±0.05 throughout ½ MS media fortified with varying concentrations BAP. BAP was beneficial in 98.59 % of cultures at 13.37µM, resulting in shoot induction at 13.60±0.04 days and total of 4.62±0.06 shoots. The number of shoots produced by BAP and KIN in ½ MS media ranges from 1.66±0.03 to 4.62±0.06, and their percentage response vary from 40.72±0.15 to 98.59±0.05 (Plate 6.4). In 98.59% of cultures, a mean of 4.62±0.06 shoots were generated, when media was supplemented with BA at a concentration of 13.37 µM. In contrast, only 46.31% of cultures generated a mean of 3.24±0.04 shoots, when ½ MS media was supplemented with BAP (4.5 µM). Media supplemented with varying amounts of KIN resulted in inferior shoot induction, with a mean shoot number of 1.69±0.01 to 2.72±0.04 and a mean percentage response of 41.38± 0.06 to 41.72± 0.15. 72% of cultures showed shoot regeneration when BAP and KIN were both applied at a dosage of 13.37 µM. The average number of days for shoot induction was 22. The induction of shoots was seen regardless of the concentration of BAP used in the media (Table 6.8). On media with 13.37 µM of both BAP and KIN, 72.00±0.58 % of cultures developed 3.41±0.05 mean number of shoots, while only 58.22±0.40 % of cultures created 2.18±0.10 number of shoots on media

with 13.37 µM BAP and 22.25 µM KIN. 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. Our results are in accordance with Krishnan et al. (2018) and Roja (2008) that attained shoot formation from *O. mungos* var *angustifolia* nodal and shoot tips explants and *O. rugosa* var *decumbans* on media augmented with BAP. Shahnaz Beegum et al. (2007) and Jaishma et al. (2010) too obtained direct shoot regeneration in *O. prostrata* and *O. eriantha* on ½ MS media amended with BAP. In various earlier reports BAP along with KN has been used commonly for direct shoot initiation (Gopalkrishnan & Shanker, 2014; Krishnan et al., 2018a). In a study comparing BAP, KN, and IAA for direct shoot regeneration from *O. prostrata* seedlings explants, Gopalkrishnan et al. (2018) showed that BAP-amended medium resulted in the highest rates of shoot regeneration. Direct shoot regeneration from *Viola odorata* crown explants was maximized on MS media containing BAP (3 or 4 mg/L) and IBA (0.5 mg/L). Kumar et al. (2017) regenerated *D. deltoidea* shoots from nodal segments in medium supplemented with BAP, IBA, NAA, and TDZ, with the combination of BAP and IBA inducing greatest regeneration. Using direct organogenesis, Hesami et al. (2019) found that the maximum shoot initiation occurred in media supplemented with BAP at 1.5 mg/L of concentration and 0.15 mg/L IBA, with a mean number of 6.26 and 10.13 shoots/explant, respectively.

**Table 6.8** Effect of different concentration of cytokinins on shoot induction on ½ MS after 4 weeks of culture.

| **BAPµM** | **KINµM** | **Mean no. of days for shoot induction** | **Mean no. of shoots/explant** | **Percentage of shoot induction (%)** |
|---|---|---|---|---|
| 0 | 0 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 4.5 | - | 22.39±0.20$^{f}$ | 3.24±0.04$^{b}$ | 46.31±0.06$^{m}$ |
| 8.94 | - | 15.83±0.17$^{h}$ | 4.34±0.03$^{a}$ | 85.68±0.19$^{b}$ |
| 13.37 | - | 13.60±0.04$^{i}$ | 4.62±0.06$^{a}$ | 98.59±0.05$^{a}$ |
| 17.22 | - | 16.31±0.06$^{h}$ | 2.31±0.06d$^{e}$ | 84.18±0.10$^{c}$ |
| 22.25 | - | 20.39±0.20$^{g}$ | 4.41±0.05$^{a}$ | 54.83±0.44$^{k}$ |
| - | 4.5 | 34.34±0.09$^{a}$ | 1.69±0.01$^{f}$ | 41.38±0.06$^{op}$ |
| - | 8.94 | 22.67±0.33$^{ef}$ | 2.52±0.08$^{cde}$ | 41.56±0.29$^{o}$ |
| - | 13.37 | 20.28±0.04$^{g}$ | 2.44±0.08$^{cde}$ | 47.10±0.10$^{l}$ |
| - | 17.22 | 22.67±0.33$^{ef}$ | 2.72±0.04$^{e}$ | 41.72±0.15$^{o}$ |
| - | 22.25 | 26.17±0.10$^{b}$ | 1.66±0.03$^{f}$ | 40.72±0.15$^{p}$ |
| 8.94 | 4.5 | 24.00±0.58$^{d}$ | 3.28±0.04$^{b}$ | 42.49±0.11$^{n}$ |
| 8.94 | 8.94 | 23.62±0.23$^{de}$ | 3.33±0.33$^{b}$ | 62.49±0.11$^{f}$ |
| 8.94 | 13.37 | 20.00±0.58$^{g}$ | 4.28±0.15$^{a}$ | 66.52±0.08$^{e}$ |
| 8.94 | 17.22 | 22.52±0.08$^{f}$ | 3.62±0.06$^{b}$ | 54.41±0.10$^{k}$ |
| 8.94 | 22.25 | 20.59±0.05$^{g}$ | 3.59±0.05$^{b}$ | 55.77±0.15$^{j}$ |
| 13.37 | 4.5 | 25.18±0.10$^{c}$ | 2.66±0.03$^{cd}$ | 62.50±0.29$^{f}$ |
| 13.37 | 8.94 | 22.44±0.29$^{f}$ | 3.38±0.06$^{b}$ | 61.57±0.30$^{g}$ |
| 13.37 | 13.37 | 22.00±0.58$^{gef}$ | 3.41±0.05$^{b}$ | 72.00±0.58$^{d}$ |
| 13.37 | 17.22 | 23.00±0.58$^{ef}$ | 2.33±0.33$^{de}$ | 60.52±0.08$^{h}$ |
| 13.37 | 22.25 | 25.11±0.59$^{c}$ | 2.18±0.10$^{e}$ | 58.22±0.40$^{i}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

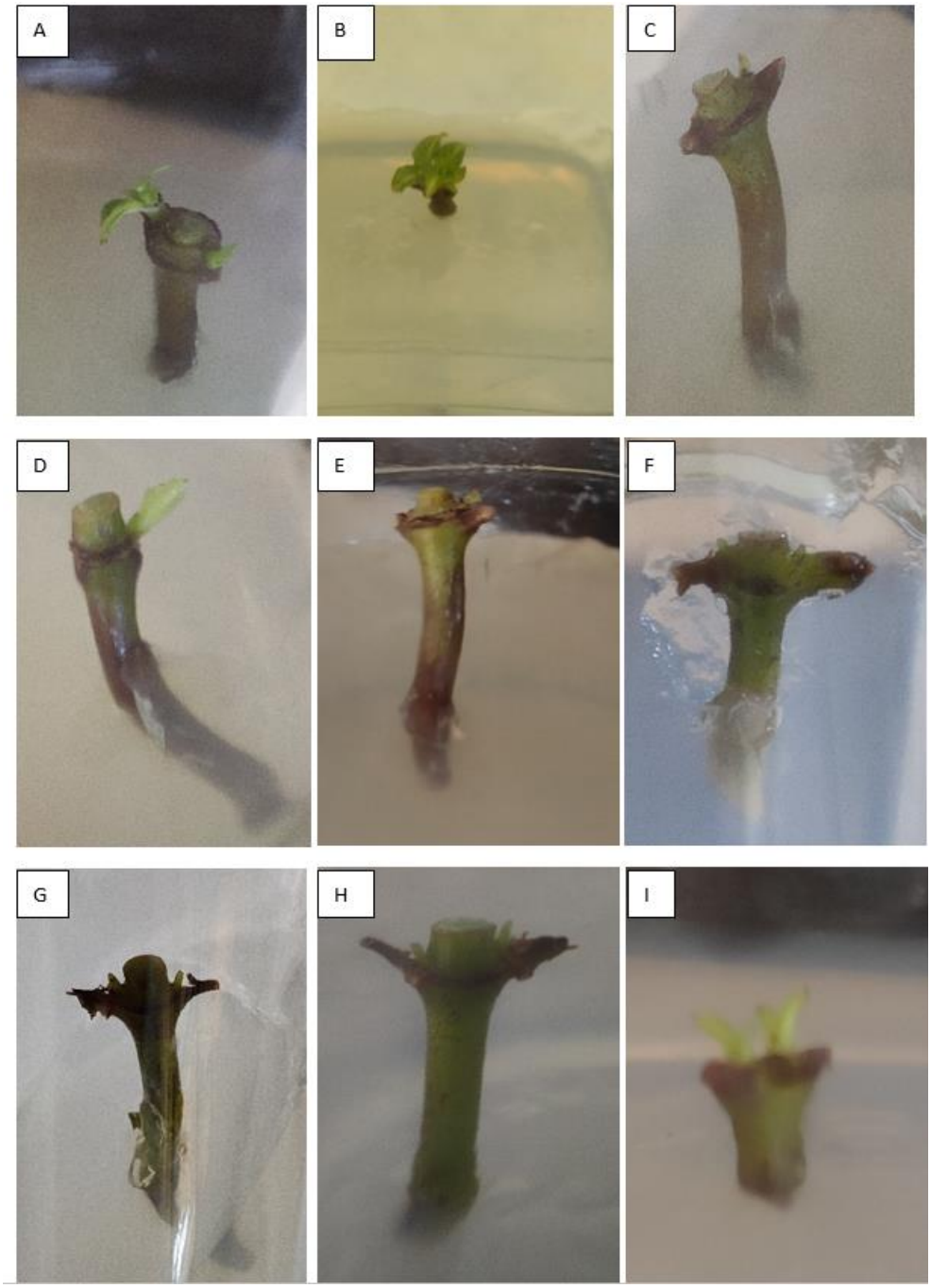



**Plate 6.3:** Effect of different growth regulators on shoot induction on ½ MS media

***(A)*** *BA 8.95µM* ***(B)*** *BA 13.37µM* ***(C&D)*** *BA 17.22 µM* ***(E-G)*** *BA 22.25 µM* ***(H)*** *KIN 13.37 µM* ***(I)*** *BA 13.37 µM + KIN 13.37 µM*

#### 6.3.1.3.2 Shoot multiplication

After the initiation stage, the young shoots were transplanted into various media, including solid ½ MS media (with agar) and liquid ½ MS media (without agar) supplemented with varying doses of BA, IBA and mT. Data was collected about the percentage of multiplication response, the number of shoots/ explants, and the average shoot length following subculture in multiplication media after 4 weeks of culture. (Table 6.9, 6.10 and 6.11).

