

**EFFECT OF BIOTIC AND ABIOTIC FACTORS INFLUENCING
SAPONIN CONTENT IN *IN-VITRO* AND *IN-VIVO* CULTURED
PLANTS OF *Chlorophytum borivilianum***

**Thesis Submitted for the Award of the Degree of
DOCTOR OF PHILOSOPHY**

**in
BOTANY**

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2026



DECLARATION

I hereby declare that the work presented in the thesis titled “**Effect of Biotic and Abiotic Factors Influencing Saponin Content in *In-Vitro* and *In-Vivo* Cultured Plants of *Chlorophytum borivilianum***” has been accomplished and submitted by me for the award of the degree of Doctor of Philosophy in Botany from the Department of Botany, 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 “**Effect of Biotic and Abiotic Factors Influencing Saponin Content in *In-Vitro* and *In -Vivo* Cultured Plants of *Chlorophytum borivillianum***” being submitted by Mr. Rohtas Kumar under my supervision for the award of Doctor of Philosophy in the discipline of Botany is an original work done by his.

It is further certified that:

- a) The scholar has worked under my supervision for the requisite time period under statutes.
- b) The scholar has the required percentage of attendance.
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Rohtas Kumar

ABBREVIATIONS

°C	Degree Celsius
2, 4- D	2, 4-Dichlorophenoxyacetic acid
ABA	Absciscic 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	Liter
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification

MAE	Microwave-assisted extraction
MHz	Megahertz
mL	Milli-liter
mm	Milli-meter
min	Minute
μL	Micro-liter
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
UV	Ultra-violet

ABSTRACT

Safed musli (*Chlorophytum borivillianum* L.) is an important medicinal plant famous for its saponin compounds and use in Ayurveda and Traditional Chinese Medicine. However, scientific evidence supporting the enhancement of saponin production during stress condition is limited. This study successfully established and validated an HPTLC method for the simultaneous detection and quantification of diosgenin and stigmasterol in *C. borivillianum*. Well-resolved peaks at R_f values 0.67 and 0.72 confirmed the identity of the biomarkers, and the developed method showed excellent linearity, low LOD and LOQ, and high recovery rates, thereby validating its robustness for routine analysis. TLC fingerprinting further established the phytochemical profile of the plant extracts, while overlay UV spectra and densitometric scanning confirmed the presence of target compounds. The optimized formulation demonstrated high composite desirability, indicating the effectiveness of the experimental design approach. The validated HPTLC method, combined with statistical optimization, provides a reliable analytical and formulation strategy for standardization and quality control of herbal preparations containing diosgenin and stigmasterol.

A highly effective method has been developed to examine the impact of different combination of meta-topolin and various cytokinins on *Chlorophytum borivillianum* using apical bud explants. The effects of benzyl adenine purine (BAP), kinetin (Kin), meta-topolin (mT), and thidiazuron (TDZ) were explored separately and in different concentrations (ranging from 0.5-3.0 mg L⁻¹) on shoot proliferation, as well as the influence of auxins like Indole-3-acetic acid (IAA), α -Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) on rooting. When grown on MS-medium supplemented with mT, highest growth rates were observed followed by BAP. Within the permutations tested, 2.0 mg L⁻¹ of mT and 1.0 mg L⁻¹ of BAP produced the best results with 96.3% success rate and average of 19.2 ± 1.92 shoot measuring 6.72 ± 0.49 cm in length after 8 weeks of culture. Among the various auxins used for *in vitro* root formation, 95.5% of multiple shoots effectively established roots on MS medium supplemented with 1.0 mg L⁻¹ of IBA resulting in an average of 14.0 ± 1.39 root numbers and 3.36 ± 0.27 cm in length after 4 weeks of culture. Furthermore, 94% of the acclimated plants not only survived but also thrived when grown in a mixture of Loam, sand and Fym (in 1:2:1 ratio) with an

average weight of 16 g. High-performance thin layer chromatography (HPTLC) analysis revealed the presence of diosgenin and stigmasterol in both *in vitro* regenerants and mother plant, although there were variations in their content. To ensure genetic fidelity among regenerants, ISSR and SCoT markers were used, but no somatic differences were detected. Therefore, the established procedure seems to be easy and consistent for large scale development of genetically identical clones with a greater content of bioactive compounds. Optimization of drought induced (PEG6000) and elicitor (chitosan) were added to the MS medium for enhanced production of diosgenin and stigmasterol. The validated HPTLC method, combined with statistical optimization, provides a reliable analytical and formulation strategy for standardization and quality control of herbal preparations containing diosgenin and stigmasterol. The integration of validated HPTLC analysis with RSM-based optimization provides a powerful framework for phytochemical standardization, quality control, and formulation development. These findings contribute to the broader goal of advancing phytopharmaceutical research and open avenues for scale-up and metabolic engineering strategies targeting steroidal metabolites in medicinal plants.

This study aimed to assess the effects of drought on *Chlorophytum borivillianum* saponin production and explore comprehensively for the first time the potential impacts of various biostimulants. A controlled pot experiment was conducted with drought stress, alongside treatments of *Bacillus* spp. (phosphate solubilizer), *Glomus* spp (plant mycorrhiza) and *Azotobacter* spp. (nitrogen fixers). The purpose of this work is to optimize the treatment conditions of microbes using a full factorial design. Quantitative analysis of saponins was performed through HPTLC analysis. Among all treatments, the T7 combination (*Azotobacter* + *Bacillus* + *Glomus*) showed maximum growth (28 leaves, 9 roots) and high diosgenin content (0.67%). Single treatments showed moderate responses. Drought stress led to zero growth but increased diosgenin (0.68%), indicating stress-induced metabolite accumulation. ANOVA confirmed significant main and interaction effects ($P < 0.001$) for growth and diosgenin. For stigmasterol, only phosphate solubilizers showed significance. These findings underscored the potential of biostimulants to mitigate the adverse effects of drought stress, thereby enhancing Safed musli resilience and productivity in drought environments.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Medicinal plants are crucial for maintaining the primary health care. According to UNO report nearly about 80% of the world population of world is dependent on medicinal plants for their healthcare (Farnsworth et al., 1985).

Chlorophytum borivilianum Santapau and Fernandes an important medicinal plant native to India is commonly known as Safeed Musli, is an annual herb belonging to family Liliaceae. *C. borivilianum* is regarded as most valuable medicinal plant used since long in traditional Indian medicinal system 'Ayurveda'. It has found its mention in traditional Indian literature such as *Bhavapraksh*, *Raja Ballabh Nighantu*, *Rasendra sarsangrah* as *vajikaran* which means, it is a significant immunomodulator. It is regarded as 'white gold' or some called this plant as Indian Viagra. Due its huge medicinal importance the plant is being harvested ruthlessly posing a threat to its occurrence and biodiversity. It is mainly discovered as 'Herbal viagra'. Safed musli is discovered in deciduous forests in tropical moist and dry region. In India it spread across Southern Rajasthan, Western Madhya Pradesh, North Gujarat and few parts of Karnataka. But due to its continuous exploration, its frequency, distribution has decreased (Singh et al., 2012). Its roots are of much medicinal value as various extracts from them are used in the preparation of more than 100 ayurvedic drug formulations. It is regarded as a potent source of manufacture of various herbal drugs. As we all know due to the side effects posed by the use of chemically manufacture drugs, most of the people in the world has turned to herbal drugs for maintaining their health relating issues and curing various ailments. But problem with the herbal drug formulations is their proper standardization. As according to UNO, appropriate standardization of herbal drugs is essential to enhance their efficacy towards illness. From studies it has been observed that greater than 60% of the commercialised drugs are collected from plant sources (Cragg and Newman, 2013). The general concept of herbal medicine gives prominence to health promotion, disease control, prompt diagnosis and personalized treatment (Patwardhan, 2014).

In India, ayurvedic formulations been used from the ancient times. The most ancient use of medicinal plants has been reported in various Hindu scriptures like *Rigveda* (4500-1600 BC), *Charaka Samhita* (1000-800 BC), *Sushruta Samhita* (800-700 BC), and others. Most of the tribal communities are reliant on traditional medicines methods for their health care. In India

approximately 75% population is dependent on herbal medicines for their primary healthcare. The Eastern Himalayas, Western Ghats and Andaman and Nicobar Islands are reported as the hot-spots of medicinal plant diversity (The Wealth of India 1988; Indian Herbal Pharmacopoeia 2002).

Chlorophytum borivilianum is a wonder herbal drug. In India it is used as Rasyan drug. Rasyan signifies the property that helps it to rejuvenate, i.e. it is having significant adaptogenic, aphrodisiac and immunomodulatory activity (Thakur et al., 2007).

This plant is crucial due to presence of bioactive compounds like saponins and alkaloids present in root of plant. Roots of plant is composed of various bioactive compounds and their proportion is proteins 80-90%, Carbohydrates 42%, Saponins 2-17%, alkaloids 15-25% and fibers 3-4%. Plant holds a great potential for being used as an immune strengthening agent. Study on various extracts from roots of plant has been found it can be used as an effective immunomodulating agent.

Saponins are natural compounds found in many plants and can have various pharmacological properties, such as anti-inflammatory, antimicrobial, and immunostimulatory effects.

Chlorophytum borivilianum, commonly known as Safed Musli, is an important medicinal plant belonging to the family Asparagaceae. Increasing commercial demand and indiscriminate harvesting from natural habitats have led to depletion of natural populations, making the species near threatened and necessitating urgent conservation and sustainable utilization strategies.

Table1.1 Distribution of saponins in medicinal plants

S/N	Plant	Common Name	Saponin Type	Use/Effect	References
1	<i>Quillaja saponaria</i>	Soapbark tree	Triterpenoid saponins	Used in cosmetics, medicine, and as a foaming agent	de Andradei et al., 2020
2	<i>Glycyrrhiza glabra</i>	Licorice	Glycosides (Triterpenoid saponins)	Anti-inflammatory, anti-viral, digestive aid	Fiore et al., 2008
3	<i>Aesculus hippocastanum</i>	Horse Chestnut	Aescin (Triterpenoid saponin)	Vascular health, anti-inflammatory	DeLuca et al., 2019
4	<i>Sapindus mukorossi</i>	Soapnut	Saponins (Triterpenoid and steroidal)	Used as a natural detergent, cleansing agent	Wojtoń et al., 2021

5	<i>Saponaria officinalis</i>	Soapwort	Saponins (Triterpenoid saponins)	Used for its foaming properties and in traditional medicine	Chandra et al., 2021
6	<i>Chenopodium quinoa</i>	Quinoa	Saponins (Triterpenoid saponins)	Natural detergent, anti-inflammatory	Dini et al., 2005
7	<i>Pulsatilla vulgaris</i>	Pasque Flower	Steroidal saponins	Anti-inflammatory, toxic in high doses	Li et al., 2021
8	<i>Tribulus terrestris</i>	Puncturevine	Saponins (Steroidal)	Enhances athletic performance, libido	Zuo et al., 2021
9	<i>Panax ginseng</i>	Ginseng	Ginsenosides (Triterpenoid saponins)	Anti-fatigue, adaptogen, cognitive booster	Kim et al., 2020
10	<i>Ilex paraguariensis</i>	Yerba Mate	Saponins (Triterpenoid saponins)	Antioxidant, stimulant properties	Canto et al., 2010
11	<i>Asparagus racemosus</i>	Shatavari	Saponins (Steroidal)	Adaptogen, supports female reproductive health	Hayes et al., 2021
12	<i>Camellia sinensis</i>	Green Tea	Saponins (Triterpenoid saponins)	Antioxidant, anticancer properties	Yu et al., 2019

1.2 Biotic and Abiotic factors influencing saponin production in medicinal plants

Biotic elicitors like jasmonic acid, chitosan, and pathogen stresses trigger plant defense pathways, often leading to elevated saponin synthesis. Abiotic elicitors, ranging from heavy metals to physical stimuli like ultrasound and UV can significantly boost saponin accumulation or secretion, depending on dosage, exposure, and plant type. Many elicitors show dose- or duration-dependent effects, including both increases and potential decreases in tissue saponin content or shifts in related metabolites like sterols. Plant species and tissue type (e.g., root vs leaf, hairy root cultures vs whole plants) critically influence responses.

Table 1.2 Abiotic and biotic factors enhancing or modulating saponin production

S/N	Factor	Effect on Saponin Production	Notes	Reference
1	Pathogen or herbivore attack	Increases saponin content in plant tissues	Observed in pea cultivars exposed to <i>Aphanomyces euteiches</i> , <i>Fusarium</i> , etc.	Lozovaya et al., 2004 a, b; Papadopoulou et al., 1999
2	Jasmonic acid (JA) and methyl jasmonate (MeJA)	Strongly increases accumulation and secretion of triterpenoid saponins	Studied in <i>Calendula officinalis</i> hairy root cultures	Fuchs and Kutchan, 1999; Ahlawat et al., 2014
3	Chitosan (fungal elicitor)	Slight increase in both accumulation and secretion of saponins, plus sterols	<i>Calendula officinalis</i> hairy roots	Ramirez-Estrada et al., 2016
4	Heavy metals (Ag^+ , Cd^{2+})	Greatly enhance secretion of oleanolic acid saponins	Up to 12-fold secretion in <i>Calendula officinalis</i> ; Cd^{2+} response proportional, Ag^+ inversely proportional	Cusidó et al., 1999
5	Ultrasound	Enhances secretion (up to 11-fold); exposure time dependent	Short 20-min exposure induced 4–11-fold secretion; some decrease in tissue saponins & sterols	Zhao et al., 2005
6	UV-C irradiation	Slight increase in tissue saponin; secretion enhanced up to 8.5 folds	Observed in two hairy root lines of <i>Calendula officinalis</i>	Ramirez-Estrada et al., 2011
7	Osmotic stress, temperature, light, Cu^{2+} , oxygen	Varying positive or negative impacts on saponin levels	General summary of multiple abiotic elicitors in diverse cultures	Goossens et al., 2003
8	Selenium (Se) and Nickel (Ni^{2+})	Moderate increases (~1.2-1.3 folds) in ginseng saponin content	<i>Panax ginseng</i> hairy root cultures	Liang et al., 2010
9.	Drought stress	Increases saponin or related secondary metabolite content	Mild water stress boosted saikosaponins in <i>Bupleurum chinense</i>	Guo et al., 2010

10	UV-B radiation (ambient sunlight)	Suppresses plant growth; may increase saponin accumulation; saponins may act as growth inhibitors	In cotton, removing UV-B decreased saponins and boosted growth	Li et al., 2010
11	Salicylic acid, jasmonic acid, UV, mechanical damage	Induces saponin accumulation in <i>Quillaja brasiliensis</i> leaves	Demonstrates multiple abiotic elicitors enhancing saponins	Santos et al., 2017
12	Temperature / precipitation (climate)	Affect saponin content & composition over time	Dynamic, environment-dependent variation in pulse crops	Muir et al., 2003
13	Pathogen or herbivore attack	Increases saponin content in plant tissues	Observed in pea cultivars exposed to <i>Aphanomyces euteiches</i> , <i>Fusarium</i> , etc.	Lozovaya et al., 2004 a,b; Papadopoulou et al., 1999

1.3. In vitro biotechnological methods for saponin production

In vitro biotechnological methods used for plants containing saponins. These approaches are widely applied to enhance production, conserve germplasm, or obtain bioactive saponins for pharmaceuticals, nutraceuticals, and cosmetics. Hairy root cultures are considered the most reliable and productive method for saponin biosynthesis *in vitro*. Elicitation (JA, SA, chitosan, heavy metals, UV, ultrasound) is critical for boosting yields. Bioreactor systems are necessary for scaling up production to industrial levels. Metabolic engineering (CRISPR/Cas, overexpression of *CYP450s*, *glycosyltransferases*) is the future direction for targeted saponin biosynthesis.

