BIOTIC FACTORS INFLUENCING PLUMBAGIN PRODUCTION IN *IN VIVO* AND *IN VITRO* CULTURED *PLUMBAGO* SPECIES

Thesis Submitted For the Award of the Degree of

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By

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DECLARATION

I hereby declare that the work presented in the thesis titled "Biotic factors influencing plumbagin production in *in vivo* and *in vitro* cultured *Plumbago* species" has been accomplished and submitted by me for the award of the degree of Doctor of Philosophy in Botany from the Department of Biotechnology, Lovely Professional University, Phagwara, India. There is no previous record of similar work done by another researcher.

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CERTIFICATE

This is to certify that the thesis entitled "Biotic factors influencing plumbagin production in *in vivo* and *in vitro* cultured *Plumbago* species" is being submitted by Ms. Kajal Katoch under my supervision for the award of Doctor of Philosophy in the discipline of Botany is an original work done by her.

It is further certified that:

a) The scholar has worked under my supervision for the requisite period under statutes.

b) The scholar has the required percentage of attendance.

c) The candidate has fulfilled the requirements of the UGC regulations for the Degree of Doctor of Philosophy.

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ABBREVIATIONS

°C	Degree Celsius
2, 4- D	2, 4-Dichlorophenoxyacetic Acid
ABA	Abscisic Acid
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
BA/BAP	6-Benzylaminopurine
BBD	Box-Behnken Design
Bp	Base pair
CC	Column chromatography
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribose Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic Acid
GC-MS	Gas chromatography-mass spectrometry
Gm	Gram
Hr	Hour
HPLC	High-performance liquid chromatography
HPLC-ESI-MS/MS	High-performance liquid chromatography/electrospray ionization tandem mass spectrometry
HPTLC	High-performance thin-layer chromatography
$HgCl_2$	Mercuric Chloride
IAA	Indole-3-Acetic acid
IBA	Indole-3-Butyric acid
ICH	International Council for Harmonisation
ISSR	Inter Simple Sequence Repeats
L	Litre
LC-MS	Liquid chromatography-mass spectrometry

LOD	Limit of Detection
LOQ	Limit of Quantification
MAE	Microwave-Assisted Extraction
MHz	MegaHertz
mL	Milli-litre
Mm	Milli-meter
Min	Minute
μL	Micro-litre
μM	Micro-mole
MP	Mother Plant
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
Nm	Nano-meter
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
PDB	Potato Dextrose Broth
PGR	Plant Growth Regulators
PVP	Polyvinyl Pyrrolidine
RAPD	Random amplified polymorphic DNA
RFLP	Random Fragment Length Polymorphism
Rpm	Revolutions per minute
RSM	Response Surface Methodology
ROS	Reactive oxygen species
SD	Standard Deviation
TAE	Tris Acetate EDTA buffer
TDZ	Thiadizuron

TLC	Thin-layer chromatography
UAE	Ultrasound-Assisted Extraction
UFLC	Ultra-Fast Liquid Chromatography
UPLC	Ultra-performance liquid chromatography
UV	Ultra-violet
VASE	Vacuum-assisted sorbent extraction

ABSTRACT

Plumbago species belong to the family Plumbaginaceae. It is one of the most studied medicinal plants recently because of the presence of an important naphthoquinone, plumbagin. The roots of *Plumbago* are used traditionally to cure ailments such as piles, diarrhea, leprosy, inflammations, scabies, ulcers, and so on. Besides traditional use it is also extensively studied for pharmaceutical activity and is found to be anti-microbial, anti-malarial, antifungal, anti-inflammatory, anti-carcinogenic, anti-tumor, anti-fertility, antiplasmodial, antioxidant, anti-diabetic. *Plumbago* species are commonly distributed among the central and south Indian states growing in wild. Due to over-exploitation to meet the increasing demand, it is placed under the endangered species in the IUCN (International Union for Conservation of Nature) Red Data Book. The current status of *Plumbago* inspired me to develop alternative biotechnological techniques to combat the rising obstacles. Plant tissue culture is one of the alternatives which can be used to meet the market demand without over-exploiting the natural habitat. An exclusive investigation was designed and executed to optimize the extraction techniques and mass-produce *Plumbago* species via plant tissue culture assisted with elicitation.

Plumbago species were collected from various regions to identify the elite species based on the presence of plumbagin. The plants were collected from the regions of Jammu and Kashmir, Punjab, Haryana, Himachal Pradesh, and Uttarakhand. The plant samples collected were screened by the HPTLC method of quantification. All samples collected contained plumbagin but the content varied according to the geographical region. The difference in the content could be because of many factors that can influence the yield of plumbagin. Different factors such as pre-harvest, post-harvest, and extraction methods were optimized to establish a simple, cost-effective, and accessible extraction method. Various pre-harvest factors (plant part, age of plant, harvesting season), post-harvest factors (drying method, storage time of raw material, extraction method), and extraction methods were evaluated. The microwave-assisted extraction factors were further optimized by RSM (Response surface methodology).

In vitro regeneration procedure for *P zeylanica* and *P auriculata* was established. The elicitation of plumbagin in *in vitro* shoot cultures and potted plants of *P zeylanica* was studied. The nodal and leaf explants were used for the regeneration of plants. A wide range of PGRs was tested to determine the most effective concentration and combinations. The adventitious root culture of *P. zeylanica* was successfully established in leaf explants. Hairy root culture was established with leaf and nodal explant of *P zeylanica*. Genomic DNA was extracted from the transformed and non-transformed roots and shoot cultures to check the presence of *rol* B genes in transformed cultures.

The shoot cultures of *P zeylanica* were treated with different biotic elicitors (*A rhizogenes, T viride,* and yeast extract) and the experiment was performed and analyzed by RSM. All of the elicitors tested were found to be significant. The plants were treated with biotic elicitors under greenhouse conditions also aiming to validate the effect of biotic elicitors in natural conditions. The independent variables used were lysate of *A rhizogenes*, *T viride,* and vermicompost.

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CHAPTER 1

Introduction

Plants have essentially created sustainable ecosystems, the photosynthetic plants and organisms provide the energy and raw material and hence the basics of survival such as the production of breathable air, water, food, and shelter. The entire animal kingdom depends directly or indirectly on plants and trees for their survival. Domestication of plants played a major role in world civilization, basically the first step towards civilization. Progress came with an understanding of healing properties exhibited by medicinal plants. The knowledge was passed on from generation to generation and documented over time. The earliest written traces of plant-based medicine are clay tablets from Mesopotamia dating back to 2600 B.C. The first Ayurveda document can be found in the form of Susruta and Charaka dating back to 1000 B.C. Wu Shi Er Bing Fang is the first Chinese medicine record dating back to about 1100 B.C. Ebers Papyrus is an Egyptian medicinal record dating back to 1500 B.C. (Fallarino, 1994). According to Hexa Research, the global plant-based medicine market was estimated at USD 71.19 billion in 2016 and it is expected to value around USD 117.02 billion by 2024 (https://www.hexaresearch.com/research-report/global-herbal-medicine-market).

1.1. Indian Medicinal Plants

According to India's Fifth National Report to the Convention on Biological Diversity 2014, there are a total of 34 biodiversity hotspots all over the world out of which 4 are present in India covering an area of over 692,027 km^2 which is 21.05% of the total geographical area of India. These four hotspots are the Himalayas, the Western Ghats, the Northeast, and the Nicobar Islands. These hotspots are represented by rich biodiversity and gold mine of endemic species. There are 4045 species, 141 genera belonging to 47 families of flowering plants that are endemic to India (https://www.cbd.int/doc/world/in/in-nr-05-en.pdf).

Various threats are endangering the biodiversity of India such as unauthorized use of forest land, overexploitation of natural resources, haunting of unique animals, forest fire, and climate change.

To conserve biodiversity the Wildlife Institute of India has set up 104 National Parks covering an area of 43,716 km^2, 566 sanctuaries covering 122,420 km^2 (http://www.wiienvis.nic.in/Database/Protected_Area_854.aspx). About 70% of Indian medicines consist of natural derivatives which come directly or indirectly from plants (Verma and Singh, 2008).

Table 1.1: Major medicinal plants used in India and their medicinal use

Plant species			Therapeutic and Medicinal use	References
Botanical name	Family	Common name		
Adhatoda vasica	Acanthaceae	Adosa	Anti-asthmatic, bronchodilator activity, wound healing, anti-bacterial, anti-ulcer, anti-allergy, anti-tubercular, abortifacient, uterotonic	Gangwar and Ghosh, 2014
Allium sativum	Liliaceae	Lasun	Anti-microbial, cardio-protective, anti-cancer	Singh and Singh, 2008
Aloe vera	Asphodelaceae	Aloe	Wound healing, anti-inflammatory, anti-aging, anti-tumor, laxative, immunity booster, antiseptic, anti-diabetic, anti-cancer, anti-oxidative, anti-microbial	Sahu *et al.*, 2013
Berberis	Berberidaceae	Daruharid	Hepatoprotective, anti-	Potdar *et*

aristata		ra	diarrheal, cardiotonic, anti-diabetic, anti-cancer, ophthalmic	*al*., 2012
Cyperus rotundus Linn	Cyperaceae	Motha	Anti-inflammatory, antipyretic, analgesic, anticonvulsant, tranquilizing, hypolipidaemic, anti-emetic	Sivapalan, 2013
Embelia ribes	Myrsinaceae	Vidanga	Anthelmintic, anti-anxiety, anti-microbial, anti-cancer, antihistamine, cosmetic agent	Souravi and Rajasekharan, 2014
Glycyrrhiza glabra	Fabaceae	Mulethi	Anti-microbial, anti-inflammatory, anti-Alzheimer, anti-diabetic, anti-carcinogenic	El-Saber Batiha *et al*., 2020
Holarrhena antidysenterica	Apocynaceae	Kurchi	Anti-inflammatory, anti-diabetic, anti-urolithic property, anti-haemorrhoidal, anthelminthic, anti-microbial	Sinha *et al*., 2013
Murraya koenigii	Rutaceae	Karipatta	Vasodilating, anti-diabetic, anti-ulcer, anti-diarrheal, Anti-lipid peroxidative, wound healing, memory enhancer, analgesic and antinociceptive, anti-microbial, anti-oxidative, cytotoxic, anti-tumor, radioprotective and	Handral *et al*., 2012

			chemoprotective	
Ocimum sanctum	Lamiaceae	Tulsi	Antidiabetic, radio-protective, genotoxicity, gastroprotective, antimicrobial, immunomodulatory, anticancer	Pattanayak *et al.*, 2010
Phyllanthus emblica	Euphorbiaceae	Amla	Anti-oxidative, anti-diabetic, anti-cancer, anti-hypcrlipidemic, hepatoprotective, anti-bacterial, anti-inflammatory,	Mirunalini and Krishnaveni, 2010; Dhale and Mogle, 2011; Dang *et al.*, 2011
Piper longum	Piperaceae	Pipar	Anticancer, anti-oxidant, hepatoprotective, immunomodulatory, anti-inflammatory, anti-obesity, coronary vasodilation, bioavailability-enhancing, radioprotective, antiplatelet anti-fertility, anti-hyperlipidemic analgesic, adulticidal, melanin-inhibiting, cardioprotective, antimicrobial, antidepressant	Kumar *et al.*, 2011
P zeylanica	Plumbaginaceae	Chitra	Anticancer, anti-diabetic, hypocholesterolemic, anti-	Ganesan and Gani,

			inflammatory, antimicrobial, abortifacient, anti-fertility	2013
Terminalia chebula	Combretaceae	Hara	Anti-oxidative, anti-microbial, anti-carcinogenic, radio-protective, reno-protective, anti-inflammatory, anti-arthritic, anti-anaphylactic, hypolipidemic	Bag *et al.*, 2013
Tinospora cordifolia	Menispermace ae	Gurcha	Anti-allergic, anti-inflammatory, anti-arthritic, anti-osteoporotic, anti-oxidant, antineoplastic and radio-protective, anti-hyperglycemic, anti-leprotic, hepato-protective, anti-fertility, cardio-protective,	Upadhyay *et al.*, 2010
Trachysper mum ammi	Apiaceae	Ajwain	Antihypertensive, antispasmodic, hepatoprotective, Antilithiasis, diuretic, abortifacient, galactogogic, anti-inflammatory, antitussive, antifilaria, gastro-protective, anti-microbial, Anti-hyperlipidemic, anthelmintic	Bairwa *et al.*, 2012
Withania somnifera	Solanaceae	Ashwagan dha	Anti-microbial, antidiabetic, neuroprotective, anti-stress,	Dar *et al.*, 2015

anti-Alzheimer**,** anti-
ischemic, and anti-hypoxic**,**
anti-carcinogenic,
antihypertensive

1.2. Secondary metabolites

Besides carbohydrates, amino acids, pigment, etc. plants synthesize low molecular weight compounds essentially to defend the plant from environmental (biotic and abiotic) stress and unsure plants survival (Bennett and Wallsgrove, 1994). These compounds are known as secondary metabolites, they exhibit specific biological activities, odor, color, and taste. The unique properties of secondary metabolites contribute to their use in the pharmaceutical, cosmetic, textile, and food industries. They are synthesized in extremely low quantity; often less than 1%, there are three major groups of secondary metabolites: alkaloids, phenolics and terpenes and steroids (Bourgaud *et al.*, 2001). Their synthesis is an evolutionary trait acquired by specific plant species over time to counteract specific stress (Pichersky and Gang, 2000). The pathway for secondary metabolites is very complex and not completely understood yet. Their production is often organ, tissue, and cell-specific and their quantity may differ in individuals of even the same species (Pagare *et al.*, 2015). The extraction and purification are extensive processes and depend on a lot of factors such as the nature of secondary metabolites (hydrophilic, lipophilic), extraction solvent, plant material, extraction method, etc. Despite careful extraction, there is a risk of contamination of extract with interfering compounds such as lipids, pigments, tannins, and plasticizers. These risks can be eliminated via chromatographic separation, solvent-solvent separation, use of distilled solvents, quantitative and qualitative analysis (Jones and Kinghorn, 2006). Over time many researchers have developed and optimized the extraction methods to ensure complete extraction of secondary metabolites from plant material. These methods involve conventional techniques and some recently developed newer and non-conventional ones. Some of the recent developments include microwave-

assisted extraction (MAE) anthraquinones (Suktham *et al.*, 2021), phenols (Baltacıoğlu *et al.*, 2021), essential oils (Boudraa *et al.*, 2021); ultrasound-assisted extraction (UAE) of phenolic compounds (Tzima *et al.*, 2021), flavonoid (Nguyen *et al.*, 2021); supercritical fluids extraction of phenols (Al-Maqtari *et al.*, 2020), nicotine (Tita *et al.*, 2021); vacuum-assisted sorbent extraction (VASE) of terpenes (Jeleń *et al.*, 2021), among others. The preliminary screening of secondary metabolites can be done by thin-layer chromatography (TLC) and column chromatography (CC), further analysis for identification and determination can be done by advanced techniques such as High-performance liquid chromatography (HPLC), High-performance thin-layer chromatography (HPTLC), ultra-performance liquid chromatography (UPLC), Ultra-Fast Liquid Chromatography (UFLC), Liquid chromatography-mass spectrometry (LC-MS), Gas chromatography-mass spectrometry (GC-MS), etc.

1.3. *In vitro* culture techniques and approaches

Overexploitation and climate change have put the majority of medicinal plants under threatened or extinct categories. Various worldwide conservation measures have been developed and employed to preserve botanical diversity. *In-vitro* or biotechnological approaches are one of the most effective and prominent techniques for rapid multiplication and maintenance of critical genotype of medicinal plants. These techniques include *in-vitro* propagation, mycorrhization, genetic transformation, cryopreservation, DNA banks, etc. (Jain *et al.*, 2012).

1.3.1. In-vitro propagation

In-vitro propagation is possible because of the unique property of plants cells i.e., totipotency; the ability of a plant cell to develop into a whole plant via somatic embryogenesis (Su *et al.*, 2021). *In-vitro* propagation relies on various controlled factors. The pH of the growth media should be between 5.0-6.0, culture medium constituents (macronutrients, micronutrients, and vitamins), plant harmones, and sucrose (carbon source). Agar, gelatin, agarose, alginate, and gelrite are gelling agents used for the

preparation of semisolid or solid culture media (Singh and Singh, 2021). Some of the advantages of *in-vitro* propagation are mass production, all-year-round production, disease-free plants, conservation of genotype, genetic engineering, etc.
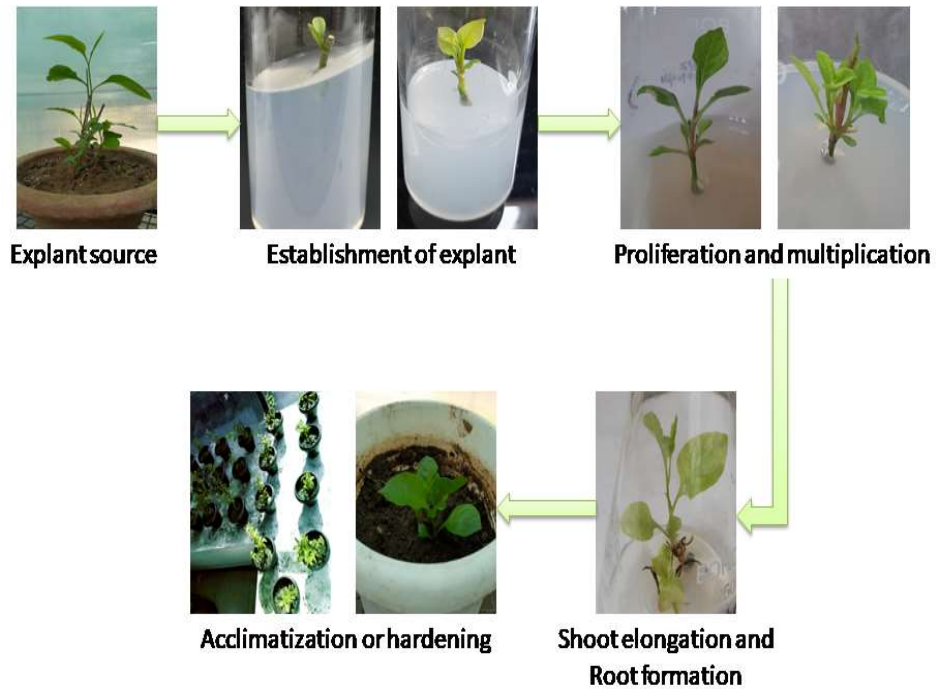
Fig. 1.1: Different stages of micro-propagation or *in-vitro* propagation for the majority of plants

Depending on the type of explants in-vitro propagation can be categorized into different cultures such as;

Meristem/shoot tip culture, meristems are the center of plant growth which is present in the tip of young shoot and leaves. It is an effective technique to eradicate disease and maintain genetic stability (Salokhe, 2021). Successful meristem culture protocols have been developed by researchers over decades for the production of various plants such as; *Zingiber officinale* (Bhagyalakshmi and Singh, 1988), *Allium sativum* (Conci and Nome, 1991), *Curculigo orchioides* (Francis *et al*., 2007), *Withania somnifera* (Sabir *et al*.,

2008), *Sacchar um* spp (Ramgareeb *et al*., 2010), *Ipomoea batatas* (Alam *et al*., 2009), *Citrus aurantifollia* (Sarker *et al*., 2015), *Hosta capitata* (Pe *et al*., 2020), etc.

Callus culture, callus is a non-differentiated meristemic mass of cells that can be differentiated into a whole new plant under appropriate growth conditions. Callus culture is ideal for genetic engineering to produce secondary metabolites and stress and disease-resistant plants (Efferth, 2019). Some examples of callus cultures are; *Triticum aestivurn* L. (Ahloowalia, 1982), *Tylophora indica* (Faisal and Anis, 2003), *Stevia rebaudiana* (Patel and Shah, 2009), *Curcuma aromatica* (Mohanty *et al*., 2008), *Arnebia hispidissima* (Shekhawat and Shekhawat, 2011), *Satureja khuzistanica* (Sahraroo *et al*., 2014), *Gynochthodes umbellate* (Anjusha and Gangaprasad, 2017), *Vernonanthura patens* (Chóez-Guaranda *et al*., 2021), etc.

Somatic embryogenesis involves the development of a somatic embryo from a non-zygotic cell and essentially the formation of a whole new plant (Von Arnold *et al*., 2002). It has been successful in many plants such as; *Zea mays* L. (Conger *et al*., 1987), *Pinus strobes* (Klimaszewska *et al*., 2001), *Solanum melongena* L. (Kantharajah *et al*., 2004), *Jatropha curcas* L. (Baran Jha *et al*., 2007), *Stewartia* species (Gladfelter *et al*., 2021), etc.

Suspension culture is a culture of a cell or a cluster of cells suspended in an agitated liquid medium. It is appropriate for the study of elaborate physiological phenomenon at the microscopic levels because of homogeneity, large quantity, fast cell growth, and reproducibility of conditions (Moscatiello *et al*., 2013). Such examples are; *Lithospermum erythrorhizon* (Fujita *et al*., 1981), *Taxus* species (Tabata, 2006), *Withania somnifera* (Nagella and Murthy, 2010), *Artemisia absinthium* (Ali and Abbasi, 2013), *Vernonia anthelmintica* (Hassanpour and Niknam, 2020), etc.

Anther culture is a culture of anthers containing uninucleate microspores excised from closed flowers (Sopory and Munshi, 1996). It has been successfully done in various plants such as; *Cucumis sativus* L. (Kumar *et al*., 2003), *Azadirachta indica* A. Juss.

(Chaturvedi *et al*., 2003), *Curcuma attenuate* (Kou *et al*., 2013), *Borago officinalis* L. (Hoveida *et al*., 2017), *Cicer arietinum* L. (Abdollahi and Rashidi, 2018), etc.

1.3.2. Genetic fidelity

Somaclonal variation is the phenomenon of occurrence of spontaneous variations among the subclones of a single parent. These variations are not specific to any species or organ and can be either temporary or permanent (Larkin and Scowcroft, 1981). Epigenetic or physiological effects result in temporary changes which can not be inherited and can be reversed (Kaeppler *et al*., 2000). The permanent changes are heritable and may occur because of previous variations in the parent plant or any uncertain genetic changes (Larkin and Scowcroft 1981). To avoid the somaclonal variations in plants, researchers have developed various techniques to detect such variations such as; morphological detection based on the morphological characteristics, physiological/biochemical detection based on the response to PGRs, and light and molecular detection based on the changes at the molecular level (Bairu *et al*., 2011). Molecular detection is the most reliable method to analyze genetic variation among *in vitro* grown plants. Restriction fragment length polymorphism (RFLP) has been successfully employed to assess the molecular diversity in soybean (Keim et al., 1989), *Brassica rapa* (Song *et al*., 1991), alfalfa (Tavoletti *et al*., 1996), etc. RFLP have limitations such as it requires a large quantity of plant, radioactive/toxic reagents, time-consuming and costly. Some more effective and less complex methods are developed over time known as PCR-based techniques. Random amplified polymorphic DNA (RAPD) has been used for tomato (Klein-Lankhorst *et al*., 1991), *Isotoma petraea* (Bussell, 1999), rice (Ikhajiagbe and Omoregie, 2020), etc. Amplified fragment length polymorphism (AFLP) been used for *Moringa oleifera* (Muluvi *et al*., 1999), *Sesamum indicum* L. (Laurentin and Karlovsky, 2006), *Onobrychis viciifolia* ScoP (Bhattarai *et al*., 2017), *Iris* (Morgil *et al*., 2020), etc. Inter simple sequence repeat (ISSR) has been employed in *Oryza* species (Blair 1999; Joshi *et al*., 2000), *Cymbidium goeringii* (Wang *et al*., 2009), *Salvadora persica (*Monfared *et al*., 2018), Camellia oleifera (*Xiao *et al*., 2020), etc.