##### 6.3.1.3.2.1 Impact of BA, IBA and metatopolin on shoot proliferation

For the micropropagation of the *O. mungos* plant, a reliable and effective regeneration protocol was created. 13-22 days after culture, shoot initiation from nodal segments was noticed. Tables 6.9, 6.10 and 6.11 illustrate the effects of several PGRs on shoot regeneration. The highest frequency of shoot initiation (85%) and shoot multiplication (98%) were seen in a ½ MS medium along with 8.94 µM BAP and 13.37 µM BAP, respectively (Fig. 6.2& Fig. 6.3). The preexisting axillary primordia may be responsible for the direct shoot proliferation from nodal explants, which multiplied into shoots as a result of the media addition of cytokinin (BAP 4.5 µM-22.25 µM). At an optimum concentration of 13.37 µM BAP with 98% of shoot induction, effective shoot regeneration (4.62± 0.06 shoots per explant) was seen. A reduction in shoot growth was seen when BAP and IBA concentrations were increased. Other *O. mungos var angustifolia* species showed similar outcomes, according to reports ((Krishnan et al., 2018)). According to studies, *O. decumbans, O. eriantha*, and *O. japonica* incubated with combination of IBA and NAA showed promoter effects on shoot production whereas *O. mungos* var. *angustifolia* showed commencement of shoot proliferation from nodal explants by the effect of IBA BAP (Gopalkrishnan and Shanker, 2014; Jaimsha et al., 2010; Kai et al., 2008; Krishnan et al., 2018a).

Liquid ½ MS media was fortified with different combinations of BA (4.5 µM-22.25 µM) and IBA (0.49-2.46 µM). Shoot multiplication was observed in all the BA and IBA combinations and the percentage of response culture ranges from 30.00±0.29 to 99.51±0.06 % and the shoot numbers ranges from 4.33±0.33 to 16.97±0.26 with an average length of 0.29±0.01 to 0.89 ± 0.01 cm (Table 6.10). When BA was 4.50µM in combination with IBA (0.49 – 2.46 µM) on the ½ MS media, 30.00 ± 0.29 to 35.27 ± 0.12% of cultures were

responded with 4.33 ± 0.33 to 6.03 ± 0.15 mean number of shoots/explant were produced having average shoot length 0.29 ± 0.01 to 0.39 ± 0.01 cm. ½ MS media augmented with a combination of BA 8.94 µM and IBA (0.49 – 2.46 µM) produced 7.70 ± 0.35 to 16.17 ± 0.44 mean number of shoots/explant with 0.48 ± 0.02 to 0.87 ± 0.03 cm average shoot length (Figure 6.4). The response percentage of shoot multiplication in this media was in the range of 64.86 ± 0.09 to 99.51 ± 0.06%. ½ MS media fortified with BA 13.37 in combination with various IBA concentrations regenerated 9.97 ± 0.09 to 16.97 ± 0.26 mean number of shoots with 0.51 ± 0.01 to 0.89± 0.01 cm average shoot length (Plate 6.6). The best percentage of culture responses 99.43 ± 0.19% were recorded in media containing BA 13.37 µM and IBA 1.96 µM and produced highest number of shoots/ explants 16.97 ± 0.26 with 0.88 ± 0.02cm shoot length (Plate 6.6: G-I). This combination was considered the optimum PGR combination for shoot multiplication in *O. mungos* among all treatments. When concentration of BA was increased to 17.22 µM and used in combination with IBA there was decline in the number of shoots and percentage of response. The response percentage was recorded as 57.90 ± 0.15 to 73.00 ± 0.03% and shoot numbers per explant as in the range of 6.00 ± 0.58 to 12.67 ± 0.33 with 0.36 ± 0.02 to 0.59 ±0.01 cm average shoot length. Some plant species have been observed to benefit from a combination of auxins and cytokinins in previous research (Khan et al., 2011; Revathi et al., 2020; Singh et al., 2014; Xu et al., 2008). Our findings are in agreement with those Krishnan et al. (2018), who cultivated plants in solid ½ MS media supplemented with BAP (8.8 µM) and IBA (1.96 µM) and found a mean of 18.67±0.58 shoots per plant with a mean shoot length of 0.4±0.1 cm. In another study, Shahnaz Beegum et al. (2007) recorded maximum numbers of multiple shoots (76) per explant on media fortified with 8.87 µM BA and 2.46 µM IBA. Roja (2008) obtained multiple shoots/explant of *O. rugosa* var *decumbens* on the MS medium, ½ MS and Zenk's production medium (Z), either with no hormone or combination of BA and NAA. Using liquid MS media, Piatczak et al. (2005) successfully multiplied *Centaurium erythraea* shoots into 60 microshoots in under 4 weeks, although they did it by adding IAA rather than IBA. Shoot tip explants of *Phoenix dactylifera* L. were cultivated by Mazri (2015) in both liquid and semi-solid half MS media modified with several plant hormones, with the highest shoot multiplication being observed in semi-solid half MS media fortified with 3 µM IBA and 3 µM BAP. *Curcuma zedoaria* explants grown in multiplication media supplemented with 13.31 µM $L^{-1}$ BAP and 2.85 µM $L^{-1}$ IBA produced an average of 5.6 shoots per explant, according to Loc et al. (2005). On Schenk and Hildebrandt (SH) medium fortified with 5 µM BA and 1 µM IBA, Brassard et al.

(1996) successfully induced and propagated *Larix x eurolepis* Henry shoots. With 8 mg/L of BA and 1 mg/L of IBA added to the multiplication media, the average number of lateral branches per stem for *Ficus anastasia* shoots multiplied in vitro was 20 (Al Malki & Elmeer, 2010).

After four weeks of culture, the effects of mT (0-12.25 µM) on shoot proliferation in vitro are shown in Table 6.11. Maximum shoot number (14.33±0.88) and shoot length (0.78±0.02 cm) seen at a concentration of 0.75 µM and 0.5µM mT respectively (Fig.6.5), above which mT gradually inhibited shoot proliferation in *O. mungos*. When compared to BA and the control, the shoot regeneration rate is significant at all concentrations. These findings are consistent with recent research showing that mT has significant shoot proliferation potential in several plant species, including *Scaevola taccada* (Gaertn.) Roxb. (Shekhawat et al., 2021), *C. malabaricum* (Chahal et al., 2022), *C.brachynema* (Kaur et al., 2022) and *Dioscorea pentaphylla* L. (Manokari et al., 2022). As previously mentioned, mT has been shown to significantly induce shoot proliferation in a wide variety of plant species, including *Aloe* spp. (Bairu et al., 2007), *Pelargonium* cultivars (Wojtania, 2010), *Huernia hystrix* (Hook.f.) N.E.Br. (Amoo & Van Staden, 2013).

**Table 6.9** Impact of solid ½ MS media containing BA and IBA on in vitro shoot multiplication from nodal explants of *O. mungos* after 4 weeks of culture.

| **Solid ½ MS + growth regulators µM** | | **% response** | **Mean no. of shoots/explant** | **Shoot length (cm)** |
|---|---|---|---|---|
| **BA** | **IBA** | | | |
| 0 | 0 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00±0.00 |
| 4.50 | 0.49 | $35.00\pm0.29^{r}$ | $4.67\pm0.33^{m}$ | $0.33\pm0.03^{h}$ |
| 4.50 | 0.98 | $31.68\pm0.34^{s}$ | $5.67\pm0.33^{klm}$ | $0.27\pm0.01^{h}$ |
| 4.50 | 1.47 | $30.40\pm0.21^{t}$ | $5.67\pm0.33^{klm}$ | $0.34\pm0.02^{h}$ |
| 4.50 | 1.96 | $36.43\pm0.35^{q}$ | $7.33\pm0.33^{ij}$ | $0.29\pm0.01^{h}$ |
| 4.50 | 2.46 | $29.43\pm0.23^{t}$ | $5.33\pm0.33^{lm}$ | $0.33\pm0.04^{h}$ |
| 8.94 | 0.49 | $66.57\pm0.34^{i}$ | $8.67\pm0.33^{hi}$ | $0.49\pm0.01^{fg}$ |
| 8.94 | 0.98 | $74.47\pm0.48^{e}$ | $9.33\pm0.33^{gh}$ | $0.57\pm0.02^{cdef}$ |

| | | | | |
|---|---|---|---|---|
| 8.94 | 1.47 | 75.97±0.26$^{d}$ | 12.33±0.33$^{de}$ | 0.76±0.02$^{b}$ |
| 8.94 | 1.96 | 99.17±0.60$^{a}$ | 18.00±0.58$^{a}$ | 0.84±0.01$^{a}$ |
| 8.94 | 2.46 | 83.27±0.37$^{c}$ | 15.00±0.58$^{c}$ | 0.76±0.03$^{b}$ |
| 13.37 | 0.49 | 69.47±0.48$^{g}$ | 10.33±0.33$^{fg}$ | 0.55±0.03$^{def}$ |
| 13.37 | 0.98 | 72.03±0.15$^{f}$ | 11.00±0.58$^{ef}$ | 0.53±0.01$^{ef}$ |
| 13.37 | 1.47 | 75.57±0.32$^{d}$ | 12.33±0.33$^{de}$ | 0.63±0.01$^{cd}$ |
| 13.37 | 1.96 | 99.73±0.15$^{a}$ | 19.00±0.58$^{a}$ | 0.85±0.03$^{a}$ |
| 13.37 | 2.46 | 93.43±0.54$^{b}$ | 16.33±0.67$^{b}$ | 0.85±0.08$^{a}$ |
| 17.22 | 0.49 | 60.40±0.31$^{k}$ | 7.67±0.88$^{ij}$ | 0.44±0.03$^{g}$ |
| 17.22 | 0.98 | 57.97±0.15$^{l}$ | 7.67±0.33$^{ij}$ | 0.35±0.01$^{h}$ |
| 17.22 | 1.47 | 68.10±0.44$^{h}$ | 11.33±0.33$^{ef}$ | 0.51±0.02$^{fg}$ |
| 17.22 | 1.96 | 72.23±0.50$^{f}$ | 13.33±0.33$^{d}$ | 0.56±0.01$^{def}$ |
| 17.22 | 2.46 | 62.87±0.41$^{j}$ | 11.67±0.33$^{ef}$ | 0.45±0.02$^{g}$ |
| 22.25 | 0.49 | 42.97±0.32$^{n}$ | 7.00±0.58$^{jk}$ | 0.62±0.01$^{cd}$ |
| 22.25 | 0.98 | 38.53±0.26$^{p}$ | 6.67±0.33$^{jkl}$ | 0.55±0.01$^{def}$ |
| 22.25 | 1.47 | 36.77±0.15$^{q}$ | 7.67±0.33$^{ij}$ | 0.64±0.02$^{c}$ |
| 22.25 | 1.96 | 44.90±0.15$^{m}$ | 7.67±0.67$^{ij}$ | 0.56±0.02$^{def}$ |
| 22.25 | 2.46 | 40.33±0.20$^{o}$ | 6.67±0.33$^{jkl}$ | 0.60±0.01$^{cde}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