Table 1.3 Analytical methods and applications for saponins in plants

S/N	Method	Description	Application to Saponins	Example Plants	References
1	Callus Culture	Induction of undifferentiated tissue on nutrient medium with plant growth regulators (PGRs)	Provides biomass for saponin extraction; useful for genetic transformation	<i>Panax ginseng</i> , <i>Bupleurum chinense</i>	Zhao et al., 2011
2	Cell Suspension Culture	Liquid cultures of dispersed	Large-scale production of triterpenoid and	<i>Panax notoginseng</i> ,	Rao and Ravishankar, 2002

		cells derived from callus	steroidal saponins; suitable for elicitation studies	<i>Medicago truncatula</i>	
3	Hairy Root Culture (<i>Agrobacterium rhizogenesis</i> -mediated)	Stable, fast-growing root cultures with high genetic/biochemical stability	Highly efficient for saponin accumulation, secretion, and metabolic engineering	<i>Panax ginseng</i> , <i>Glycyrrhiza uralensis</i> , <i>Calendula officinalis</i>	Srivastava and Srivastava, 2007
4	Somatic Embryogenesis	Development of embryos from somatic tissues under <i>in vitro</i> conditions	Generates uniform plantlets with high saponin content; used for synthetic seed technology	<i>Panax ginseng</i> , <i>Chlorophytum borivillanum</i>	Komath and Kavikishor, 2000
5	Elicitation (Aiotic and abiotic)	Application of elicitors (JA, SA, chitosan, heavy metals, UV, etc.)	Enhances biosynthesis and secretion of saponins via secondary metabolism activation	<i>Panax ginseng</i> , <i>Calendula officinalis</i>	Gadzovska et al., 2007
6	Precursor Feeding	Supplying pathway precursors (e.g., mevalonic acid, squalene)	Boosts triterpenoid saponin production in cultures	<i>Medicago truncatula</i> , <i>Glycyrrhiza glabra</i>	Kim et al., 2005
7	Metabolic Engineering	Genetic modification of key enzymes in saponin biosynthesis (e.g., β -amyrin synthase, CYP450s)	Tailored production of specific saponins; higher yields	<i>Panax ginseng</i> , <i>Medicago truncatula</i>	Moses et al., 2014
8	Polyploid Induction (<i>In vitro</i>)	Colchicine/oryzalin used to induce polyploidy	Enhances secondary metabolite (saponin) content	<i>Stevia rebaudiana</i> , <i>Chlorophytum borivillanum</i>	Thakur and Sharma, 2016
9	Bioreactor Culture	Large-scale cultivation of cells/roots/callus in liquid media	Industrial-scale saponin production under optimized conditions	<i>Panax ginseng</i> (ginsenosides)	Choi et al., 2010
10	Synthetic Seeds	Encapsulation of somatic embryos or shoot buds	Propagation and conservation of elite high-saponin lines	<i>Chlorophytum borivillanum</i>	Rout et al., 2000

11	Conventional breeding	Cross-breeding and selection of superior genotypes	Development of high-saponin cultivars	<i>Medicago sativa</i> , <i>Quillaja saponaria</i>	Szakiel et al., 2011
12	Agronomic practices (Soil/Fertilization)	Optimization of soil nutrients (N, P, K, Se, Si, organics)	Certain nutrients (e.g., Se) enhance steroidal/triterpenoid saponins	<i>Panax ginseng</i> , <i>Chlorophytum borivilianum</i>	Hu et al., 2003
13	Elicitor sprays (Field)	Exogenous JA, SA, chitosan, yeast extract sprays	Stimulates defense pathways → upregulates saponin biosynthesis	<i>Glycyrrhiza uralensis</i> , <i>Bupleurum chinense</i>	Li et al., 2016
14	Environmental Stress Induction	Exposure to drought, salinity, heat, UV-B, wounding	Moderate stress enhances saponin accumulation	<i>Bupleurum chinense</i> (drought ↑ saikosaponins), Cotton (UV-B ↑ saponins)	Guo et al., 2010; Li et al., 2010
15	Plant Growth Regulators (Field)	GA ₃ , cytokinins, ABA, etc. applied in field	Improves biomass and secondary metabolite (saponin) levels	<i>Panax notoginseng</i>	Chen et al., 2009
16	Endophytic Microbes / Mycorrhiza	AMF fungi (<i>Glomus</i> , <i>Piriformospora</i>), rhizobacteria	Enhance nutrient uptake and trigger saponin biosynthesis	<i>Medicago truncatula</i> , <i>Panax ginseng</i>	Jiao et al., 2018
17	Genetic Engineering (Whole Plant)	Transformation or CRISPR genome editing in planta	Overexpression of β-amyrin synthase, CYP450s ↑ saponins	<i>Medicago truncatula</i> , <i>Panax ginseng</i>	Moses et al., 2014
18	Polyploidization (<i>In vivo</i>)	Chromosome doubling in seedlings/regenerants	Higher biomass + saponin yield vs diploids	<i>Chlorophytum borivilianum</i> , <i>Glycyrrhiza glabra</i>	Lavania et al., 2012
19	Harvest Stage Optimization	Identification of optimal growth stage for highest saponins	Saponin content varies with age and organ.	<i>Panax ginseng</i> (4-6 yr roots richest), <i>Quillaja brasiliensis</i>	Yang et al., 2012

1.4 Aims and objectives of the study

Objectives of the study

Chlorophytum borivilianum is an economically important medicinal plant valued for its rich saponin content and diverse therapeutic applications. Due to indiscriminate harvesting, low seed viability, and increasing commercial demand, the species is considered near threatened in its natural habitat. Considerable research is still required for the collection and evaluation of elite germplasm, development of efficient propagation systems, and enhancement of bioactive compounds through biotechnological approaches. Therefore, the present investigation was undertaken to collect germplasm of *Chlorophytum borivilianum* from different geographical to assess saponin content through HPTLC analysis for identification of superior chemotypes. The study also aims to establish an efficient *in vitro* mass propagation protocol for rapid multiplication and production of clonally uniform plants. Further, the investigation focuses on evaluating the role of biotic and abiotic elicitors in enhancing saponin accumulation under both *in vitro* and *in vivo* conditions, thereby contributing toward sustainable utilization, conservation, and quality improvement of this valuable medicinal species.

- To collect the germplasm of *Chlorophytum borivilianum* from Akola (Maharashtra), Punjab and Haryana and estimation of saponin by HPTLC method.
- Mass propagation of *Chlorophytum borivilianum* by *in-vitro* methods.
- Role of biotic factors on enhancement of saponin in *in-vitro* and *in-vivo* cultured plants.
- Role of abiotic factors on enhancement of saponin in *in-vitro* and *in-vivo* cultured plants.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Introduction

2.1.1. Taxonomy, phylogeny, diversity and distribution

In old biological classification, the genus *Chlorophytum* was placed under the family Liliaceae, but now it has been reassigned to the family Anthericaceae of the series *Coronariae* within the class Monocotyledons (Bentham and Hooker, 1883). The name *Chlorophytum* is derived from the Greek words *chloros* (green) and *phyton* (plant). Hooker and Jackson (1895) reported over 300 species, with approximately 85% occurring in tropical and subtropical Africa, suggesting that Africa is the probable center of origin and diversification. However, according to Poulsen (pers. comm.), the catalog of *Chlorophytum* species remains incomplete, with an estimated 125-150 species possibly existing.

The majority of species within the genus are tuberous geophytes, typically found in habitats such as savanna grasslands or open woodlands (Kumar et al., 2017). *Chlorophytum* species are mainly distributed in tropical regions worldwide, with several species also cultivated as ornamentals due to their attractive flowers. Approximately 16 species of *Chlorophytum* have been documented in India (Sharma & Balakrishnan, 1993). A new species, *C. kolhapurens*, was identified in the Kolhapur region of Maharashtra (Yadav et al., 2009).

Several species, including *C. arundinaceum* Baker, *C. attenuatum* Baker, *C. borivilianum*, *C. brevescapum* Dalaz, *C. laxum* R.Br., and *C. tuberosum* Baker, are commonly traded under the name ‘Safed Musli’ (Kothari et al., 2005). In India, Safed Musli is known by multiple vernacular names: *Shweta musli* (Sanskrit), *Taniravi thang* (Tamil), *Khiruva* (Hindi, U.P.), *Safed musli* (Marathi/Hindi), *Dholi musli* (Gujarati), *Tella nela tadi gaddalu* (Telugu), and *Shedheveli* (Malayalam) (Singh and Chauhan, 2003). Among these species, *C. arundinaceum* is especially recognized for its tuber characteristics in Ayurvedic texts (Nadkarni, 1954). However, *C. borivilianum* has recently gained prominence as the primary Safed Musli crop, supported by modern phytochemical and pharmacological research (Thakur et al., 2016).

2.1.2 Botanical Description of *Chlorophytum borivilianum*

Chlorophytum borivilianum Santapau & Fernandes is a small annual herb. Plant grows well in sub-tropical and tropical areas upto an altitude of 1500 meters. Plant is short in length and

grows only upto height of 1.5 ft. Plant consist of tuberous roots which can grow in soil upto a depth of about 8-10 inches, sub erect stem disc mounted with bunch of leaves. The general botanical description of plant can be described as:

Taxonomy of *Chlorophytum borivilianum*

Scientific classification

Kingdom	Plantae
Clade	Angiosperm
Family	Asperagaceae
Genus	<i>Chlorophytum</i>
Species	<i>borivilianum</i>

Root:

Plant consists of tuberous root system which is white at juvenile stage of plant and turns into pale brown as the plant matures. Roots are tasteless and possess a characteristic odour. Roots originate directly from stem disc and are fleshy devoid of any fibrous structures. Tuberous roots are cylindrical in shape having a thickness of about 0.9 -1 cm and grows upto the length of 7-8 cm on average. Tuberous roots are attached to stem disc and generally tapering towards lower side. It has been observed that on an average plant possesses 5-30 tubers. These roots possess so many medicinal values as their extracts are used up in the preparation of more than 100 rasayan drug formulations (Panda et al., 2011)

Stem:

The plant consists of a thin stem disc. It is usually a false stem. Tuberous roots directly originate from this stem disc.

Leaf:

Leaves are attached slightly narrowing at base and tapering at apex having broader middle part. Leaves are spread horizontally having parallel venation and wavy margins. Leaves get dried or wilted during the months of October- February and starts to appear again during the month of March.

Flowers:

Chlorophytum borivilianum consists of flowers which are small, whitish in colour. Flowers are zygomorphic, pedicellate, bracteates and are usually arranged in clusters. The flower clusters are usually found positioned alternate to each other and dense cluster of flowers are observed on the upper part of scape. Floral bracts are linear, purplish and somewhat papery

usually having a length of 1.0, 10.5 centimeters. Pedicle is about 6-10 mm in length and whitish in colour. Plant generally begins to flower during the month of April after overcoming the dormant phase during winters. At each flower stalk, about 6 petals are arranged. Stalk arises from the center of plant. Plant has solitary and scape inflorescence, which is terminal, long and unbranched. Each inflorescence consists of 10 - 25 flowers (Biradar, 2011). Androecium of flower consists of 6 stamens in two whorls of 3 each, which lay opposite to tepals, united to the perianth. Anthers are yellowish in colour, linear and dehisce by longitudinal slits. Gynoecium of flower consists of an ovary which is superior, syncarpous, trilocular, and tricarpeal with axile placentation. Two ovules are present in each locule of ovary. Ovary consists of a style which is single and swollen at apex. Perianth of flower consists of 6 tepals which are arranged two in each whorl. They are whitish in colour. Perianth have tepals which are linear, acute and membranous, having imbricate aestivation.

Floral formula of plant is as, $\oplus$, ♂, ♀, P (3+3), A (3+3), G (3) (Nikam and Chauhan, 2009).

Fruit:

Fruit is generally green to yellow in colour having uniform length and breadth.

Seed:

Seed is small and black. It is enclosed in the holes; holes consist of seeds and in each whole contains about 10-12 seeds. Seeds are like that of onion, which are black in colour, endospermic and angular in shape.

2.1.3. Distribution of *C. borivilianum*

C. borivilianum is a medicinally important plant. It is key member of genus *Chlorophytum*. 256 species of this genus are known and are spread all along the tropical and subtropical regions of world. It is native to India. In India, the plant was first reported from Aravalli hills (Purohit et al., 1994). It was also identified in Dang forests. It can be found growing at an altitude of over 1500 meters from sea level (Raghavendra et al., 2005; Nayar and Sastry, 1988). Warmth and humidity are essential for the its's growth and development. It is generally found in those areas where annual rain fall is in the range of 50- 150 cm, the normal temperature range for optimum growth and development is about 35°C. Temperature more than this does not favour the normal growth of plant. In India it is mainly distributed and grown in the states of Madhya Pradesh, Uttar Pradesh, Gujarat, Haryana, Jarkhand, Rajasthan Andhra Pradesh, Madhya Pradesh, Bihar and Maharashtra (Singh A et al., 2003). In India 13 species of genus *Chlorophytum* have been found (Mandal and Nandi, 2012). As the medicinal importance of this plant has gone up to many fold due to the new research findings evaluating its importance in many health related issues mankind is coping with, the plant is being

cultivated and grown in many other states like Punjab and Orissa. Due to the importance of this plant in pharmacological industries, it has been transferred from its native places and cultivated in many countries of world.

2.1.4 Distribution of saponins in the plant kingdom

Saponins is a widespread and found in many medicinal plants.

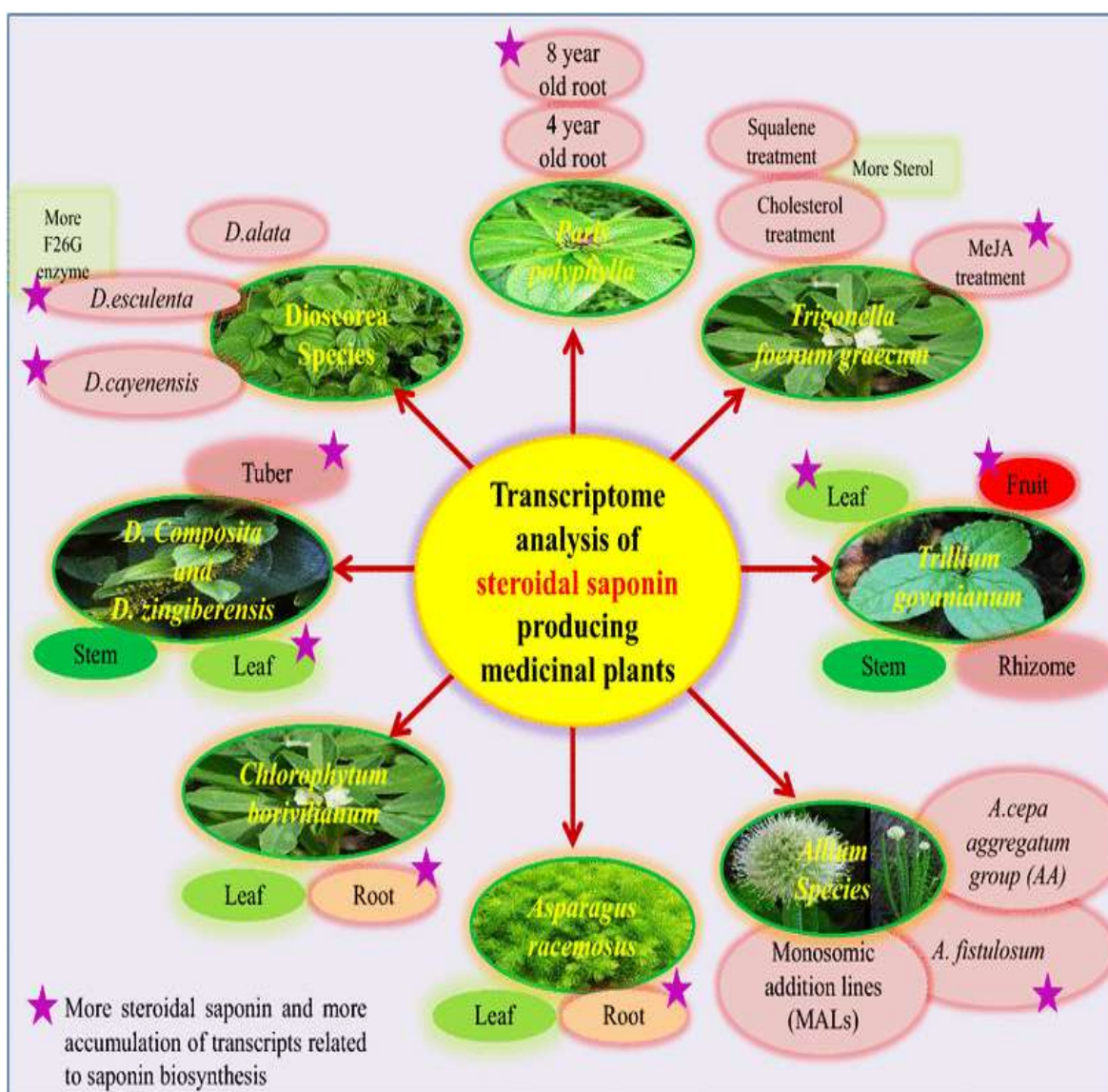


Fig. 2.1 Distribution of saponins in medicinal plants

Table 2.1 Saponins reported in *Chlorophytum borivilianum*

S/N	Saponin / Sapogenin	Type / Structural class	Reported Biological Activities	References
1	Diosgenin	Spirostanol sapogenin (steroidal nucleus)	Precursor for steroidal drugs, aphrodisiac, hypocholesterolemic, anti-inflammatory	Kothari et al., 2013; Dixit et al., 2005
2	Hecogenin	Spirostanol sapogenin	Anti-inflammatory, hepatoprotective, precursor for semi-synthetic steroids	Acharya et al., 2008
3	Tigogenin	Spirostanol sapogenin	Anti-hyperlipidemic, potential aphrodisiac	Acharya et al., 2009
4	Borivilianosides A-H	Furostanol and spirostanol saponins (glycosides)	Aphrodisiac, adaptogenic, immunomodulatory	Acharya et al., 2009
5	Borivilianoside C1, C2	Steroidal saponin glycosides	Antioxidant, immunostimulant	Patel et al., 2016
6	Chloromaloside A	Furostanol glycoside	Antioxidant, cytoprotective	Gupta et al., 2018
7	Total steroidal saponins (crude fraction)	Mixture (spirostanol/furostanol glycosides)	Aphrodisiac, anti-stress, immunomodulatory, antioxidant, anti-diabetic	Shukla et al., 2010; Thakur et al., 2022

2.1.5 Structure of Steroidal saponins (Stigmasterol) and Sapogenin (Diosgenin)

Tubers of all *Chlorophytum* species generally contain steroidal and triterpenoidal saponins as their primary phytochemical constituents. Steroid saponins are significant secondary metabolites of plants that are reputed for their pharmacological significance (Thakur et al., 2009c; Kalra et al., 2013a). It has been shown that the roots of every species in this genus are thick, somewhat meaty, and white (Maiti and Geetha, 2005). Its larger roots or tubers are said to provide a variety of pharmacological qualities in the traditional medical system. Glucuronic acid, glucose, galactose, xylose, and other monomeric sugar moieties are thought to create a glycosidic bond with aglycone, which is hydrophobic and contributes to the chemical structure of saponins (Kaushik, 2005). First and foremost, glucopyranoside, a sugar containing compound, was extracted from *C. arundinaceum* fruits (Gupta et al., 1979).