1.3.3. Elicitation

Plant tissue culture provides an advanced method for the production of useful secondary metabolites. Controlled culture accelerates the secondary metabolites yield as compared to naturally growing plants. To maximize the production of secondary metabolites several culture factors can be optimized such as the strength of basal media, carbon source, PGRs, photoperiod, temperature, etc. (Zobayed *et al*., 2005; Cui *et al*., 2010; Amoo and Van Staden, 2013; Fazal *et al*., 2016; Gao *et al*., 2018).
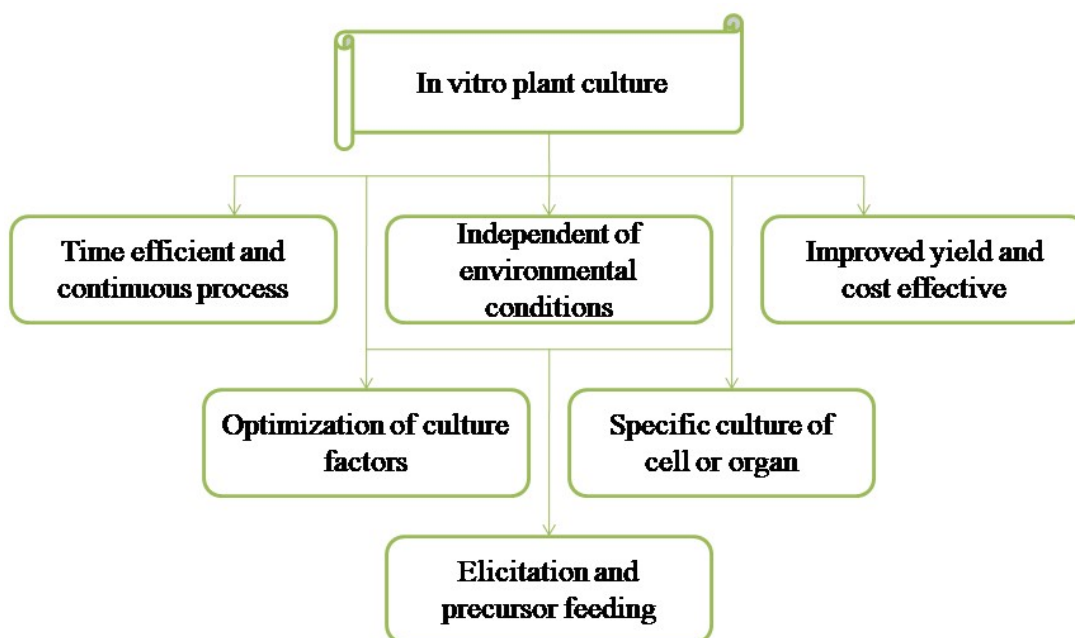
Fig. 1.2: Advantages of *in vitro* culture for production of Secondary metabolites

Recent advances in science have incorporated bioengineering techniques and transformed *in vitro* technology into industrially feasible products. For instance, the anticancer medication Taxol manufactured by an American-based pharmaceutical company (Bristol-Myers Squibb) is procured by plant tissue culture technique (Narayani and Srivastava, 2017). Besides culture factors elicitation is one of the commonly used stimulation for enhanced production of secondary metabolites. Elicitation is the application of biotic or abiotic elicitors to the plant or plant cells which triggers the plants' defense system. The

plants undergo a sequence of structural and biological changes when exposed to biotic and abiotic stress (Potters et al. 2007). Thus, one of the primary strategies for increasing the production of secondary metabolites is to expose the plants/cells to elicitors (Javed *et al*., 2018; Gonçalves *et al*., 2019; Makowski *et al*., 2020; Ayoola-Oresanya *et al*., 2021). The molecular system of elicitation involves identification of elicitors by the receptors present in the plasma membrane which stimulate the ion fluxes, Calcium ion (Ca^{2+}) burst, acidifed cytoplasm, reactive oxygen species (ROS) rupture, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and G-protein, and mitogen-activated protein kinase phosphorylation (Zhao *et al*., 2005). It also stimulates directing a series action messengers. Messengers set off transcription factors and gene expression, which results in rearranged secondary metabolism (Vasconsuelo and Boland, 2007).
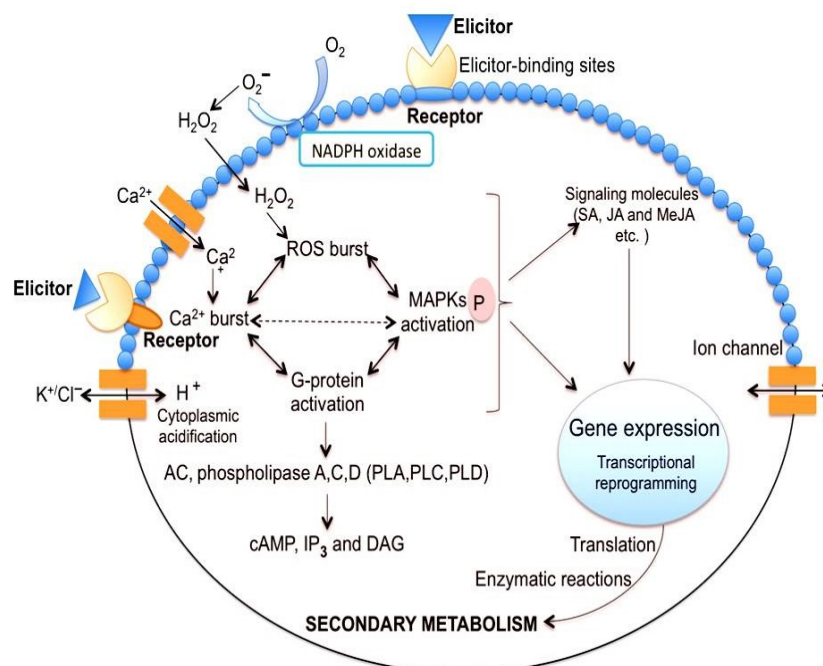
Fig. 1.3: The molecular mechanism of elicitation (Shakya *et al*., 2019)

Elicitors can be categorized as endogenous and exogenous because of their origin and abiotic and biotic elicitors because of their nature (Goel *et al*., 2011). Tables 1.2 and 1.3

summarize the effect of different abiotic and biotic elicitors used so far in the plant cell/tissue culture.

Table 1.2: Abiotic elicitors employed to increase the *in vitro* secondary metabolite yield

Abiotic elicitors				
Elicitor	Plant species	Secondary metabolite	Increase in fold	Reference
Cadmium chloride	*Gymnema sylvestre*	Gymnemic acid	6.8	Ch *et al*., 2012
NaCl	*Rauwolfia serpentine*	Ajmalicine	14.8	Srivastava *et al*., 2016
NaCl	*Solanum khasianum*	Solasodine	4	Srivastava *et al*., 2016
Cobalt	*Vitis vinifera*	3-O-glucosyl-resveratrol	1.6	Cai *et al*., 2013
Ultrasound	*Valeriana glechomifolia*	Valepotriate	2	Russowski *et al*., 2013
Methyl jasmonate	*Hypericum perforatum*	Flavonoid	2.7	Wang *et al*., 2015
Heat shock	*P indica*	Plumbagin	5	Jaisi and Panichayupakaranant, 2016
Gamma rays	*Hypericum triquetrifolium*	Chlorogenic acid	4	Azeez *et al*., 2017
Salicylic acid	*Bacopa monnieri*	Bacoside	2	Koul and Mallubhotla, 2020
Methyl jasmonate	*Narcissus pseudonarcissus*	Galanthamine	5.6	Ferdausi *et al*., 2021

Table 1.3: Biotic elicitors employed to increase the *in vitro* production of secondary metabolites

Biotic elicitors				
Elicitor	Plant species	Secondary metabolite	Increase in fold	Reference
Mannan	*Hypericum adenotrichum*	Hypericin	1.7	Yamaner *et al.*, 2013
Stenotrophomonas maltophilia	*Hypericum perforatum*	Hypericin	3	Mañero *et al.*, 2012
Aspergillus niger	*Gymnema sylvestre*	Gymnemic acid	11.2	Chodisetti *et al.*, 2013
Aspergillus *niger*	*Psoralea corylifolia*	Psoralen	9	Ahmed and Baig, 2014
Aspergillus niger	*Isatis tinctoria* L.	Flavonoid	6.83	Jiao *et al.*, 2018
Mesorhizobium amorphae	*Panax ginseng*	Ginsenosides Rb_2	19.4	Le *et al.*, 2018
Yeast extract	*Azadirachta indica*	Azadirachtin	2.5	Farjaminezhad and Garoosi, 2021
Escherichia coli	*Helicteres isora*	Diosgenin	9.1	Shaikh *et al.*, 2020

1.4. Aim and objectives

The present study is focused on the study of an important napthaquinone i.e. plumbagin. Plumbagin is the most commonly occurring naphthoquinone having a molecular weight of 188.8 and melting point between 78-79°C. It was isolated from *Plumbago* species which contributed to its name, Plumbagin (van der Vijver, 1972). Plumbagin is a widespread naphthoquinone found in families like *Plumbaginaceae* (Babula *et al.,* 2009;

de Paiva *et al*., 2005), Droseraceae (Kreher, 1990; Nahálka *et al.,* 1996, 1998; Crouch *et al.,* 1990), Ebenaceae (Zhong *et al.,* 1984; Zakaria *et al.,* 1984), Euphorbiaceae (Fournet *et al.,* 1992; Babula *et al.,* 2007), Nepenthaceae (Aung *et al.,* 2002; Cannon *et al.,* 1980; Likhitwitayawuid *et al.,* 1998), Balsaminaceae (Glennie *et al.,* 1965), and many other plant species. The main sources of plumbagin are *P indica* and *P zeylanica*, the annual demand for the raw material is >100 MT (metric tonne) and 1000 MT, respectively in India (https://nmpb.nic.in/content/marketing-trade-1). *Plumbago* species are endangered medicinal plants exhibiting a wide range of therapeutic and economic value. The high demand for these plants has put them under threatened categories (Gowthami *et al*., 2021). *Plumbago* (Family- Plumbaginacea) is a perennial medicinal shurb, widely used in traditional and modern medicine.
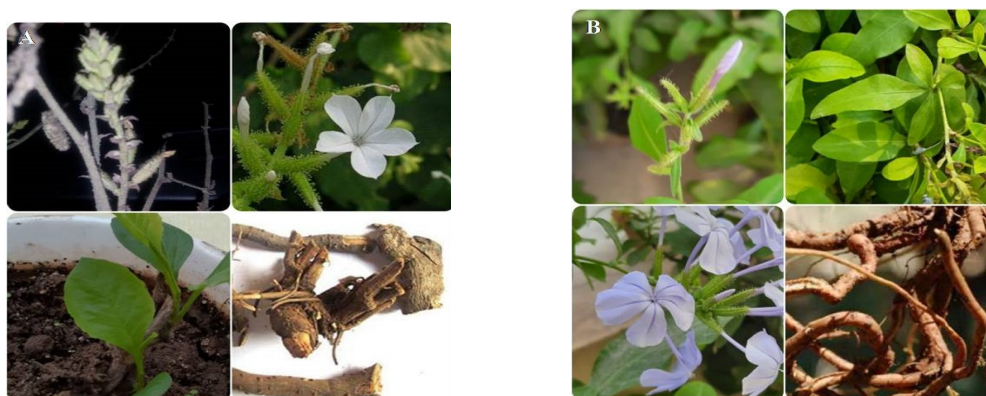
Fig. 1.4: Morphological description of the (A) *P zeylanica* and (B) *P auriculata*

Taxanomic classification (Sharma and Singh, 2015)

Kingdom	Plantae
Subkingdom	Tracheobionta
Class	Magnoliopsida
Subclass	Caryophyllidae
Superdivision	Spermatophyta

Kingdom	Plantae
Division	Magnoliophyta
Order	Caryophyllales
Family	Plumbaginaceae
Genus	*Plumbago*

Objectives of the study

- Collection of *Plumbago* species from different geographical areas of India (Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, and Uttarakhand) and screening of elite population on the basis of plumbagin content by HPTLC method.

- Optimization of various factors such as pre-harvest and post-harvest and extraction method for improved plumbagin production.

- *In vitro* and *in vivo* propagation of the elite species of *Plumbago* for the increased production of plumbagin using biotic factors i.e., polysaccharides or AM fungi, PGPR, etc.

CHAPTER 2
Review of literature

1.1. Structure of plumbagin

Plumbagin ($C_{11}H_8O_3$) is an orange pigment and dissolves easily in organic solvents (Babula *et al.,* 2009). It belongs to quinone family and is the most commonly found naphthoquinone (Rajalakshmi *et al*., 2018). Plumbagin can be toxic to normal body cells in a higher dose, to avoid the toxicity a metal complex [Cu (PLN) (PHEN)] NO_3 was created. This complex can intercalate and cleave DNA effectively (Padumadasa *et al*., 2016). Plumbagin is a plant-based secondary metabolite chemically known as 5-Hydroxy-2-methyl-1, 4-naphthoquinone, where methyl and hydroxyl groups substituted hydrogens at positions 2 and 5, respectively (Fig. 1) (https://pubchem.ncbi.nlm.nih.gov/compound/Plumbagin).
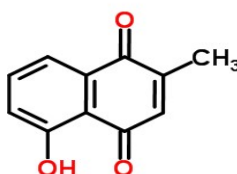
Fig. 2.1: Chemical structure of Plumbagin
(https://pubchem.ncbi.nlm.nih.gov/compound/Plumbagin)

1.2. Biosynthetic pathway

The earliest attempt to depict the biosynthesis pathway involved in the synthesis of plumbagin was an acetate feeding experiment in which precursors were incorporated into plumbagin in young shoots of *P europaea* L. (Durand and Zenk 1971). The plumbagin obtained was chemically degraded to investigate whether it was labeled or not. The results confirmed that the biosynthesis of plumbagin occurs via the polyacetate-malonate pathway (Durand and Zenk 1971; Springob *et al*. 2007). The same acetate feeding

experiment was also conducted on the leaves of *Drosophyllum lusitanicum* and similar results were obtained. A recent study conducted by Vasav *et al*., 2020 demonstrated that one molecule of Acetyl-CoA and five molecules of malonyl-CoA undergo iterative condensation and form an intermediate hexaketide backbone catalyzed by polyketide synthase (PzPKS). Aldo-keto reductase1 (PzAKR1) and cyclase1 (Pzcyclase1) catalyzes the decarboxylation and form naphthoquinone and naphthalene derivative. 3-Methyl-1,8-napthalenediol and/or isoshinanolone were synthesized from hexaketide backbone using predicted PzAKR1 and Pzcyclase1 along with PzPKS. The isoshinanolone undergo the oxidation reaction to form plumbagin which might be catalyzed by cytochrome P450 (PzCYP81B140 and PzCYP81B141) with an accessory cytochrome P450 reductase (PzCPR).
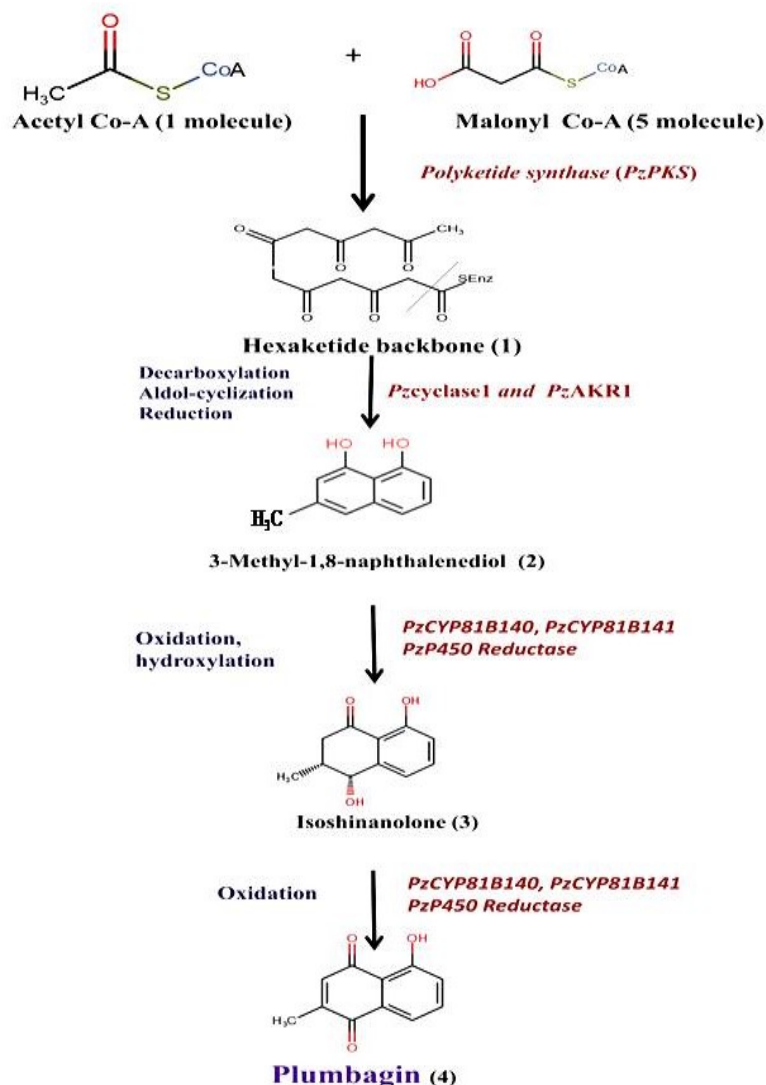
Fig. 2.2: Postulated biosynthetic pathway of plumbagin (Vasav *et al*., 2020)

1.3. Pharmacology

Plumbagin exhibit many pharmacological properties such as anti-microbial (de Paiva *et al*., 2003; Nair *et al*., 2016; Dissanayake *et al*., 2020), anti-malarial (Likhitwitayawuid *et al*., 1998), anti-plasmodial (Simonsen *et al*., 2001), antifungal (Adebanjo and Victoria, 2021), anti-inflammatory (Zheng *et al*., 2017; Checker *et al*., 2009, 2010; Oyedapo, 1996); anti-tumor (Chen *et al*., 2017); anti-carcinogenic (Parimala and Sachdanandam

1993; Sameni *et al.*, 2016; Yu *et al.*, 2018; Zhang *et al.*, 2021); anti-fertility (Bhargava, 1984; Kini *et al.*, 1996); antioxidant (Tilak *et al.*, 2004); anti-diabetic (Zarmouh *et al.*, 2010; Sunil *et al.*, 2012) and many more. Most of the recent studies focused on the therapeutic role of plumbagin against human cancer cell lines such as cervical (Jaiswal *et al.*, 2018), esophagus (Cao *et al.*, 2018), breast (De *et al.*, 2019), lung (Tripathi *et al.*, 2020), bladder (Zhang *et al.*, 2020), pancreatic (Pandey *et al.*, 2020), endometrial (Zhang *et al.*, 2021) *via* apoptosis, cell cycle arrest, autophagy, growth inhibition, *etc*.

Sheeja *et al.*, (2010) investigated various leaf extracts of *P zeylanica* for the anti-inflammatory properties using *in vivo* experimental models. When linked to the control collection, inflammation in the carrageenan-induced rats can be reduced by the acetone extract (p < 0.01). Oyedapo, (1996) evaluated the anti-inflammatory activity from the phosphate shielded saline root extracts of *P zeylanica*. Alpana in 1996 found the reducing effect of plumbagin on cholesterol and LDL- cholesterol was 53% - 86% and 61% - 91% respectively; minor cholesterol/ phospholipid ratio was reported to be 45.8%; enrapture decreased HDL-cholesterol in the rabbit The convoluted curative potential of *P zeylanica* was investigated in rats and it was found that the methanolic root extract of *P zeylanica* was reported to have a momentous wound healing action (Kodati *et al.*, 2011; Reddy *et al.*, 2002).

Table 2.1: Pharmacological activities showed by plumbagin

Biological activity/effect	Model organism/cell	Result	Reference
Larvicidal activity	*Anopheles stephensi*	100% larval mortality at 100 ppm	Pradeepa *et al.*, 2014
Nephroprotective effect	Swiss albino mice	Cisplatin-induced changes were reversed at 400mg/kg	Rajakrishnan *et al.*, 2017
Leishmanicidal activity	*Leishmania donovani*	Programmed cell death	Awasthi *et al.*, 2016
Immunosuppressive effect	Human acute T lymphoblastic	NF-$_K$B signaling pathway inhibition	Bae *et al.*, 2016

	leukemia MOLT-4 cells	and T-ALL cell proliferation suppression	
Apotosis	Human promyelocytic leukemia HL-20	Elimination of HL-20	Zhang *et al.*, 2017
Anti-carcinogenic	Human cancer cell lines Ovarian teratocarcinoma cell line and Human lung adenocarcinoma	Positive results were observed at 10-40 microgram	Lakshmanan *et al.*, 2016
Anti-inflammatory	Mice	Suppression of NF-κB in human osteoarthritis chondrocytes	Zheng *et al.*, 2017
Neuroprotective	Male Sprague-Dawley rats	Reversed the mechanical hyperalgesia caused by nerve injury.	Arruri *et al.*, 2017
Anti-obesity	-	Pancreatic lipase enzyme inhibition	Jaradat *et al.*, 2016
Anti-tumour	Human glioblastoma multiforme cells and medulloblastoma cells	Telomere shortening	Khaw *et al.*, 2015
Anti-tumour	Human glioma cell lines	Tumor decreased by 54.48%	Niu *et al.*, 2015
Anti-microbial activity	*Candida albicans, Staphylococcus aureus*	Growth Inhibition	Nair *et al.*, 2016
Anti-oxidant effect	Wistar rats liver cells	Radical scavenging and lipid peroxidation-inhibition	Tilak et al., 2004
Cytotoxic activity	MCF7(breast	Positive results	Nguyen *et al.*,

	cancer cell)		2004
	Bowes cell lines (melanoma).		
Anti-tumor activity	Human colon cancer cells	Cell proliferation and induces apoptosis	Chen *et al*., 2013
Anti-diabetic activity	Male Wistar albino rats	Increased insulin secretion	Sunil *et al*., 2012

For better understanding and treatment of diseases synergetic or combinatory analyses of plumbagin were done. The synergetic result of plumbagin and zoledronic acid on human breast cancer cells was analyzed *in vitro* which suppressed the cancer cells (Qiao *et al* 2015). Synergetic treatment affects various kinds of tumor cells (Stresing *et al*., 2007). The synergetic anti-microbial activity of plumbagin, Oxacillin and Tetracycline against was found to be effective (Rondevaldova *et al*., 2015). Celecoxib and Plumbagin combined inhibited melanoma tumor survival and growth (Gowda *et al*., 2017). Highly potent (95.167%) anti-implantation activity has been reported from hydroalcoholic extract of *P zeylanica* leaves due to anti-estrogenic property, which provokes estrogen action, leading to structural and functional changes of the uterus. The anti-estrogenic activity as well provokes decrease content of glycogen, diameter, and depth of endometrium, myometrium, condensed uterine lumen with declined pits and folds, lessened number, and size of uterine glands, vaginal opening, and cornification (Vishnukanta and Rana, 2010).