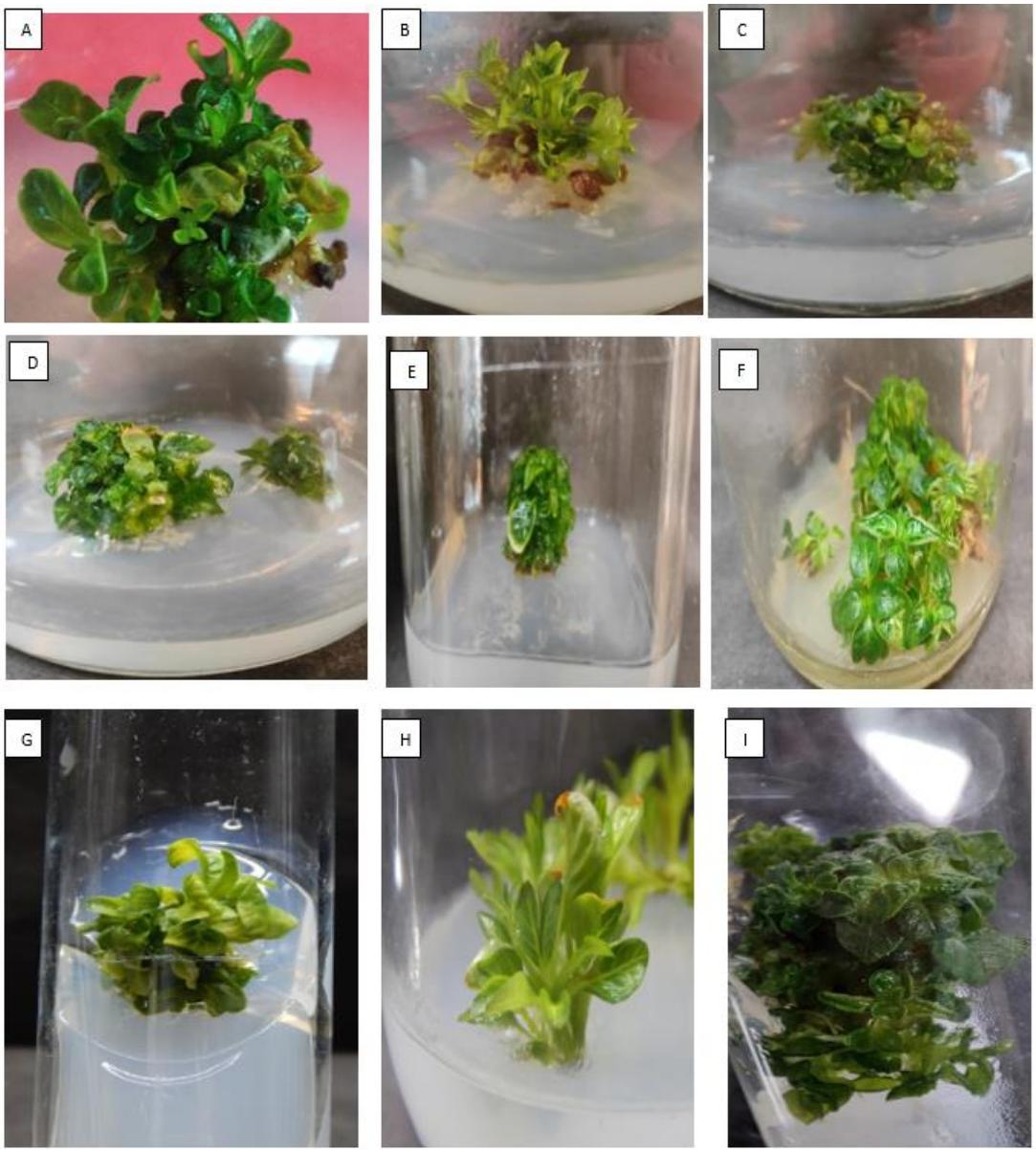



**Plate 6.4:** Shoot multiplication on solid ½ MS medium supplemented with varying combinations of BA and IBA ***(A)****8.94µM BA+0.98 µM IBA;* ***(B)*** *8.94µM BA+1.47 µM IBA;****(C)*** *8.94µM BA+1.96 µM IBA;****(D)*** *8.94µM BA+2.46 µM IBA;* ***(E)*** *13.37µM BA+1.47 µM IBA;* ***(F-G)*** *13.37µM BA+1.96 µM IBA;* ***(H-I)*** *13.37µM BA+2.46 µM IBA.*

**Table 6.10** Effect of liquid ½ MS media containing BA and IBA on in vitro shoot multiplication from nodal explants of *O. mungos* after 4 weeks of culture.

| **Liquid ½ MS + growth regulators µM** | | **% response** | **Shoots/explant** | **Shoot length (cm)** |
|---|---|---|---|---|
| **BA** | **IBA** | | | |
| 0 | 0 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 4.50 | 0.49 | 33.50±0.17$^{r}$ | 4.33±0.33$^{j}$ | 0.39±0.01$^{h}$ |
| 4.50 | 0.98 | 32.69±0.17$^{s}$ | 5.00±0.12$^{j}$ | 0.38±0.04$^{h}$ |
| 4.50 | 1.47 | 32.36±0.20$^{rs}$ | 4.77±0.15$^{j}$ | 0.30±0.01$^{i}$ |
| 4.50 | 1.96 | 35.27±0.12$^{q}$ | 6.03±0.15$^{i}$ | 0.29±0.01$^{j}$ |
| 4.50 | 2.46 | 30.00±0.29$^{t}$ | 4.63±0.07$^{j}$ | 0.35±0.03$^{hi}$ |
| 8.94 | 0.49 | 64.86±0.09$^{h}$ | 7.70±0.35$^{h}$ | 0.48±0.02$^{g}$ |
| 8.94 | 0.98 | 75.24±0.14$^{de}$ | 8.27±0.20$^{h}$ | 0.48±0.02$^{g}$ |
| 8.94 | 1.47 | 76.03±0.5$^{8d}$ | 11.23±0.34$^{f}$ | 0.69±0.01$^{c}$ |
| 8.94 | 1.96 | 99.51±0.06$^{a}$ | 16.17±0.44$^{b}$ | 0.87±0.03$^{a}$ |
| 8.94 | 2.46 | 84.63±0.15$^{c}$ | 14.17±0.09$^{d}$ | 0.86±0.02$^{a}$ |
| 13.37 | 0.49 | 68.08±0.46$^{g}$ | 9.97±0.09$^{g}$ | 0.59±0.01$^{de}$ |
| 13.37 | 0.98 | 72.72±0.12$^{f}$ | 11.17±0.09$^{f}$ | 0.51±0.01$^{fg}$ |
| 13.37 | 1.47 | 74.47±0.26$^{e}$ | 11.30±0.25$^{f}$ | 0.73±0.02$^{b}$ |
| 13.37 | 1.96 | 99.43±0.19$^{a}$ | 16.97±0.26$^{a}$ | 0.88±0.02$^{a}$ |
| 13.37 | 2.46 | 93.14±0.13$^{b}$ | 15.40±0.23$^{c}$ | 0.89±0.01$^{a}$ |
| 17.22 | 0.49 | 61.37±0.29$^{j}$ | 6.03±0.09$^{i}$ | 0.53±0.01$^{gh}$ |
| 17.22 | 0.98 | 57.90±0.15$^{k}$ | 6.00±0.58$^{i}$ | 0.36±0.02$^{h}$ |
| 17.22 | 1.47 | 67.31±0.15$^{g}$ | 10.60±0.31$^{fg}$ | 0.49±0.01$^{fg}$ |
| 17.22 | 1.96 | 73.00±0.03$^{f}$ | 12.67±0.33$^{e}$ | 0.59±0.01$^{de}$ |
| 17.22 | 2.46 | 63.30±0.33$^{i}$ | 10.40±0.21$^{g}$ | 0.49±0.01$^{fg}$ |
| 22.25 | 0.49 | 42.67±0.29$^{h}$ | 6.10±0.21$^{i}$ | 0.64±0.03$^{cd}$ |
| 22.25 | 0.98 | 37.80±0.15$^{o}$ | 6.80±0.15$^{i}$ | 0.60±0.01$^{de}$ |

| | | | | |
|---|---|---|---|---|
| 22.25 | 1.47 | 36.17±0.15$^{p}$ | 6.60±0.15$^{i}$ | 0.63±0.02$^{d}$ |
| 22.25 | 1.96 | 45.60±0.29$^{l}$ | 6.47±0.12$^{i}$ | 0.49±0.01$^{fg}$ |
| 22.25 | 2.46 | 40.61±0.29$^{n}$ | 6.37±0.09$^{i}$ | 0.55±0.02$^{ef}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