Following that, *C. malayense*, which is found in the southern east of Asia, yielded four further crude saponin derivatives: chloromaloside A, B, C, and D (Table 1) (Li et al. 1990). Methanol was employed as a promising extraction medium to separate pure saponin from *C. malayense*. This was followed by n-butanol partitioning and, finally, a chloroform-methanol-water (50:10:1-10:10:1) mixture during silica gel column chromatography. Similarly, three new sapogenins-tokorogenin, neogitogenin, and tigogenin-were discovered in *C. arundinaceum* based on mass spectral fragmentation (Tandon and Shukla, 1992). The same species was also found to include a few other biomolecules, including 4-hydroxyl-8, 11-oxidoheneicosanolnonacosane, tetracosanoic, triacontanoc, and pentacosyldocosanoate (Tandon et al., 1992). The first report of the existence of 2,2',4,4'-tetrahydrobibenzylxyloside in Liliaceae family members was credited to Tandon and Shukla (1993).

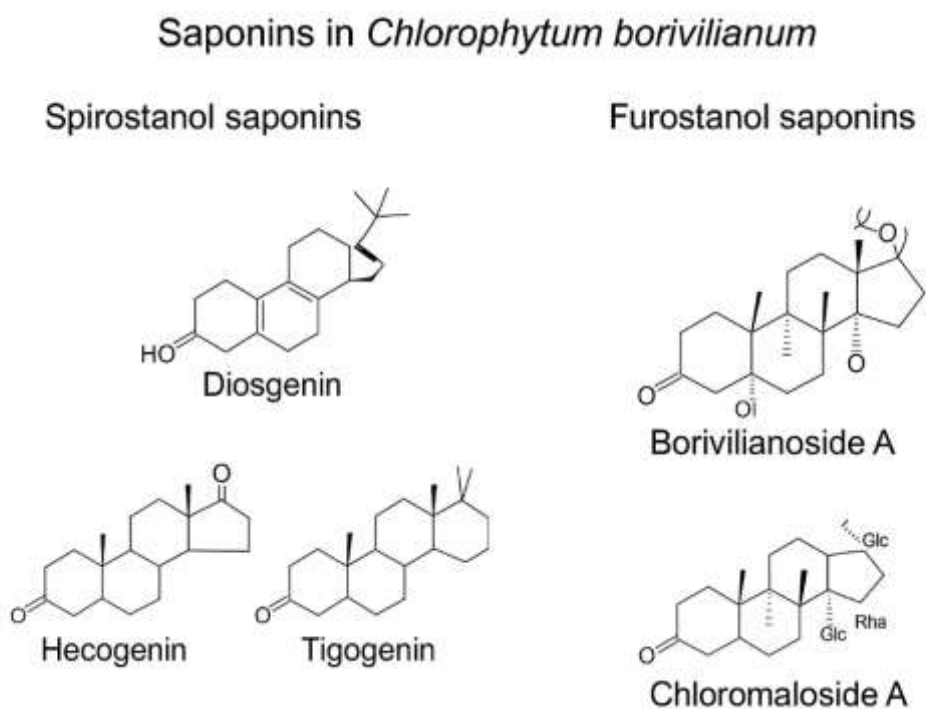


Fig. 2.2 Chemical structure of Saponins

2.1. Biosynthetic pathway

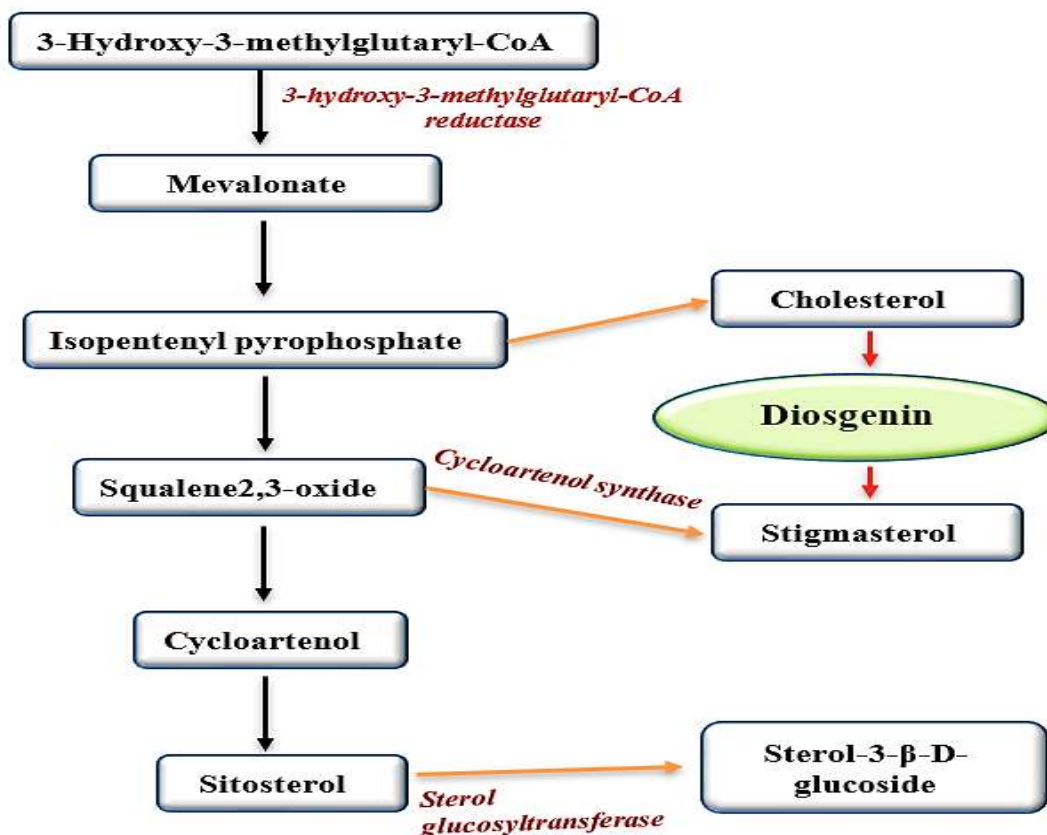


Fig. 2.3 Postulated biosynthetic pathway of saponins.

2.1.6 Ethnobotany and Pharmacology

Traditional and Ethnobotany

Chlorophytum borivillianum, commonly known as Safed Musli, is an important medicinal herb extensively used in traditional Indian systems of medicine including Ayurveda, Unani, and folk medicine. In Ayurveda, the plant is categorized under “Vajikaran Rasayana,” a class of rejuvenating herbs known to improve vitality, reproductive health, strength, and immunity. The tuberous roots have traditionally been used as an aphrodisiac, adaptogenic tonic, immunomodulator, and rejuvenating agent. Indigenous communities from Rajasthan, Madhya Pradesh, Maharashtra, and Gujarat have long utilized Safed Musli for the treatment of male infertility, sexual weakness, diabetes, arthritis, rheumatism, leucorrhoea, postnatal weakness, and general debility (Khanam et al., 2013; Thakur et al., 2009). Traditional preparations of *C. borivillianum* are commonly administered in powdered form mixed with milk, honey, ghee, or herbal formulations. In several tribal communities, the tubers are consumed as health-promoting supplements to enhance physical stamina and body immunity. The plant is also

traditionally used to improve lactation in feeding mothers and as a restorative medicine after childbirth. Due to its medicinal and commercial importance, Safed Musli is popularly referred to as “white gold” in the herbal medicine industry (Vijaya and Chavan, 2009; Khanam et al., 2013). The plant is rich in steroidal saponins, alkaloids, flavonoids, polysaccharides, vitamins, and minerals, which contribute to its antioxidant, antidiabetic, anti-inflammatory, antimicrobial, immunomodulatory, and aphrodisiac properties (Thakur et al., 2009). Modern investigations have also highlighted its nutraceutical potential and increasing demand in pharmaceutical, herbal, and wellness industries (Dhakad et al., 2024).

Pharmacology

Members of the *Chlorophytum* genus have long been used as "Rasayana drugs" in Ayurveda to treat a variety of illnesses and conditions. They are also used as nutritional supplements, immunomodulators, rejuvenators, and aphrodisiacs (Tandon et al., 1992; Govindarajan et al., 2005a; Thakur and Dixit, 2006). Additionally, it was demonstrated that they possessed chemo-modulatory, anti-oxidant, anti-tumor, and anti-diabetic properties (Li et al., 1990; Mimaki et al., 1996; Deore and Khadabadi, 2010; Kumar et al., 2010). To assess the aphrodisiac potential of *C. borivillianum* aqueous extracts, several in vitro studies were conducted (Thakur et al., 2007; Thakur et al., 2009a). A few male albino rats of the Wistar strain were given a crude extract of it orally in a similar investigation, and their pendiculatory activity and other characteristics were then monitored. These rats subsequently showed a notable improvement in sperm count and pendiculatory activity. Additionally, it was observed that the albino rats' diabetes-induced sexual dysfunction may be lessened by the aqueous extract of *C. borivillianum* (Thakur et al., 2009b; Giribabu et al., 2014).

2.2. Extraction and analysis of saponins

The primary step in the investigation of medicinal plants for separation, purification, isolation, and recovery is the extraction of bioactive chemicals. The plant material can be extracted in a variety of ways. The main elements that determine the effectiveness of the extraction process and the characteristics of the end product are the extraction conditions, which include PH, temperature, time, solvent to solid ratio, extraction solvent, composition, and particle size. Extraction solvents, sample pre-treatments, and extraction techniques were some important elements in the extraction process that are crucial to its use and activity.

Table. 2.2 Extraction of saponins

S/N	Method / Approach	Solvent(s) Used	Key Steps / Process	Yield / Remarks	References
1	Soxhlet Extraction	Methanol, Ethanol	Defatting (hexane/petroleum ether) → Soxhlet (6–10 h) → Concentration via rotary evaporator	High saponin yield; commonly used; reproducible	Gupta et al., 2013; Patel et al., 2012
2	Maceration	70–80% Ethanol (Hydroalcoholic)	Defatting → Soaking for 24–48 h → Filtration → Solvent evaporation	Moderate yield; simple but time-consuming	Sharma et al., 2010; Singh and Chauhan, 2003
3	Aqueous Extraction	Water (hot/cold)	Root powder soaked/boiled in water → Filtration → Concentration	Low saponin yield; mainly polysaccharides	Singh and Chauhan, 2003
4	Liquid-Liquid Partition	n-Butanol fraction	Partitioning crude alcoholic extract with solvents (chloroform, ethyl acetate, n-butanol)	n-Butanol enriches saponins	Goyal et al., 2010; Chauhan et al., 2014
5	Column Chromatography	Silica gel; Methanol-Water mix	Crude extract loaded on silica → Gradient elution → Fraction collection	Isolation of pure steroidal saponins	Chauhan et al., 2014; Maurya and Yadav, 2005
6	HPLC / LC-MS	Methanol-Water (analytical)	Extract analyzed by RP-HPLC, LC-MS for quantification and structural identification	Identifies and quantifies individual saponins	Patel et al., 2012; Gupta et al., 2013
7	Ultrasound-Assisted Extraction (UAE)	Ethanol, Hydroalcoholic	Ultrasonication of root powder in solvent → Filtration → Concentration	Improved penetration, eco-friendly; limited studies	Gupta et al., 2013
8	Microwave-Assisted Extraction (MAE)	Ethanol, Methanol	Microwave heating accelerates solvent extraction → Filtration → Concentration	Faster, less solvent use; risk of degradation	Patel et al., 2012
9	Supercritical Fluid Extraction (SFE)	CO ₂ + co-solvent (ethanol)	High-pressure extraction with supercritical CO ₂ and ethanol co-solvent	Green method, high selectivity; rarely reported	Sangwan et al., 2007

Tables 2.3 Analytical methods for determination of saponins from *Chlorophytum* spp.

Analytical technique	Species/Material Used	Sample preparation	Bioactive compounds/metabolites	Instrument condition	Key Findings / Notes	References
Column Chromatography	<i>Chlorophytum borivilianum</i> (roots)	Crude methanolic extract partitioned with n-butanol. N-butanol layer used for column chromatography	Furostane-type steroidal saponins (e.g., borivilianins G, H, I, J)	Stationary phase: ODS (Octadecylsilyl silica gel)	Isolated and characterized four new furostane-type steroidal saponins	Acharya et al., 2008
HPTLC	<i>C. borivilianum</i> (In vitro vs. in vivo roots)	Extraction solvent: 80% methanol	Saponins, Sarsasapogenin, Tigogenin, Ruscogenin	Saponins: Chloroform: Methanol: Acetic acid: Water (60: 30: 10: 8); Sarsasapogenin: Benzene : Isopropanol (70 : 30); Tigogenin, Ruscogenin: Benzene : Methanol (70 : 30) Amino acids: Butanol: Acetic acid: Water (4: 1: 2)	Developed root cultures, HPTLC for saponins, amino acids, sugars	Basu and Jha, 2013
HPLC	<i>C. borivilianum</i> (wild accessions)	Dried powdered sample Extraction solvent: 70% methanol Purification: dried fraction of saponin	Saponins	Column: ODS, 4.5mm × 250mm Mobile phase: Acetonitrile-water (40:60 v/v). Flow rate: 1.0 ml/min. wavelength: 203 nm.	Saponin content range (12.66 – 2.06) %	Dwivedi et al., 2013
HPLC	<i>C. borivilianum</i> (roots)	Dried roots peeled, dried at 60°C then sun-dried. Extraction solvent: 85% ethanol.	Triterpenoid oleanone type saponins	Column: 250 x 4.8 mm SS column; amino packing 5-micron particle size. Mobile phase: Water and acetonitrile (60:40 v/v).	Resolved chromatograms obtained Saponin content (2.06%)	Pandey et al., 2015

		Purification: Column chromatography, TLC		Flow rate: 1 ml/min. Injection size: 20 µL. Detector: UV. Wavelength: 203 nm	to 12.66%)	
HPTLC	<i>C. borivillianum</i> (tubers)	Dried and powdered tubers defatted petroleum ether Extraction solvents: Methanol Extraction procedure: maceration, Soxhlet, sonication, or Microwave-Assisted Solvent Extraction	Saponins	Stationary phase: Precoated silica gel 60F254 plates. Mobile phase: Chloroform: Glacial acetic acid: Methanol: Water (16:8:3:2 v/v). Detection: Anisaldehyde-sulphuric acid reagent and heating	Microwave-Assisted Solvent Extraction showed significantly higher yield (5.11%)	Patil et al., 2015
GC/MS	<i>C. borivillianum</i> (<i>in vitro</i> callus and root cultures, mother plant roots)	Powdered dried samples Extraction solvent: 80% methanol	Saponins	Stationary Phase: Silica gel 60 F254 plates. Solvent system: Chloroform-methanol-water (60:30:10), chloroform-Gl. acetic acid-methanolwater (16:8:3:2) and n-hexane-ethyl acetate (9:1)	Showing saponin presence in both <i>in vitro</i> cultures and mother plant	Jha and Bansal, 2018
TLC	<i>C. borivillianum</i> (leaves)	Extraction solvent: methanol: water (1:1)	Saponins	Mobile phase: butanol: water: acetic acid (84:14:7)	Method is simple, specific, convenient, time saving	Sharma et al., 2012
HPLC	<i>C. borivillianum</i> (root extract)	Dried roots peeled and dried. Powdered. Extracted with 85% ethanol. Purified by column chromatography and TLC.	Saponins, Betulin	Column: ODS C18 (4.5 mm × 250 mm). Mobile Phase: Acetonitrile-water (40:60 v/v). Detector: UV. Wavelength: 203 nm. Injection volume: 20 µL. Flow rate: 1 ml/min	HPLC used to identify and quantify saponins and betulin. The chromatogram	Zarin and Ahmad, 2020