1.4. Extraction of plumbagin

The extraction process needs to be precise; proper protocol should be followed for specific a secondary metabolite, the extraction solvent should be chosen carefully depending upon the solubility of the compound to be extracted, there should be no mixing of other compounds with the desired one, shun all kinds of contaminations and prevent degradation of the secondary metabolite. Extraction of air-dried roots of *P zeylanica* using the Soxhlet method with chloroform for 4 hours gave the highest yield of plumbagin as compared to using *n*-hexane (Gupta *et al*., 1993). In another study, it was

again concluded that the soxhlet method with chloroform as the solvent, process duration of 5 hours is the most efficient as compared to other methods studied; static maceration, dynamic maceration, and ultrasound-assisted extraction (Paiva *et al*., 2004). Plumbagin is also extracted from methanol crude extract of *P rosea* using the Soxhlet method with silica column and hexane/ethyl acetate (Komaraiah *et al*., 2001). In another study, plumbagin was isolated from chloroform/dichloromethane (1:1) root extract of *P zeylanica* using the cold maceration method (Bothiraja *et al*., 2011). Plumbagin was isolated from the aged root extract of *P scandens* using *n*-hexane and chloroform by the soxhlet method (Rodrigues *et al*., 2006).

A reverse phase HPLC method with UV detection was demonstrated by Unnikrishnan *et al.,* 2008 for the determination of plumbagin. HPTLC utilizing hexane: ethyl acetate as a mobile phase was also used to quantify plumbagin. It was observed that higher plumbagin content in roots of *P indica* as compared to the roots of *P zeylanica*. Both the methods were simple, accurate, and sensitive. TLC densitometric method was used to quantify plumbagin content, the solvent system was toluene: glacial acetic acid and plumbagin was identified as yellow spots on the TLC plate (Yogananth *et al.,* 2009). The plumbagin from roots of *P zeylanica* was determined and identified by (LC-MS/MS) Liquid Chromatography with tandem mass spectrometry (Hsieh *et al.,* 2005). HPTLC was used to determine the plumbagin content in three different species of *Plumbago*; *P zeylanica, P capensis*, *P rosea,* the quantity was recorded as 0.569, 0.274 and 0.429, % respectively (Dorni *et al.,* 2007).

Table 2.2: Analytical methods for determination of Plumbagin and its related compounds

Analytic-al techniqu-e	Plant species/part	Instrument condition	Sample preparation/Extraction solvent	Reference
HPLC	*P zeylanica* Roots	Mobile phase: Aqueous methanol. Retention time- 4.34 min.	Extracted in ethyl acetate by soxhlet extractor.	Nayak *et al*., 2015
HPTLC	*P zeylanica*	Mobile phase: toluene and formic	Powdered roots (0.5 g) were extracted	Panda and Kamble,

	L. Roots	acetic acid (99:01). Spots visualized under short wavelength UV 254 nm light. Rf value - 0.35 ± 0.02.	using 10 ml acetone for 12 hrs thrice. The extract was concentrated to 10 ml.	2016.
HPTLC	*P zeylanica, P rosea* and *P capnesis* Roots	Mobile phase: toluene, ethyl acetate, and methanol (8:1:1). Rf -0.78	Cold maceration extraction with 50% methanol.	Dorni *et al*., 2010
HPLC	*Drosera peltata*	Mobile phase –water: methanol (65:35) Retention time-7.9 min Detection wavelength-254nm	Sonication with methanol for 30 min twice.	Tian *et al*., 2014
HPLC	*P indica* Root	Mobile phase: methanol and 5% aqueous acetic acid (80:20) Detection wavelength-260 nm	Powdered root extracted in ethanol by heat reflux method	Jaisi and Panichayu pakaranan t, 2016
RP-HPLC ^{1}H NMR (Proton nuclear magnetic resonance)	*P zeylanica* Linn. Roots	Rf value- 0.785 Retention time-4.088 min ^{1}H NMR recorded on a NMR-400 MHz confirmed chemical constituent of Plumbagin is 2-methyl-5-hydroxyl-1, 4-naphthoquinone ($C_{11}H_8O_3$)	Extraction by soxhlet extractor using methanol as solvent.	Pradeepa *et al*., 2014
RP-HPLC	*P scandens* Roots	Mobile phase: methanol and water (50:50 v/v)	Extraction done in laboratory-built high-pressure stainless steel cell.	Rodrigues *et al*., 2006
UPLC-UV and HPTLC	*P auriculata, P indica,* and *P*	HPTLC Mobile phase: toluene and ethyl acetate (80:20)	Extraction by sonication in methanol for 30 minutes	Galal *et al*., 2013

	zeylanica	Rf-value -0.2–0.8 UPLC-UV Mobile phase: water and acetonitrile flow rate of 0.23 mL/min.		
Liquid chromatography with tandem mass spectrometry(LC-MS/MS)	*P zeylanica* L. Roots	Mobile phase: 90 % methanol	Heat maceration in aqueous ethanol	Hsieh *et al*., 2005

1.5. Plumbagin production in *in vitro* culture

Due to the declining wild population of plant species producing plumbagin, the development of alternative techniques to conserve these plants is very critical. *In vitro* culturing enables mass production of plants and conservation of many endangered and other important plants. Plumbagin has been isolated from different plant species, the content also varies in different plant parts. In *P zeylanica* higher amount of plumbagin accumulates in the roots (Mallavadhani *et al*., 2002), plant tissue culture provides an alternate source of plumbagin by root culture technique. Secondary metabolites are produced in response to stresses (physical, chemical, or biological). For example, chemical and osmotic conditions affected the biomass growth and secondary metabolite production (plumbagin) in suspension culture of *Drosophyllum lusitanticum*, it was reported that pH 3.5 and 10g sucrose/l was optimum (Nahálka *et al*., 1996) for higher yield of plumbagin. The cell suspension culture of *D lusitanticum* under the influence of optimal exogenous concentrations and media resulted in 10 time higher plumbagin production than in the naturally growing plant (Nahálka *et al*., 1996). Rooting was induced in *P zeylanica* in *in vitro* culture on ½ basal MS medium containing 0.25mg dm^{-3} IBA (Saxena *et al*., 2000).

1.5.1. Conventional methods

Conventional methods comprise general or basic methods used to propagate or produce plants like callus culturing, organogenesis (direct and indirect), embryogenesis (zygotic and somatic), organ culture, etc. Several *in vitro* studies were conducted and it was found out that *in vitro* grown plant/cultures accumulated more plumbagin. A study conducted by Satheeshkumar and Seeni (2002), concluded that cell suspension culture is a better way as compared to callus culturing because its less time-consuming.

1.5.1.1. In-direct organogenesis or Callus culture

In-direct organogenesis involves the formation of callus before shoot or root formation, the organs can be regenerated from the callus via optimum application of PGRs. Lubaina and Murugan (2012) studied the influence of plant growth regulators on *P zeylanica* growth and plumbagin content. Surface sterilized leaf and stem explants were used for the experiment inoculated in MS media with 3% sucrose gelled with 0.8% agar. The cultures were incubated at room temperature (25°C-27°C) under 3000 lux intensity white light with 16h light and 8h dark period. Different concentrations of different PGRs were tested for best results, it was concluded that for callus induction the combination of 2,4-D (1mg/L) and BAP (0.5mg/L) was best and for shoot regeneration BAP (1mg/L) was best, the plumbagin content was also found higher than *in vivo* grown plant species.

Table 2.3: Selective protocols for callus culture of *Plumbago species*

Species	Explant	Culture	MS+PGR			References
			Callus induction	Shooting	Rooting	
P rosea L.	Stem	Callus culture	MS+2.5 mg/L 2,4-D+1.5 mg/L kinetin	MS+2.0 mg/L BAP+1.0 mg/L NAA	MS+1.5 mg/L IBA+3% sucrose	Kumar and Bhavanadan 1988
Drosophyllum	Nodes	Suspension	Gamborg B5(1968)			Nahálka *et al.*, 1996

lusitanic um		culture	medium+1.0 mg/L IBA+0.5 mg/L NAA			
P zeylanica	Leaves and stem	Callus culture	MS basal medium+3% sucrose+11.4 2uM IAA+2.22u M BAP(better callus from leaf explant)	MS basal medium+3% sucrose+4.44 uM BAP+1.42u M IAA(Better shoot induction from leaf explant)	½ strength MS+0.57u M IAA+2% sucrose	Rout *et al.*, 1999
P rosea L.	Leaves	Callus culture	MS+1mg/L IAA+0.5mg/ L NAA+0.3mg /L BAP+3% sucrose	-	-	Komaraiah *et al.*, 2001
P zeylanica	Stem	Callus	MS media + BAP (2.0 mg/L) + IAA (1.5 mg/L)	-	-	Sivanesan *et al.*, 2009

1.5.1.2. Direct organogenesis

Direct organogenesis is as the name suggests is a technique for direct organ regeneration from explants, it is a better alternate of growing plantlets in a short period. Das and Rout in 2002 used leaf explants to successfully induced shoots from *P zeylanica* and *P rosea*

with a 90 % survival rate of the developed plantlets. For optimum shoot proliferation, the explant was inoculated in MS medium comprising of 1.5 mg/L BAP, 0.75 mg/L IBA, 0.75 mg/L adenine sulfate, and 10 % coconut milk. The micro-shoots were rooted in half-strength MS media supplemented with only 0.75 mg/L IBA. The rooting response was found 100% and the survival rate of plantlets in the natural environment was also 100%. Kanungo *et al*., (2012) reported 95 % multiple shoot growth from the nodal explants of *P zeylanica* on MS basal medium supplemented with BAP (2.0 mg/L), IAA (1.5 mg/L), and IBA (1.0 mg/L). The micro-shoots rooted in MS medium supplemented with NAA (1.5 mg/L), IAA (1.5 mg/L) and IBA (2.0 mg/L). All the regenerated plantlets survived under the natural environment. ¼ MS medium without any plant growth regulator was found optimum for rooting and shooting in *Drosera intermedia* (Grevenstuk *et al*., 2010).

Table 2.4: Direct organogenesis studies in *Plumbago species*

Species	Explant	Culture	MS+PGR		References
			Shooting	Rooting	
Drocera capensis	Leaves	Bud culture	½ MS+0.100 mg/L NAA+0.100 mg/L BAP	½ MS+0.100 mg/L NAA+0.0125 mg/L BAP	Crouch *et al*., 1990
D natalensis	Leaves	Bud culture	½ MS+0.025 mg/L NAA+0.1 mg/L lBA	½ MS+0.1 mg/L NAA+0.125 mg/L BAP	Crouch *et al*., 1990
P zeylanica L.	Nodes	Bud culture	MS+3% sucrose+27.2 µM AdSO$_4$+2.46 µM IBA	MS +4.92 µM IBA	Selvakumar *et al*., 2001
P rosea	Leaves	Root culture	-	Root Solid B5 medium+0.5-2.0 mg/L NAA+0.1-0.5 mg/L kinetin	Panichayupakaranant and Tewtrakul 2002

			Root growth-Liq. B5 medium+1 mg/L NAA+0.1 mg/L kinetin		
P rosea	Leaves	Shoot culture	Shoot induction- MS+6.66 μM BAP+2.69 μM NAA Shoot elongation- 1.11 μM BAP+1.44 μM GA$_3$	½ strength MS	Gopalakrishnan *et al*., 2009
P auriculata	Leaves, nodal and apical shoots	Root culture		MS media 1.5 mg/L IBA IAA and NAA 1.5 mg/L and 0.1 mg/L	Deshpande *et al*., 2015
P rosea	Internodes	Cell suspension culture	-	1.5 mg/L NAA 1.0 mg/L IAA	Silja *et al*., 2016

1.5.2. Contemporary or advanced methods

Contemporary methods are the advance experimental approaches that involve the application of elicitors (biotic and abiotic), synthetic seeds, genetic manipulation, hairy root culture, bio-reactors, metabolic engineering, use of endophytes, etc. The development of these modern systems of propagation allows cultivators to obtain desirable traits in plants. There are a lot of chemical compounds, microorganisms, and

physical stresses which trigger the plant defense system, these compounds or factors are called elicitors and the process is known as elicitation. Recent studies for the production of plumbagin were mainly centered on the implementation of elicitors. Transformed hairy root culture accumulated more plumbagin as compared to non-transformed roots (Nayak *et al.*, 2015). The experiment included *Agrobacterium rizogenes* which were injected in internodes and leaf midrib and co-cultivated for 5-6 days. The transformation of the roots was proved by PCR amplification, the highest plumbagin content was found in A4 transformed rhizo-clone HRA2B5 that was 2.26mg/g DW.

1.5.2.1. Elicitation

A lot of plant species are cultured for their secondary metabolites/secondary metabolites (Vanisree *et al.*, 2004). Various biotechnological techniques were used for better yield of plumbagin from several plant species. Elicitation is one of the techniques which have been proved to be very effective (Gonçalves and Romano, 2018). There are some factors (biotic and abiotic) that influence the production of secondary metabolites, these factors are designated as elicitors and the phenomenon i.e. the improved biosynthesis of precise compounds is elicitation (Namdeo, 2007). Various fertilizers (chemical and natural) were applied to *P zeylanica* to study the effect on the plumbagin content, it was found that *Azospirilum* enhanced the concentration of plumbagin in roots (0.027% w/w) as compared to the control (Patel *et al.,* 2016).

Table 2.5: Effect of various elicitors used *in vitro* culture to enhance plumbagin production

Species	Explant	Elicitors	Achievements	References
Drosophyllum lusitanicum	Nodal part	Chitin/pectin	*N*-Acetylchitooligosacchari des enhanced the biosynthesis of plumbagin	Nahálka *et al.*, 1998
P rosea	Leaf expalnt	Fungi, yeast extract, and	Plumbagin yield increased in Chitosan	Komaraiah *et al.*, 2002

30

		chitosan	treated cells by 6.71-fold	
P rosea	Leaf derived cell culture	Synergetic effect of chitosan, immobilizati on, and in situ extraction	Plumbagin yield:- 92.13 mg g $^{-1}$ DCW	Komaraiah *et al*., 2003
P rosea	Leaf	Acetylsalicyl ic acid, Ammonium	3 fold increase	Komaraiah *et al*., 2004
Drosera burmanii	*In vitro* culture d stem segmen ts	Methyl jasmonate, yeast extract, chitosan, or salicylic acid	Yeast extract increased plumbagin production by 3.5 fold	Putalun *et al*., 2010
Drocera indica	Seeds	Yeast extract, methyl jasmonate, chitosan, salicylic acid	5.4 fold increase	Thaweesak *et al*., 2011
P indica	Leaf	Gamma rays	Increased plumbagin yield	Jaisi *et al*., 2013
P rosea L.	Nodal part	Jasmonic acid, yeast extract, NAA	Embryogenic cell suspension cultures treated with NAA increased plumbagin production by 5.59 fold	Silja *et al*., 2014

			compared to control plant	
P rosea	Nodal part	Jasmonic acid, sodium salicylate, yeast extract	Increased plumbagin content increased by the addition of jasmonic acid to 1.23% DW.	Silja and Satheeshkumar, 2015

1.5.2.2. Genetic transformation/engineering

Genetic engineering includes the technology by which a gene is introduced in a plant cell. The foreign gene (introduced gene) exhibits a specific desired trait in the regenerated plant, resulting in the manipulation of the original genetic make-up (Mittler and Blumwald, 2010). The significance of this technique is the considerable enhanced secondary metabolite yield. There are several methods of genetic transformation, for example; *Agrobacterium*-mediated transformation (Hooykaas and Schilperoort, 1992), biolistics (Maqbool *et al*., 2004), poly ethylene-mediated gene transfer (Zarei *et al*., 2011), microinjection (Neuhaus *et al*., 1987), and electroporation (Prasanna and Panda, 1997). The available literature of *in vitro* plumbagin production only includes *Agrobacterium*-mediated gene transfer, which paves the way to new techniques/experimental setup for sustainable production of plumbagin. *Agrobacterium tumefaciens* and *A. rhizogenes*are considered the natural metabolic engineers of plants.

Table 2.6: Enhanced plumbagin production following *Agrobacterium*-mediated transformation

Species	Explant	Strain	References
P zeylanica	Leaves	*A. rhizogenes* strain A4	Verma *et al*., 2002
P zeylanica	Cotyledon, hypocotyl and petiole	*A. tumefaciens* strains AGL1 and LBA4404	Wei *et al*., 2006

P indica	Hairy roots	A. rhizogenes strain ATCC 15834 pH 5.6 and 3 % sucrose	Gangopadhyay et al.,2008
P indica	Hairy root	A. rhizogenes strain ATCC 15834	Gangopadhyay et al., 2010
P indica	Hairy root	A. rhizogenes strain ATCC 15834	Gangopadhyay et al., 2011
P indica L.	Leaf and internode explants	A. rhizogenes strain A4M70GUS And elicitation by Methyl jasmonate (MJ) and acetylsalicylic acid (AS)	Martin et al., 2011

1.5.2.3. Bioreactors

For large-scale production of plumbagin *in vitro* special culture vessels are designed keeping in mind the different optimal conditions needed for the high plumbagin accumulation. These vessels are called bioreactors, which are customized according to the requisite optimum conditions for secondary metabolite production. One such bioreactor or reaction kettle was customized by Jose *et al.*, (2016), which accumulated 1.5% DW plumbagin and 12- times increased hairy root biomass.

1.6. Genetic fidelity

Isozymes and DNA sequences are used as molecular markers in genetic assessment to analyze the diversity among species and within the population of a specific species. Genetic diversity is defined based on polymorphism i.e., the difference in the DNA sequence of a DNA segment (Rani and Raina, 2000). Several polymorphic molecular markers can be considered to analyze the diversity: RFLP, RAPD, AFLP and STSs.

Table 2.7: Molecular markers used for genetic assessment

Species	Methods /tools	Results	References
Ten accession of *P zeylanica* L. were collected from different locations in Western Ghats	RAPD-PCR Fingerprint	Genetic distance within population- 0.1178 to 0.8109	Britto *et al*., 2009
5 different populations of *P zeylanica were* collected from the Western Ghats	ISSR markers (6 primers)	The population collected from Anchetty showed the highest level of polymorphism	Dharmar and John De Britto, 2012
P zeylanica, P auriculata, P rosea	RAPD markers	*P auriculata* showed a high level of polymorphism	Gadge and Nathar, 2014
5 different populations of *P zeylanica* acquired from Western Ghats	ISSR markers using 20 primers	The population collected from Kallar-Ooty exhibit the highest level of polymorphism	Dharmar and John De Britto, 2015

CHAPTER 3

Collection of *Plumbago* species from different geographical areas of India (Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, and Uttarakhand) and screening of elite population on the basis of plumbagin content by HPTLC method

3.1. Introduction

There are three species of *Plumbago* available in India, namely *P auriculata, P zeylanica,* and *P rosea*. *P auriculata* is an ornamental plant that can be easily found in gardens throughout the country. *P zeylanica* is mostly distributed across Assam, Uttar Pradesh, Bihar, Madhya Pradesh, Maharashtra, Rajasthan, Karnataka, Tamil Nadu, and Kerala. *P rosea* is rare and can only be found in Kerala and Tamil Nadu (India Biodiversity Portal). The multi-utility of plumbagin has raised its market value enormously and still rising every day. The plumbagin content varies according to the plant species and plant part, it was found highest in roots of *P rosea* (19.13×10^{-2}%), followed by the stem of *P zeylanica* (1.1×10^{-2} %) and *P auriculata* (2.1×10^{-2} %) leaf (Mallavadhani *et al*., 2002). The plant samples collected were screened by HPTLC method of quantification. It is a TLC (thin-layer chromatography) based automated technique that includes an adsorbent film, a stationary phase, mobile phase, development chamber, and samples. This technique is applicable for both quantitative and qualitative analysis whereas quantitative analysis is more optimized (Shewiyo *et al*., 2012). HPTLC has been used for the quantification of plumbagin by many researchers over time and comparative studies are also done with other methods such as HPLC (Unnikrishnan *et al*., 2008), HPTLC method was reported to be a more specific and cost-effective method as compared to HPLC.

3.2. Material and method

3.2.1. Chemicals and standard compound

Standard (Std) compound plumbagin was procured from Sigma-Aldrich Company, USA, and was stored at 4 °C. Solvents such as methanol, ethanol, chloroform, were of HPLC grade and were bought from Himedia, India.

3.2.2. Collection of plant material

The plants were collected from the regions of Jammu and Kashmir, Punjab, Haryana, Himachal Pradesh, and Uttarakhand. The plant samples were identified based on morphological characteristics and phytochemical analysis based on presence of standard (marker) compound plumbagin. Various books and papers were followed as well as interviews of distributors of local medicinal markets were conducted to study the distribution of plants. The plants were collected during the monsoon season (July-August) of 2018.

3.2.3. Sample preparation

The plant material was washed properly and shade dried to minimize the loss of plumbagin content due to heat. The dried material (1 g) was grounded finely and then extracted by maceration with different solvents such as ethanol, methanol and chloroform to determine best extraction solvent. The samples were then filtered with Whatman No. 1 paper and dried completely at 40ºC in a rotary evaporator (Buchi, R-300, India). Each extract was then dissolved in 1mL of methanol and passed through a syringe filter of 0.45 µm size to eliminate fine particles. The prepared samples were then kept in amber-colored glass vials at 4ºC until further use.

3.2.4. HPTLC analysis

The HPTLC analysis consists of standard and sample preparation, instrument settings and development of the mobile phase. The developmental method was carried out as per guidelines given in the ICH (The International Council for Harmonisation of Technical

Requirements for Pharmaceuticals for Human Use) quality guidelines. The analysis was done on pre-coated silica gel 60 F_{254} plates (E. Merck) with dimensions 20×10 cm and 0.2 mm uniform thickness. CAMAG Linomat 5 sample applicator with 100 μL micro-syringe was used to apply extract sample and standard sample in band length 8.0 mm and 14.1 mm apart with the application rate 150 nls^{-1}. The mobile phase consisted of toluene-ethyl acetate (8:2) saturated in a CAMAG twin trough glass chamber. Analysis was carried out at 272 nm in absorbance remission mode by CAMAG TLC scanner along with winCATS software.