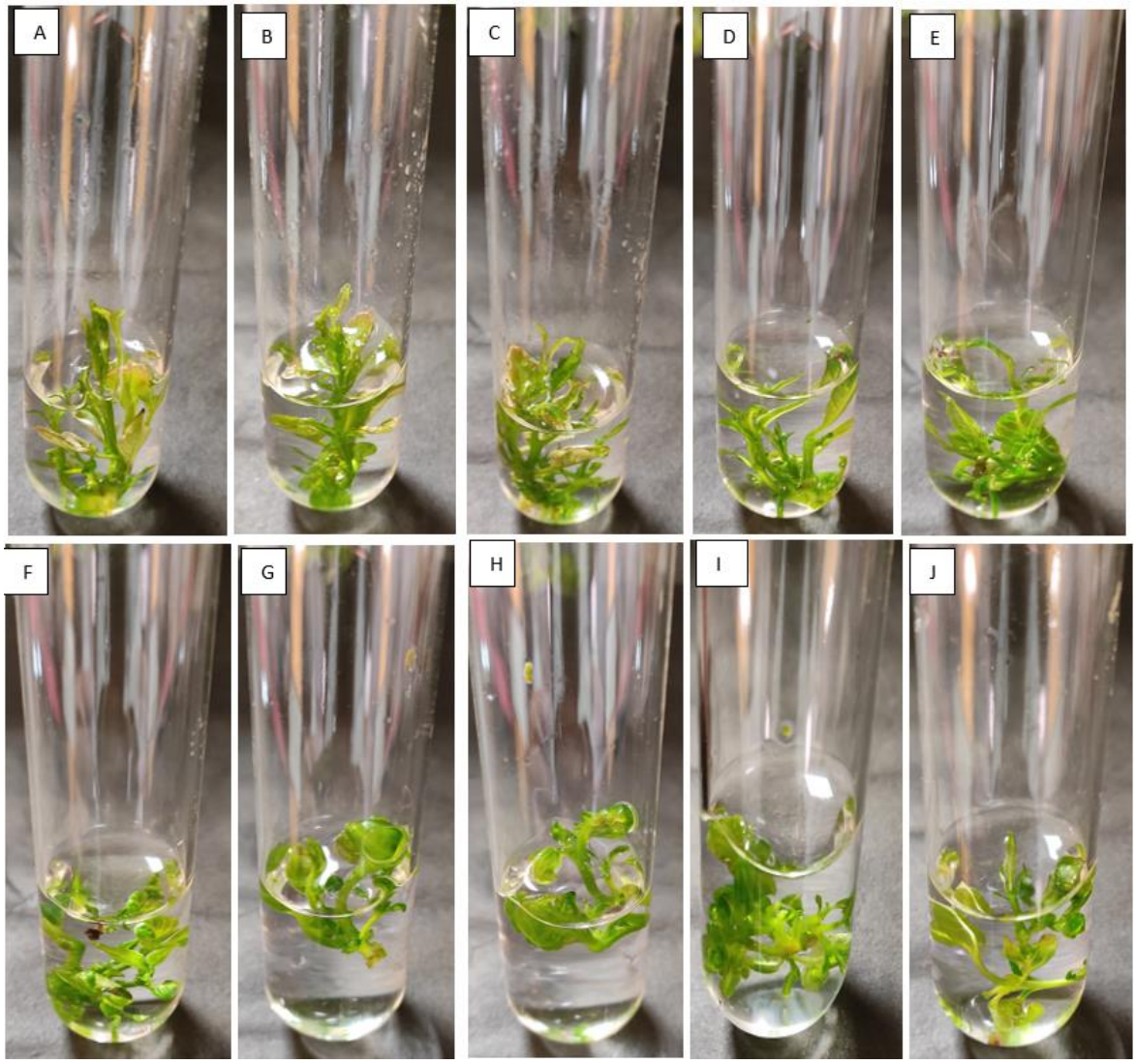



**Plate 6.5:** Shoot multiplication on liquid ½ MS medium supplemented with varying combinations of BA and IBA.

***(A-B)****8.94µM BA+0.98 µM IBA;* ***(C)*** *8.94µM BA+1.47 µM IBA;****(D)*** *8.94µM BA+1.96 µM IBA;****(E)*** *8.94µM BA+2.46 µM IBA;* ***(F)*** *13.37µM BA+1.47 µM IBA;* ***(G-I)*** *13.37µM BA+1.96 µM IBA;* ***(J)*** *13.37µM BA+2.46 µM IBA.*

**Table 6.11** Impact of different concentration of metatopolin (mT) on solid ½ MS media on in vitro shoot and root multiplication from in vitro shoots of *O. mungos* after 4 weeks of culture.

| mT conc. | No. of shoots/explant | Shoot length | No. of roots/plant | Root length |
|---|---|---|---|---|
| 0 µM | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 0.25µM | 11.67±0.33$^{bc}$ | 0.54±0.01$^{c}$ | 2.67±0.67$^{a}$ | 2.78±1.21$^{a}$ |
| 0.5µM | 14.00±0.58$^{a}$ | 0.78±0.02$^{a}$ | 3.67±0.33$^{a}$ | 2.48±0.13$^{ab}$ |
| 0.75µM | 14.33±0.88$^{a}$ | 0.72±0.02$^{b}$ | 2.33±0.88$^{a}$ | 1.37±0.44$^{bc}$ |
| 1.0µM | 12.93±0.52$^{ab}$ | 0.48±0.02$^{d}$ | 2.33±0.88$^{a}$ | 1.13±0.37$^{bc}$ |
| 1.25µM | 11.67±0.33$^{bc}$ | 0.45±0.01$^{d}$ | 0.67±0.33$^{b}$ | 0.63±0.45$^{c}$ |
| 2.5µM | 10.67±0.33$^{cd}$ | 0.44±0.03$^{d}$ | 0 | 0 |
| 5µM | 10.00±0.00$^{d}$ | 0.54±0.01$^{c}$ | 0 | 0 |
| 7.5µM | 9.29±0.46d$^{e}$ | 0.56±0.01$^{c}$ | 0 | 0 |
| 10µM | 8.17±0.44$^{e}$ | 0.55±0.01$^{c}$ | 0 | 0 |
| 12.5µM | 8.39±0.06$^{e}$ | 0.47±0.01$^{d}$ | 0 | 0 |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

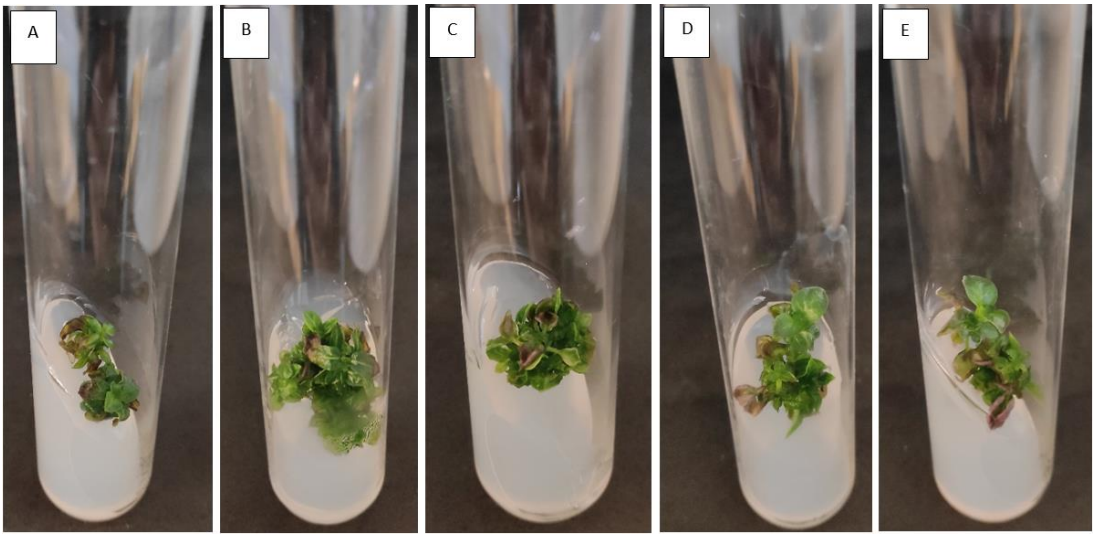



**Plate 6.6:** Effect of metatopolin (mT) on shoot multiplication and rooting on solid ½ MS medium

***(A)*** *0.25µM mT* ***(B)*** *0.50µM mT* ***(C)*** *0.75µM mT* ***(D)*** *1.00µM mT* ***(E)*** *1.25µM mT.*

#### 6.3.1.3.3 Shoot elongation

Multiple shoots regenerated in ½ MS media supplemented with BAP ana IBA after four weeks where subcultured in ½ MS media fortified with varying concentrations of GA3(5-30µM) for shoot elongation. Longest shoot length (5.05±0.06cm) was seen in 20 µM GA3 and shortest(2.42±0.04cm) in 5 µM. GA3 is the most common hormone used for shoot elongation in plant tissue culture. In an experiment, Kaushik et al. (2015) used GA3 for shoot elongation in *O. mungos* and reported 1mg/L as the most suitable concentration for shoot elongation.

**Table 6.12** Impact of GA3 on in vitro shoot elongation.

| GA3µM | No.of shoots±SE | Shoot length±SE(cm) |
|---|---|---|
| 0 | 0.00±0.00 | 0.00±0.00 |
| 5 | $7.67\pm0.33^{bc}$ | $2.42\pm0.04^{c}$ |
| 10 | $9.00\pm0.58^{ab}$ | $4.68\pm0.04^{b}$ |
| 20 | $9.90\pm0.21^{a}$ | $5.05\pm0.06^{a}$ |
| 30 | $7.00\pm0.58^{c}$ | $4.67\pm0.12^{b}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

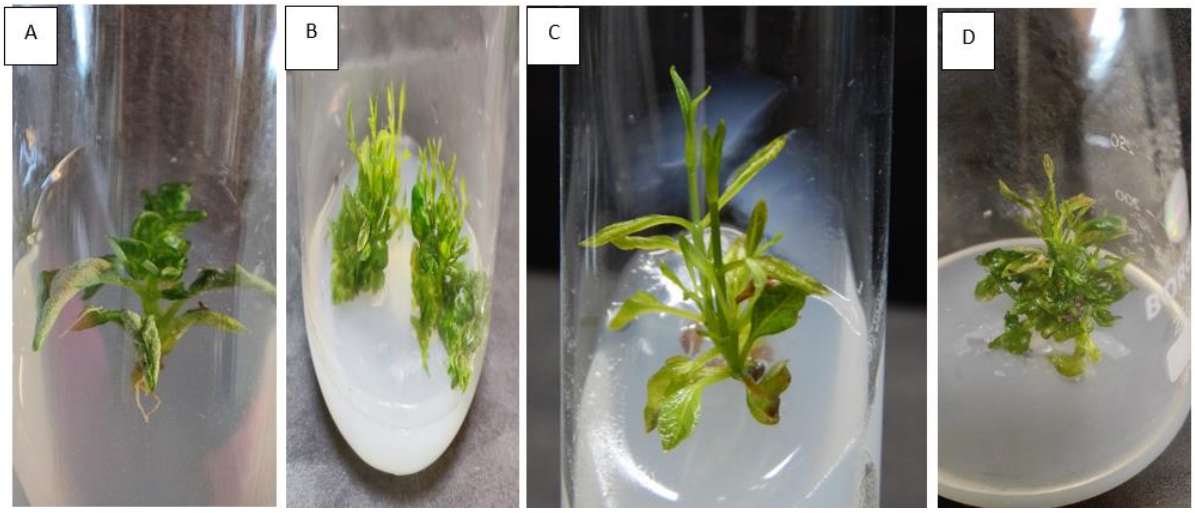



**Plate 6.7:** Shoot elongation from multiple shoots on solid ½ MS medium ***(A)****10µM GA3;* ***(B-C)*** *20µM GA3;* ***(D)*** *30µM GA3.*