		Sample dissolved in acetonitrile.			showed clear peaks. Saponins found to be 1.25% (dry weight).	
GC-MS	<i>C. borivilianum</i> (root extract)	Dried root powder (25g) extracted with 250ml of deionized water for 3 hours. Solution filtered and incubated at 37°C for 3 days. Filtered again. Methanol used as a solvent for GC-MS analysis	5-methyl hex-2-yl pentadecyl ester, 9,10-Anthracenedione, Phthalic acid, Betulin, etc. (Note: Direct saponins typically require derivatization for GC-MS)	Instrument: Shimadzu GCMSQP2010 Ultra system. Column: Rtx-5MS (30 m x 0.25mm x 0.25 µm). Carrier gas: Helium (1.00 ml/min). Injector temperature: 280°C. Oven temperature program: initial 60°C (2 min), ramp 10°C/min to 260°C (10 min hold). MS ionization: 70 eV. Mass scan range: 40-550. NIST database used	Complements HPLC for broader phytochemical screening.	Zarin and Ahmad, 2020
GC-MS	<i>C. borivilianum</i> (methanolic extract of tubers)	Methanolic extract of tubers	Phenol, Tannins, Flavonoids, Alkaloids, Saponins, Glycosides (qualitatively identified). Specific	GC system coupled with MS. Chromatogram interpreted by MS comparing with NIST database	Identified numerous compounds. Confirmed presence of saponins and other bioactive compounds. Reported	Kushwaha et al., 2021

			cally, over 200 compounds identified; 21 major compounds (e.g., 4-mercaptophenol, Betulin, Octadecanal)		antimicrobial, analgesic, anti-inflammatory, anti-neoplastic activities based on identified compounds.	
GC-MS	<i>C. comosum</i> (leaves)	Methanolic extract of leaves. Water fraction was analyzed.	19 compounds identified (e.g., fatty acids, isoprenoids, steroid compounds)	MALDI-TOF mass spectrometry used for water fraction	Identified several known and one previously undetected compound (4'-methylphenyl-1C-sulfonyl-β-d-galactoside). Provided a phytochemical profile	Dvornyk et al., 2024
LC-Q-TOF-MS	<i>C. borivillianum</i> , <i>C. glaucoides</i> , <i>C. glaucum</i> , <i>C. Bharuchae</i> (leaves, tubers)	Metabolite profiling of leaves and tubers using specific extraction protocols for saponins (e.g., solvent extraction, filtration).	Identification of 13 saponins across the different Chlorophytum species (e.g.,	LC-MS System: High-resolution LC system coupled with Q-TOF-MS (e.g., Agilent Q-TOF-MS). ESI mode.	Provided comprehensive saponin profiles. Powerful for identifying and characterizing diverse	Khedkar et al., 2024

			Tokorogenin, diosgenin, sarsapogenin).		saponin structures, offering detailed molecular information. <i>C. glaucum</i> found abundant in saponins.	
MALDI TOF	<i>C. comosum</i> (leaves)	leaves freeze-dried, then ground to powder Extraction solvent and condition: methanol for 14 days Dry extract fractionated successively with n-hexane, chloroform, and n-butanol, then dried. Pellets dissolved in DMSO.	9,12-octadecadienoic acid (Z, Z)- (41.27 %), neophytadiene (9.57%), and n-hexadecanoic acid (7.66%)	MALDI-TOF mass spectrometry used for water fraction Spectra recorded positive linear mode	Methanolic extract of leaves can be a valuable source of bioactive constituents	Rzhepakovsky et al., 2022
TLC	<i>C. borivilianum</i> (leaves)	Extraction solvents: methanol: water (1:1)	Saponin-enriched fraction	Mobile phase: n-butanol: water: acetic acid (84:14:7) TLC plates processed by ethanol: sulfuric acid solution spraying		Singh et al., 2022

2.3. *In vitro* culture

The majority of their planting materials were vegetative propagules of *C. borivilianum* (Bordia et al., 1995; Kaur et al., 2009). The poor viability and vigor of their seeds is a major factor in the utilization of vegetative propagules as a primary source of planting stock. According to reports, only 8-13% of *C. borivilianum* seeds are viable, and they are also unsuitable for

producing high-quality seedlings with the intended growth potential (Bordia et al., 1995; Kaur et al., 2009). Some plant growth-promoting substances, such as testosterone and cholesterol, were tested to speed up and enhance the rate of *C. borivillianum* seed germination in an attempt to overcome these obstacles, but they only partially succeeded. The *in vitro* culture of *C. borivillianum* attempted due to their aphrodisiac potential. Several researchers have already attempted a variety of *in vitro* culture techniques, including meristem culture, somatic embryo creation, organogenesis, and tuber induction, in an effort to micropropagate this significant genus. However, people have also tried to cultivate *C. borivillianum* commercially using bioreactors due to its extremely high market (Rizvi and Kukreja, 2014).

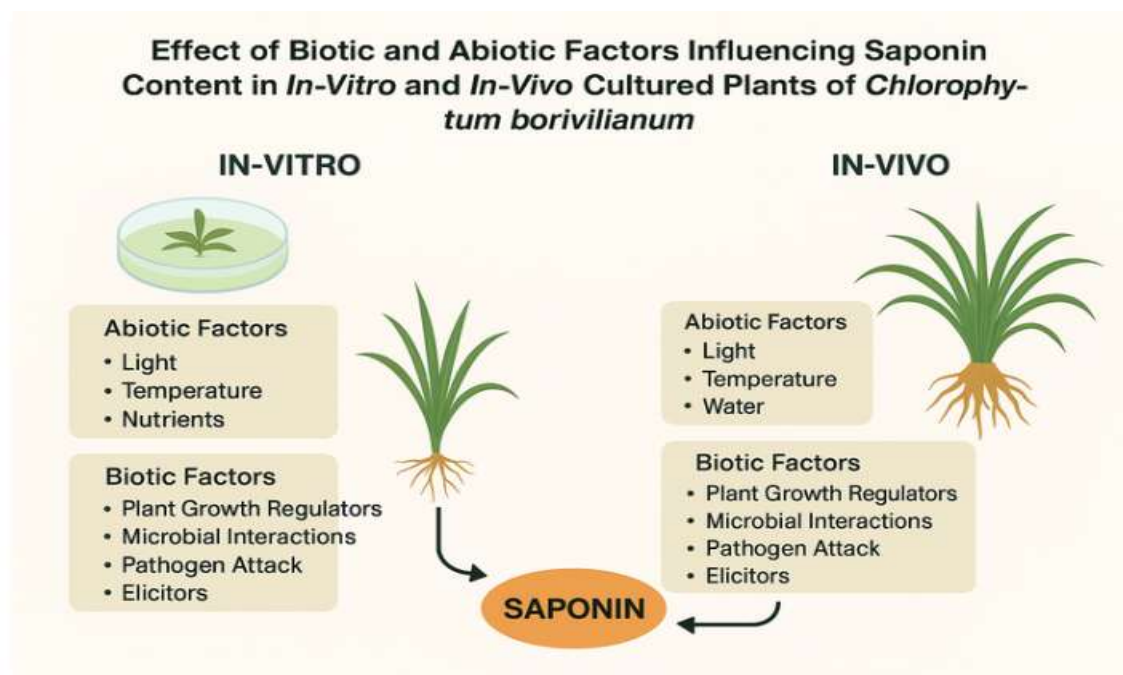


Fig. 2.4 Biotic and abiotic factors influencing saponin content in *Chlorophytum borivillianum*.

Table 2.4 *In vitro* culture of *Chlorophytum borivillianum* for mass multiplication

Species Name	Explant	MS Medium	Result	Reference
<i>C. borivillianum</i>	Hypocotyl, Shoot cultures	MS + 2.25 μ M 2,4-D + 1.15 μ M kinetin (for somatic embryogenesis); Optimal N ratio (1:4 ammonium: nitrate) at low total N (250/500 mgNL ⁻¹); 2-iP > TDZ > kinetin > BAP for embryogenesis; MS-9 + 100 mgL ⁻¹ proline + 1.12 μ M 2,4-D + 7.42 μ M 2-iP (max 63 embryos/inoculum); MS-9 + 17.6 μ M BAP (for 48% embryo germination). For stable cultures: B5 medium + 22 μ M TDZ + 0.18 mM adenine (from stem-disc callus)	Improved method for large-scale rapid multiplication through somatic embryogenesis; 90% survivability on transfer to soil	Arora et al., 1999
<i>C. borivillianum</i>	Stem-disc explants	B5 medium + 0.18 mM adenine + 22 μ M BAP (for high frequency shoot regeneration); Increased (NH ₄) ₂ SO ₄ and KNO ₃ for long-term cultures; Optimal for long-term maintenance: B5 + 2880 mgL ⁻¹ KNO ₃ + 471.4 mgL ⁻¹ (NH ₄) ₂ SO ₄ + 0.18 mM adenine + 22 μ M 2-iP; B5 liquid medium + 0.49 μ M IBA (for rooting); Medium for tuberous roots: IBA (0.49 μ M) + glucose (10 gL ⁻¹) + sucrose (80 gL ⁻¹)	Large number of shoots (15/explant initially, 58/inoculum in 3rd passage); Organogenetic potential declined after 10 subcultures; Optimal medium produced 58 shoots/inoculum; Optimal rooting; Tuberous roots formed (3/plant); 100% survival on soil transfer.]	Suri et al., 1999
<i>C. borivillianum</i>	Encapsulated shoot buds (4 mm long)	3.0% sodium alginate matrix + 100 mM CaCl ₂ ·2H ₂ O; Standard multiplication (SM) medium: MS salts + 5.0 mg L ⁻¹ BAP (for regrowth). Alginate matrix supplemented with sucrose and MS salts (ALMSS)	80% sprouting within 3 weeks (wet agar-gel, culture room); >90% sprouting after 7 days storage (dark, 4°C); 2.5-fold multiplication rate per 21-day subculture; >70% rooting on $\frac{3}{4}$ MS	Dave et al., 2004

			salts + 2.0 mg L ⁻¹ IBA	
<i>C. borivilianum</i>	Immature floral buds along with inflorescence axis	MS + 2 mg L ⁻¹ kinetin + 0.1 mg L ⁻¹ 2,4-D (for multiple shoot induction); MS + 2 mg L ⁻¹ benzylaminopurine (for maximum shoot production); Knops medium (with MS iron/vitamins) + 2 mg L ⁻¹ IBA + 0.1% activated charcoal (for rooting)	Maximum 35 shoots per explant; 86.7% rooting with maximum 5 fasciculated roots; 80% plant survival after hardening.	Sharma and Mohan, 2006
<i>C. borivilianum</i>	Shoot bases; Immature floral buds along with inflorescence axis	MS + 2 mg L ⁻¹ BAP + 0.1 mg L ⁻¹ IBA (from shoot bases); MS + 2 mg L ⁻¹ Kin + 0.1 mg L ⁻¹ 2,4-D (from inflorescence axis); Best multiplication rates from both explants on MS + 2 mg L ⁻¹ BAP. For vitrification reduction: aerated caps, reducing BAP to zero, 0.5-2 mg L ⁻¹ Kin. Rooting: Knop salts (with MS iron/vitamins) + 2 mg L ⁻¹ IBA + 0.1% activated charcoal	Vitrification appeared at multiplication stage, reduced by aerated caps (80%) and BAP reduction/Kin use; 80% plantlet survival on soil transfer	Sharma and Mohan, 2006
<i>C. borivilianum</i>	(Initial explant not specified, but focuses on liquid culture for cost-effectiveness)	Liquid MS medium	7.5-fold increase in shoot multiplication in liquid medium vs. 4.5-fold on agar solidified medium; Up to 92.31% reduction in single shoot production cost compared to solid medium	Rizvi et al., 2007
<i>C. borivilianum</i>	Short segments of inflorescence axis bearing flower bud; Young shoot buds	MS + 2mg/L (9.1µM) 2,4-D + 0.2mg/L (0.9µM) BA (for callus induction); MS + 4mg/L (17.6µM) BA (for multiple shoot proliferation from callus); MS + 2mg/L (9.9µM) IBA (for rooting)	Maximum callusing (100%) from young shoot buds and inflorescence axis; Maximum shoot multiplication on MS with 5mg/l BA (after 2nd subculture); 87% rooting; 60%	Debnath et al., 2007

			survival after acclimatization.	
<i>C. borivilianum</i>	Seedling-derived embryonic callus	MS medium with varying culture age mentioned	High morphological and cytological variation observed, increasing with culture age; Chromosomal variation $2n=3x-3$ to $2n=7x+2$; Reduced variation (6.15%) by re-initiating cultures from genetically stable plants	Suri and Saini, 2007
<i>C. borivilianum</i>	explant used with liquid medium,	MS medium supplemented with cytokinins (BAP 0.5-5mg/L, Kinetin 0.1-0.5mg/L), alone or combined with auxins (NAA 0.1-1 mg/L), 3% sucrose and 4.5 gm/L agar (for initial solidification, though liquid medium was also key)	Better shoot growth and multiplication response in liquid medium compared to solid medium.	Hajaam et al., 2007
<i>C. borivilianum</i>	Micro-cloned plantlets/ Un-rooted shoots (post-micropropagation, focus on hardening)	Bio-inoculants: <i>Glomus aggregatum</i> , <i>Trichoderma harzianum</i> , <i>Piriformospora indica</i> , <i>Azospirillum</i> sp., <i>Actinomyces</i> sp.	>95% establishment in soil with <i>Glomus aggregatum</i> , <i>Trichoderma harzianum</i> , <i>Piriformospora indica</i> ; Up to 85% with <i>Azospirillum</i> sp. and <i>Actinomyces</i> sp.; 50% in vivo rooting of un-rooted shoots with VAM and <i>Trichoderma harzianum</i>	Mathur et al. 2008
<i>C. borivilianum</i>	Shoot cultures (pre-established)	BA supplemented MS medium (optimal at 40.0 g M ⁻³ CO ₂ in chamber and 3.0% sucrose in medium)	Growth comparable to 3.0% sucrose medium at 40.0 g M ⁻³ CO ₂ ; Number of shoots nearly doubled compared	Joshi et al., 2009

			to natural CO ₂ and sucrose-supplemented medium	
<i>C. borivilianum</i>	Immature inflorescence explants	Half-strength MS + 3.0 mg L ⁻¹ BA + 150 mg L ⁻¹ Ads + 0.1 mg L ⁻¹ NAA + 3% (w/v) sucrose (for shoot regeneration); Half-strength basal MS + 0.1 mg L ⁻¹ IAA + 2% (w/v) sucrose (for rooting)	Direct shoot regeneration achieved; High frequency of shoot bud regeneration; 90% plant survival after hardening	Samantaray et al., 2009
<i>C. borivilianum</i>	Crown explants	Solidified modified MS media with various hormonal combinations; Optimal for establishment: 2 mg/l Kinetin and 1 mg/l IBA; Optimal for multiplication: 3 mg/l Kinetin and 2 mg/l BAP; Rooting: Half strength modified MS basal medium + 30gm/l sucrose + 1mg/l IBA	Average 73 shoots generated; Rooting initiated effectively; More than 95% success in hardening	Haque et al., 2009
<i>C. borivilianum</i>	Young shoot buds	MS + 3.0 mg/l BAP (for shoot induction); MS + 1.0 mg/l IBA + 30 g/l sucrose (for rooting)	Highest mean number of shoots per explant (18.90) and mean length of shoots (6.0 cm); Regenerated shoots rooted successfully	Kemat et al., 2010
<i>C. borivilianum</i>	<i>In vitro</i> response of these accessions studied	Overall rates of shoot multiplication and rooting are reported as varying between accessions	<i>In vitro</i> response varied significantly among accessions; Best shoot multiplication rate in PBL-4 and PBL-3; Maximum rooting in PBL-4. Useful for selecting accessions for propagation and conservation	
<i>C. borivilianum</i>	Shoot explants, for multiplication and rooting	MS medium; BA better than KIN for shoot multiplication; 116-290 mM sucrose and heat shock (50°C, 1h) promoted shoot multiplication;	Maximum plant survival (>95%) in vermicompost directly transferred to net-house. Genetic similarity	Kumar A. et al. 2010