3.2.5. Preparation of calibration curve of plumbagin

The calibration curve of plumbagin was prepared by using standard plumbagin. To prepare a stock solution 10 mg plumbagin was dissolved in 10 ml ethyl acetate and serially diluted to 200-1000 μg/ml. The standard was analyzed in triplicate, the peak area obtained was plotted against concentration and the calibration curve was made along with correlation coefficient (R^2) was determined.

3.2.6. Method validation

The ICH quality guidelines were followed to check the linearity range, precision, limit of detection (LOD), limit of quantification (LOQ), accuracy, robustness and specificity.

3.3. Result and discussion

3.3.1. Quantification of plumbagin

The phytochemical screening was conducted based on the presence of plumbagin in the collected plant material. The aerial part of the plants was collected because of the threatened status of the *Plumbago* species. The collected samples revealed numerous peaks which were compared with the standard plumbagin (Std) observed at R*f* 0.69, and matched with the samples to verify the presence of plumbagin. The solvent screening showed the highest yield was obtained from absolute ethanol. The further study was continued using absolute ethanol as an extraction solvent.
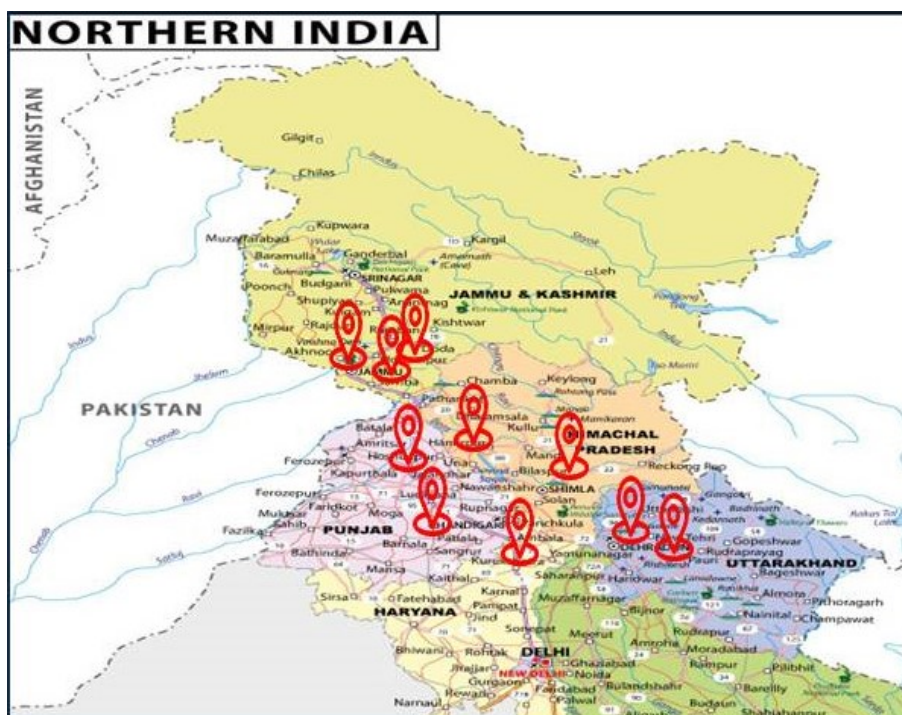
Fig.3.1: Collection sites of *P auriculata* and *P zeylanica*

All samples collected contained plumbagin but the content varied according to the geographical region. In the case of *P auriculata*, three regions were visited to collect the plant samples from Jammu and Kashmir namely, Chatha, Jammu, and Udhampur. The highest amount of plumbagin was observed in plant samples collected from Chatha (0.83µg/g). The plant samples collected from Udhampur (0.57µg/g) and Jammu (0.58 µg/g) did not show much difference in the content of plumbagin. In Punjab, the plant samples were collected from Jahaldhar and Patiala, the plumbagin content was found to be 0.70µg/g and 0.35µg/g, respectively. Two regions were visited in Himachal Pradesh namely, Kangra and Shimla, the plumbagin content obtained was 0.73µg/g and 0.41µg/g respectively. The other regions visited were Kurukshetra (Haryana), Rishikesh, and Dehradun (Uttarakhand), the plumbagin content was found to be 0.53µg/g, 0.73µg/g and 0.61µg/g, respectively.

Fig.3.2: Collected *P auriculata* from different locations

Table 3.1 Collection regions of *P auriculata* with geographical details and plumbagin content

Plant samples	Collection site	Elevation (m)	Geographical coordinates	Plumbagin content (µg/g)
A$_1$	Chatha, Jammu and Kashmir	327	32.6837° N, 74.8243° E	**0.83 ± 0.33**
A$_2$	Jammu, Jammu and Kashmir	336	32.7286° N, 74.8471° E	0.58 ± 0.24

A_3	Udhampur, Jammu and Kashmir	755	32.9160° N, 75.1416° E	0.57 ± 0.31
A_4	Jalandhar, Punjab	228	31.2560° N, 75.7051° E	0.70 ± 0.37
A_5	Patiala, Punjab	250	30.3589° N, 76.4497° E	0.35 ± 0.41
A_6	Shimla, Himachal Pradesh	2276	31.1110° N, 77.1394° E	0.41 ± 0.36
A_7	Kangra, Himachal Pradesh	774	32.2245° N, 76.1566° E	0.73 ± 0.46
A_8	Kurukshetra, Haryana	260	29.9565° N, 76.8173° E	0.53 ± 0.33
A_9	Rishikesh, Uttarakhand	340	30.1238ºN, 78.3043ºE	0.73 ± 0.58
A_{10}	Dehradun, Uttarakhand	640	30.2690° N, 78.0443° E	0.61 ± 0.39

P zeylanica was collected from six different regions including Chatha, Jammu, Udhampur (Jammu and Kashmir), Jalandhar (Punjab), Shimla (Himachal Pradesh), and Dehradun (Uttarakhand). The highest yield of plumbagin was obtained from the samples collected from Jammu (2.23µg/g) followed by Chatha (1.15µg/g) located in Jammu and Kashmir. The collection of *P zeylanica* was much laborious than *P auriculata* as it is an ornamental plant that can be spotted more easily. The phytochemical screening is important to determine the elite chemotype for industrial as well as research purposes. In an earlier study, HPLC analysis of *P zeylanica* collected from different regions of Himachal Pradesh revealed the samples collected from Hamirpur contain the highest amount of plumbagin as compared to other regions (Sharma and Agrawal, 2018). The difference in plumbagin content may vary because of many factors such as genotypes and environmental conditions (Panda and Kamble, 2016).

Fig. 3.3: Collected *P zeylanica* plants from different locations

Table 3.2 Collection regions of *P zeylanica* with geographical details and plumbagin content

Plant samples	Collection site	Elevation (m)	Geographical coordinates	Plumbagin content µg/g
J_1	Jammu, Jammu and Kashmir	336	32.7387° N, 74.8525° E	**2.23 ± 0.43**
J_2	Chatha, Jammu and Kashmir	327	32.6837° N, 74.8243° E	1.15 ± 0.26
J_3	Udhampur, Jammu and Kashmir	755	32.7286° N, 74.8471° E	0.85 ± 0.37
J_4	Jalandhar, Punjab	228	31.2560° N, 75.7051°	0.51 ± 0.69

			E	
J_5	Patiala, Punjab	250	-	-
J_6	Kangra, Himachal Pradesh	774	-	-
J_7	Shimla, Himachal Pradesh	2276	31.1110° N, 77.1394° E	0.59 ± 0.31
J_8	Kurukshetra, Haryana	260	-	-
J_9	Rishikesh, Uttarakhand	340	-	-
J_{10}	Dehradun, Uttarakhand	640	30.3631° N, 77.8761° E	0.53 ± 0.33
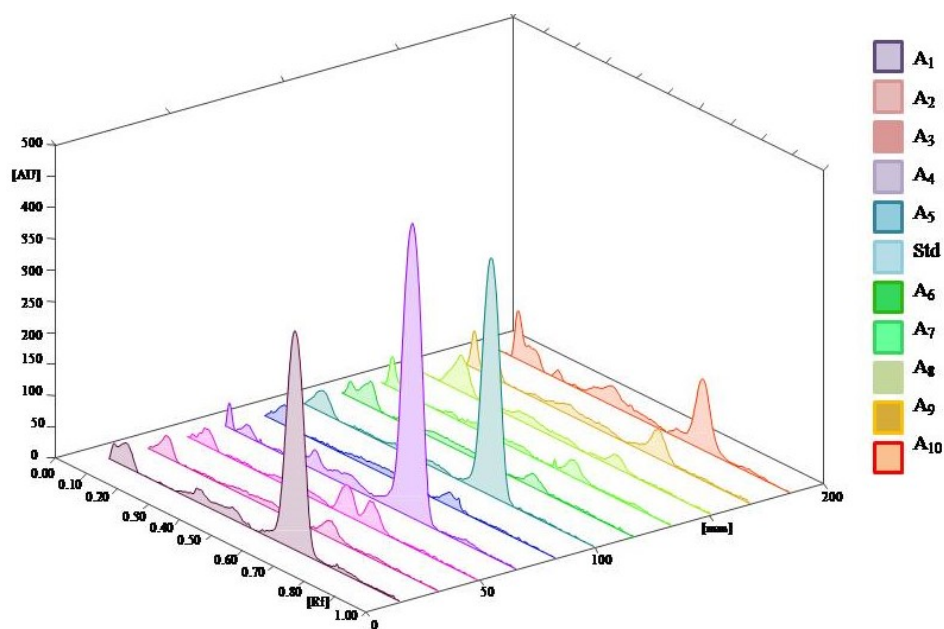
Fig. 3.4: HPTLC chromatogram of standard plumbagin (Std) and *P auriculata* plant samples (A1-A10) collected from different regions
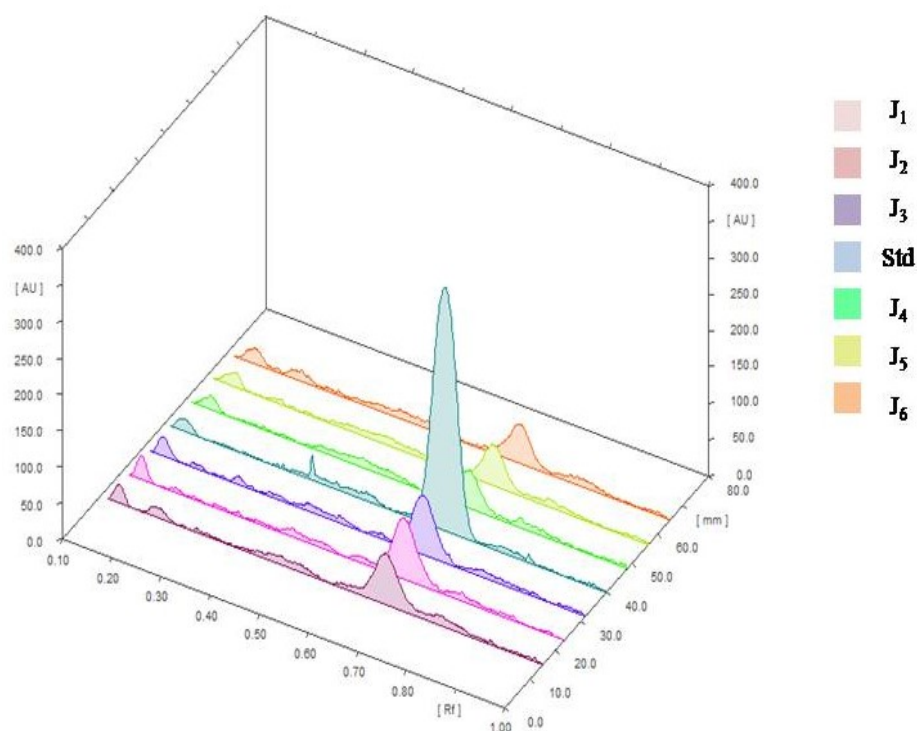
Fig. 3.5: HPTLC chromatogram of standard plumbagin (Std) and *P zeylanica* plant samples (J$_1$-J$_6$) collected from different regions

3.3.2. Method validation

The mobile phase used was toluene-ethyl acetate (8:2) that produced fine separation and peaks. R$_f$ of plumbagin was determined to be 0.69 which was then compared to the test samples. The mobile phase used resolved plumbagin from other compounds of extract efficiently. The calibration curve was drawn for reference plumbagin and a linear equation y = 2.060x + 652.4 (where = response as peak area and x = concentration) was derived with correlation coefficient R^2 = 0.993 (Fig. 3.3).
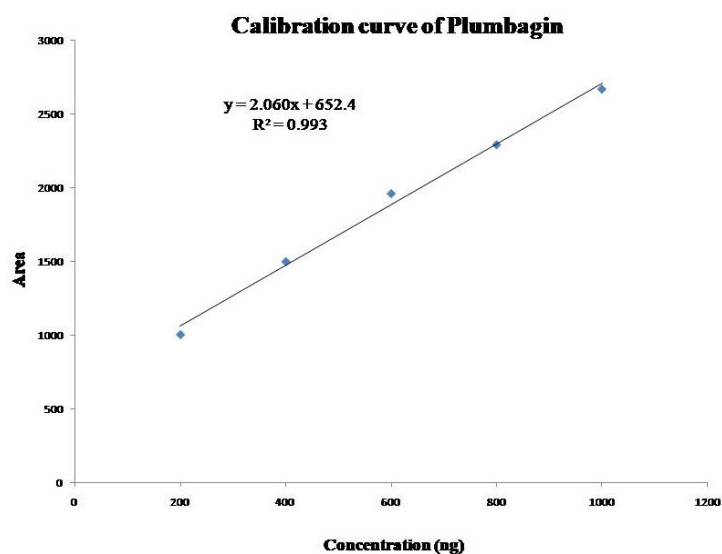
Fig. 3.6: Calibration curve of plumbagin

To establish the efficiency of the method, a suitability test was performed on pre-analyzed extract samples mixed with freshly prepared reference stock solution. The results obtained are listed in Table 4. The recovery percentage was found to be between 99.47 – 99.74 % which demonstrate the reliability and reproducibility of the method. The overall average recovery % was 99.57 %. Intra-day precision (%RSD) and inter-day precision (%RSD) on the basis of content of plumbagin was found to be between 0.61 – 1.01 and 0.97 – 1.06 respectively. The LOD (1.321 µg/spot) and LOQ (1.991 µg/spot) values reveal that the developed method shows very good sensitivity

Table 3.3: Method validation parameters by the proposed TLC-densitometric method

Parameters	Results
Linearity range (µg/spot)	0.2 – 1.0
Regression equation (n=5)	y = 2.060x + 652.4
Correlation coefficient (R^2)	0.993

% RSD intra-day precision	0.61 – 1.01
% RSD inter-day precision	0.97 – 1.06
Recovery (%)	99.50 ± 1.71
LOD (µg/spot)	1.321
LOQ (µg/spot)	3.091

3.4. Conclusion

The HPTLC method is cost and time-effective process for identification and quantification of plant-based medicines and formulations. The stated method was found to be highly precise and simple saving a lot of time and cost. The plant samples collected from different regions narrowed down the elite population of *Plumbago* species. The collected samples presented a wide range of difference in the plumbagin yield. The difference in the content could be because of many factors that can influence the yield of plumbagin. Further study can be done on a broader geographical region and the identified elite population can be mass propagated to fulfill the rising demand of the raw material for plumbagin.

CHAPTER 4

Optimization of various factors such as pre-harvest and post-harvest and extraction method for improved plumbagin production

4.1. Introduction

Extraction is one of the most important preliminary processes for the determination and application of plumbagin. The optimum extraction factors should fit in the various criteria such as economical, feasible, and effective. The extraction efficacy is influenced by various factors such as solubility of secondary metabolite, solvent composition, extraction time, temperature, solvent volume ratio, sample size, and many more (Tanko *et al*., 2005). Statistical optimization procedures enable the production of the best possible response from various variables. Response surface methodology (RSM) is the most applicable multivariate statistical technique (Box and Wilson, 1992). RSM has been applied to optimize the extraction of oils (Belhachat *et al*., 2018; Ibrahim and Zaini, 2018; Khalili *et al*., 2018; Liu *et al*., 2017; Liu *et al*., 2018), flavonoids (Ouédraogo *et al*., 2018; Huang *et al*., 2017), polyphenols (Bilgin *et al*., 2018), triterpenoids (Pandey and Kaur, 2018) and many more.

This study aimed to assess the yield of plumbagin in response to different extraction factors (pre-and post-harvest) and techniques. There are various pre-harvest factors (age of plant, plant part, harvesting season) and post-harvest factors (drying method, storage time of raw material, extraction method) to be evaluated. Furthermore, plumbagin was extracted by microwave-assisted extraction method using ethanol as solvent. RSM was applied to optimize the process parameters/variables (extraction time, solvent volume ratio, and particle size) which influence the extraction of plumbagin. Each parameter was tested at three levels by Box-Behnken design to optimize the extraction of plumbagin.

4.2. Material and method

4.2.1. Chemicals

HPLC grade solvents were used for extraction were obtained from Himedia, India, authentic plumbagin standard compound was procured from Sigma-Aldrich Company, USA.

4.2.2. Sample collection and preparation

P zeylanica and *P auriculata* was collected from the field station of Council of Scientific and Industrial Research–Indian Institute of Integrative Medicine (CSIR-IIIM) Jammu, India. The plant material was categorized into leaf, stem, and root then grounded finely. The plant material (1 g) was extracted using 10 ml absolute ethanol by sonication for half an hour. The samples were further subjected to maceration overnight for extraction. The solvent was dried completely and the extract was dissolved in 1mL of methanol for LC-MS analysis.

4.2.3. Experimental design
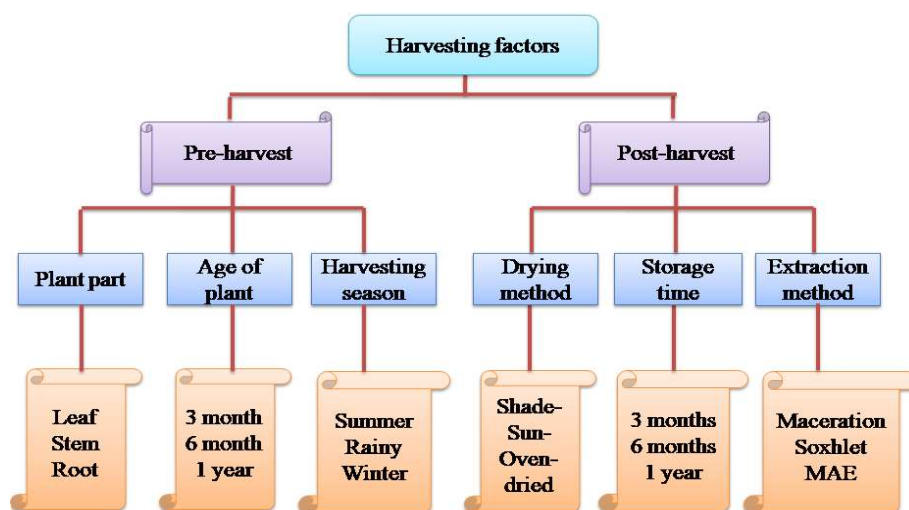
Fig. 4.1: Pre- and post-harvest factors analyzed for the plumbagin yield

47

The two species of *Plumbago* were compared based on the amount of plumbagin yield. The harvest factors were applied one at a time while keeping other factors constant wherever possible such as the screening of plant parts were carried out with three different plant parts while keeping the age (6 months), harvesting season (autumn), drying method (shade-dried), storage time (nil) and extraction method (maceration) constant. For screening the age of the plant, the aerial part of the plant was taken along with other constant factors.

4.2.3.1. Pre-harvest factors

The plant material collected was separated and categorized into leaf, stem, and root to study variation among specific plant parts. The plant material was also collected from plants of different ages i.e., 3 months, 6 months, and 1 year to study the difference in the accumulation of plumbagin over time. The harvesting was also done for the seasonal study at the end of each season i.e., summer, rainy, and winter.

4.2.3.2. Post-harvest factors

Freshly harvested plant material was dried under shade, sun, and oven to study the impact of heat on plumbagin content. The dried plant material was stored in amber bottles for different time duration (3 months, 6 months, and 1 year) to study the deterioration of plumbagin over time as it is reported as sublimating in nature (Mallavadhani *et al*., 2002).

4.3. HPLC-ESI-MS/MS analysis

The analyses were performed using an Agilent 1260 Infinity (Agilent, USA) HPLC system equipped with 1260VL infinity quaternary pumps, autosampler, a thermostat compartment. The samples were separated on a Chromolith RP-18e column (100 x 4.6mm). Mobile phases consisted of 0.1% (*v/v*) formic acid in water (eluent A) and acetonitrile (eluent B). An isocratic elution was performed 85:15 (A: B *v/v*). The flow rate was adjusted to 0.5 mL min^{-1} and column temperature was maintained at 30^{0}C. Triple-quadrupole tandem mass spectrometry (MS/MS) was carried out on Agilent G6410A tandem triple quadrupole mass spectrometer (TQD-MS) equipped with an ESI

ion source operating in both positive ion mode and negative mode. ESI source was operated in positive ionization mode. Quantification was performed in MRM mode. The MS parameters optimized were: capillary, voltage 3.5 kV and gas temperature, 300^0C. Nitrogen was used as desolvation gas at the rate of 13 L min^{-1} and nebulizer pressure was maintained at 50 psi. Nitrogen was also used as the collision gas (Sajad et al., 2017). The precursor-to-product ion pairs, fragmentor voltage (V) and collision energy (CE) for Plumbagin 250V and collision energy 45eV, respectively. All the data was collected in the centroid mode and acquired and processed using Mass Hunter work station software (Agilent).

MS scan mode conditions were optimized using the reference compound. Higher sensitivity and clear mass spectra were observed in analyses conducted in the positive ion mode. In positive ion mode, quasi-molecular ions $[M+H]^+$ of Plumbagin was generated, whose product ions were high and having good specificity.

Quantification was performed in MRM mode having the ion transitions Plumbagin m/z 189.0/160.6, 189.0/132.8 and, 189.0/120.8. The developed method showed retention time of Plumbagin 4.5 min. Compound was identified by comparison of molecular ion, fragmented ions (MRM) and retention time with that of the standard compound.