#### 6.3.1.3.4 Root formation

One of the most crucial steps in micropropagation is rooting, which involves inducing roots in previously produced shoots in vitro. Rooting media were needed for the successfully regenerated shoots to develop. The ability of plant tissues to promote the induction of adventitious roots is dependent on the interplay of a number of endogenous and exogenous variables. Several researchers have confirmed that auxins (IBA and NAA) and cytokinin (BAP) play a crucial role in inducing roots to grow (Scott, 1972; Gaspar et al, 1996; Gaba, 2005). The success of the acclimatization process depends in part on how well in vitro shoots are able to establish roots (Gonçalves et al., 1998). In this experiment, both BA and IBA were present in the liquid shoot multiplication medium at the same time, allowing for simultaneous rooting. IBA triggered spontaneous roots in the propagation media. Rooting could not be induced by BA alone in medium. To root well on liquid ½ MS media, either a high concentration of BA or a low concentration of IBA is sufficient. In addition, ½ MS media fortified with varying amounts of NAA was employed as a rooting medium. Table 6.13 detail the effect of various rooting media on the resulting culture response, root density per transplant, and root length. Root induction was detected within 5 weeks of subculture in all combinations tested except PGR free media (Table 6.13 and 6.14). Liquid ½ MS media fortified with BA and IBA showed best rooting (Table 6.14; Plate 6.9). Maximum response 99.54% with 20.21 ± 0.12 roots/explant (Plate 6.10: A&B) having average root length of 5.41 ± 0.06 cm was observed in liquid ½ MS media amended with BA (13.37µM) + IBA (1.96 µM). ½ MS medium augmented with BA (13.37µM) + IBA (1.47µM) also showed good culture response (82.22%) with 15.91 ± 0.11 mean number of roots having 4.39 ± 0.05 cm average root length. Moderate culture response (63.30%) was observed in media fortified with of 13.37µM BA and IBA at 2.46 µM producing 15.40 ± 0.21 mean number of roots having 4.00 ± 0.06 cm average root length (Figure 6.8). Culture response (76.04%) was obtained in media augmented with BA 13.37µM and IBA 0.98µM producing 15.67 ± 0.33 of roots/explant with 4.40 ± 0.06 cm root length. Minimum culture response 24.08% was observed in media fortified with BA 13.37µM and IBA 0.49µM producing 4.80 ± 0.06 mean number of roots with 1.30 ± 0.06 cm root length.

½ MS media augmented with higher concentration of NAA (4 µM) mean number of roots/explant 4.65 ± 0.09 with 1.16 ± 0.03 cm root length (Plate 6.9: C) was regenerated in 64% cultures within 31 days. NAA at 2 µM showed 2.65 ± 0.03 mean number of roots with 0.78 ± 0.04 cm average root length (Plate 6.9: B). ½ MS media augmented with NAA 1 µM

showed lowest culture response (37.5%) produced 1.97 ± 0.03 roots/explant having root length 0.90± 0.06 cm (Figure 6.7). In earlier reports, simultaneous production of roots was also observed in *O. prostrata* by Gopalkrishnan et al. (2018); *O. mungos* var *prostrata* (Krishnan et al., 2018a); *O. mungos* (Deepthi & Satheeshkumar, 2017b); *O. japonica* (Kai et al., 2008); *Zingiber petiolatum* (Prathanturarug et al., 2004); *D. deltoidei* (Nazir et al., 2021).

After 4 weeks of culture in ½ MS media with varying concentrations of metatopolin, multiple shoots were transferred to ½ MS fortified with melatonin (MEL) (0.25-12.5µM) for root induction. In ½ MS media with 0.75 µM, maximum 14.14±0.09 number of roots per plant and 3.87 ±0.02 root length was observed (Plate 6.11: A). MEL at 1.00 µM showed 12.75±0.20 average number of roots and 3.01±0.07 average root length (Plate 6.11: B). ½ MS with 12.5 µM produced lowest 0.85±0.03 roots per plant having root length 0.75±0.02(Fig. 6.9). In various reports, melatonin has been used for root induction in apple (Mao et al., 2020), *Prunus spp.* (Sarropoulou et al., 2012), *Arabidopsis thaliana* (Pelagio-Flores et al., 2012) and have shown positive response in root formation and increased root length.

**Table 6.13** Effect of different concentrations of auxins on in vitro rooting.

| **NAAµM** | **BAPµM** | **IBAµM** | **Mean no. of roots ±SE** | **Average root length ±SE** |
|---|---|---|---|---|
| 0 | 0 | 0 | 0.00±0.00 | 0.00±0.00 |
| 0.5 | - | - | 2.41±0.05$^{g}$ | 1.30±0.06$^{d}$ |
| 1 | - | - | 1.97±0.03$^{h}$ | 0.90±0.06$^{e}$ |
| 2 | - | - | 2.65±0.03$^{g}$ | 0.78±0.04$^{e}$ |
| 4 | - | - | 4.65±0.09$^{e}$ | 1.16±0.03$^{d}$ |
| - | 13.37 | 0.49 | 4.31±0.06$^{f}$ | 0.58±0.04$^{f}$ |
| - | 13.37 | 0.98 | 11.23±0.12$^{c}$ | 3.67±0.09$^{b}$ |
| - | 13.37 | 1.47 | 13.72±0.15$^{b}$ | 3.61±0.07$^{b}$ |
| - | 13.37 | 1.96 | 16.69±0.17$^{a}$ | 4.47±0.04$^{a}$ |
| - | 13.37 | 2.46 | 10.38±0.06$^{d}$ | 3.07±0.06$^{c}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

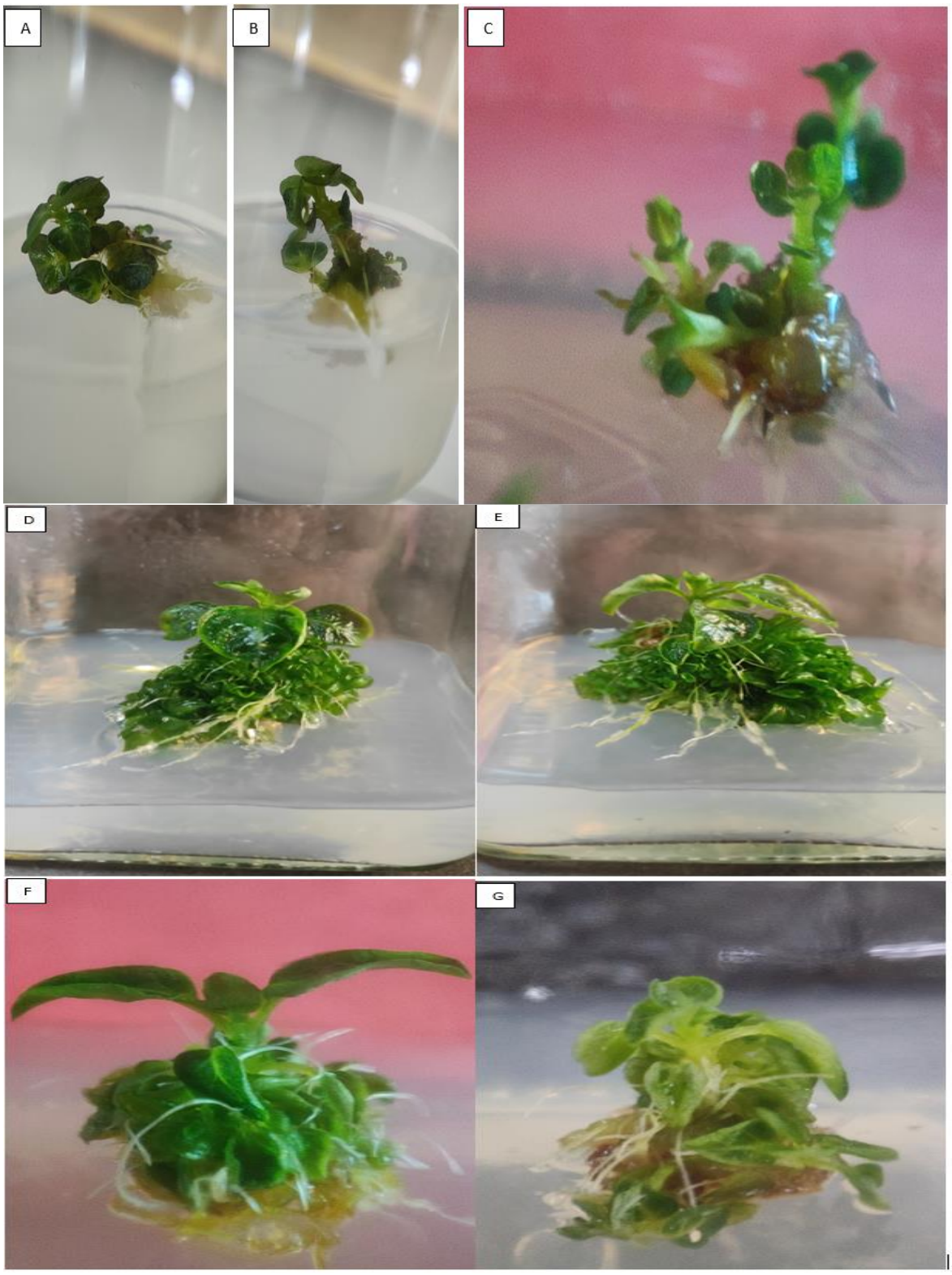



**Plate 6.8:** Induction of root from multiple shoots on solid ½ MS medium

*(A) NAA 0.5 µM (B) NAA 2 µM (C) NAA 4 µM (D) BAP 13.37 + IBA 1.47µM (E-F) BAP 13.37 + IBA 1.96µM (G) BAP 13.37 + IBA 2.46µM*

**Table 6.14** Impact of liquid ½ MS with PGRs on in vitro rooting of *O. mungos.*

| NAAµM | BAPµM | IBAµM | No. of roots ±SE | Average root length ±SE |
|---|---|---|---|---|
| 0 | | | 0.00±0.00 | 0.00±0.00 |
| 0.5 | 0 | 0 | 2.70±0.06$^{e}$ | 1.44±0.03$^{e}$ |
| 1 | - | - | 2.40±0.06$^{e}$ | 1.06±0.07$^{f}$ |
| 2 | - | - | 2.87±0.09$^{e}$ | 0.87±0.02$^{g}$ |
| 4 | - | - | 4.73±0.12$^{d}$ | 2.60±0.06$^{d}$ |
| - | 13.37 | 0.49 | 4.80±0.06$^{e}$ | 1.30±0.06$^{e}$ |
| - | 13.37 | 0.98 | 15.67±0.33$^{bc}$ | 4.40±0.06$^{b}$ |
| - | 13.37 | 1.47 | 15.91±0.11$^{b}$ | 4.39±0.05$^{b}$ |
| - | 13.37 | 1.96 | 20.21±0.12$^{a}$ | 5.41±0.06$^{a}$ |
| - | 13.37 | 2.46 | 15.40±0.21$^{c}$ | 4.00±0.06$^{c}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.