		Sucrose also promoted rooting with IBA; Phytigel better gelling agent than agar	to mother plant confirmed by RAPD and ISSR	
<i>C. borivilianum</i>	Shoot buds/microtubers	Solid and stationary liquid MS media + 60 g l ⁻¹ sucrose + 950, 1265, or 1580 µM 2-chloroethyl-trimethylammonium chloride (CCC). Liquid culture with shaking (80 r.p.m.) also tested	High in vitro tuberization rate; 950 µM CCC with 60 g l ⁻¹ sucrose was best for tuberization. Liquid shake culture resulted in >2.5-fold increase in tuber production vs. solid	Farshad Ashraf et al. 2013
<i>C. borivilianum</i>	Material for microtuberization in RITA system	Microtuberization medium (MM); Liquid hormone-free MS (MSO) medium for substitution	Significantly higher mean number (16) and length (52 mm) of microtubers obtained with 15 min immersion/60 min rest	Ashraf et al., 2013
<i>C. borivilianum</i>	Tissue culture plants	CO ₂ enriched environment, liquid culture system, culture vessel ventilation; Media can be sucrose-devoid	Cultures grew better in CO ₂ -enriched environment and on sucrose-devoid media; Liquid cultures with borosilicate glass beads promoted growth; Proper ventilation beneficial; Enhanced growth and survival with <i>Piriformospora indica</i> inoculation during hardening	Purohita and Habibi, 2014
<i>C. borivilianum</i>	Basal/crown plate explants	MS + 3.0 mg/L BAP + 2.0 mg/L KIN (for shoot multiplication); Half-strength MS basal medium (without PGRs) + 3% sucrose (for rooting)	Highest number of shoots (21.67), maximum shoot length (13.14 cm), 85% shoot induction response. 85% survival rate in greenhouse. No morphological variation	Minakshi et al., 2014

<i>C. borivilianum</i>	Young shoot bases	MS medium + 22.2 μ M benzyladenine (for shoot multiplication); MS medium with 3/4-strength inorganic and organic constituents + 9.8 μ M indolebutyric acid (for rooting)	Shoots multiplied at 4-fold every 3 weeks; All shoots rooted; 67% establishment in pots; Produced normal fasciculated storage roots	Ashraf et al., 2014
<i>C. borivilianum</i>	Young shoot buds	MS + 5 mg/L 2,4-dichlorophenoxyacetic acid (for callus induction); MS + 6-benzylaminopurine or kinetin (for shoot regeneration from callus); MS + 1.0 mg/L indole-3-butyric acid (for rooting)	Callus induction better with 2,4-D; Callus differentiated into shoots with BAP or kinetin (TDZ failed); 88.3% survivability of rooted plantlets after acclimatization	Nakasha et al., 2016
<i>C. borivilianum</i>	Shoot explants	Murashige and Skoog medium + 2 mg L ⁻¹ 6-benzyladenine + 0.2 mg L ⁻¹ naphthalene acetic acid (for multiplication); Medium with 120 g L ⁻¹ sucrose (for slow-growth storage); Rooting: 2 mg L ⁻¹ indole-3-butyric acid.	Continuous shoot proliferation up to 7th subculture; 100% survival after 4 months of slow-growth storage; 100% regeneration from stored cultures; 95% survival after acclimatization.	Chauhan et al., 2016
<i>C. borivilianum</i>	Nodal segments	MS + 2.0 mg L ⁻¹ BAP + 1.0 mg L ⁻¹ NAA + 20.0 mg L ⁻¹ AdSO ₄ (for shoot multiplication); Semi-solid medium + 2.0 mg L ⁻¹ IBA + 50.0 mg L ⁻¹ Putrescine (for rooting)	Promoted multiple shoot induction and proliferation; 90% maximum field survival rate for rooted plants	Khatri et al., 2019
<i>C. borivilianum</i>	Basal plate	MS + 2.0 mg/L 6-benzyl aminopurine (BAP) (for shoot induction)	Maximum 80% regeneration with 2.9 average shoots per explant after 28 days	Dhanda et al., 2022
<i>C. borivilianum</i>	Shoot proliferation cultures	Alternative MS medium (AMS) prepared using ammonium hydroxide and nitric acid instead of commercial ammonium nitrate	<i>In-vitro</i> shoot multiplication rate on AMS was statistically not different from standard MS medium	Singh et al., 2021

<i>C. borivilianum</i>	Apical bud	MS + 2.0 mg L ⁻¹ meta-topolin (mT) + 1.0 mg L ⁻¹ BAP (for shoot proliferation); MS + 1.0 mg L ⁻¹ IBA (for rooting)	96.3% success rate with an average of 19.2 ± 1.92 shoots (6.72 ± 0.49 cm length); 95.5% rooting with 14.0 ± 1.39 roots (3.36 ± 0.27 cm length)	Karwasra et al., 2024
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2.4 Contemporary or advanced methods

Contemporary methods refer to advanced experimental approaches that utilize elicitors (both biotic and abiotic), synthetic seeds, genetic manipulation, hairy root cultures, bioreactors, metabolic engineering, and the application of endophytes, among others. 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 saponins were mainly centered on the implementation of elicitors. Transformed hairy root culture accumulated more saponins 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 saponins content was found in A4 transformed rhizo-clone HRA2B5 that was 2.26mg/g DW.

2.5. 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. Several polymorphic molecular markers can be considered to analyze the diversity: RFLP, RAPD, AFLP and STSs.

Table 2.5. Summary of genetic fidelity

Species name	Marker	Result	References
<i>C. borivilianum</i>	RAPD	RAPD primers displayed the <i>same banding profile</i> within all micropropagated plants and the <i>in vivo</i> explant donor.	Lattoo et al., 2006
<i>C. borivilianum</i>	AFLP	Assessed genetic similarity among 34 accessions from Central India; 246 polymorphic fragments	Tripathi et al., 2012

		out of 612 amplified; indicated a small amount of genetic diversity; low PIC values (0.125-0.379).	
<i>C. borivilianum</i>	RAPD	RAPD confirmed genetic similarity to mother plants.	Samantaray and Maiti, 2010
<i>C. borivilianum</i>	RAPD, ISSR	Assessed genetic diversity among 21 accessions from different geographical regions of India. RAPD showed 92.26% polymorphism; ISSR showed 82.76% polymorphism. RAPD was found to be more effective. Combined RAPD + ISSR analysis showed genetic distance from 0.788 to 1.000 (mean 0.89) for tissue culture-derived plants. High genetic diversity was recorded among natural populations.	Samantaray and Ngangkham, 2017
<i>C. borivilianum</i> , <i>C. arundinaceum</i> , <i>C. laxum</i> , <i>C. capense</i> , <i>C. comosum</i>	Plastid rbcL region sequences	Species-specific markers identified; similarity coefficient ranged from 0.6 to 0.85. Effective for determining genetic relationship at species level and authentication	Katoch et al., 2010
<i>C. borivilianum</i> , <i>C. tuberosum</i> , <i>C. attenuatum</i>	AFLP	polymorphic bands (99.91%), Shannon's information index (0.3592) and Nei's gene diversity (0.2085)	Patil et al., 2015
<i>C. comosum</i>	Complete Chloroplast Genome Sequence	<i>C. comosum</i> is closely related to <i>C. rhizopendulum</i> but distinguishable from other plants.	Peng et al., 2020
<i>C. comosum</i> , <i>C. gallabatense</i> , <i>C. rhizopendulum</i>	Chloroplast (cp) genome	Phylogenetic analysis clustered these species together in the same clade with a closer relationship to <i>Anthericum ramosum</i> .	Munyao et al., 2020
<i>C. borivilianum</i>	RAPD	Assessed genetic diversity in 9 genotypes. 26 polymorphic bands out of 43 obtained (67.49% polymorphism)	Dwivedi and Sharma, 2011
<i>C. laxum</i>	Complete Chloroplast Genome Sequence	Phylogenetic analysis placed <i>C. laxum</i> as sister to <i>C. comosum</i> , supported the monophyly of the genus <i>Chlorophytum</i> within Asparagaceae	Li et al., 2024

CHAPTER 3

Collection of *Chlorophytum borivilianum* from different geographical areas of India (Punjab, Haryana, Maharashtra) and screening of elite population on the basis of saponin content by HPTLC method

3.1 Introduction

Chlorophytum borivilianum (Safed Musli) is a medicinally important plant valued in Ayurveda, Unani, and modern phytomedicine. Its tuberous roots are rich in steroidal saponins, which exhibit aphrodisiac, immunomodulatory, anti-inflammatory, and adaptogenic activities (Kothari et al., 2013; Shukla et al., 2010). Due to rising demand in pharmaceutical, nutraceutical, and herbal industries, *C. borivilianum* has become a threatened species in India, largely because of overexploitation, habitat loss, and limited natural populations (Dixit et al., 2005; Thakur et al., 2022). Identifying elite germplasm with higher saponin content is critical for conservation, genetic improvement, and commercial cultivation. The plant is native to India, occurring in tropical and sub-tropical regions, but its populations exhibit variability in morphology and phytochemistry (Kumar et al., 2011). Steroidal saponins are the principal compounds responsible for pharmacological activities such as aphrodisiac, immunostimulant, and anti-stress effects (Gupta et al., 2018). Considerable variation in saponin content among natural populations has been reported, but systematic evaluation across Jammu and Kashmir, Himachal Pradesh, Punjab, and Haryana is lacking (Patel et al., 2016). High-performance thin layer chromatography (HPTLC) is a reliable, rapid, and cost-effective technique for qualitative and quantitative analysis of saponins (Wagner and Bladt, 2009; Sharma et al., 2020). The present study collection of *C. borivilianum* germplasm from diverse geographical regions of North India (Punjab, Haryana) to evaluate inter-population variability in saponin content. The study leads to identify elite populations with high saponin content for conservation, breeding, and large-scale cultivation programs.

3.2 Material and method

3.2.1 Chemicals and marker compound (diosgenin and stigmasterol)

Marker compound diosgenin and stigmasterol 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, Punjab, Haryana, and Maharashtra. The plant samples were identified based on morphological characteristics and phytochemical analysis based on presence of marker compound saponins. The plants were collected during the autumn season (October-November) of 2018. The collection sites were selected based on cultivation practices, availability of natural populations, and reported medicinal importance of the species. Disease-free and morphologically uniform plants were carefully uprooted along with intact tubers to avoid mechanical injury. The collected germplasm was identified by the Dr. Devendra Kumar Pandey, Professor, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara properly labeled according to accession number, location, and date of collection for further identification and characterization. The plants were properly marked and kept in the green house. The temperature inside the greenhouse was maintained at $25 \pm 2^{\circ}\text{C}$ with relative humidity ranging between 60-70%. Plants were exposed to natural photoperiod conditions with adequate sunlight supplemented by diffused light to avoid excessive heat stress.



Fig. 3.1 *Chlorophytum borivillianum* plants collected from different locations of India (Punjab, Haryana, Maharashtra).

3.3. Phytochemical analysis

3.3.1 Sample preparation

Plant samples of *C. borivillianum* first collected and dried under shade. After that dried samples finely powdered using electric mixer grinder. For the extraction of samples from each soil type plants, 1 g of powdered samples were used to maceration with 30 mL ethanol (50%) for 24 h followed by sonication for 10 min. After that each extracted samples in solvent strained through Whatman filter paper No.1 followed by dehydrated in rotary evaporator (Buchi, R-300, India) at 40 °C. The dried residues of each sample were then mixed with hydrochloric acid (20 mL of 10%) (HPLC grade, E. Merck, India). These samples were subjected to acid hydrolysis using water bath for 1hr at 98 °C to isolate the saponins (Nazir et al., 2022). After the acid hydrolysis was completed, Chloroform was added to each sample after acid hydrolysis, for the development of distinct layers. Whereas upper layer was removed. After that lower layer was rewashed with chloroform afterward drying with rotary evaporator. Residue from rotary evaporator than dissolved in methanol and utilized for HPTLC analysis.

3.3.2 HPTLC analysis of diosgenin and stigmasterol

The analysis was performed using HPTLC (Linomat5 applicator CAMAG; Switzerland), a 100 ml syringe, and a TLC scanner-3, as well as Win CATS software (Scanner_180710" S/N 180710 (2.01.02)) with 8 tracks, application position 15.0 mm, solvent front 75.0 mm, track distance 7.5 mm, slit dimensions 4.00 × 0.30 mm (Micro Light), and scanning speed 20 mm/s. Mobile phase used here is toluene: ethyl acetate (7:3 v/v) with precoated silica gel 60 F₂₅₄ 8 × 10 cm TLC plates as stationary phase. Dissolved samples of *C. borivillianum* and marker compounds (diosgenin and stigmasterol) of 3µl were administered to the plates. For derivatization, anisaldehyde reagent (1 mL anisaldehyde, 20 mL glacial acetic acid, 170 methanol, and 10 mL conc. sulphuric acid) was sprayed on the plates (Nazir et al., 2021b). Plates were derivatized and then dried in a hot air oven (Bionics Scientific, India) for five minutes at 100 °C. CAMAG TLC scanner 3 and Win Cats software were scanned the plates at 515 nm wavelength. The concentration was calculated from the calibration curves by comparing the retardation factor (R_f) of the extract peak to that of the marker chemicals.

3.3.3 Preparation of calibration curve of diosgenin and stigmasterol

The calibration curve of saponins was prepared by using marker saponins. To prepare a stock solution 10 mg saponins 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.

3.3.4 Method validation for HPTLC

The method was validated as per ICH guidelines for accuracy, precision, linearity, LOD, LOQ, repeatability, specificity, and recovery; precision, detection, and quantification limits were evaluated using replicate analyses and signal-to-noise ratios.

3.4 Result and discussion

3.4.1 Quantification of diosgenin and stigmasterol

HPTLC fingerprinting of the plant extracts revealed the presence of multiple bands corresponding to different phytoconstituents (Figure 2.1). The bands observed were well resolved and comparable with standard diosgenin and stigmasterol, confirming their presence in the samples.

The overlay UV spectral scans of all tracks showed sharp and distinct peaks corresponding to diosgenin and stigmasterol standards. Both compounds exhibited characteristic absorbance maxima at λ_{max} 515 nm, which matched with the test samples, further validating their identification (Figure 5.1). Densitometric scanning at 515 nm clearly resolved diosgenin and stigmasterol at R_f values of 0.67 and 0.72, respectively (Fig. 3.2). The peaks were well separated from other matrix components, demonstrating method specificity. The sharpness and intensity of the peaks indicated high sensitivity and suitability of the developed method for quantitative analysis.

Table 3.1 Collection regions of *Chlorophytum borivillianum* with geographical details and diosgenin and stigmasterol content

Plant samples	Collection site	Geographical coordinates	Diosgenin content (%)	Stigmasterol content (%)
A ₁	Phagwara, Punjab	31.2232°N, 75.7670° E	0.45± 0.037	0.40 ± 0.037
A ₂	Patiala, Punjab	30.3398°N, 76.3869° E	0.51± 0.023	0.45 ± 0.041
A ₃	Kurukshetra, Haryana	29.9695°N, 76.8783°E	0.47± 0.043	0.43 ± 0.033
A ₄	Hisar, Haryana	29.09°N, 75.43°E	0.56± 0.038	0.53 ± 0.058
A ₅	Akola, Maharashtra	20.17° N, 77.4° E	0.71± 0.031	0.61 ± 0.39

Chlorophytum borivillianum was collected from five different regions including Phagwara (Punjab), Patiala (Punjab), Kurukshetra (Haryana), Hisar (Haryana) and Akola (Maharashtra). The highest yield of saponins was obtained from the samples collected from Akola (Table 3.1). The difference in saponins content may vary because of many factors such as genotypes and environmental conditions (Panda and Kamble, 2016).

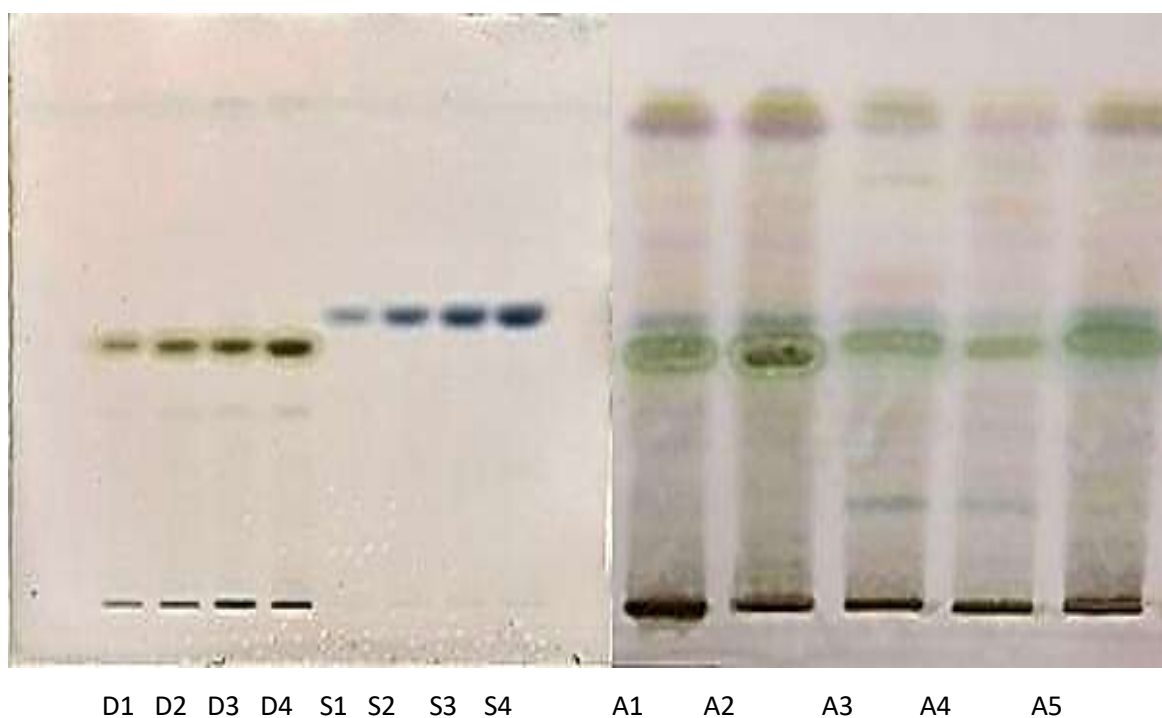


Fig. 3.2 HPTLC chromatogram of standard Diosgenin and Stigmasterol and *Chlorophytum borivillianum* plant samples (A1-A5) collected from different regions.