4.4. Optimization of Microwave-assisted extraction

A domestic microwave oven (IFB model-30SC3) with a power source of 230 V – 50 Hz, the heater output power of 1250 W, and microwave frequency of 2450 MHz was used for extraction of plumbagin using ethanol as solvent. One gram of powdered plant material was weighed and mixed with absolute ethanol; the mixture was stirred and rested for some time before extraction. The mixture was placed in the microwave oven and extraction was done under different parameters. After extraction, the mixture was filtered with Whattman paper and then centrifuged at 12,000 rpm for 20 minutes at 4°C. The filtrate was reduced to 1 ml using a rotary vacuum evaporator at 40°C. The sample obtained was transferred to amber vials and kept in a freezer at 4°C until further use.

4.4.1. Single-factor experiment

The single-factor experiment was used to screen the effect of different parameters at different levels by extracting plumbagin using one parameter each time at various levels and other parameters were taken as a constant. Extraction time was tested at six different levels, i.e., 2, 4, 6, 8, and 10 minutes while particle size and the solvent volume ratio were taken as constant. Particle size was also tested at six levels, i.e., 0.2, 0.4, 0.6, 0.8, and 1.0 mm with the other two as constant. Similarly, the solvent volume ratio was tested at six different levels, i.e., 5, 10, 15, 20, and 25 ml mixed with one gram of sample with time and particle size constant. The constants were taken the same for all tests.

4.4.2. Response surface methodology design

Based on the results of the single factor experiment three levels were selected for each variable; a higher level (+1), lower level (-1), and a middle level (0) as depicted in Table 4.1. Box-Behnken design (BBD) was applied to optimize the extraction variables, consisting of 15 experimental runs in total (Table 2). A second polynomial equation was applied to evaluate total plumbagin content:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_1{}^2 + \beta_{22}X_2{}^2 + \beta_{33}X_3{}^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3$$

$$(Equation\ 4.1)$$

Y = recovery yield of plumbagin (mg/g)

β_0 = scaling constant

X_1, X_2, and X_3 = extraction factors

β_1, β_2, and β_3 = linear coefficients

β_{11}, β_{22}, and β_{33} = quadratic coefficients

β_{12}, β_{13} and β_{23} = interactive factors.

Table 4.1: Actual and coded values for independent variables used in Box-Behnken design

Variables/factors	Levels		
	-1	0	+1
X_1, Extraction time (minutes)	2	4	6
X_2, Solvent volume ratio (1 mg/mL)	10	15	20
X_3, Particle size (mm)	0.6	0.8	1.0

4.4.3. HPTLC analysis

As earlier described in section- 3.2.4

4.5. Result and discussion

4.5.1. Pre-harvest factor analysis

The study of pre-harvest factors included the screening of plant parts, the age of the plant, and the harvesting season Table 4.2. The main source of plumbagin was found to be the roots as it has the highest yield (1170.93µg/g) followed by the stem (243.66µg/g) and the least amount of plumbagin was found in the leaves (173.06µg/g). Similar observations were made in the previous studies which showed that the highest amount of plumbagin accumulates in the roots of *P zeylanica* (Mallavadhani et al., 2002). Further study indicated that plumbagin accumulates with time in the plant as the highest plumbagin content was found in older plants than younger ones. The highest plumbagin content was found in the twelve-month-old (216.06µg/g) plants followed by six-month-old (94.34µg/g) and the least content was observed in three-month-old plants (46.29µg/g). As far as the seasonal variations are concerned the plumbagin content variation was not much, plumbagin content was found highest in winter (64.39µg/g) and summer (63.39 µg/g), and the least was found in rainy season harvest (55.55µg/g).

Table 4.2 Screening of pre- and post harvest factors and plumbagin content

Pre-harvest factors	Plumbagin content (µg/g)	Post-harvest factors	Plumbagin content (µg/g)
Plant part		Drying method	
Leaf	173.06	Shade-dried	**85.76**
Stem	243.66	Sun-dried	64.32
Root	**1170.93**	Oven-dried	58.17
Age of plant (months)		Storage time (months)	
3	46.29	3	113.52
6	94.34	6	**159.69**
12	**216.06**	12	36.94
Harvesting season		Extraction method	
Rainy (July)	55.55	Maceration	192.59
Summer (May)	63.39	Soxhlet-assisted	981.56
Winter (December)	**64.39**	Microwave-assisted	**1250.84**

4.5.2. Post-harvest factor analysis

For the screening of drying method six-month-old plants were harvest and dried by different methods such as shade-dried (room-temperature), sun-dried (34± 2℃), and oven-dried (50 ℃). The highest plumbagin content was found in the shade-dried samples (85.76 µg/g) followed by sun-dried (64.32 µg/g) and the least content was found in the oven-dried samples (58.17 µg/g). It was observed that with an increase in the drying temperature there was a degradation of the phytochemical. The storage time analysis showed that with passing time plumbagin content was found to be decreased which indicate that plumbagin is sublimating in nature (Mallavadhani *et al*., 2002). The screening of extraction methods revealed that the microwave-assisted extraction (MAE) had a better yield (1250.84µg/g) than the soxhlet-assisted method (981.56µg/g) and maceration (192.59µg/g). The maceration was done overnight at room temperature, it is

the simplest method of extraction and is based on the natural leeching out of the phytochemicals therefore it is neither time-effective nor cost-effective. The soxhlet-assisted extraction was conducted via., soxhlet-apparatus at 50°C for 6 hours using 50 ml absolute ethanol, the extraction mainly occurs by the heat applied which is a conduction-convection event where most of the heat energy is lost to the surroundings. Whereas, in MAE the microwave heating occurs in a targeted and selective manner and almost no heat is lost to the surroundings (Letellier and Budzinski, 1999; Mandal *et al.*, 2007). MAE method is superior to the conventional methods of extraction in terms of time which is typically less than 30 minutes (Huie, 2002), and utilization of less solvent. MAE has been proved to be more effective as compared to the conventional methods for the extraction of bioactive compounds in *Vernonia amygdalina* (Alara *et al.*, 2019), *Helichrysum stoechas* (Zengin *et al.*, 2020), *Lagenaria siceraria* (Abbas *et al.*, 2021).

4.5.3. Optimization of MAE method

4.5.3.1. Single-factor experiment

According to the single factor experiment conducted, the range for the variables was set as follows; extraction time of 2-6 minutes, solvent 10-20 ml, and sample particle size of 0.6-1.0 mm (Fig. 4.3). With increasing extraction time, the plumbagin yield also increased but after 8 min the yield plummeted (Fig. 4.3 A). The optimum solvent volume was found to be 15 mL, further increase in the volume was found to be not significant (Fig. 4.3 B). An initial increase in particle size didn't show any significant difference but further increase decreased the plumbagin yield (Fig. 4.3 C).
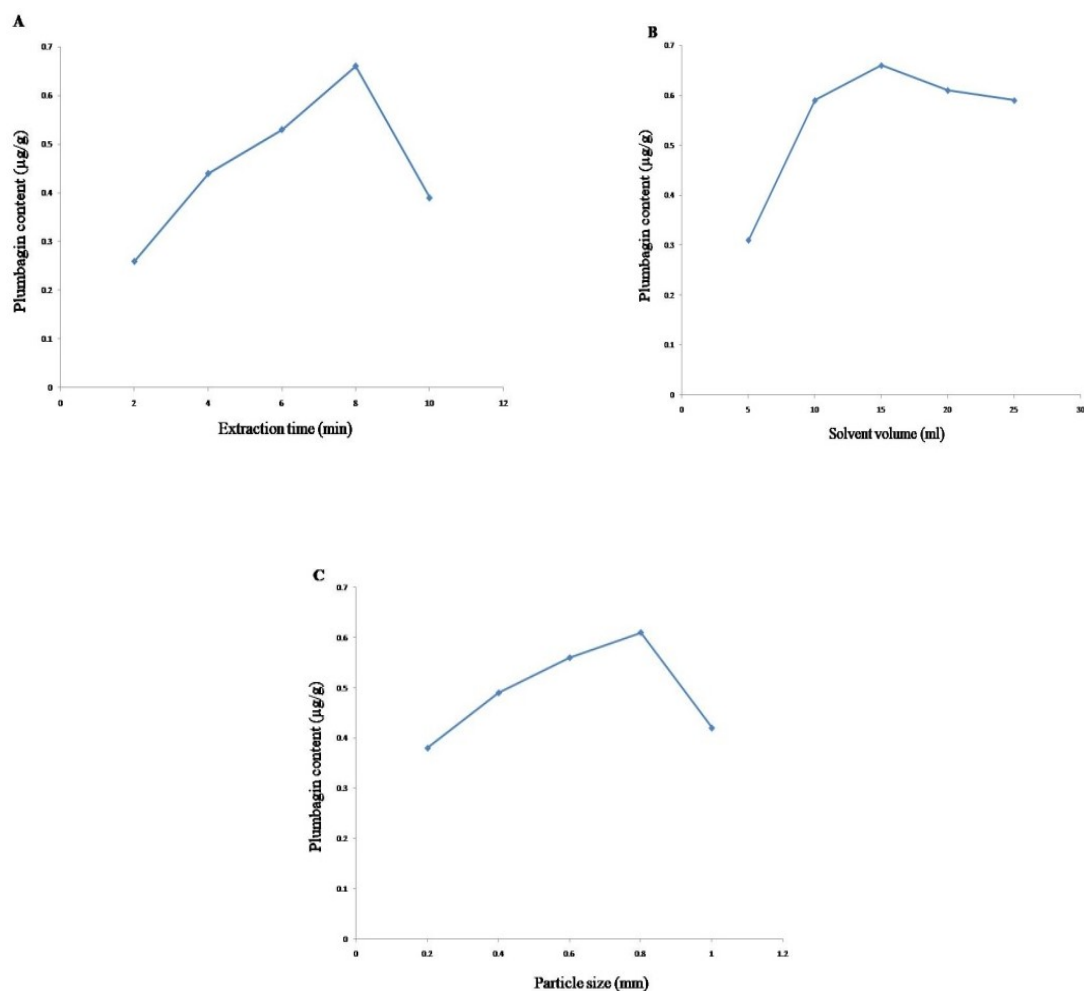
Fig. 4.2: Effect of single factors on the extraction of plumbagin (A) Extraction time, (B) Solvent volume, and (C) Particle size

4.5.3.2. Variables and response

The yield of plumbagin from samples at various experimental parameters (extraction time, solvent volume, and particle size) was given in Table 4.3. The regression model was depicted by the quadratic polynomial equation obtained:

$$Y = 2.209 \quad - 0.3147 \, X_1 \quad + 0.3196 \, X_2 \quad - 8.325 \, X_3 \quad + 0.01192 \, X_1{}^2 \quad - 0.005303 \, X_2{}^2$$
$$+ 6.123 \, X_3{}^2 \qquad + 0.00503 \, X_1 X_2 \qquad + 0.2406 \, X_1 X_3 \qquad - 0.2155 \, X_2 X_3$$

(Equation 4.2)
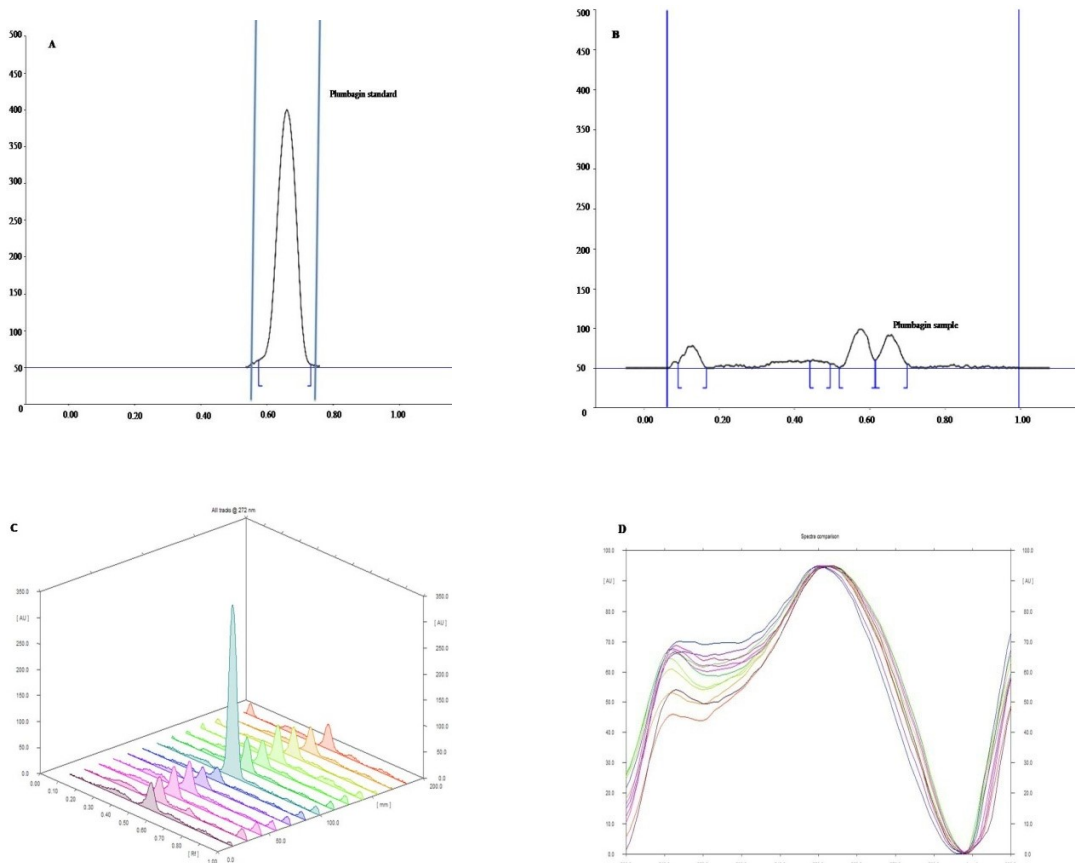
Fig. 4.3: HPTLC chromatograms of plumbagin (A) plumbagin standard, (B) *P zeylanica* sample (C) HPTLC chromatograms of the samples of experimental runs at 272 nm wavelength, and (D) Overlay spectra

Table 4.3 sums up the experimental design, experimental response, and predicted values from 15 experimental runs. The level and range of each variable were coded as mentioned earlier. The highest experimental value for plumbagin yield was observed in experimental run 10 with 0.992 % plumbagin content and the least was observed in experimental run 12 with 0.270 % plumbagin content.

Table 4.3: Box-Behnken design with experimental and predicted values of plumbagin content

Run order	Variables			Experimental Value (%)	Predicted value (%)
	X_1	X_2	X_3		
1	-1	-1	0	0.298	0.313
2	+1	-1	0	0.405	0.407
3	-1	+1	0	0.288	0.295
4	+1	+1	0	0.606	0.590
5	-1	0	-1	0.959	0.938
6	+1	0	-1	0.947	0.939
7	-1	0	+1	0.419	0.426
8	+1	0	+1	0.792	0.812
9	0	-1	-1	0.497	0.502
10	0	+1	-1	**0.992**	**1.015**
11	0	-1	+1	0.637	0.613
12	0	+1	+1	0.270	0.265
13	0	0	0	0.481	0.486
14	0	0	0	0.495	0.486
15	0	0	0	0.484	0.486

4.5.3.3. Model adequacy checking

The difference between the actual response value (obtained from the experimental run) and the predicted value (obtained from Equation 4.2 using regression analysis) is defined as residuals. The model adequacy checking depends on three statements of the residuals, (1) the residuals should be normally distributed, (2) residuals should be spread out randomly around zero, and (3) the residuals should be independent (Un *et al*., 2015). The normal probability plot (Fig. 4.5 A) indicated almost all the residual points were distributed along the straight line which in turn fulfilled the first statement for model

adequacy. The random distribution without any significant structure was observed in the residual versus fitted value plot (Fig. 4.5 B) which proves the second statement for model adequacy. The histogram plot was observed to be almost symmetrical which further supports the first statement (Fig. 4.5 C). The completely random residual points observed in the residual versus observation order plot indicate that the residuals were independent with each other, therefore proved the third statement (Fig. 4.5 D).
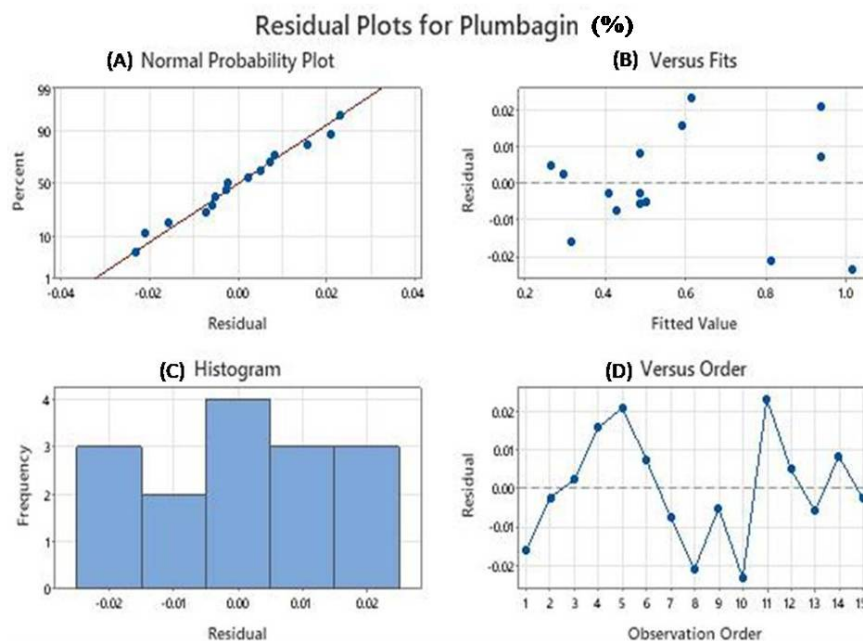
Fig. 4.4: Residual plots of model adequacy checking (A) Normal probability plot (B) Residual versus fitted value plot (C) Histogram of frequency versus residual (D) Residuals versus time sequence or observation order plot

4.5.3.4. Analysis of Variance (ANOVA)

The analysis of variance for the fitted quadratic model was given in Table 4.4. F-value was given which predicts whether the variables were associated with the response. The higher F-value indicated statistical significance. The p-value indicates the probability that processes the data against the null hypothesis. The significance of each coefficient was checked by the p-values given; a high F-value and a low p-value indicated a significant

coefficient (Luo *et al*., 2020). The low p-value (0.05) provides confirmation against the null hypothesis. The low p-value (< 0.0001) given in Table 4.4 that suggested the regression model was highly significant. In the case of lack-of-fit, the p-value (0.059) is higher than the significance level which means that the model was sufficient for predicting plumbagin yield (R^2 = 0.9967, Adj R^2 = 0.9497).

Table 4.4: The ANOVA analysis for response surface quadratic model of plumbagin yield

Source	F-Value	P-Value
Model	170.21	< 0.0001***
Linear	178.31	< 0.0001***
X_1	137.59	< 0.0001***
X_2	24.73	0.004*
X_3	372.60	< 0.0001***
Square	190.41	< 0.0001***
X_1^2	15.33	0.011*
X_2^2	118.64	< 0.0001***
X_3^2	404.84	< 0.0001***
2-Way Interaction	141.91	< 0.0001***
X_1X_2	18.46	0.008*
X_1X_3	67.73	< 0.0001***
X_2X_3	339.55	< 0.0001***
Lack-of-Fit	16.12	0.059ns
R^2	0.9967	
Adj R^2	0.9497	

Note: X_1 = extraction time (min), X_2 = solvent volume (mL), and X_3 = particle size (mm), ns = not significant, * = significant ($p < 0.05$), *** = highly significant ($p < 0.001$).

4.5.3.5. Response surface plots

The effect of extraction factors on plumbagin yield can be predicted by the three-dimensional surface plots for plumbagin yield based on the interaction between variables X_1, X_2, and X_3. The interactions between the variables can be determined by the shapes of contour plots (Prakash *et al.*, 2008). The highest plumbagin content in each plot suggested the optimum point of two independent factors. The interaction between extraction time and solvent volume presented in Fig. 4.4 (A) show an initial increase of plumbagin yield with an increase in both variables but consequently decreased, probably because of extent extraction time which may degrade compound. Extraction time and particle size interaction presented by Fig. 4.4 (B) suggest that the maximum plumbagin yield was observed at a minimum particle size and extraction time and with an increase in the variables the plumbagin yield decreased drastically. The interaction between solvent volume and particle size (Fig. 1.5 C) suggest that the increase in solvent volume along with minimum particle size gave the highest plumbagin content.
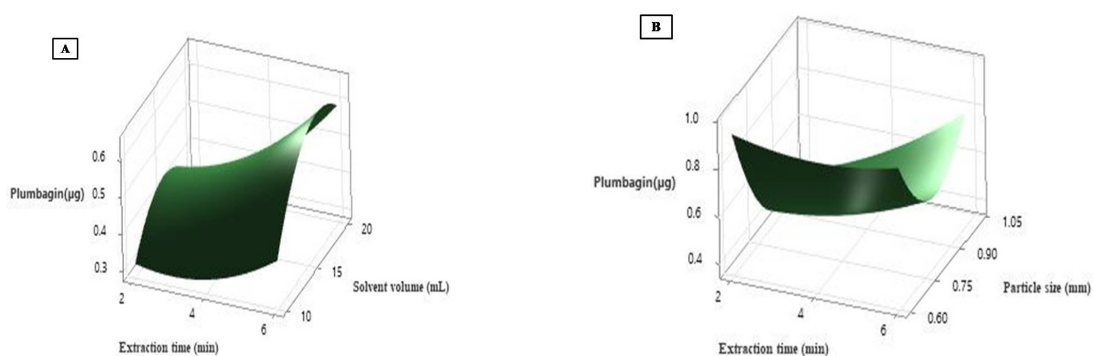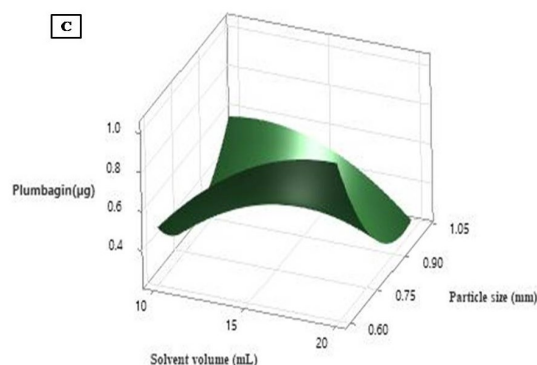
Fig.4.5: The response surface plots of the three factors interaction on the plumbagin yield. (A) Extraction time and solvent volume (fixed condition: particle size 0.8 mm); (B) Particle size and extraction time (fixed condition: Solvent volume 15 mL); (c) Solvent volume and particle size (fixed condition: Extraction time 4 min)

4.6.Conclusion

There is an uprising demand for plumbagin in the pharmaceutical industry. This study was carried out to optimize the extraction method for a time-efficient and cost-effective process. The preliminary tests revealed that the roots of *P zeylanica* contain the highest amount of plumbagin as compared to other parts and also *P auriculata*. The older plants should be harvested during the month of December. The harvested plants should be shade dried and used at the earliest to prevent the loss of plumbagin during storage time. The most suitable solvent for extraction was found to be absolute ethanol. MAE was found to be more efficient as compared to conventional methods. MAE was employed and optimized successfully by using RSM; this is the first-ever reported optimization of MAE for plumbagin. The best parameters found were; extraction time of 4 minutes, solvent volume 20 mL, and particle size 0.6 mm. Further studies can help to evaluate the industrial viability of this method.