**Plate 6.9:** Induction of root from multiple shoot on Liquid ½ MS medium

***(A&B)*** *13.37 µM BAP+1.96µM IBA*

**Table 6.15** Effect of different concentration of melatonin (MEL) on solid ½ MS media on in vitro roots of *O. mungos.*

| **MEL conc.** | **No.of root /plant±SE** | **Average root length±SE** |
|---|---|---|
| 0.00µM | 0±0.00 | 0±0.00 |
| 0.25µM | 4.30±0.06$^{e}$ | 0.73±0.03$^{g}$ |
| 0.50µM | 4.30±0.10$^{e}$ | 0.86±0.02$^{fg}$ |
| 0.75µM | 14.14±0.09$^{a}$ | 3.87±0.02$^{a}$ |
| 1.00µM | 12.75±0.20$^{b}$ | 3.07±0.07$^{b}$ |
| 1.25µM | 10.07±0.07$^{c}$ | 1.37±0.09$^{e}$ |
| 2.5µM | 6.27±0.15$^{d}$ | 2.10±0.06$^{c}$ |
| 5µM | 4.24±0.14$^{e}$ | 1.90±0.06$^{d}$ |
| 7.5µM | 2.68±0.09$^{f}$ | 0.93±0.02$^{f}$ |
| 10µM | 1.24±0.03$^{g}$ | 0.84±0.01$^{fg}$ |
| 12.5µM | 0.85±0.03$^{h}$ | 0.75±0.02$^{g}$ |

Results are presented as Mean ± SE (standard error) of 12 explants per treatment across 3 independent repeats. Duncan's multiple range at 95% confidence interval finds no statistically significant difference between the means denoted by the same letter across successive columns.

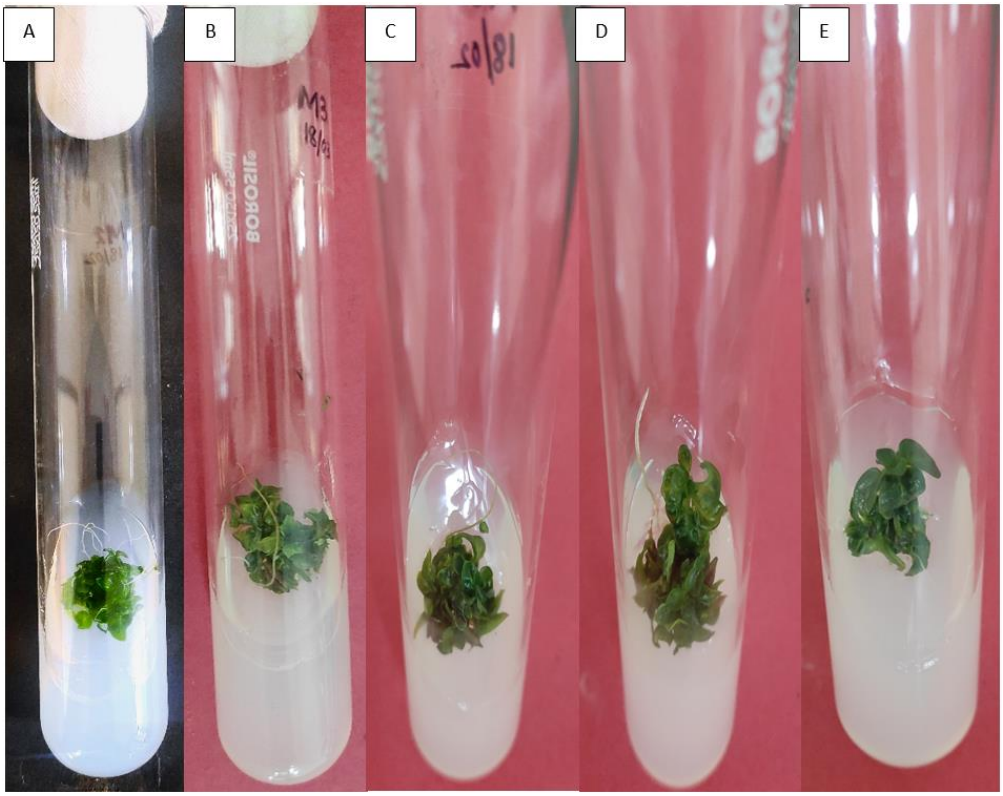



**Plate 6.10:** Induction of root from multiple shoots on solid ½ MS medium.

***(A)*** *0.75µM MEL* ***(B)****1.00 µM MEL* ***(C)****1.25 µM MEL* ***(D)****2.5 µM MEL* ***(E)****5.00 µM MEL*

### 6.3.2 Acclimatization of in vitro raised plants

Sequentially, the in vitro-grown seedlings were transferred to a hardening-off chamber. Within three weeks of being grown in sand, sterile soil, and manure (1:1:1) in pots, plantlets with fully developed leaves and roots were acclimatized under ex-vitro settings (Plate 6.12). After a short time in the greenhouse, the hardened plants had reached a 95% survival rate. No phenotypic differences were observed between the regenerated plants and their wild-grown counterparts.

### 6.3.3 Genetic fidelity study

To obtain consistent plants, micropropagation is employed. However, verifying the protocol's reliability for mass multiplication requires verifying the clonal fidelity of in vitro-regenerated plants. Numerous scientists have turned to the RAPD technique for evaluating the genetic stability of micropropagated plants due to its ease of use and low cost (Agnihotri et al., 2009; Chalageri & Babu 2012; Kaushik et al., 2015; Paul et al., 2010). Therefore, we used RAPD analysis in our work to ensure that the micropropagated plants and the mother

plant were genetically identical. Only six of the ten RAPD primers employed generated bands that could be scored and replicated. On average, nine scoreable bands were obtained from each primer, for a grand total of 58 (Table 6.16). Maximum number of scoreable bands was generated by using primers OPA 11(13bands) and OPD 18(11bands) (Plate 6.13 C & B). The RAPD study results suggested genetic stability among the plants by showing no polymorphisms or alterations between the micropropagated plants and the mother plants of *O. mungos*.

As for the monomorphism study of micropropagated plants analyzed with RAPD markers, our results are consistent with those of numerous writers. Using RAPD markers, researchers evaluated the genetic fidelity of in vitro regenerants of *Citrus limon* L. cv. Kaghzi Kalan (Goswami et al., 2013) and *Ocimum gratissimum* L. (Soumen et al., 2011) and found that both were genetically identical. When Chalageri and Babu (2012) utilized 25 RAPD primers to multiply in vitro seedlings of *Viola patrinii* regenerated by petiole callus, they observed a homogeneous amplification profile and obtained a total of 86 amplicons. *Chlorophytum borivilianum* in vitro plantlets were tested for genetic stability using RAPD markers, and all of the regenerating specimens demonstrated complete genetic continuity (Basu & Jha, 2014). Effective evaluation of the genetic homogeneity test in *Ophiorrhiza mungos* has been conducted by Kaushik et al. (2015).

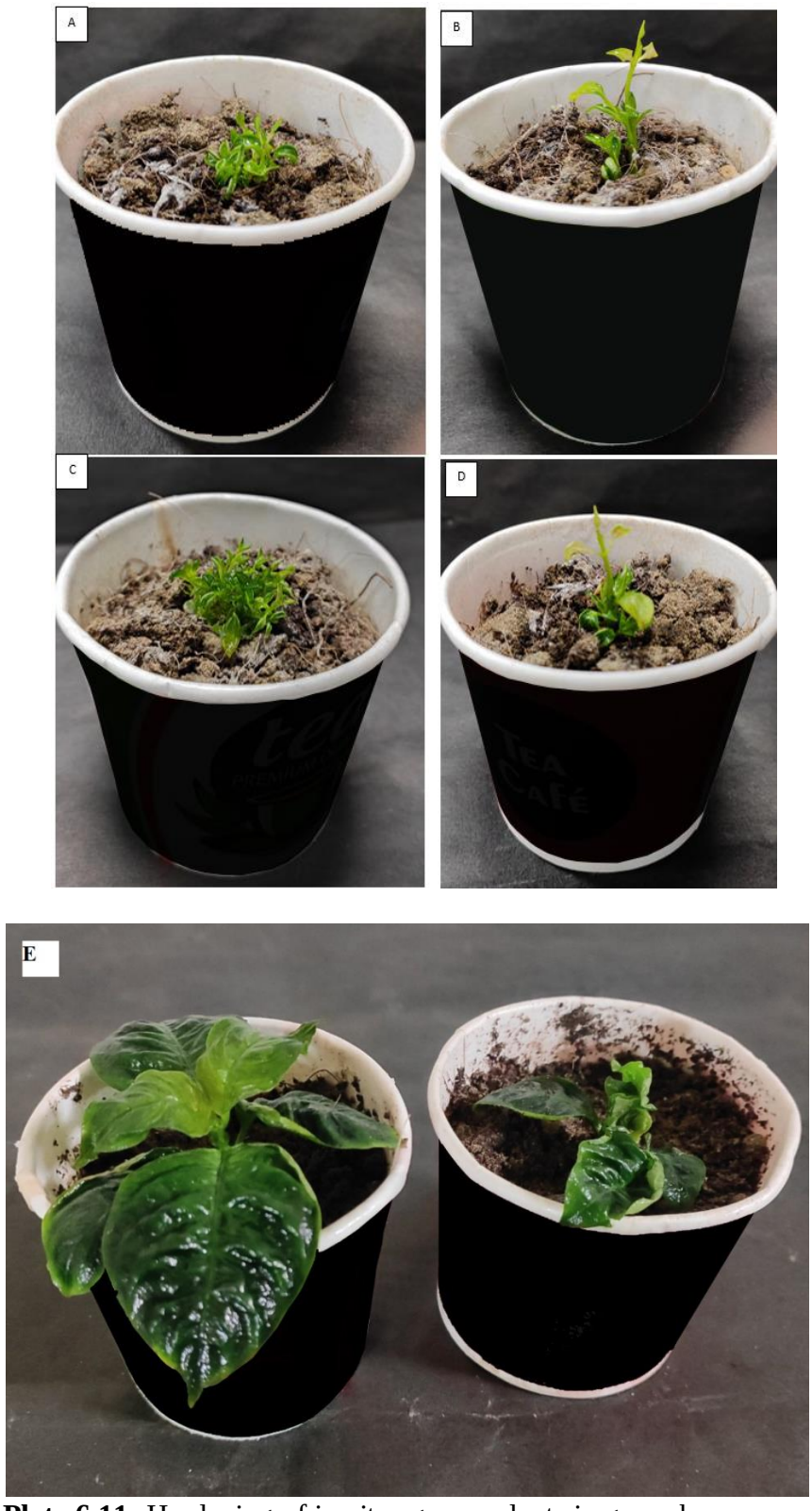



**Plate 6.11:** Hardening of in vitro grown plants in green house.

**Table 6.16** RAPD analysis banding pattern of in vitro, hardened and parent plant.

| Sl.no | Primer code | Bands number | Size of bands(bp) |
|---|---|---|---|
| 1. | OPD 20 | 7 | 750-2000 |
| 2. | OPD 18 | 11 | 250-1800 |
| 3. | OPA 11 | 13 | 200-2000 |
| 4. | OPA 18 | 10 | 250-1800 |
| 5. | OPA 20 | 8 | 750-2000 |
| 6. | OPC 02 | 9 | 200-1700 |