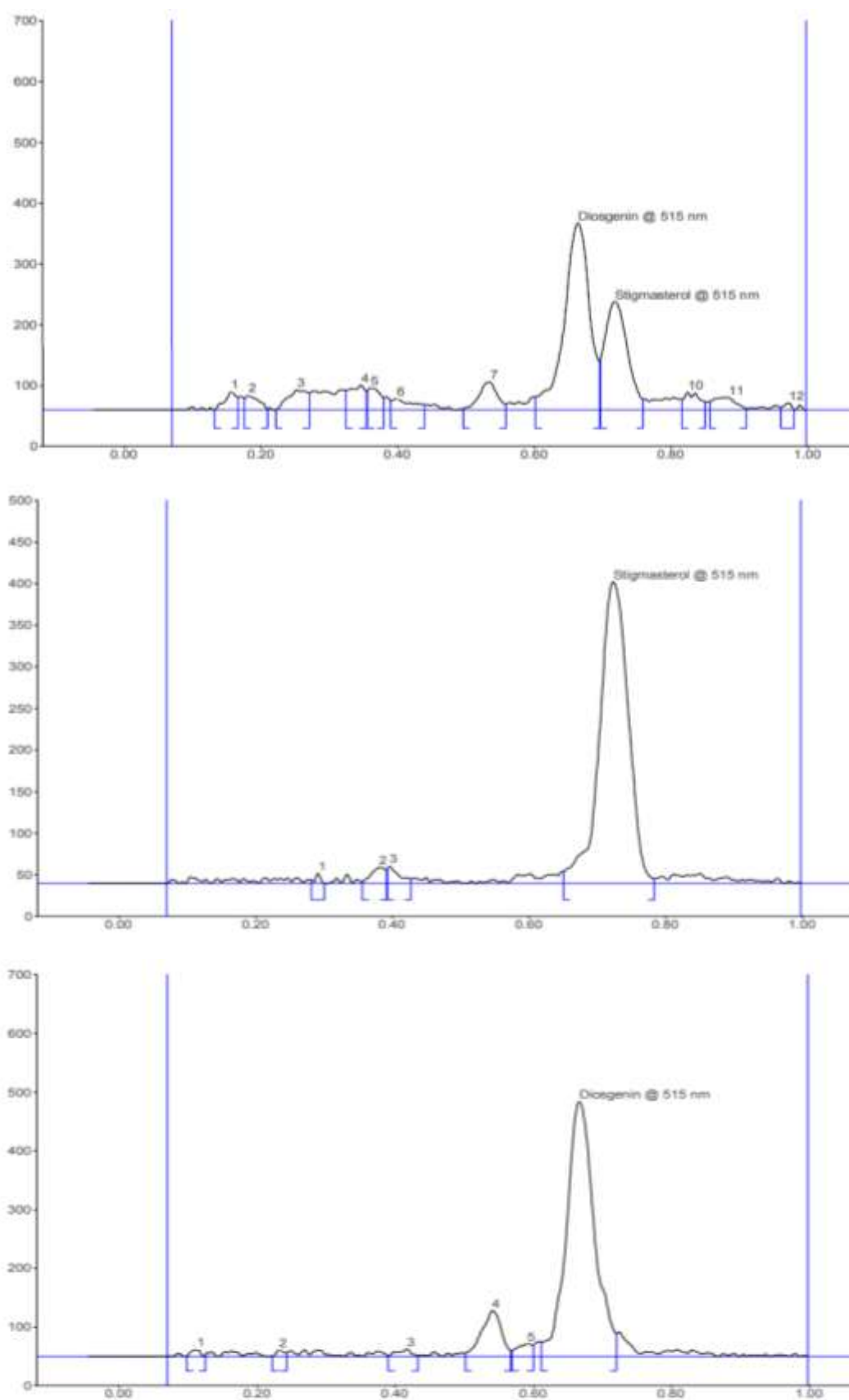


Fig. 3.3 HPTLC chromatogram of standards Diosgenin and Stigmasterol (Std) and *Chlorophytum borivillianum* plant samples (A1-A10) collected from different regions.

3.4.2. Method validation

In the present study, HPTLC analysis was employed for the quantification of diosgenin and stigmasterol in plant samples. Well-resolved spots were observed at R_f values of 0.65 and 0.70 with $\lambda_{\max}$ of 436 nm and 312 nm, respectively. The calibration curve showed good linearity in the range of 300–1200 ng/spot with correlation coefficients (r^2) of 0.992 for diosgenin and 0.997 for stigmasterol, and regression equations $Y = 10.21X + 1250$ and $Y = 9.381X + 97$. The LOD and LOQ were 35 ng and 105 ng for diosgenin, and 30 ng and 90 ng for stigmasterol, respectively. The method exhibited high precision, with intra-day and inter-day RSD values below 2%, and recovery studies showed mean recovery percentages of 99.79% for diosgenin and 99.15% for stigmasterol, confirming accuracy and reliability of the developed method (Table 3.2; fig. 3.1).

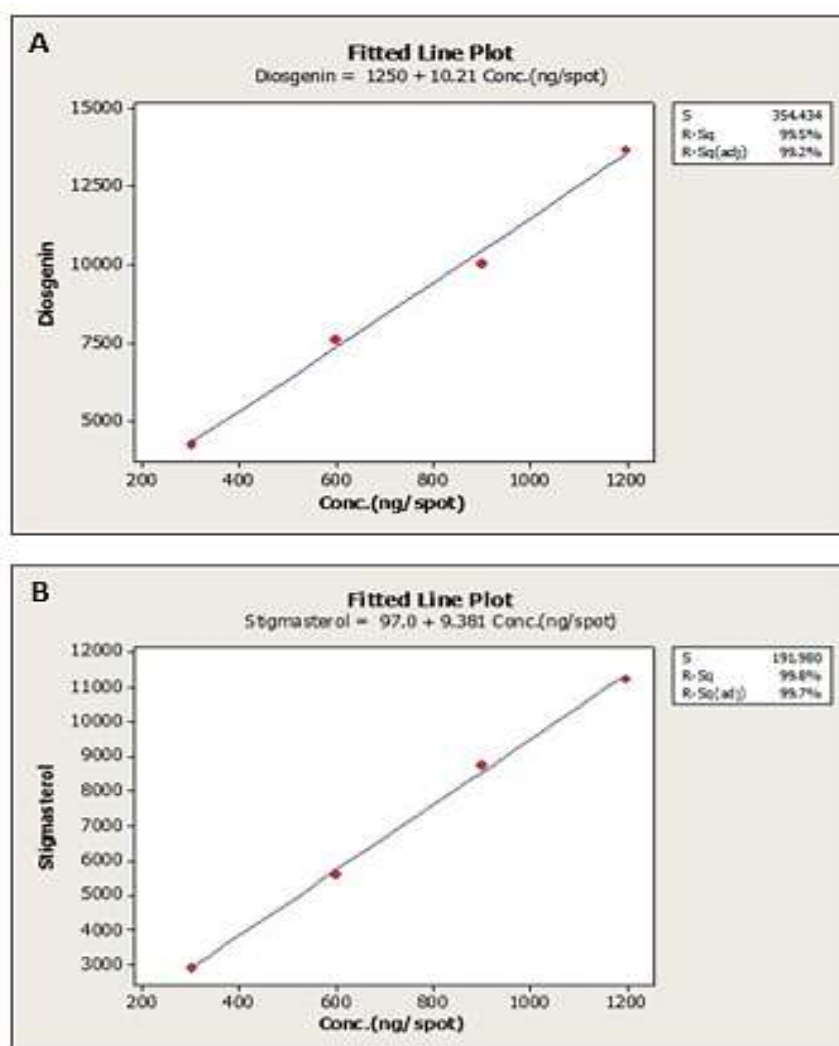


Fig. 3.4 Calibration curve of saponins (A. Diosgenin; B. Stigmasterol)

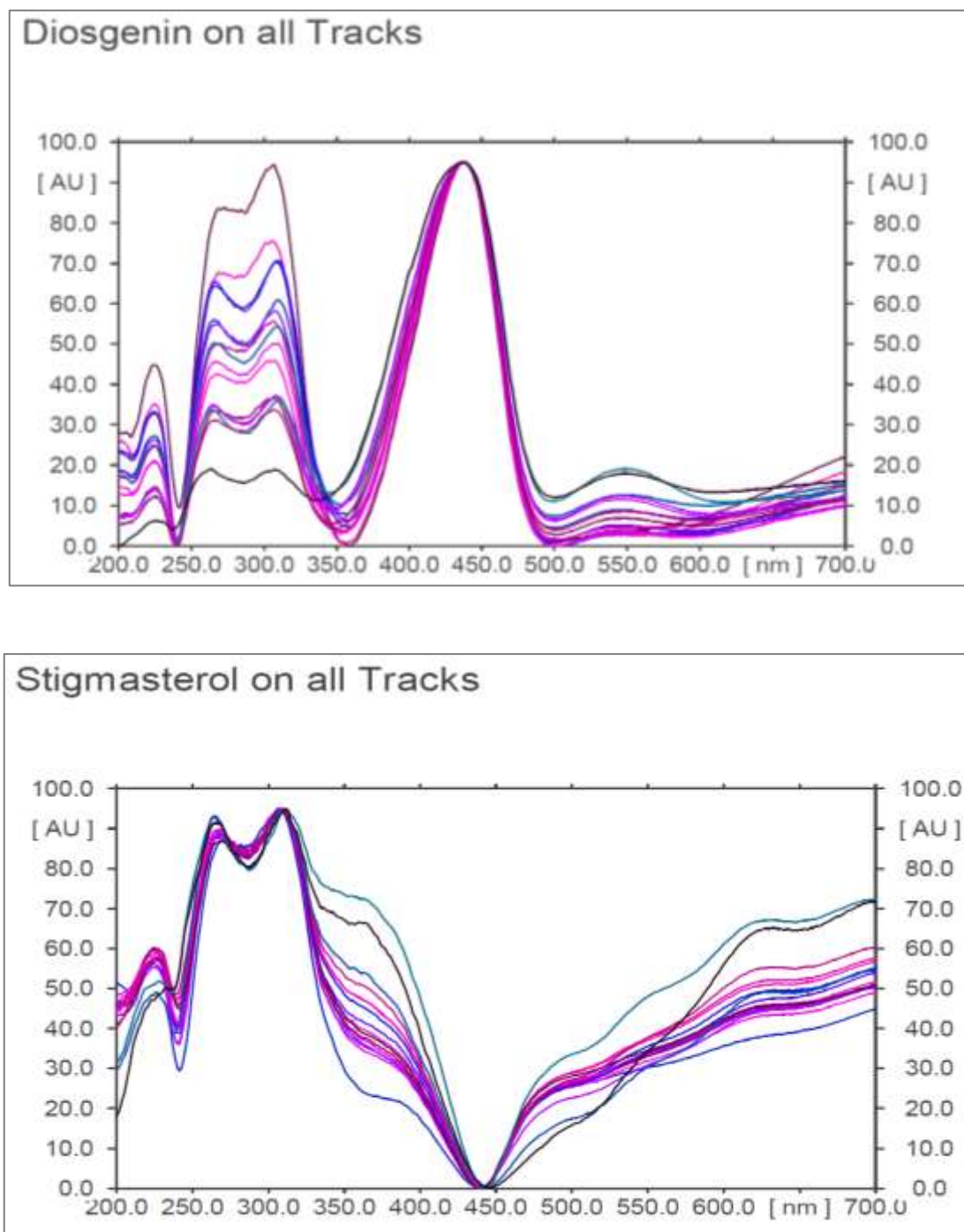


Fig. 3.5 Overlay spectra λ_{max} of diosgenin (436 nm) and stigmasterol (312 nm)

To evaluate the efficiency of the method, a suitability test was conducted using pre-analyzed extract samples combined with freshly prepared reference stock solution. The results are shown in Table 3.2. The recovery percentage (%) was found in the range of 99.47 – 99.74 %

which implies 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 saponins. The LOD (105 ng/spot) and LOQ (90 ng/spot) values implies very good sensitivity of the developed method.

Table 3.2 Method validation for diosgenin and β -sitosterol quantification

S/N	Parameters	Diosgenin	Stigmasterol
1	Linearity range (ng/spot)	300–1200	300–1200
2	Correlation coefficient (r^2)	0.992	0.997
3	Regression equation	$Y = 10.21X + 1250$	$Y = 9.381X + 97$
4	Calculated SD value	1.43	1.35
5	^b Limit of detection (LOD) (ng) [$3 \times \text{SD/S}$]	35	30
6	^b Limit of quantification (LOQ) (ng) [$10 \times \text{SD/S}$]	105	90
7	R_f	0.65	0.70
8.	λ_{max} (nm)	436	312
Precision and accuracy			
8	Intra-day RSD (%), $n = 5$	1.21	1.36
9	Inter-day RSD (%), $n = 5$	1.62	1.71
Recovery			
10	Amount of standard in plant samples ($\mu\text{g mg}^{-1}$) having maximum bioactive compounds	9.6	7.8
11	Amount of standards added in plant samples ($\mu\text{g mg}^{-1}$)	9, 18, 27	7, 14, 21
12	Amount of standard found ($\mu\text{g mg}^{-1}$)	18.62, 27.53, 36.43	14.61, 21.62, 28.68
13	Recovery (%)	100.10, 99.74, 99.53	98.71, 99.17, 99.58
14	Mean Recovery (%)	99.79	99.15

^a Four concentration levels in triplicate. ^b SD is the standard deviation of the blank response, and S is the slope of the calibration plot.

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 *Chlorophytum* species. The collected samples presented a wide range of difference in the saponins yield. The difference in the content could be because of many factors that can influence the yield of saponins. Further study can be done on a broader collection region and identified elite population can be mass propagated to fulfill the rising demand of the raw material for saponins.

3.5. Conclusion

The present study collection of *C. borivillianum* germplasm from diverse geographical regions of North India (Punjab, Haryana, Maharashtra) to evaluate inter-population variability in saponin content. The plant collected from Akola; Maharashtra has the maximum quantity of diosgenin and stigmasterol. The study leads to identify elite populations with high saponin content for conservation, breeding, and large-scale cultivation programs.

CHAPTER 4

Mass propagation of elite population of *Chlorophytum borivillianum* by *in-vitro* methods

4.1 Introduction

Throughout prehistoric times, therapeutic plants have served as a valuable reservoir of natural and man-made drugs to treat illnesses. There are 20,000 species that have been used for healing. China and India have long been pioneers in the use of herbal medicines for therapeutic and prophylactic medicinal procedures. Many eminent and famous scientists have developed herbal medicines, and because they are still widely available, traditional medicine practitioners continue to use them (Devendra et al., 2012). Traditional and modern medicinal products are produced using medicinal plants as natural resources and therapeutic agents. A significant quantity of foreign currency can be generated by commercializing the extraction of pharmaceuticals from herbal plants, in addition to exporting medicinal plants. This then gives indigenous herbal species an economic perspective, encouraging the conservation and development of the plantation regions as well as contributing to the local economy.