CHAPTER 5

In vitro and *in vivo* propagation of the elite species of *Plumbago* for the increased production of plumbagin using biotic factors

5.1. Introduction

P zeylanica produces and accumulates an extensive range of secondary metabolites (Lin *et al*., 2003; Vanitha *et al*., 2020). Plumbagin mainly accumulates in the roots of *P zeylanica*. Therefore it is acquired following the destructive harvest of the naturally grown plants. It cannot be chemically synthesized because of little knowledge regarding its biosynthetic pathway. *P zeylanica* can not be grown from the seeds because of the low viability and slow growth. The conventional production of plumbagin does not fulfill the demand. Moreover, it has put the plant in the endangered plants' category due to over-exploitation. Plant tissue culture offers an alternative supply of plumbagin. It is cost and time-effective. The cultures reported a higher yield of plumbagin as compared to the naturally grown plants but not enough to meet the rising market demand. Further development in *in vitro* studies of plumbagin production involved elicitation (biotic and abiotic). Elicitation has been used for increased production of secondary metabolites in many medicinal plants such as *Plumbago rosea* (Silja *et al*., 2014; Jaisi and Panichayupakaranant, 2017), *P zeylanica* (Roy and Bharadvaja, 2019, Singh *et al*., 2020), *Bacopa monnieri* (Sharma *et al*., 2015), *Salvia virgata* (Dowom *et al*., 2017), *Narcissus pseudonarcissus* (Ferdausi *et al*., 2021), *Swertia paniculata* (Kaur *et al*., 2020), *Trigonella foenum* (Mala *et al*., 2021), etc. Biotic elicitors are organic substances derived from micro-organisms and plants. Various works have been done to examine the effect of biotic elicitors on secondary metabolite yield in plants. Such studies showed that the biotic elicitors considerably increased the secondary metabolite yield as compared to the abiotic elicitors and control. For instance, plumbagin content was reported to be increased 6.5 fold when subjected to yeast extract elicitation and 3.4 fold with salicylic acid as

compared to control (Sharma and Agarwal, 2018). Some of the biotic elicitors were used in combinations which further increased plumbagin production as compared to individual elicitors. Chitosan and yeast extract when used in combination increased plumbagin production by 12.08 fold as compared to their individual effect i.e., 4.58 and 6.50 fold, respectively (Singh *et al.*, 2020).

In the present study, *in vitro* regeneration procedure was established. The elicitation of plumbagin in shoot cultures using biotic elicitors in the form of lysates was done. The effect of three different biotic elicitors on the production of plumbagin was studied with the help of RSM. BBD was applied to determine the optimum concentration and combination of biotic elicitors. To our best knowledge, this is the first experiment conducted to study the effects of biotic elicitation on shoot cultures of *P zeylanica* by lysates.

5.2. Material and method
5.2.1. Collection of plant material

The fresh shoots of *P zeylanica* were collected from the field station of Council of Scientific and Industrial Research–Indian Institute of Integrative Medicine (CSIR-IIIM) Jammu, India.

5.2.2. Explants preparation and culture conditions

The nodal and leaf explants were used for the regeneration of plants; the sterilization was done after washing the explants thoroughly under tap water. The washed explants were treated with 1% Teepol detergent solution for 15 minutes and rinsed thoroughly. Further sterilization was done in aseptic conditions. Explants were treated with 1% sodium hypochlorite for 5 minutes and rinsed properly. The explants were also cured with 0.2 % Bavistin for 5 minutes and rinsed thoroughly. Finally, the explants were treated with freshly prepared 0.1% mercuric chloride for 40 sec onds and rinsed thoroughly and the moisture on the surface of explants was eliminated with whattman paper.

The sterilized nodal explants were placed in MS basal (Murashige and Skoog, 1962) media complimented with various concentration and a combination of BAP and NAA. A wide range of concentrations of PGRs (1-10μM) was tested to determine the most effective concentration to further apply the combinations. The cultures were maintained under light with 16 hours photoperiod at 25± 2°C. Each PGR treatment was tested on twenty explants and repeated thrice. The treatments were compared to control explants cultured on MS medium without any PGRs. Direct shoot regenerated from the explants without callus phase, percentage of shoot initiation, and no. of shoots regenerated showed variation with different concentrations of PGRs. The leaves were cut and cultured on solid ½ MS medium with 3% sucrose, 0.7% agar and 1.0-6.0 μM BAP and NAA were used. The cultures were maintained under a white light incubator with photoperiod of 16 hour at temperature range 25 ± 1 °C.

5.2.3. Hairy root culture

Agrobacterium rhizogenes LBA 9402 was used for hairy root formation in *P zeylanica* leaf and nodal explants. The bacteria were cultured in liquid lysogeny broth (LB) medium fortified with rifampicin (50 mg/L). The bacterial culture was prepared according to Sharafi et al., 2014 with modifications. The culture was incubated in darkness on a rotary shaker (120 rpm) until an OD_{600} of 0.8–1.0 was achieved. The cultured bacteria was centrifuged at 6500 rpm for 10 minutes and washed with autoclaved double distilled water. The bacteria were re-suspended in half-MS medium (pH- 5.6) supplemented with 2 % sucrose, 100 μM acetosyringone and MES buffer (0.2 M). The bacterial culture was rested for 1 hour in complete darkness on a rotator shaker (80 rpm) at 28 °C before inoculation. The explants were injured with a needle and inoculated for half an hour using immersion method. The explants were dried out using Whattman paper and cultured on half-strenght MS medium with 3 % sucrose for 72 hours in darkness. Controlled explants without the bacterial treatment were also cultured on half-strenght MS medium.

Genomic DNA (deoxyribose nucleic acid) was extracted from the transformed and non-transformed roots and shoot cultures using cetyl trimethyl ammonium bromide (CTAB) technique (Doyle and Doyle, 1987) with few alterations. The plasmid DNA from *A. rhizogenes* strain was extracted using a conventional alkaline lysis approach (Sambrook *et al.*, 1998). The transformed cultures were screened for the presence of *rol B* gene (Forward-5 ' TCACAATGGATCCCAAATTG 3 ' Reverse- 5 ' TTCAAGTCGGCTTTAGGCTT 3 ')The PCR reaction consisted of PCR premix (10 µL), rol B forward (1 µL), rol B reverse (1 µL), template DNA (1 µL) and 3 µL distilled water to makeup the final volume. The reaction was done in a thermal cycler (Applied Biosystems, 9700 CA, USA) following series: initial denaturation at 95 °C for 5 min, followed by 34 cycles of 95 °C for 1 min, annealing 50 °C for 2 min and 72 °C for 1 min followed by final extension at 72 °C for 5 min. The PCR amplified products were observed on 1.5% w/v agarose gel stained with ethidium bromide.

5.2.4. Elicitor treatment

A rhizogenes (CFU count: 2.5×10^6 mL^{-1}) and *T viride* (CFU count: 3×10^5 mL^{-1}) were grown in LB medium at 28°C and PDB medium at 37°C, respectively for 3 days. The cells lyses were done with liquid nitrogen and the mix obtained was then autoclaved. Yeast extract was prepared in distilled water (10 mg ml^{-1}) and autoclaved for further use. Different concentration and combination of elicitors were added to the shoot cultures maintained in a liquid medium. The effective range of the elicitors was tested before applying RSM. Control cultures were maintained without any elicitors and the experiment was performed according to BBD. Cultures were harvested after 2 days and extracted to determine plumbagin production. Plumbagin was extracted from finely grounded shoots overnight after sonication for half an hour in methanol.

5.2.5. Box-Behnken Design (BBD)

Response surface methodology (RSM) is a system derived from statistics and mathematics to determine the relationship between the response of interest and multiple

variables used (Khuri and Mukhopadhyay, 2010). RSM also determines the interactions between variables and their effect on response with a minimal number of experiments (Bezerra et al., 2008). Box-Behnken design (BBD) is one of the most efficient techniques. BBD is a three-level (–1, 0, +1) at a three-factor design which was employed for quadratic response and generates a second-degree polynomial equation (Eq. 5.1). The independent variables were *A rhizogenes, T viride* and yeast extract designated as A, B and C, respectively. A total of 15 experimental runs were carried out in triplicates to study the effects of independent variables (biotic elicitors) on plumbagin production.

5.2.6. HPLC-ESI-MS/MS analysis

As ealier described in section- 4.3

5.2.7. Statistical analysis

The individual and interactive effects of biotic factors were assessed by analysis of variance (ANOVA). The effects for optimal plumbagin production were statistically analyzed using MINITAB 18.1 software.

5.3. Results and discussion

5.3.1. *In vitro* propagation of *P zeylanica*

5.3.1.1. Direct shoot culture

For direct shoot initiation, the nodal explants were inoculated onto MS medium fortified with different PGRs. The explants resulted in single shoot cultures when treated with BAP individually. It was found that BAP induced a significant response in the range of 4-7 µM. The increasing concentration of BAP showed shoot initiation earlier as compared to the lower concentration. The explants supplemented with above or below the range mentioned showed delayed or no response and ultimately turned brown. The explants were treated with BAP in combination with and NAA for multiple shoot development. The nodal explants of *in vitro* raised plants showed vigorous growth as compared to the explants obtained from field raised plants for further studies. Previously, shoot cultures of *Plumbago* species have been obtained with BAP (Roy and Bharadvaja, 2018), adenine

sulfate, and indole-3-butyric acid (Selvakumar *et al.*, 2001), thidiazuron (Sharma and Agrawal, 2018) resulting in 4-6 shoots per explants.

The combination of BAP and NAA has been proved to be optimum for multiple shoot proliferation in many medicinal plants such as *Vitex agnus-castus* L. (Skrzypczak *et al.*, 2018), *Echinops kebericho* (Enyew and Feyissa, 2019), *Lippia rotundifolia* Cham. (de Hsie *et al.*, 2019), *Psoralea corylifolia* (Gajula *et al.*, 2021). The consequence of various blends of BAP and NAA on regeneratation of shoots is summarized in Table 5.1. Shoot induction was observed to be successful in all cultures but there were variations in terms of the number of shoots and shoot length. Among the combinations tested, approximately 97% of cultures induced buds fortified with 5.5 µM BAP and 3 µM NAA after 10 days. After another 1 week, the shoots elongated 1-2 cm and the final observations were recorded after 30 days.
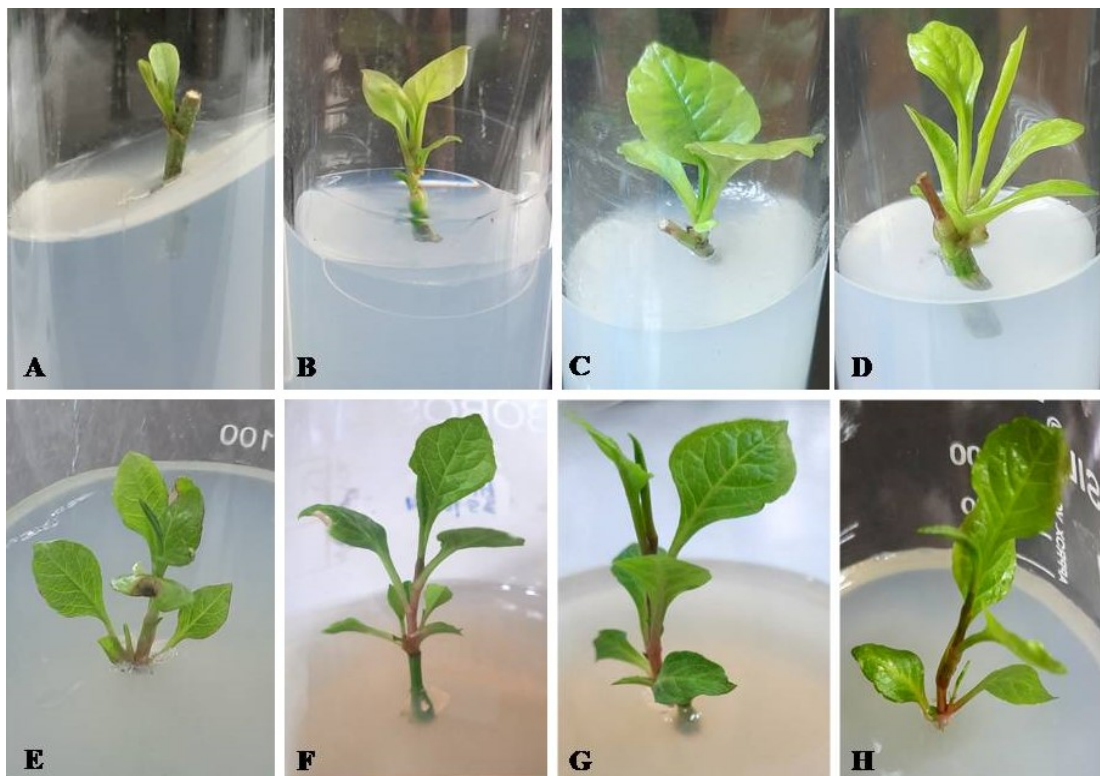
Fig. 5.1: Direct shoot induction and growth observed one week apart achieved in MS medium supplemented with 5.0 µM BAP

Table 5.1: Effect of different concentrations of BAP and NAA on *in vitro* shoot multiplication analyzed after 30 days of culture

Treatment	PGRs combination		Percentage of shoot induction	Number of shoots	Shoot length (cm)
	BAP(µM)	NAA(µM)			
1	4.0	1.5	34.9 ± 1.6^h	2.0 ± 0.10^f	2.9 ± 0.10^e
2	4.0	3.0	45.1 ± 0.8^{fg}	2.0 ± 0.20^f	3.1 ± 0.50^e
3	4.0	4.5	41.2 ± 1.2^h	4.5 ± 0.50^e	3.0 ± 0.10^{cd}
4	5.5	1.5	56.4 ± 0.7^d	5.3 ± 0.40^d	4.2 ± 0.20^{bc}
5	5.5	3.0	99.2 ± 0.2^a	7.0 ± 0.50^a	3.7 ± 0.50^a
6	5.5	4.5	89.2 ± 1.1^b	4.0 ± 0.40^b	4.9 ± 0.10^b
7	7.0	1.5	62.3 ± 0.9^c	5.9 ± 0.60^c	4.5 ± 0.20^c
8	7.0	3.0	48.5 ± 1.2^e	5.2 ± 0.41^d	4.0 ± 0.10^{cd}
9	7.0	4.5	46.3 ± 1.1^{fg}	5.2 ± 0.4^d	4.1 ± 0.40^{cd}
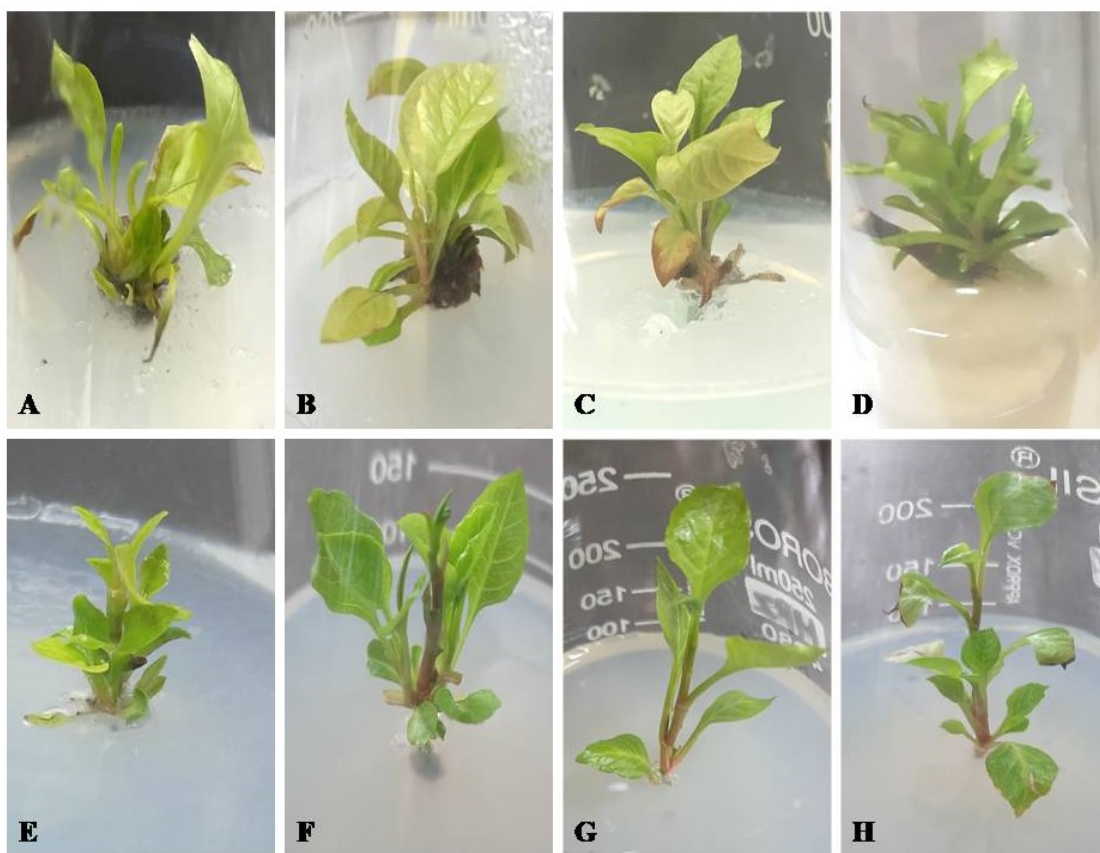
Fig. 5.2: Initiation and proliferation of shoots from nodal explants of *P zeylanica* (A) Treatment 2, (B) Treatment 3, (C) Treatment 4, (D) Treatment 5, (E) Treatment 6, (F) Treatment 7, (G, H) 5.0 µM BAP

The maximum number of shoots obtained per nodal explants was 7.0±0.5 with almost 100 % shoot induction on medium with 5.5 µM BAP and 3.0 µM NAA. The second-highest numbers of shoots were obtained with medium with 5.5 µM BAP and 4.5 µM NAA resulting in almost 90 % shoot induction and 3-5 shoots per explants. The PGRs combination with lower BAP (4.0 µM) and NAA (1-3 µM NAA) formed callus followed by shoot induction with the least number of shoots and growth was much slower. The optimum concentration and combination were found to be 5.5 µM BAP and 3.0 µM NAA which gave the maximum number of shoots in the minimum number of days. The shoot regenerated were separated and sub-cultured after 30 days as compared to other PGRs

treatments the shoots were sub-cultured after 45 days. Upon sub-culturing, the shoots elongated more rapidly as compared to cluster culture.

5.3.1.2. Direct root regeneration

The adventitious root culture of *P. zeylanica* has been successfully established (Fig. 2). The biomass of root, culture response (%), and number of days required for root culture were investigated and observations were observed and documented after 6 weeks of culture. The roots emerged directly from the cut sides of leaves (Fig. 1g), in other cases, roots emerged from initial callus formation (Fig. 1c, d, e, f, h). The earliest roots started emerging from the leaf explants within 28-30 days of inoculation and maximum days reached 46 for root culture (Table 1). A higher concentration of BAP in combination with a lower concentration of NAA produced more roots as compared to other combinations. However, lower concentrations of BAP and NAA (Fig. 1c, d) showed lower root biomass. When the concentration of both PGRs was increased root biomass was also increased. The best result was observed in 6.0 µM BAP and 2.5 µM NAA with 89.47 % culture response produced maximum biomass (3.43% FW) in less no. of days (28 days) (Table 1). Further increase in PGRs decreased the biomass and eventually, no response was observed. Culture response percentage was found in the range of 19.93 % to 89.47 % and biomass in the range of 1.30 g FW to 3.43 g FW in cultures treated with different combinations of BAP and NAA. It demonstrates that the production of adventitious root biomass is PGRs dependent. When NAA was used at 1.0 µM no adventitious root induction was observed even if the concentration of BAP was increased to 6.0 µM. Moreover, root morphology was found diverse but the color remained same during the experiment.

Adventitious root formation have been reported in various plants such as *Morinda citrifolia* (Baque *et al*., 2010), *Withania somnifera* (Praveen and Murthy, 2010), *Gynuraprocumbens* (Saiman *et al*., 2012), *Prunella vulgaris* L. (Fazal *et al*., 2014), *Tripterygium wilfordii* (Zhang *et al*., 2020), *Morinda coreia* (Kannan *et al*., 2021), *Gentiana kurroo* Royle (Alphonse and Thiagarajan, 2021). However, the growth hormones were different for different species. Rose *et al*., (2006) reported root initiation

from the callus obtained from the leaf explants in *Medicago truncatula* when auxin was added. NAA in lower concentrations and combination with BAP accelerated the production of adventitious roots in *Aloe vera* (Lee *et al*., 2011). NAA was found the best option for the production of adventitious rooting in *Eurycoma longifolia* (Hussein *et al*., 2012), however, in our study NAA alone failed to produce adventitious roots. A combination of BAP/KIN was also found favorable for the induction of adventitious roots in *Ophiorrhiza prostrate* (Martin *et al*., 2008).

Fig.5.3 Root culture of *P. zeylanica* L. using leaf explants (a) Narurally grown *P. zeylanica*; (b) leaf explants; (c) Treatment I (d) Treatment III (e) Treatment V (f) Treatment VI (g) Treatment VIII (h) Treatment IX

Lower concentrations of BAP and NAA (Fig. 1c, d) showed lower root biomass. While increasing the concentration of both PGRs showed increased root biomass. Root morphology was also different in different combinations of PGRs. The best result was observed in 6.0 µM BAP and 2.5 µM NAA concerning biomass, root length, number of roots, and earliest response (Fig. 1g). Further increase in PGRs decreased the biomass

and eventually, no response was observed. The roots appeared from the cut sides of leaves (Fig. 1g), in other cases, roots emerged from initial callus formation (Fig. 1c, d, e, f, h).

Table 5.2: Adventitious root formation on the leaf explants of *P zeylanica* fortified with BAP and NAA.

Treatment	PGRs (µM)		Culture response (%)	No. of days	Biomass (g FW)
	BAP	NAA			
I	1.0	1.0	-	-	-
II	1.0	2.5	19.93 ± 0.15^f	46	1.30 ± 0.20^e
III	1.0	3.5	35.33 ± 0.15^e	42	1.67 ± 0.15^d
IV	3.0	1.0	-	-	-
V	3.0	2.5	$42.23 \pm 0.15^{c,d}$	35	$2.3 \pm 0.20^{b,c}$
VI	3.0	3.5	53.47 ± 0.35^c	32	2.63 ± 0.25^b
VII	6.0	1.0	-	-	-
VIII	6.0	2.5	$\mathbf{89.47 \pm 0.40^a}$	28	3.43 ± 0.30^a
IX	6.0	3.5	76.53 ± 0.40^b	34	2.63 ± 0.38^b

Data represent mean ± standard deviation of three replicates. Different letters indicate significant differences ($P < 0.05$) within the column using Tukey's Test.