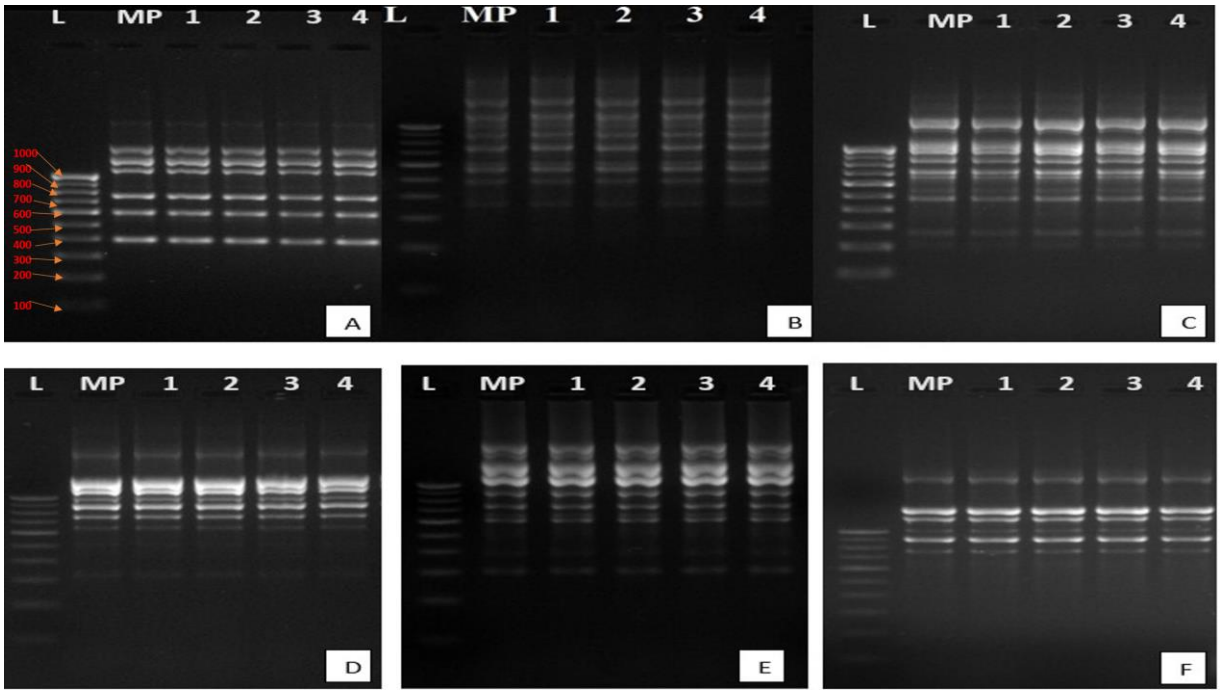



**Plate 6.12;** Gel picture of amplicons amplified using six RAPD primers *(A) OPA-18 (B)OPD-18 (C)OPA-11 (D)OPA-20 (E) OPC-02 (F)OPD-20*; where L represents ladder (100 bp) and 1-4 are the randomly selected regenerated plants and MP is mother plant.

Numerous studies have shown that in vitro regeneration can result in somaclonal variants in a variety of plant species, and these variations are both genetic and phenotypic (Kaeppler et al., 2000). Current RAPD results can be employed for commercial purposes by breeders to evaluate the genetic homogeneity of large-scale in vitro preserved regenerants of *O.mungos*, simplifying the crop enhancement programme in *Ophiorrhiza* species.

### 6.3.4 Biochemical fidelity study

Whether or not a proposed tissue culture technique ends up being successful depends on biochemical homogeneity assessments of mother plants and in vitro regenerants. The plants' medicinal benefits come from their secondary metabolites. In this study, HPTLC was used to conduct a quantitative analysis of the pentacyclic alkaloid camptothecin. The camptothecin content of ethanol extracts of the leaves, stems, and roots of wild plants (parent plant) and in vitro regenerants of *O. mungos* was estimated based on peak area. According to Table 6.17, HPTLC analysis revealed homogeneity in both in vitro regenerants and field-grown plant tissues. This study confirmed that the newly discovered method had no unfavorable impacts on camptothecin production in the plants it was applied to. *O. mungos* biochemical fidelity has been evaluated in similar studies using HPLC. This has been shown to be true by Kaushik et al. (2015).

## 6.4 Conclusion

In this study, we present a strategy that has been shown to be effective for direct shoot regeneration in *O. mungos*. Micropropagated plants tested by RAPD analysis, which measures genetic uniformity, have been shown to be completely homogeneous. Camptothecin concentrations in the parent plant and the micropropagated progeny were also similar. The in vitro-grown cells could constitute a massive reservoir of the bioactive molecule (camptothecin). This reliable plant regeneration technology is therefore useful for other genetic alteration experiments in addition to the commercial multiplication of *O. mungos* elite clones. The developed method has the potential to be employed for germplasm preservation and rapidly produces large numbers of true-to-type offspring for the commercialization of this valuable camptothecin-rich species.

**Table 6.17** Camptothecin content in in vivo and in vitro raised plants of *O. mungos.*

| **Part used** | **In vivo camptothecin (µg/g, dr.wt)** | **In vitro camptothecin (µg/g, dr.wt)** |
|---|---|---|
| Leaf | 694.02±0.005 | 723.80±0.008 |
| Stem | 490.93±0.008 | 528.04±0.009 |
| Root | 582.15±0.007 | 597.41±0.006 |

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

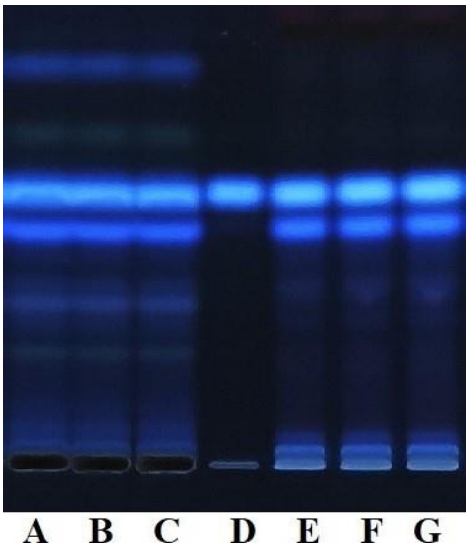



**Plate 6.13:** HPTLC fingerprinting of camptothecin **A** = in vivo Leaf, **B** = in vivo stem, **C** = in vivo root, **D** = standard (camptothecin), **E** = in vitro leaf, **F** = in vitro stem, **G** = in vitro root.

# CHAPTER 7

## *Summary and Conclusion*

The unique, high-value medicinal plant *Ophiorrhiza mungos*, more commonly known as Indian snake root, has enormous economic significance. The natural germplasm of *Ophiorrhiza mungos* is on the danger of extinction due to overexploitation and carelessness in harvesting its wild environment (Swamy et al., 2018). In light of these realities, research has recently begun on biotechnological techniques for micropropagation, the creation of clonally identical in vitro regenerants, the continuous production of camptothecin via elicitation and precursor feeding, and the development of molecular and chromatographic fingerprinting methods. The HPTLC and RAPD fingerprinting techniques developed in this study hold promise for future use as a reliable reference kit in screening of elite varieties with high drug producing potential and, more generally, in the detection of commercial trade adulteration.

For the present study, plant accessions of *Ophiorrhiza spp.* were collected from six different geographical zones of Kerala namely Thiruvananthapuram, Kottakal, Idukki, Kollam, Pathanamthitta and Ernakulam have been used for the screening of elite population on the basis of bioactive compounds. An innovative and improved method of HPTLC was developed and validated for the simultaneous quantification of camptothecin. The plants collected from Kottakal was found potent sources of camptothecin.

This study found that the abundance and prevalence of secondary metabolites are significantly influenced by environmental factors. In addition, the HPTLC approach was shown to be easy to use, accurate, and sensitive, suggesting its potential application in drug standardization and quality assurance.

The quality and amount of bioactive chemicals are highly dependent on the extraction method chosen. Although it comes early in the research process, extraction is crucial to the success of any medicinal plant. In **Chapter 3**, *O. mungos* was screened as the elite species from the selected species. Furthermore, in plant part selection, leaves (0.96%) of *O. mungos*

showed the maximum content of CPT, followed by inflorescence (0.64%), root (0.51%), and stem (0.49%). In addition, conventional and non- conventional methods such as hot water bath extraction (HWB), maceration and microwave-assisted extraction (MAE) methods, Ultrasonic-assisted extraction (UAE) methods and solvent selection {methanol (100%), methanol (60%) and water} has been compared to determine the proficient method for camptothecin extraction. UAE with 60% methanol showed very good yield of camptothecin (0.96%) in less time duration (10 min) as compare to HWB, maceration and MAE. HPTLC analysis was performed for all the extraction methods.

In **Chapter 4,** genetic diversity assessment of the selected *Ophiorrhiza* spp. using molecular marker (RAPD) and phytochemical diversity assessment of the elite species was performed. Based on the dendogram developed from RAPD data for diversity assessment, *O. mungos* was found to be more closely related to *O. hirsutula* than *O. rugosa* var *prostrata*. In phytochemical analysis, KE16 accessions in Kottakal, Kerala, with its highlands and wet marine environment of the Western Ghats of India, had the highest concentration (511.20 µg/g, dr.wt.).