Chlorophytum borivillianum is an uncommon aphrodisiac plant valued for its desiccated fasciculate roots and is in risk of extinction. Earlier it was a member of family Liliaceae but currently it is categorized under the Asparagaceae family (Shinde et al., 2016). In India it is recognized as Safed musli, in England and the United States referred as Indian spider plant, spider plant, and white musale in France, and shaqaqule in Saudi Arabia (Devendra et al., 2012). In terms of pharmacological properties, *C. borivillianum* (Root tubers) demonstrated a wide range, including antimicrobial, larvicidal activities, antioxidant hypolipidemic, anthelmintic, analgesic, anticancer, antipyretic, anti-inflammatory, hepatoprotective, antiarthritic, antidiabetic and immunomodulatory (Khanam et al., 2013). The safed musli roots are widely employed in the Ayurveda and Unani medical system for a range of remedial purposes due to the presence of saponins being one of the key phytochemical components (Basu and Jha, 2014). Besides saponins, the roots of *Chlorophytum* plants represent a substantial reservoir of carbohydrates, amino acids, polysaccharides, steroids, alkaloids, vitamins, resins, and phenol (Thakur et al., 2009a). *C. borivillianum* is a highly valuable and well-known medical shrub. In the Indian market, the desiccated fasciculate roots sell for an alluring price of \$30 USD per kg (Dave et al., 2003). Overharvesting of the species from the

natural has resulted from constantly rising requirement in domestic (35000 t) and international (300 to 700 t) marketplaces (Khanam et al., 2013). Safed musli rarely propagates through seeds, but rather vegetatively through its thick roots. The gap between supply and demand has been made wider by the rapid loss of habitat, destructive harvesting methods, extended quiescence (Kaur et al., 2009), and limited seed germination (Rizvi et al., 2007). Therefore, high-quality planting material is needed in large quantities for the marketing of this crop, which calls for the development of an efficient, rapid, and profitable propagation technique. The use of biotechnological techniques can provide the perfect way to get around the current issues. The reports that describe tissue culture-based propagation of *C. borivilianum* are mostly focused on proliferation of shoots derived from shoot base explants or stem disc (Dave et al., 2003; Kumar et al., 2010; Samantaray and Maiti, 2010) or somatic embryogenesis arising from callus tissue derived from shoot meristematic or stem disc (Arora et al., 1999). Several studies have contributed significantly to the understanding and advancement of tissue culture techniques in *Chlorophytum* species, particularly *Chlorophytum borivilianum*, an endangered medicinal herb. Rizvi et al. (2010) demonstrated successful micropropagation of *C. borivilianum*, providing a foundation for further research in this area. Samantaray and Maiti (2010) expanded upon this work by not only propagating the plant *in vitro* but also assessing the genetic fidelity of the regenerated plants, ensuring their authenticity. Joshi and Purohit (2012) optimized factors that *in vitro* propagation of *C. borivilianum*, further refining the tissue culture techniques for this species. Additionally, Khatri et al. (2006) focused on the effect of additives on enhanced micropropagation aspect, providing insights into the optimal conditions for mass multiplication of this valuable medicinal herb. These studies collectively highlight the diversity of approaches employed in tissue culture research of *Chlorophytum* species and underscore their significance for the conservation and sustainable utilization of these valuable plants. However, there are very few reports available on direct organogenesis-based *in vitro* growth of *C. borivilianum*.

It is crucial to emphasize that, even though roots of *C. borivilianum*'s abundant in saponin are the main ingredient in numerous herbal preparations and are commonly employed in their raw form, comprehensive data regarding the quantification of sapogenins, saponins, and other bioactive compounds specific to root remain undisclosed for various populations growing in distinct agroclimatic regions across India. To keep consistency, guarantee quality, and maximize the effectiveness of the production of herbal drugs, *in vitro* clonal multiplication of highly productive varieties or populations holds significant appeal from an economic and pharmacological standpoint. Thus, in the current investigation, efforts were made to establish

a dependable procedure for direct *in vitro* regeneration of Safed musli along with genetic and phytochemical assessment of the *in vitro* regenerants, taking into consideration the gaps and inadequacies of the prior research.

4.2 Material and Methods

4.2.1 Plant Material and culture conditions

In the September, 2018, *C. borivillianum* plants were collected from Haryana, India. On the basis of morphology, identification was done by Dr. Devendra Kumar Pandey, Professor, School of Bioengineering and Biosciences, Lovely professional University, Punjab and specimens were deposited in Herbarium of Lovely Professional University, Punjab, (Voucher Specimen No.35698).

The explants apical buds taken from the mother plant were extensively cleaned in distilled water after being thoroughly washed in flowing tap water for 20 min, cleansed for 10 min with Tween-20 (Sisco Research Laboratories Pvt. Ltd, India). Finally, externally disinfected for 3-5 min with mercuric chloride 0.1% (Merck Specialties Pvt. Ltd, Mumbai, India) and rinsed repeatedly in Laminar Air Flow Chamber with distilled water 3- 4 times and inoculated on MS (Murashige and Skoog, 1962) supplemented with 0.7% agar and 3% sucrose and different plant growth regulators. Medium pH was attuned to 5.8 and autoclaved 121 °C for 15 min at 105 kPa. The cultures were sustained in a growth chamber having 16h light and 8h dark cycle receiving light from cool-white fluorescent lamps with intensity $45\text{-}50\ \mu\text{mol m}^{-2}\text{s}^{-1}$ at incubation temperature $25 \pm 1^\circ\text{C}$ and relative humidity 40-50%.

4.2.2 Shoot bud induction and multiplication

The morphogenic response of the apical bud explants was assessed using MS medium that had been enriched with various growth hormones such as BAP, Kin, TDZ or mT (Sigma-Aldrich and Himedia India) at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L⁻¹) either separately or in conjunction for axillary shoot induction and multiplication (Table 4.1). Five explants were placed in each Erlenmeyer flasks (250 cm³) with 50 cm³ experimental media. Plantlets that had been successfully initiated from the most effective medium were transferred to the same medium for shoot multiplication, and after 4 weeks of culture, the number and length of shoots were recorded based on the response percentage (%) of explants.

4.2.3 Rooting, tuberization and acclimatization

The 8-week-old fully developed shoots of average length 5.5 cm were shifted to rooting media augmented with varying concentrations of IAA, NAA and IBA for 4 weeks (Table 4.2) and after 4 weeks, culture response (%), root number per shoot and length were recorded. After proper root formation, the *in vitro* rooted plants were transplanted to tuber media enriched

with IBA (0.75 mg L⁻¹), NAA (0.5 mg L⁻¹) and varying amount of sucrose (20-60 g L⁻¹) for 6 weeks. Culture media devoid of any plant hormone were set as controls.

In vitro grown healthy plantlets of 14 weeks old with well-developed roots were rinsed thoroughly to completely remove residues of agar and planted in plastic pots containing different combination of sand, fym, loam, perlite and vermicompost in various ratios as presented in Table 4 and kept in growth chamber for 15-20 days. After required time period the plantlets were shifted to experimental plots and the percentage of their survival was recorded.

4.3 Phytochemical analysis

4.3.1 Sample preparation

C. borivilianum sample was dried under shade and finely powdered using electric mixer grinder (Philips JMG4-HL7581/00). 1 g sample of each soil type plants were subjected to maceration with 30 mL ethanol (50%) for 24 h and then sonicated for 10 min. The extracts of each sample were strained through Whatman filter paper No.1 and dehydrated in rotary evaporator (Buchi, R-300, India) at 40 °C. Following this, 20 mL of 10% hydrochloric acid (HPLC grade, E. Merck, India) were added to the dried remains of each sample. These samples were subjected to acid hydrolysis using water bath for 1hr at 98 °C to isolate the saponins (Nazir et al., 2022). Chloroform was added to each sample after the completion of acid hydrolysis resulting in the formation of two distinct layers, upper layer was eliminated and lower layer was washed again with chloroform and subsequent drying via a rotary evaporator. The dried residue was then treated with methanol, subjected to syringe filtration, and subsequently utilized for quantitative analysis through HPTLC.

4.3.2 HPTLC analysis of diosgenin and stigmasterol

This work made use of an HPTLC system utilizing a Linomat5 applicator CAMAG (Switzerland) equipped with a 100 ml syringe, a TLC scanner-3 run, in conjunction with WinCATS software (version:1.4.6.2002). HPTLC was performed using toluene: ethyl acetate (7:3 v/v) as the mobile phase on precoated silica gel 60 F254 20 × 10 cm TLC plates. Using a Linomat-5 applicator CAMAG fitted with a 100 ml syringe adjusted to the specified settings: band length 6 mm, application rate 10 sec/mL, 8 mm gap between bands, distance from the side edge of the plate 1.5 cm, and distance from the plate's bottom 2 cm, the 3 mL of each sample of *C. borivilianum* and marker compounds (diosgenin and stigmasterol) solution was administered to the plates in the form of bands measuring 6 mm in width. Anisaldehyde reagent (1 mL anisaldehyde, 20 mL glacial acetic acid, 170 methanol, and 10 mL conc. sulphuric acid) was sprayed on the plates to post-derivatize them (Nazir et al., 2021b). The

plates were then subjected to a 5 min drying process at 100 °C in a hot air oven (Bionics Scientific, India). Using the Camag TLC scanner 3 and Win Cats software, the plates were scanned at 433 nm reflectance wavelength after the formation of the bands. By comparing the extract peak's retardation factor (R_f) to that of the marker compounds, the concentration was determined from the calibration curves.

4.4 Clonal fidelity of *in vitro* regenerants

ISSR and SCoT markers were used to examine the genetic integrity of plants cultured under *in vitro* condition. Young, healthy leaves (100 -200 mg) of both source plants and cultured plantlets were harvested and total genomic DNA was extracted using the CTAB technique (Doyle and Doyle, 1990). Additionally, the amount and purity of DNA were assessed using a 0.8% agarose gel. After that, TE buffer (Tris-EDTA; 25 ng μL^{-1}) was added, and the samples were preserved at 4 °C before being used in subsequent studies. In order to assess the genetic stability of the plants produced from tissue culture, 5 ISSR primers and 5 SCOT primers (Merck Biosciences, Germany) were chosen, three of these primers of ISSR and SCOT (Table 4.3) displayed distinct and reproducible bands. In a Gradient Thermal Cycler (iGene Labserve or G-storm), polymerase chain reactions (PCR) were performed. The amplification cycle consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of 94 °C for 20 sec (denaturation), 58 °C for 30 sec (annealing), 72 °C for 2 min (extension) and 1 cycle of 72 °C for 10 min (final extension). With the aid of TAE 1x solution and agarose (2.5%) gel electrophoresis, amplification products were separated, and the sizes of the amplicons were measured and compared using a 100 bp DNA ladder (GeneRuler SM0321, Thermo Scientific; USA).

4.5 Statistical analysis

Each treatment included 10 explants, and all tests were carried out in triplicate. Using the SPSS 16 (SPSS Inc.; Chicago, USA) statistical analysis programme, observations of shoot induction, root development and tuber formation were periodically assessed and analysed using one-way analyses of variance (ANOVA). Mean differences were successfully achieved through the application of Duncan's multiple range test (DMRT) at a confidence level of 95%.

4.6 Result and Discussion

4.6.1 Effect of individual cytokinins on *in vitro* shoot initiation and multiplication

Explants grown in control (MS-medium) produced no morphogenic response. However, after a week of inoculation, emergence of shoot was observed in all examined doses of BAP, Kin, mT, or TDZ. In regards to promoting shoot buds per explant, mT exhibited a more robust response compared to BAP, Kin, or TDZ of the cytokinins that were tested individually.

Following 8 weeks of inoculation, it was determined that the MS medium fortified with 2.0 mg L⁻¹ mT produced the best (87.6%) morphogenic response with a maximum average shoot number (12.4 ± 1.14) and shoot length (4.76 ± 0.95) (Fig. 4.1a, b). The number of shoots reach its peak level at mT (2.0 mg L⁻¹), after which a decline in growth metrics was noticed (Table 1). The sequence of response in the shoot initiation and multiplication was noticed as mT > BAP > Kin > TDZ. Culture response (28.3% to 72.6%) with 3.8 ± 1.30 to 8.8 ± 1.30 mean shoot number/explant was recorded in the media fortified with BAP. Kin supplemented media produced 2.4 ± 1.14 to 5.8 ± 2.58 mean number of shoots having average shoot length 1.96 ± 0.91 to 2.7 ± 0.48 cm. TDZ containing media showed response in the range of 19.8% to 52.9% producing 2.2 ± 1.64 to 6.1 ± 0.83 shoots. Previous studies have indicated that the optimal medium for shoot proliferation of Safed musli includes supplementation with 3.0 mg L⁻¹ BAP, resulting in an average shoot count of 18.90 after 28 days of inoculation (Kemat et al., 2010). This finding is consistent with reports from various other studies (Ashraf et al., 2014; Khatri et al., 2019), suggesting that BAP outperforms other cytokinins in initiating and proliferating shoots. In our current investigation, BAP demonstrated superiority over Kin and TDZ, with mean shoot counts and lengths of 8.8 shoots and 3.22 cm, respectively.

The cytokinin mT has been discovered to exhibit efficacy in enhancing morphogenic processes across various species, including *Curcuma longa* (Salvi et al., 2002), *Musa* species (Escalona et al., 2003), *Aloe* spp. (Bairu et al., 2007), *Pelargonium* varieties (Wojtania, 2010), *Pyrus communis* (Dimitrova et al., 2016), *Allamanda cathartica* (Khanam et al., 2019), *Ribes grossularia* (Kucharska et al., 2020), *Scaevola taccada* (Shekhawat et al., 2021), *Iris × hollandica* (Verma et al., 2022) and *Caralluma umbellata* (Jayaprakash et al., 2023). The efficacy of mT for promoting maximum shoot initiation is due to the existence of hydroxyl group on its side chain in its fine chemical structure, which aids in the production of non-toxic and readily interchangeable forms of cytokinin storage known as O-glucoside conjugate forms. Additionally, it moves quickly and readily within plant tissues (Werbrouck et al., 1996; Amoo et al., 2011; Kaminek et al., 1987). Cultures stimulated by mT were robust and exhibited no aberrations, such as hyperhydricity or shoot tip necrosis, over the course of extended cultures, as was seen with other cytokinins. There have been reports of Tang et al. (2002), Petri and Scorza (2010), Aremu et al. (2012), Mala et al. (2013), Naaz et al. (2019), Hussain et al. (2019), Singh and Kumaria, (2020), Manokari et al. (2022) reporting comparable morphogenic reactions induced by mT on cultures grown *in vitro*. Anis et al. (2023) found that mT at 5.0 µM significantly enhanced shoot induction and proliferation in *Feronia limonia*, outperforming BA. In a study by Kaur et al. (2022), mT proved superior in

promoting shoot regeneration in *Crinum brachynema* cultures, with 5.0 μM mT resulting in the highest shoot regeneration frequency (11.10) compared to the control. No reports on the application of topolins in the micropropagation of *Chlorophytum* species exist as of yet. This study represents the first investigation on the use of mT on *C. borivillianum* that we are aware of.

4.6.2 Effect of optimized concentration with other cytokinins

The establishment of a successful procedure for the large-scale multiplication and preservation of elite genotypes depends critically on the choice of cytokinin type, concentration, and combination with other cytokinins. The combination of mT with BAP was determined to be more proficient in shoot elongation and proliferation than any other combinations examined. After 8 weeks of culture, the MS medium enriched with 2.0 mg L^{-1} mT and 1.0 mg L^{-1} BAP exhibited the best response (96.3%) with a mean of shoots 19.2 ± 1.92 and 6.72 ± 0.49 cm in length (Table 2, Fig. 1C). The sequence of response in the shoot initiation and multiplication was noticed as mT + BAP > mT + Kin > mT + TDZ. In the mT and Kin combination, 88.7% response was observed at 1 mg L^{-1} Kin with 13.7 ± 1.34 mean shoot numbers having 4.62 ± 0.89 cm average length. With TDZ, 82.6% response was noticed at 1 mg L^{-1} TDZ concentration with 12.0 ± 1.58 mean number of shoots having 3.96 ± 0.87 cm average length. With increase in the concentration of BAP, Kin, TDZ decline in the response was observed. The adventitious shoot regeneration from apical bud explants in *C. borivillianum* must therefore be maximized by cytokinin combinations. The morphogenic response in apical bud explants was recorded in Tables 2 for the mT and other cytokinin combinations, whereas Kin and TDZ combinations with mT exhibited less shoots as compared to BAP.