The present study successfully concluded with fast-growing roots from leaf explants. The optimum concentration of PGRs and the combination was established for direct adventitious root culture. This culture system can be used for industrial-scale production of the root of *P. zeylanica* and can be used for secondary metabolites instead of natural

roots. Moreover, the cultured roots can be produced in less time and are inexpensive as compared to natural roots.

5.3.2. *In vitro* propagation of *P auriculata*

The induction and growth of plants was different according to the combination and amount of PGRs supplemented. The experiment was conducted in triplicate and repeated twice to validate the results obtained.

Table 5.3: *In vitro* shoot multiplication from nodal explants of *P zeylanica* fortified with BAP and NAA

PGRs combination		Number of days for	Percentage of shoot
BAP(µM)	NAA(µM)	shoot induction	induction (%)
2.2	5.5	25 ± 2	33.0 ± 1
2.2	10	22 ± 2	35.1 ± 1
2.2	16	30 ± 2	40.2 ± 1
6.6	5.5	27 ± 2	50.4 ± 1
6.6	10	10 ± 2	**99.0 ± 1**[**]
6.6	16	19 ± 2	70.2 ± 1[*]
12	5.5	17 ± 2	60.3 ± 1[*]
12	10	30 ± 2	39.4 ± 1
12	16	35 ± 2	42.0 ± 1

** Excellent growth * moderate growth

The optimal combination of PGRs obtained was BAP (6.6 µM/L) and NAA (10 µM/L) with almost 100 % shoot induction. The shoot induction was observed after 10 - 12 days of culture. Plants cultured with 6.6µM/L BAP and 16µM/L NAA showed almost 70 % successful shoot induction but it took two-fold more time. It was followed by 60 % shoot induction at 12µM/L BAP and 5.5µM/L NAA after 17 ± 2 days. The rooting was initiated on the elongated shoots when grown on ½ strength MS media supplemented with 2 % sucrose (w/v) and IAA. The optimum root formation was obtained in media

supplemented with 4µM/L IAA with 28 – 30 roots and length ranging from 4.5 – 5.0 cm. The rooted regenerated plants were transfered to a mixture of vermicompost and sand (50/50 w/w). Almost 75 % plantlets survived in the green house.

Table 5.4: Effect of various concentrations of IAA on *in vitro* root regeneration

IAA concentration(µM)	Number of roots	Average length of roots (cm)
1.2	3.05 ± 0.43	2.65 ± 0.61
3	12.00 ± 0.50	3.90 ± 0.25
4	**28.65 ± 0.82**	**4.50 ± 0.26**
6	21.50 ± 0.50	2.50 ± 0.25
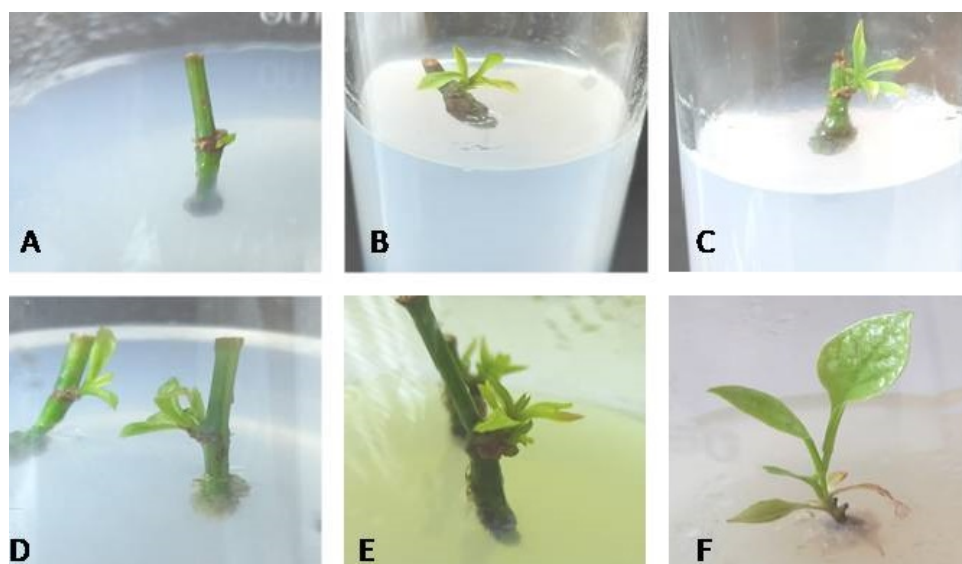
Fig. 5.4 Direct shoot growth in *P auriculata* cultured on MS medium fortified with NAA (6.6µM/L) and BAP (10µM/L) (A-E); Root regeneration on half-strength medium with 4 µM/L IAA (F)

5.3.3. Hairy root formation and shoot regeneration in *P zeylanica*

Almost all the explants co-cultured with *A rhizogenes* formed the hairy roots at the site of injury after 7-9 days. The hairy root was cut from the explant and cultured on the fresh half-MS media every 15 days for better growth. Shoot buds were observed in the hairy roots after 2 weeks. The shoot buds were separately grown on the half-MS medium without any growth regulators. Hairy root formation was observed from the shoot buds after one week. The growth rate of transformed plants was much higher than the un-transformed plants. The observartion determined a successful genetic transformation, whereas, in the non-transformed *in vitro* control culture there was no amplification (Fig. 5.6). Similar observations were reported in *P indica* (Gangopadhyay *et al*., 2010) and *Hypericum tomentosum* (Henzelyová and Čellárová, 2018).
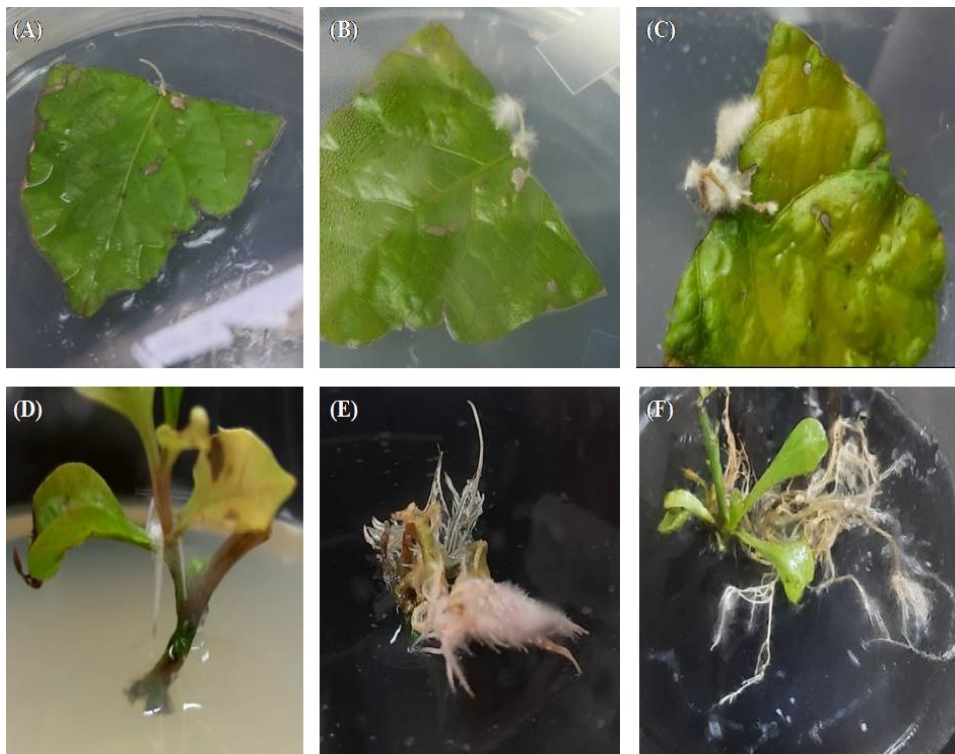
Fig.5.5 Hairy root inititation from leaf and nodal explants after co-culturedwith *A rhizogenes* (A-C) Hairy root initiation and growth from the leaf explants (D) Hairy root initiation and growth from the nodal explants (E-F) Shoot regeneration from cut hairy root
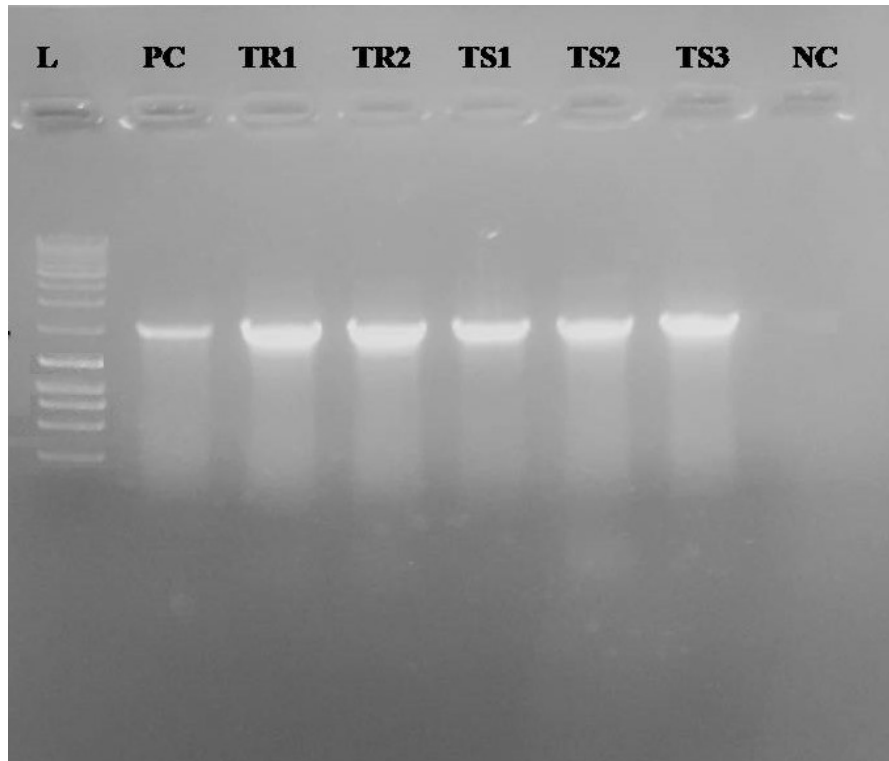
Fig. 5.6 Confirmation of *A rhizogenes* mediated transformation by PCR using *rol B* (L) 100 bp DNA ladder, (PC) positive control/ *A rhizogenes* plasmid DNA, (TR1, TR2) transformed root DNA, (TS1, TS2, TS3) transformed shoot DNA, (NC) negative control/non-transformed DNA

The plumbagin yield in the regenerated plants was quantified by LC-MS. It was observed that the highest plumbagin yield was obtained from the transformed roots (2216.54 ± 0.09) followed by transformed shoots (1613.34 ± 0.15) of *P zeylanica*. The plumbagin yield in the roots of *P auriculata* was observed to be slightly higher (1165.73 ± 0.20) as compared to the shoots (979.309 ± 0.17). The plumbagin yield in transformed roots increased 1.6-fold as compared to the non-transformed roots. The growth rate was also higher in the transformed roots as compared to non-transformed ones.

Table 5.5: Plumbagin yield in 3-month old *in vitro* cultured plants

Plant species	Plant part	Plumbagin yield (ng/g)
P zeylanica	Shoot	1026.01 ± 0.14
	Root	1368.34 ± 0.14
	Transformed roots	**2216.54 ± 0.09**
	Transformed shoot	1613.34 ± 0.15
P auriculata	Shoot	979.309 ± 0.17
	Root	1165.73 ± 0.20

5.3.4. *In vitro* elicitor treatment to shoot cultures

The elicitors were individually tested to determine the range in which there was a positive effect. The range obtained was set for the BBD-RSM design. With an increase in *A rhizogenes* treatment the plumbagin yield also increased but after 2 % plumbagin yield decreased. In the case of *T viride,* the plumbagin yield increased until the 3 % volume of fungal extract. The plumbagin yield increased with increase in yeast extract, however, after 5 % the yield decreased.
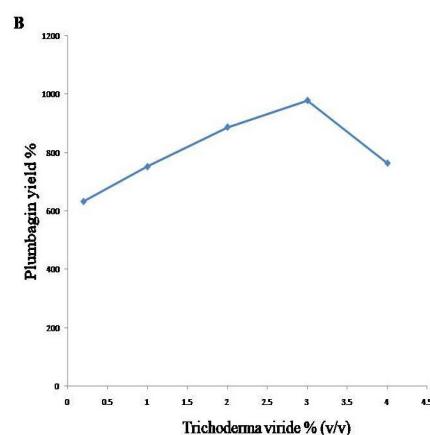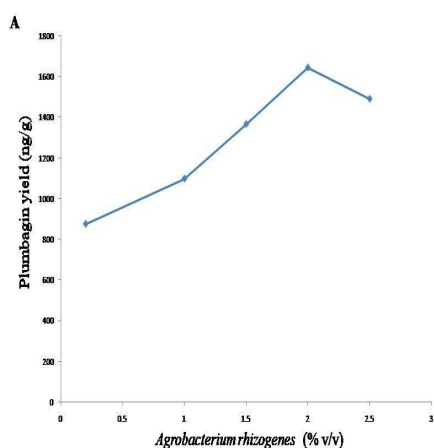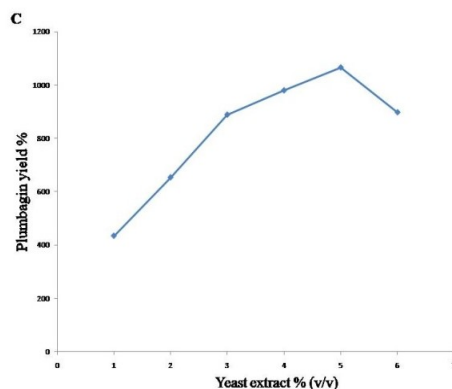
Fig. 5.7 Effect of single elicitor on the extraction of plumbagin (A) *A rhizogenes*, (B) *T viride*, and (C) Yeast extract

A three factor with three levels BBD-RSM was employed to determine the response (plumbagin yield) of the biotic elicitor treatment. Total 15 experimental runs obtained based on the BBD are given in Table 5.7.

Table 5.6: Actual and coded level of different variables for Box-Behnken Design

Variables (% v/v)	Factors	Low level (-1)	Intermediate level (0)	High level (+1)
A rhizogenes	A	0.2	1.1	2
T viride	B	1	2	3
Yeast extract	C	1	3	5

The second-order polynomial obtained by multi-regression analysis of experimental data represents the mathematical relationship between independent variables and the response (Eq. 5.1).

Plumbagin (µg) = -2.5110 + 2.1994 A + 2.0151 B + 0.3394 C - 0.5614 A^2 - 0.4240 B^2 - 0.01325 C^2 - 0.0225 AB - 0.20597 AC - 0.02450 BC   (Equation 5.1)

Table 5.7: Design and experimental results of response surface analysis

Run order	Variables			Experimental Value (%)	Predicted value (%)
	A	B	C		
1	0.2	1	3	0.212	0.194
2	2.0	1	3	0.792	0.777
3	0.2	3	3	0.663	0.677
4	2.0	3	3	1.162	1.179
5	0.2	2	1	0.469	0.467
6	2.0	2	1	**1.756**	**1.751**
7	0.2	2	5	1.142	1.146
8	2.0	2	5	0.946	0.947
9	1.1	1	1	0.852	0.870
10	1.1	3	1	1.423	1.410
11	1.1	1	5	0.893	0.905
12	1.1	3	5	1.268	1.249
13	1.1	2	3	1.583	1.586
14	1.1	2	3	1.577	1.586
15	1.1	2	3	1.598	1.586

The Pareto chart depicts the absolute values of the standardized effects from the highest to the lowest. The bars that cross the reference line are significant and determine the magnitude and importance of effects. Fig. 5.5 depicts that all the factors are significant as the bars cross the reference line represented by the red dotted line except the interaction term AB. The normal probability plot of residual (Fig. 5.6 A) follows a straight line indicating the model adequacy. The residual versus fitted value (Fig. 5.6 B) points do not follow any recognizable patterns thus verifying the model adequacy. The residual versus order (Fig. 5.6 D) was used to verify the assumption that the residuals are independent.

The residuals on the plot fall randomly around the centre line thus it confirms that the residuals are independent.
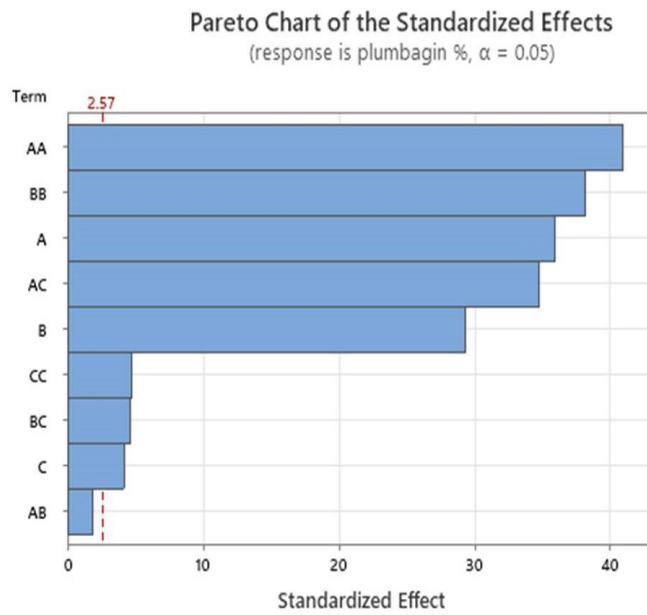
Fig. 5.8: Pareto chart of the standardized effect of independent variable on response
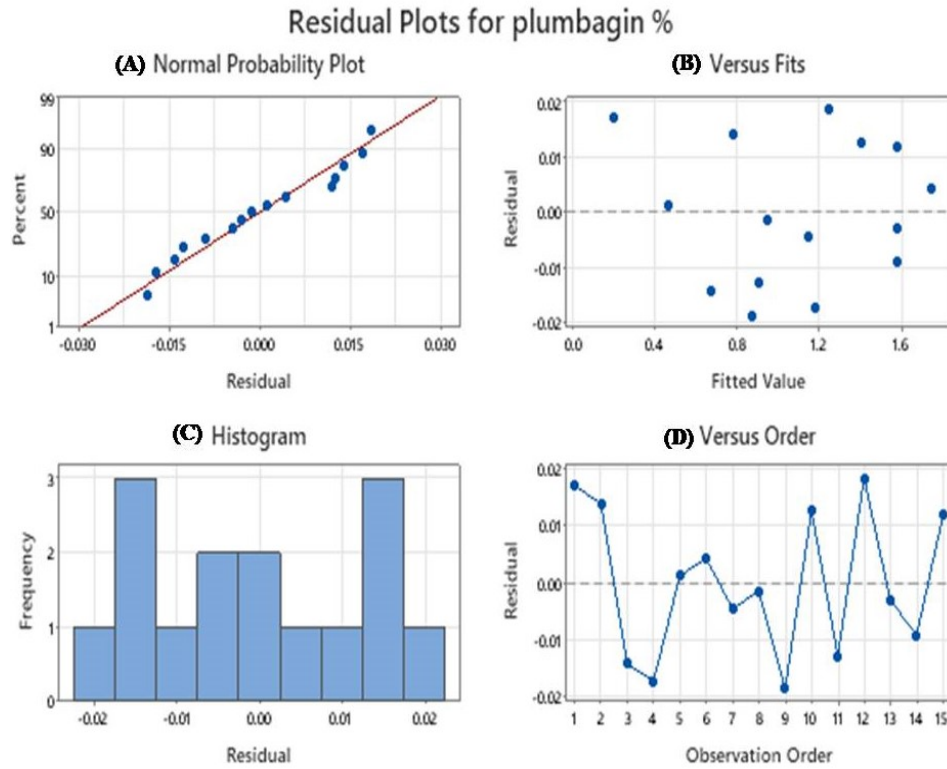
Fig. 5.9: Residual plots of model adequacy checking (A) Normal probability plot (B) Residual versus fitted value plot (C) Histogram of frequency versus residual (D) Residuals versus time sequence or observation order plot

Analysis of variance (ANOVA) is used to analyze the independence and interaction of variables on the response and significant results are documented in Table 5. The significance of each independent variable and their interaction was determined by the P-value. The considerably large F-value indicates that the model is significant. The P-value which determines the relationship between the response and each variable should be equal to or less than the significance level (0.05). The regression model was found to be highly significant based on the F and P-value obtained as 702.96 and <0.0001, respectively. The linear model variables (A, B and C), quadratic model variables (A^2, B^2 and C^2) were found to be statistically significant for the response. The variable interaction was also found to be significant except AB. The most significant variables are with P-value <0.0001.

Table 5.8: Analysis of variance (ANOVA) results of the regression equation

Source	F-Value	P-Value
Model	702.96	<0.0001
Linear	724.10	<0.0001
A	1295.79	<0.0001
B	859.19	<0.0001
C	17.34	0.009
Square	973.05	<0.0001
A^2	1680.93	<0.0001
B^2	1461.28	<0.0001
C^2	22.83	0.005
2-Way Interaction	411.72	<0.0001
AB	3.61	0.116
AC	1210.40	<0.0001
BC	21.14	0.006
Lack-of-Fit	5.80	0.150
R^2	0.9992	
Adj R^2	0.9978	

Theoretically, the optimal variable level for bacterial lysate (*A rhizogenes*), fungal lysate (*T viride*) and yeast extract obtained was 1.2, 2 and 3 %, respectively. The highest plumbagin yield predicted by the model was 1.751 %. The design was validated by comparing the predicted data with the experimental data. The optimal conditions were adjusted at bacterial lysate 1 %, fungal lysate 2 % and yeast extract 3 %, considering the practicality of the experiment. The experiment was repeated for verification, the experimental plumbagin yield confirmed the predicted value. The P-value obtained in

Table 5.7 indicates that the interactive effect of the bacterial lysate (*A rizhogenes*) and yeast extract is of the highest significance, followed by fungal lysate (*T viride*) and yeast extract and lastly the bacterial lysate and fungal lysate.

To further study the interactive effect of independent variables with the response, response surface plots and contour plots (Fig. 5.7, 5.8, 5.9) for the responses were obtained based on the regression equation (Equation 5.1). The significance of the interactive effect of the variables can be determined by the shape of the contour plots. The elliptical shape indicates the significiant interactive effect of the variables and circular shape is considered as not significiant (Muralidhar *et al*., 2001).