Due to the high medicinal value of bioactive compound camptothecin, *O. mungos* has attained much importance in the scientific community over the past few years. In **Chapter 5** genuine efforts have been made to enhance the yield of secondary metabolites in *in vitro* regenerants through biotechnological strategies which are elicitation and precursor feeding. RSM using mintab software was employed for these experiments. Methyl Jasmonate, Jasmonic acid, salicylic acid and Chitosan was used as elicitors and L-tryptophan and geraniol as precursors at different concentrations and exposure times. Elicitor treatment was given for 4 days and precursor treated cultures were harvested after 14$^{th}$ day of treatment. The outcomes of this interactive effect of elicitors on camptothecin synthesis reveals that the concentrations 150µM each of all elicitors viz MeJA, JA, SA and CH had the best yield (666.390-675.830µg/g) out of the rest combinations followed by 559.240 µg/g at 150 µM MeJA, 100 µM JA, 200 µM SA and 150 µM CH and for precursors the highest content of camptothecin (1678.53-1747.63µg/g) was recorded in combination of 1.5mM each of both L-Tryptophan and Geraniol, followed by 1486.48 µg/g in 1.5mM L-Tryptophan and 2.2 mM Geraniol.

**Chapter 6** describes the micropropagation of elite population of *O. mungos* by direct organogenesis. For the explants surface sterilization, labolene (3-4drops) for 15mins, tween

20 (2-3drops) and Bavistin (1%) for 30mins, sterilized with $HgCl_2$ for 3 minutes showed very low contamination (1.56 ± 0.25) with high survival rate (92.43 ± 0.25). Therefore, this treatment was found to be an effectual treatment in respect of survival rate and establishment. Nodal segments were shown to be the most sensitive explants in terms of direct regeneration, while leaf explants only respond to callus development. It was shown that regenerant in vitro response was considerably impacted by media composition, PGR type, and PGR concentration. Out of the growth hormones used, 2, 4- D and NAA in different concentration proved effective in regeneration of callus from explant. Among different concentration of BAP (20µM), IAA (20µM), GA3(10µM) produced maximum amount of callus with 71% culture response in 27 days followed by 65% culture response in 31days in BAP (30µM), Piclo (10µM), GA3 (10µM). Callus generated in both the cases was green friable. For direct shoot initiation, BA alone or in combination with IBA, and KIN was used. For shoot induction BAP was beneficial in 98.67 percent of cultures at 13.37µM, resulting in shoot induction at 13.67±0.33 days and total of 4.67±1 shoots. While both solid and liquid ½ MS media supplemented with BA and IBA were employed for shoot multiplication, the liquid ½ MS medium was determined to be the most effective. Liquid ½ MS medium augmented with BA 13.37µM and IBA 1.96 µM produced maximum number of shoots/explant 19.00±0.58 with 99.73% culture responses. This combination was considered the optimum PGR combination for shoot multiplication in *O. mungos* among all treatments. It was discovered that simultaneous rooting does occur in shoot multiplication medium, which is an added benefit of the large-scale micropropagation technique. Another cytokinin metatopolin was also employed for shoot multiplication in ½ MS media. Highest number of shoots/explant (13.67±0.33) was seen in 0.5 µM of mT, while 13.33±0.67 number of shoots/explant was recorded in 0.75 µM of mT. After multiple shoot formation, the explants were sub-cultured for shoot elongation in ½ MS media fortified with different concentrations of GA3(5-30 µM). Longest shoot length (5.18±0.20cm) was seen in 20 µM GA3 and shortest(2.35±0.02cm) in 5 µM. Besides that, another ½ MS media augmented with different concentrations of NAA, IBA and BAP was also used as rooting media and liquid ½ MS media fortified with BA and IBA showed best rooting. Maximum response 99.54% with 20.43 ± 0.33 roots/explant having average root length of 5.42 ± 0.06 cm was observed in liquid ½ MS media amended with BA (13.37µM) + IBA (1.96 µM). ½ MS medium augmented with BA (13.37µM) + IBA (1.4713.37µM) also showed good culture response (82.22%) with 16.03 ± 0.43 mean number of roots having 4.46 ± 0.02 cm average root length. For root induction the effect of melatonin was

also checked in *O. mungos* explants. Root induction occurred within a week after subculture. ½ MS media supplemented with 1.25 µM MEL showed a maximum of 14.11±0.33 roots/plant with average root length of 3.86±0.01. Plants with fully formed root systems were acclimatized with a 95% success rate on a medium consisting of equal parts sterile soil, sand, and manure (1:1:1). Using RAPD and HPTLC analyses, we checked the micropropagated plants for genetic and biochemical faithfulness. RAPD study showed the genetic similarity of the randomly selected in vitro raised plants to their parent plant, and HPTLC data validated the existence of CPT in both the micropropagated and mother plant, indicating biochemical identity and no change owing to tissue culturing. This ensures that the plants grown through micropropagation are similar both genetically and biochemically.

The current study concludes that a promising species, KE16 accessions in Kottakkal, Kerala, is the elite population demonstrating clear superiority in terms of secondary metabolite (camptothecin) content compared to other investigated varieties. A promising population may be cultivated on a large scale in the future. Laboratory examination of camptothecin-containing products can benefit from the developed HPTLC approach, which was found to be straightforward, accurate, and sensitive. Using the profiles will also help in screening for and distinguishing *O. mungos* from other species in the genus *Ophiorrhiza*. Using UAE as an alternative to traditional methods of extracting desirable compounds from plant matrices for their usage in various industrial fields could be a competent and environmentally friendly option. This research presents feasible alternatives to traditional methods for the widespread production and preservation of genetically identical plant germplasm. This technique may prove useful in the future thanks to the DNA and HPTLC fingerprinting it generates. Also, the use of bioreactors in conjunction with elicitor and precursor treatment can significantly increase the production of bioactive substances and the mass multiplication of medicinal plants. Nonetheless, more research is needed to develop metabolic engineering approaches that could boost in vitro culture production of valuable secondary metabolites. Researchers hoping to apply biotechnological methods to boost plant viability and yield bioactive chemicals may find this work helpful.

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# Publications

1. Konjengbam, M., Kumar, V., Dwivedi, P., Gangaprasad, A., Dey, A., & Pandey, D. K. (2023). Identification of elite species of *Ophiorrhiza* utilizing HPTLC analysis and camptothecin as a phytochemical marker: Assessment of extraction effectiveness and organ selection. *Biocatalysis and Agricultural Biotechnology*, *48*, 102632. https://doi.org/10.1016/j.bcab.2023.102632
2. Pandey, D. K., Konjengbam, M., Ghorai, M., Dwivedi, P., Roy, D., Kant, N., ... & Dey, A. (2022). Biotechnology for micropropagation and camptothecin production in *Ophiorrhiza sp*. Applied Microbiology and Biotechnology, 106(11), 3851-3877. https://doi.org/10.1007/s00253-022-11941-y

3. Konjengbam, M., Kumar, V., Dey, A., Pandey, B., & Pandey, D. K. (2023). A response surface-based approach to optimize precursor feeding in *Ophiorrhiza mungos* shoot cultures for the enhancement of camptothecin. *Industrial Crops and Products* (Under revision)
4. Konjengbam, M., Kumar, V., & Pandey, D. K. (2023). In vitro regeneration of near threatened and native Indian medicinal plant *Ophiorrhiza mungos* (Rubiaceae) via meta-Topolin and melatonin. (communicated at Scientia Horticulturae)

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MINI-REVIEW

# Biotechnology for micropropagation and camptothecin production in *Ophiorrhiza* sp.

Devendra Kumar Pandey[1] · Merinashwari Konjengbam[1] · Mimosa Ghorai[2] · Padmanabh Dwivedi[3] · Debleena Roy[4] · Nishi Kant[5] · A. Gangaprasad[6] · Abhijit Dey[2]

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**Abstract**

Camptothecin (CPT) is a monoterpenoid-alkaloid, an anticancer compound from plant. Ever since its discovery in 1996 from the bark of *Camptotheca acuminata*, various researches have been conducted for enhancing its production. CPT has also been reported in several other species belonging to the plant families *Icacinaceae*, *Rubiaceae*, *Apocynaceae*, *Nyssaceae*, *Betulaceae*, *Violaceae*, *Meliaceae*, and *Gelseminaceae*. Out of these, *Ophiorrhiza* sp. (*Rubiaceae*) is the next possible candidate for sustainable CPT production after *C. acuminata* and *Nothapodytes nimoonia*. Various biotechnological-studies have been conducted on *Ophiorrhiza* sp. for searching the elite species and the most optimal strategies for CPT production. The genus *Ophiorrhiza* has been used as medicines for antiviral, antifungal, antimalarial, and anticancer activities. Phytochemical analysis has revealed the presence of alkaloids, flavonoids, triterpenes, and CPT from the plant. Because of the presence of CPT and its herbaceous habit, *Ophiorrhiza* sp. has now become a hot topic in research area. Currently, for mass production of the elite spp., tissue culture techniques have been implemented. In the past decades, several researchers have contributed on the diversity assessment, phytochemical analysis, mass production, and in vitro production of CPT in *Ophiorrhiza* sp. In this paper, we review the on the biotechnological strategies, optimal culture medium, micropropagation of *Ophiorrhiza* sp., effect of PGR on

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# Identification of elite species of Ophiorrhiza utilizing HPTLC analysis and camptothecin as a phytochemical marker: Assessment of extraction effectiveness and organ selection

Merinashwari Konjengbam [a, b], Vijay Kumar [a, b], Padmanabh Dwivedi [c], A. Gangaprasad [d], Abhijit Dey [e], Devendra Kumar Pandey [a, b, *]

[a] School of Bioengineering and Biosciences, Lovely Faculty of Technology and Sciences, Lovely Professional University, Phagwara, Punjab, India
[b] Plant Biotechnology Lab, Division of Research and Development, Lovely Professional University, Phagwara, Punjab, 144411, India
[c] Department of Plant Physiology, Institute of Agriculture Sciences, Banaras Hindu University, Varanasi, 221005, India
[d] Centre for Biodiversity Conservation, Department of Botany, University of Kerala, Kariyavattom, Thiruvananthapuram, Kerala, 695581, India
[e] Department of Life Sciences, Presidency University, 86/1 College Street, Kolkata, 700073, India

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ABSTRACT

*Objective:* The main objective of this experiment is to find the camptothecin (CPT) content in different parts of three *Ophiorrhiza* spp.
*Methods:* In this study, three species of *Ophiorrhiza* viz., *O.mungos* L., *O. hirsutula* Wight ex Hook.

# Conferences

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*This is to certify that Mr./Ms./Dr.* Meninashwari Konjengbam

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