Fig. 5.10: The Contour plots depicting the interaction between (A) *A rhizogenes* and *T viride* (B) *A rhizogenes* and vermicompost (C) *T viride* and vermicompost

5.3.5. *In vivo* elicitor treatment

In order to observe if the positive effects obtained in *in vitro* conditions can be repeated in the potted plants in natural environment, the plants were treated with biotic elicitors under green house conditions aiming to validate the technique at the commercial level. The same BBD-RSM design was used for *in vivo* study. The independent variables used

were lysate of *A rhizogenes*, *T viride,* and vermicompost (substitute for yeast extract). The plants were grown in the greenhouse maintained by Lovely Professional University, Punjab. The plumbagin yield was studied both in the aerial parts and roots. One-year-old plants were used for the experiment and the treatment was given in the first week of March. The plants were harvest after one month for plumbagin quantification which was conducted by LC-MS. A total of 15 experimental runs were performed as per BBD-RSM design and the experimental and predicted plumbagin yield is outlined in Table 5.8. The model validation was confirmed by ANOVA and the quadratic effect of the independent variables on the plumbagin 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 plumbagin yield in shoot (Equation 5.2) and in root (Equation 5.3).

$$Y_1 = -1.796 + 2.250\,A + 1.301\,B + 1.142\,C - 0.8116\,A^2 - 0.4172\,B^2 - 0.2448\,C^2 - 0.3086\,AB - 0.0065\,AC + 0.1752\,BC \qquad \text{(Equation 5.2)}$$

$$Y_2 = 3.978 + 4.185\,A + 2.049\,B - 2.204\,C - 1.249\,A^2 + 0.575\,B^2 + 1.154\,C^2 - 1.298\,AB + 0.605\,AC + 0.505\,BC \qquad \text{(Equation 5.3)}$$

Table 5.9: Design along with experimental and predicted values results of response surface analysis for shoot and root elicitation

Run order	Variables			Experimental value %	Predicted value %	Experimental value %	Predicted value %
	A	B	C	Shoot		Root	
1	0.2	1	3	1.212	1.188	4.507	4.362
2	2.0	1	3	1.530	1.432	7.666	7.679
3	0.2	3	3	1.283	1.380	10.872	10.858
4	2.0	3	3	0.490	0.513	9.826	9.970
5	0.2	2	1	0.734	0.676	6.968	7.169
6	2.0	2	1	0.373	0.389	7.362	7.404

7	0.2	2	5	0.784	0.767	9.032	8.989
8	2.0	2	5	0.376	0.433	11.386	11.184
9	1.1	1	1	1.225	1.305	7.033	6.976
10	1.1	3	1	0.281	0.241	10.740	10.552
11	1.1	1	5	0.632	0.672	8.771	8.958
12	1.1	3	5	1.090	1.009	**14.113**	**14.169**
13	1.1	2	3	**2.090**	**2.203**	8.825	8.763
14	1.1	2	3	2.200	2.203	8.651	8.763
15	1.1	2	3	2.320	2.203	8.815	8.763

From the Pareto chart of standardized effects of factors on plumbagin yield presented in Fig. 5.10, it can be determined that the independent factor vermicompost represented by C and interactive effect of *A rhizogenes* and vermicompost represented by AC in shoots are not significant as the bars did not cross the reference line (red dotted line). The extents of bars indicate highest effect of represented variable on response. While in roots the response of all the independent variables were found to be significant.

The model adequacy statements were fulfilled by both the shoot as well as root data as per the residual plots (Fig 5.11 and Fig. 5.12). Most of the residual points in the normal probability plots were scattered along the straight line which in turn fulfilled the first statement for model adequacy. The random distribution without any significant structure was observed in the residual versus fitted value plots which proves the second statement for model adequacy. The histogram plot was observed to be almost symmetrical which further supports the first statement. The completely random residual points observed in the residual versus observation order plot indicate that the residuals were independent of each other, therefore proved the third statement.
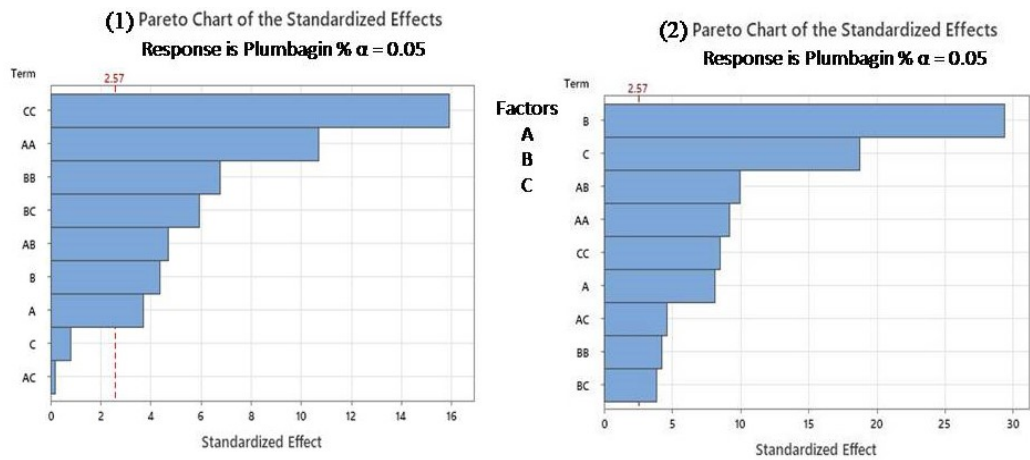
Fig. 5.11: Pareto chart of the standardized effect of independent variable on response (1)
Shoot (2) Root
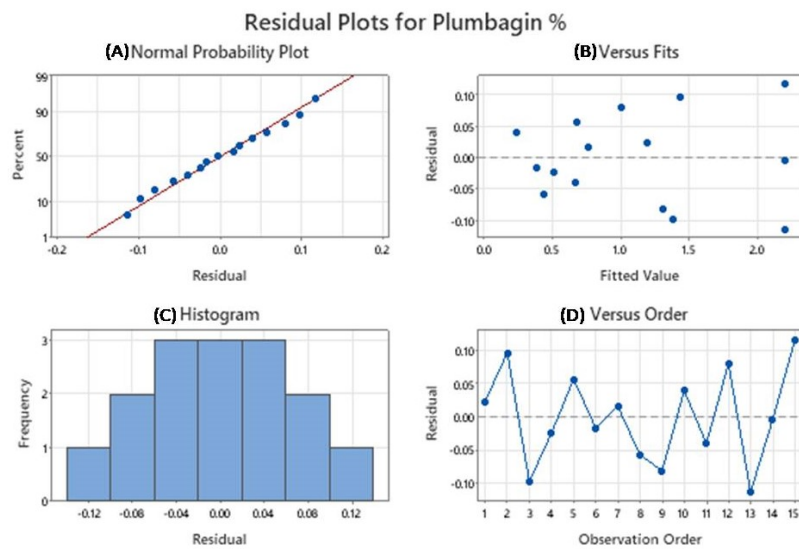
Fig. 5.12: Residual plots of model adequacy checking for shoot (A) Normal probability
plot (B) Residual versus fitted value plot (C) Histogram of frequency versus residual (D)
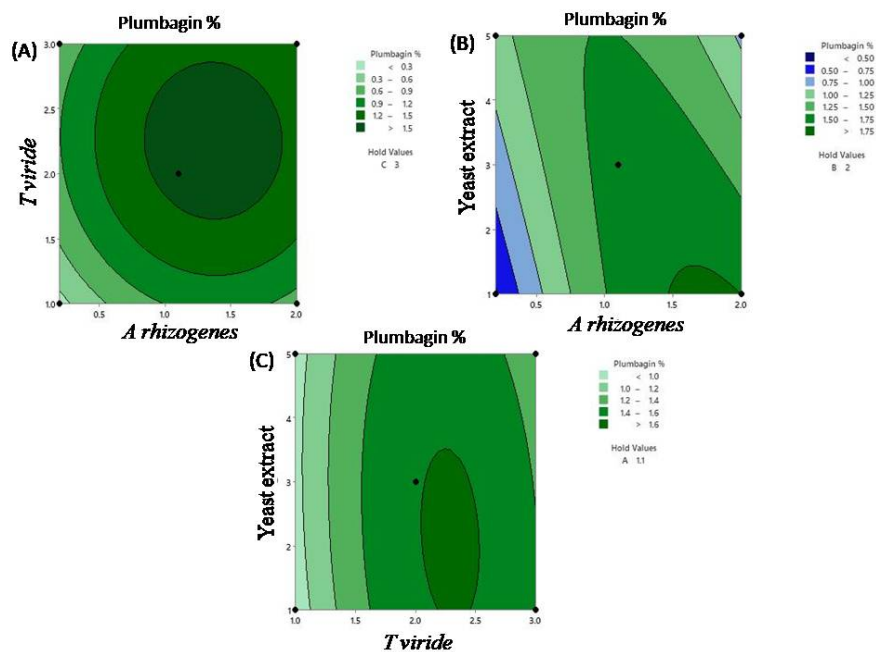Residuals versus time sequence or observation order plot
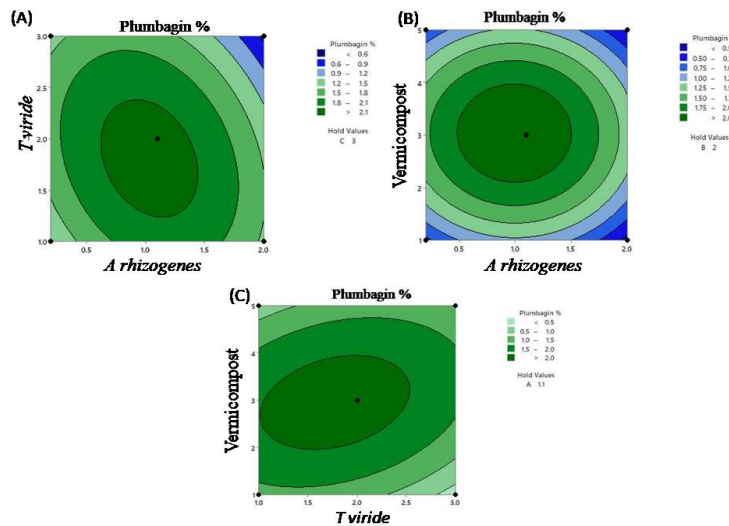
Fig. 5.13: The Contour plots depicting the interaction between (A) *A rhizogenes* and *T viride* (B) *A rhizogenes* and vermicompost (C) *T viride* and vermicompost in shoot
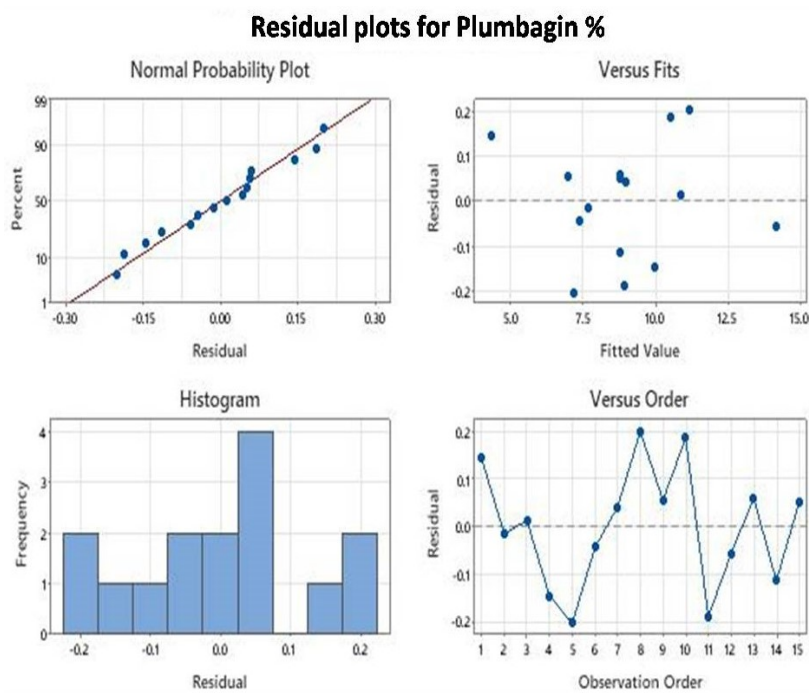
Fig. 5.14: Residual plots of model adequacy checking for root (A) Normal probability plot (B) Residual versus fitted value plot (C) Histogram of frequency versus residual (D) Residuals versus time sequence or observation order plot
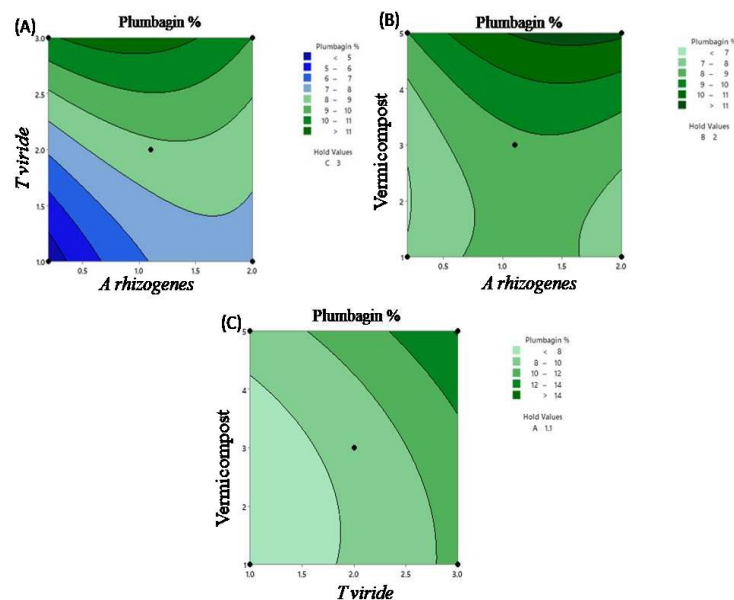
Fig. 5.15: Contour plots showing the interaction between (A) *A rhizogenes* and *T viride*

(B) *A rhizogenes* and vermicompost (C) *T viride* and vermicompost in root

The analysis of variance for the fitted quadratic model was given in Table 5.9. The significance of each coefficient was checked by the P-values. A high F-value and a small P-value indicated a significant coefficient (Luo *et al.*, 2020). The observations of the Pareto chart (Fig. 5.10) were confirmed by ANOVA. The P-value of the independent factor vermicompost represented by C (0.457) and interactive effect of *A rhizogenes* and vermicompost represented by AC (0.850) in shoots were more than the significant value (0.05). The P-value of the independent variables and their interactive effect was found to be less than the significance value in case of the ANOVA of root data.

Table 5.10: The analysis of variance (ANOVA) for plumbagin yield in shoots and root

Shoot			Root		
Source	F-Value	P-Value	Source	F-Value	P-Value
Model	51.00	<0.0001	Model	178.52	<0.0001
Linear	11.16	0.012	Linear	427.52	<0.0001
A	13.87	0.014	A	66.19	<0.0001

B	18.97	0.007	B	865.09	<0.0001
C	0.65	0.457	C	351.30	<0.0001
Square	122.70	<0.0001	Square	62.87	<0.0001
A^2	114.39	<0.0001	A^2	84.67	<0.0001
B^2	46.06	0.001	B^2	17.94	0.008
C^2	253.76	<0.0001	C^2	72.32	<0.0001
2-Way Interaction	19.13	0.004	2-Way Interaction	45.18	<0.0001
AB	22.12	0.005	AB	99.06	<0.0001
AC	0.04	0.850	AC	21.52	0.006
BC	35.22	0.002	BC	14.98	0.012
Lack-of-Fit	5.80	0.150	Lack-of-Fit	5.80	0.150
R^2	0.9892		R^2	0.9969	
Adj R^2	0.9698		Adj R^2	0.9913	

5.4. Conclusion

The present study enabled us to mass produce the plants in a small period of time in controlled conditions. The *in vitro* propagated plants were treated with biotic elicitors using a BBD-RSM design. The experimental data was found to be in agreement with the predicted data and the biotic factors used were found to be significant. The *in vivo* elicitation was also found to be significant. The lysates of *A rhizogenes* and *T viride* were found to be significant on plumbagin yield in both *in vitro* as well as *in vivo* treatments.

SUMMARY AND CONCLUSION

An exclusive investigation was designed and executed to optimize the extraction factors/techniques and mass-produce *Plumbago* species *via* plant tissue culture assisted with elicitation.

Plumbago species were collected from various regions to identify the elite species based on the presence of plumbagin. The plants were collected from the regions of Jammu and Kashmir, Punjab, Haryana, Himachal Pradesh, and Uttarakhand. The plant samples collected were screened by HPTLC method of quantification. All samples collected contained plumbagin but the content varied according to the geographical region. In the case of *P auriculata*, the highest amount of plumbagin was observed in plant samples collected from Chatha, Jammu and Kashmir (0.83μg/g). The highest yield of plumbagin in case of *P zeylanica* was obtained from the samples collected from Jammu, Jammu and Kashmir (2.23μg/g). The difference in the content could be because of many factors that can influence the yield of plumbagin.

Different factors such as pre-harvest, post-harvest and extraction methods were optimized to establish a simple, cost-effective and accessible extraction method. Various pre-harvest factors (plant part, age of plant, harvesting season), post-harvest factors (drying method, storage time of raw material, extraction method) and extraction methods were evaluated. The pre-harvest study stated that the roots had the highest plumbagin content of the twelve-month-old plants harvested in winter. The highest plumbagin content during post-harvest factors was found in the shade-dried samples, 3-month storage time and microwave-assisted extraction. The microwave-assisted extraction factors were further optimized by RSM. BBD was applied to optimize the extraction variables (extraction time, solvent volume and particle size). The best parameters found were; extraction time of 4 minutes, solvent volume 20 mL, and particle size 0.6 mm.

In vitro regeneration procedure for *P zeylanica* and *P auriculata* was established. The elicitation of plumbagin in *in vitro* shoot cultures and potted plants of *P zeylanica* was studied. The effect of three different biotic elicitors on the production of plumbagin was

studied with the help of RSM. BBD was applied to determine the optimum concentration and combination of biotic elicitors. The nodal and leaf explants were used for the regeneration of plants; the sterilization was done with 1% Teepol detergent solution, 1% sodium hypochlorite, 0.2 % Bavistin and freshly prepared 0.1% mercuric chloride. A wide range of concentrations of PGRs were tested to determine the most effective concentration and combinations. The cultures were maintained under light with 16 hours photoperiod at 25± 2℃. Approximately 97% of nodal explants of *P zeylanica* induced buds fortified with 5.5 µM BAP and 3 µM NAA after 10 days. The adventitious root culture of *P. zeylanica* was successfully established in leaf explants. The best response was observed in explants cultured on half-strength MS media with 6.0 µM BAP and 2.5 µM NAA. *P auriculata* shoot cultures was achieved with BAP (6.6 µM/L) and NAA (10 µM/L) on full-strength MS medium and 3 % sucrose. The rooting was initiated on the elongated shoots with 4µM/L IAA on half-strength MS media supplemented with 2 % sucrose (w/v). Hairy root culture was established with leaf and nodal explant of *P zeylanica*. Genomic DNA was extracted from the transformed and non-transformed roots and shoot cultures to check the presence of *rol* B genes in transformed cultures.

The plumbagin yield was found highest in the shoot cultures of *P zeylanica* which were used for further study. The shoot cultures were trested with different biotic elicitors (*A rhizogenes, T viride* and yeast extract) and the experiment was performed and analysed by RSM. All of the elicitors tested were found to be significant. Theoretically, the optimal variable level for bacterial lysate (*A rhizogenes*), fungal lysate (*T viride*) and yeast extract obtained was 1.2, 2 and 3 %, respectively. The highest plumbagin yield predicted by the model was 1.751 %. The design was validated by comparing the predicted data with the experimental data. The optimal conditions were adjusted at bacterial lysate 1 %, fungal lysate 2 % and yeast extract 3 %, considering the practicality of the experiment. In order to observe if the positive effects obtained in *in vitro* conditions can be repeated in the potted plants in natural environment, the plants were treated with biotic elicitors under green house conditions aiming to validate the technique at the commercial level. The independent variables used were lysate of *A rhizogenes*, *T*

viride, and vermicompost. The effect of vermicompost and interactive effect of *A rhizogenes* and vermicompost in the shooting part of the plant on plumbagin yield were found to be not significant. While in roots the effect of all the elicitors on plumbagin yield were found to be significant.

The screening of *Plumbago* species for the elite population and the screening of harvesting factors showed the variation of plumbagin yield. The mass propagation of the *Plumbago* species and elicitation enhanced the plumbagin yield. The techniques developed in the present study can be utilized by researchers and cultivators associated with the plumbagin production. It is important to further study the influence of various other elicitors and precursors that can be utilized to up-regulate the plumbagin yield. Genetic transformation methods and molecular insight can also be explored to further enhance the secondary metabolite production.

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1. **Katoch, K.,** Gupta, S., Nazir, R., Kumar, V., Sanyal, R., Dey, A., & Pandey, D. K. (2021). Establishment of adventitious root culture from leaf explants of *Plumbago zeylanica*: an endangered medicinal plant. *Vegetos*, 1-5. https://doi.org/10.1007/s42535-021-00300-3

2. Kumari, P., Kaur, P., Kumar, V., Pandey, B., Nazir, R., **Katoch, K.,** Dey, A., Dwivedi, P., & Pandey, D. K. (2022). Response surface methodology and artificial neural network modelling for optimization of ultrasound-assisted extraction and rapid HPTLC analysis of asiaticoside from *Centella asiatica*. *Industrial Crops and Products*. https://doi.org/10.1016/j.indcrop.2021.114320

3. **Katoch, K.,** Gupta, S., Gupta, A. P., Goyal, P., Devi, R., Dey, A., & Pandey, D. K. Biotic elicitation for enhanced production of plumbagin in regenerated shoot cultures of *Plumbago zeylanica* using Response Surface Methodology. *Plant Cell, Tissue and Organ Culture.* **(Under review)**

4. **Katoch, K.,** Gupta, S., Gupta, A. P., Verma, B., Dey, A., & Pandey, D. K. Biotic elicitation of *Plumbago zeylanica* for enhanced production of plumbagin using response surface methodology under natural conditions. *Industrial Crops and Products*. (**Under review**)

5. **Katoch, K.,** Gupta, S., & Pandey, D. K. (2021). Establishment of direct regeneration protocol for *Plumbago auriculata* plantlets, determination of clonal fidelity using ISSR markers and comparative HPTLC analysis of plumbagin. *Nucleus*. (**Under review**)

6. **Katoch, K.,** Gupta, S., Gupta, A. P., Dey, A., & Pandey, D. K. Optimization of various factors such as pre-harvest and post-harvest for optimum extraction of plumbagin. *South African Journal of Botany*. (**Under review**)

7. **Katoch, K.,** Dey, A., & Pandey, D. K. Optimization of Microwave-assisted extraction (MAE) of plumbagin from *Plumbago zeylanica* by response surface methodology (RSM). *Industrial Crops and Products*. **(Under review)**