Table 4.1 Effect of different cytokinins on *in vitro* shoot induction and multiplication of *Chlorophytum borivillianum* after 8 weeks of culture

MS + growth regulators (mg L ⁻¹)				Culture response (%)	Mean no of shoots/explant produced	Mean shoot length (cm)
BAP	Kin	TDZ	mT			
0	0	0	0	00.0	0.0 ± 0.0	0.0 ± 0.0
0.5				28.3 ^{s, t}	3.8 ± 1.30 ⁿ	2.40 ± 1.14 ^m
1.0				38.7 ^p	5.6 ± 0.89 ^j	2.60 ± 0.89 ⁱ
1.5				64.2 ^{e, f}	6.7 ± 0.89 ^f	3.32 ± 0.45 ^e
2.0				72.6 ^c	8.8 ± 1.30 ^d	3.22 ± 0.31 ^{e, f}
2.5				69.1 ^{d, e}	8.1 ± 1.22 ^e	2.92 ± 0.19 ^f
3.0				61.8 ^g	6.0 ± 0.70 ^{g, h}	1.88 ± 0.51 ^r
	0.5			23.0 ^t	2.4 ± 1.14 ^s	1.96 ± 0.91 ^q
	1.0			29.5 ^s	2.8 ± 1.48 ^r	2.14 ± 0.87 ^o
	1.5			47.3 ^k	3.5 ± 1.14 ^p	2.26 ± 0.56 ⁿ
	2.0			62.9 ^f	5.8 ± 2.58 ⁱ	2.70 ± 0.48 ^{h, i}
	2.5			56.0 ^h	5.6 ± 1.14 ^j	2.50 ± 0.45 ^k
	3.0			45.2 ^l	3.6 ± 3.05 ^o	1.96 ± 0.30 ^q
		0.5		19.8 ^u	2.2 ± 1.64 ^t	1.68 ± 0.26 ^s
		1.0		30.5 ^r	3.1 ± 0.83 ^q	2.56 ± 0.35 ^j
		1.5		39.3 ^o	3.7 ± 0.83 ^{n, o}	2.71 ± 0.45 ^h
		2.0		52.9 ⁱ	6.1 ± 0.83 ^g	2.78 ± 0.68 ^g
		2.5		50.4 ^j	5.9 ± 1.58 ^h	2.46 ± 0.49 ^l
		3.0		44.7 ^m	4.4 ± 0.83 ^l	2.06 ± 0.72 ^p
			0.5	33.3 ^q	3.9 ± 1.14 ^m	2.56 ± 0.47 ^j
			1.0	41.7 ⁿ	4.8 ± 1.30 ^k	3.76 ± 0.43 ^d
			1.5	69.2 ^d	9.1 ± 2.0 ^c	4.08 ± 0.13 ^c
			2.0	87.6 ^a	12.4 ± 1.14 ^a	4.76 ± 0.95 ^a
			2.5	82.1 ^b	11.8 ± 1.92 ^b	4.14 ± 0.66 ^b
			3.0	64.5 ^e	8.0 ± 2.23 ^{e, f}	2.74 ± 0.86 ^{g, h}

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

Research on the synergistic effects of meta-topolin (mT) with other cytokinins in *in vitro* shoot induction and multiplication has provided valuable insights into their combined efficacy. When combined with other cytokinins/auxins, the mT has been shown to produce the highest shoot regeneration rates in a variety of plant species, including *Pterocarpous marsupium* (Ahmad and Anis 2019); *Coleonema album* (Fajinmi et al., 2014). Nowakowska and Pacholczak (2020) investigated the effect of mT in combination with BAP on *Daphne mezereum* cultures, finding that at a concentration of 1 mg L⁻¹ showed superior effects on shoot regeneration explants compared to BA at the same concentration, indicating its potential as an alternative cytokinin. The cytokinin profiling study by Novák et al. (2008) provided further evidence of the importance of cytokinins, including meta-topolin, in plant tissues,

highlighting their diverse roles in growth and development. Anis et al. (2023) observed that combining 5.0 μM mT with 0.50 μM NAA resulted in the highest mean shoot numbers and average shoot length after 12 weeks of culture. These studies highlight the potential synergistic effects of mT with other cytokinins, emphasizing the importance of optimizing their concentrations for enhanced shoot proliferation and rooting in tissue culture systems. Furthermore, cytokinins engage with each other at different amounts in diverse species to produce the best possible physiological reaction (Coenen and Lomax, 1997).

Table 4.2. Effect of optimized level of mT (2.0 mg L^{-1}) with cytokinins after 8 weeks of culture

MS + growth regulators (mg L^{-1})				Culture response (%)	Mean no of shoots/explant produced	Mean shoot length (cm)
mT	Kin	TDZ	BAP			
0	0	0	0	00.0	0.0 ± 0.0	0.0 ± 0.0
2.0	0.5			78.3 ⁱ	$11.6 \pm 1.51^{\text{h}}$	$3.41 \pm 1.14^{\text{k}}$
	1.0			88.7 ^d	$13.7 \pm 1.34^{\text{e, f}}$	$4.62 \pm 0.89^{\text{d}}$
	1.5			83.2 ^e	$10.2 \pm 1.30^{\text{i}}$	$3.32 \pm 0.45^{\text{l}}$
	2.0			70.6 ^m	$9.8 \pm 1.30^{\text{k}}$	$3.22 \pm 0.31^{\text{m}}$
	2.5			64.1 ^p	$6.5 \pm 1.18^{\text{n}}$	$2.92 \pm 0.19^{\text{o}}$
	3.0			60.8 ^q	$5.6 \pm 1.34^{\text{p}}$	$2.38 \pm 0.51^{\text{q}}$
2.0		0.5		78.7 ⁱ	$11.8 \pm 0.83^{\text{g}}$	$3.65 \pm 0.91^{\text{i}}$
		1.0		82.6 ^f	$12.0 \pm 1.58^{\text{f}}$	$3.96 \pm 0.87^{\text{g}}$
		1.5		80.3 ^g	$10.1 \pm 0.83^{\text{i, j}}$	$3.78 \pm 0.56^{\text{h}}$
		2.0		72.9 ^k	$8.8 \pm 0.83^{\text{l}}$	$2.71 \pm 0.48^{\text{p}}$
		2.5		66.0 ⁿ	$7.8 \pm 0.83^{\text{m}}$	$2.13 \pm 0.45^{\text{r}}$
		3.0		65.2 ^o	$6.2 \pm 1.64^{\text{n, o}}$	$2.96 \pm 0.30^{\text{n}}$
2.0			0.5	89.8 ^c	$17.0 \pm 2.70^{\text{c}}$	$6.06 \pm 0.11^{\text{b}}$
			1.0	96.3 ^a	$19.2 \pm 1.92^{\text{a}}$	$6.72 \pm 0.49^{\text{a}}$
			1.5	94.5 ^b	$18.6 \pm 1.34^{\text{b}}$	$5.18 \pm 0.26^{\text{c}}$
			2.0	79.9 ^h	$16.6 \pm 1.14^{\text{d}}$	$4.54 \pm 0.42^{\text{e}}$
			2.5	75.4 ^j	$13.8 \pm 1.30^{\text{e}}$	$4.28 \pm 0.44^{\text{f}}$
			3.0	71.7 ^l	$10.0 \pm 1.58^{\text{j}}$	$3.51 \pm 0.41^{\text{j}}$

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

4.6.3. *In vitro* rooting of regenerated microshoots

Three auxins, NAA, IAA and IBA were tested for *in vitro* rooting proficiency in regenerated shoots. Microshoots failed to develop roots on the control medium even after four weeks of culture, but formation of root was aided by the introduction of various auxins to the MS medium. The most successful *in vitro* root initiation (95.5%) was accomplished by incorporating 1.0 mg L^{-1} IBA on MS liquid medium, which after 4 weeks of culture caused maximal (14.0 ± 1.39) average root number and (3.36 ± 0.27 cm) average root length (Table

3, Fig. 1D, E). Compared to IAA and NAA, IBA produced more roots (Fig. 1c). NAA at 0.5 mg L⁻¹ showed 85.3% culture response producing 10.6 ± 0.72 mean root numbers with average root length 2.16 ± 0.14 cm. Culture response ranges from 66.8% to 77.1% in media enriched with different concentrations of IAA with mean root numbers 4.8 ± 0.23 to 8.6 ± 0.91 . The optimized concentration of NAA (0.5 mg L⁻¹) was used in conjunction with IAA and IBA and in this combination, IBA at 1.0 mg L⁻¹ showed maximum response (93.1%) producing 13.2 ± 1.39 mean shoot number with mean shoot length 3.31 ± 0.27 cm. Integration of IAA and NAA (0.5 mg L⁻¹) showed culture response in the range of 72.7% to 91.2% producing 7.8 ± 1.15 to 11.0 ± 1.39 mean number of shoots.

Auxins are essential for rooting. IBA is the most prevalent natural auxin and is reported to be more stable in culture media than IAA (Pacurar et al. 2014). Different species of plants have documented similar effects of IBA on *in vitro* induction of roots in micro shoots (Spethmann and Hamzah, 1988; Ludwig-Müller, 2000; Perveen et al., 2013; Hussain et al., 2018; Hussain et al., 2019). IBA's fine structure, stability, and ease of translocation within plant tissues give it an advantage over other growth hormones in promoting robust root growth (Ludwig-Müller, 2000; Hussain et al., 2018). Another explanation why IBA gives the greatest promotional response might be because it is kept in free auxin conjugate forms, which are generated through hydrolysis and released as needed during the culture process (Nordstrom et al., 1991; Yang and Davies, 1999). Recent research has demonstrated the effectiveness of IBA in promoting root formation in tissue-cultured medicinal plants. Thorat et al. (2022) found that IBA significantly promoted root formation in tissue-cultured *Withania somnifera*. Similarly, Ahmed (2022) reported that IBA was more effective than NAA in inducing rooting in tissue-cultured *Lycium barbarum*. Additionally, Tadhani et al. (2006) and Patel and Shah (2009) observed that IBA was the most effective auxin for root induction in tissue-cultured *Stevia rebaudiana*. Anis et al. (2023) achieved the highest efficiency of *in vitro* root induction (90%) on ½ MS medium supplemented with 5.0 µM IBA, resulting in a mean root number of 3.20 ± 0.20 and mean root length of 4.52 ± 0.10 cm after four weeks of culture. These studies highlight the continued relevance of IBA as a rooting hormone in tissue culture systems for medicinal plants.

Table 4.3 Effect of different auxins on *in vitro* rooting of *Chlorophytum borivillianum* after 4 weeks of culture

MS + growth regulators (mg L ⁻¹)			% culture response	Roots/explants	Root length (cm)
NAA	IAA	IBA			
0	0	0	00.0	0.0 ± 0.0	0.0 ± 0.0
0.1			42.6 ^q	5.4 ± 0.39 ^m	1.35 ± 0.16 ^q
0.2			58.1 ^p	7.8 ± 1.14 ^j	1.91 ± 0.20 ⁿ
0.5			85.3 ^f	10.6 ± 0.72 ^e	2.16 ± 0.14 ^k
1.0			80.2 ^h	9.4 ± 1.32 ^h	2.36 ± 0.21 ⁱ
	0.5		66.8 ^m	4.8 ± 0.23 ^o	1.56 ± 0.11 ^p
	1.0		61.4 ⁿ	6.6 ± 0.98 ^l	2.52 ± 0.15 ^h
	1.5		77.1 ⁱ	8.6 ± 0.91 ⁱ	1.64 ± 1.41 ^o
0.5	0.5		72.7 ^k	7.8 ± 1.15 ^j	2.10 ± 1.59 ^l
	1.0		83.3 ^g	9.9 ± 0.74 ^f	2.04 ± 0.57 ^m
	1.5		91.2 ^c	11.0 ± 1.39 ^d	2.86 ± 0.27 ^e
		0.25	60.3 ^o	5.0 ± 0.91 ⁿ	2.64 ± 1.41 ^g
		0.5	73.1 ^j	6.8 ± 1.15 ^k	2.80 ± 1.59 ^f
		0.75	88.7 ^d	12.4 ± 0.74 ^{c, d}	3.14 ± 0.57 ^d
		1.0	95.5 ^a	14.0 ± 1.39 ^a	3.36 ± 0.27 ^a
0.5		0.5	68.9 ^l	9.5 ± 1.15 ^g	2.20 ± 1.59 ^j
		0.75	87.5 ^e	12.8 ± 0.74 ^c	3.16 ± 0.57 ^c
		1.0	93.1 ^b	13.2 ± 1.39 ^b	3.31 ± 0.27 ^b

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

4.6.4 *In vitro* tuberization of *C. borivillianum*

For the *in vitro* tuberization of *C. borivillianum*, the best rooting media NAA 0.5 mg L⁻¹ and IBA 0.75 mg L⁻¹ that produced the best quantity of roots and length were further modified with five concentrations of sucrose (20 - 60 g L⁻¹). The findings showed that the treatments had a substantial impact on the weight, and number of tubers. In comparison to the tuber count obtained through the other treatments, the maximum number of tubers (8 ± 0.69) with a weight of 6.6 ± 0.77 g was produced on 50 g L⁻¹ sucrose (Table 4). Sucrose at 40 and 60 g L⁻¹ almost showed same no of tubers 7 ± 0.67 and 7 ± 0.68 with 6.3g and 6.5 g fresh average weight. Very less difference was observed in media fortified with 40 and 60 g L⁻¹ sucrose in terms of tuberization. However, optimal concentration of sucrose for tuber formation of *C. borivillianum* was found as 50 g L⁻¹. Minimum tubers were produced in media fortified with 20 g L⁻¹ sucrose producing 4 ± 0.37 tubers with 4 ± 0.34 g average weight. These results align with the research conducted by Nazir et al. (2022), where impact of varying sucrose concentrations was investigated (40, 60 and 80 g L⁻¹) with BAP 0.9 mg L⁻¹ in *D. deltoidea* *in vitro* culture and documented 60 g L⁻¹ sucrose concentration optimum for tuber production as

compared to 40 and 60 g L⁻¹. Moreover, in another study led by Kohmura et al. (1995) also reported 60 g L⁻¹ sucrose to be more efficient than 30 g L⁻¹ concentration of sucrose for tuber formation. Enzymatic activity may get possibly induced at high sucrose supplementations for the starch production and its accrual in storage tissues (Staikidou et al., 2005). According to the current research, sucrose played a vital part in the *in vitro* tuberization process because the number and lengths of *in vitro* tubers obtained at 50 g L⁻¹ sucrose were optimal. A comparable finding was also observed by Chauhan et al. (2018) during *in vitro* tuber formation of *C. borivilianum* and microrhizome formation in *Acorus calamus* (Quraishi et al., 2017).

Table 4.4 Effect of different sucrose concentration on *in vitro* tuberization of *Chlorophytum borivilianum* after 6 weeks

$\frac{1}{2}$ MS + PGR (mg L ⁻¹)			No of rooted plants showing tuber induction	Average tuber fresh weight per plant (g)
NAA	IBA	Sucrose (g L ⁻¹)		
0.5	0.75	20	4.0 ± 0.37 ^d	4.0 ± 0.34 ^e
0.5	0.75	30	6.0 ± 0.57 ^c	5.8 ± 0.47 ^d
0.5	0.75	40	7.0 ± 0.67 ^b	6.3 ± 0.57 ^c
0.5	0.75	50	8.0 ± 0.69 ^a	6.6 ± 0.77 ^a
0.5	0.75	60	7.0 ± 0.68 ^b	6.5 ± 0.67 ^b

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

4.6.5 Acclimatization and hardening of *in vitro* plants

Any micropropagated protocol developed will be successful or unsuccessful based on the capacity of *in vitro* developed plantlets to endure in natural environmental settings (Anis and Ahmad, 2016; Naaz et al., 2019). Plantlets have to shift from a protective to a non-protective environment in the open at this crucial stage, which could be harmful to their survival. As a result, it might not be feasible to move *in vitro* developed plantlets directly to field conditions (Kant et al., 2010; Hussain et al., 2018). Plantlets with well-developed root system having three to five completely formed leaves were delicately placed in pharmacol containers filled with sterilized soil rite. The plants were moved to clay pots containing different soil types with various ratios to check their effect on survival and yield potential of *in vitro* regenerated plantlets of *C. borivilianum*. Highest survival rate 94% was observed in Loam: sand: Fym (1:2:1) with 16 g average weight and 12 g fresh weight of plants survived. Sand: vermicompost (1:1) also showed good survival percentage 92% with 12 g average weight of tubers per plant and 9.4 g fresh weight. sand: fym: perlite (2:1:1) showed 93% survival rate

with 11 g average and 8 g fresh weight of tubers per plant. Lowest survival rate 86% was observed in the soil type comprising of Sand: Fym: perilite (1:1:1) with 13 g average weight and 8.7 g fresh weight of tubers. In this study, Loam: sand: Fym (1:2:1) soil type was found best for hardening of *in vitro* regenerated plants of *C. borivilianum*. The pots were then moved to a greenhouse, where they flourished with a regular development cycle with no morphological variance (Fig. 1F).

Table 4.5 Effect of soil type on survival and yield potential on regenerated plantlets of *Chlorophytum borivilianum*

Soil Type	Percent plants survived in green house (%)	Average weight of tubers per plant	Fresh weight of tubers per plant (g)
Loam: sand: Fym (2:1:1)	88	14.0 ± 0.22 ^b	10.0 ± 0.33 ^b
Loam: sand: Fym (1:1:1)	89	12.0 ± 0.25 ^d	9.0 ± 0.39 ^d
Loam: sand: Fym (1:2:1)	94	16.0 ± 0.19 ^a	12.0 ± 0.31 ^a
Sand: Fym: perilite (2:1:1)	93	11.0 ± 0.15 ^e	8.0 ± 0.42 ^g
Sand: Fym: perilite (1:1:1)	86	13.0 ± 0.17 ^c	8.7 ± 0.32 ^e
Sand: vermicompost (2:1)	89	11.0 ± 0.23 ^{e, f}	8.4 ± 0.45 ^f
Sand: vermicompost (1:1)	92	12.0 ± 0.29 ^d	9.4 ± 0.47 ^c

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT



Fig. 4.1 (A) Shoot induction in *C. borivilianum* on MS medium augmented with 2.0 mg L⁻¹ mT after 3 weeks. (B) Shoot induction in *C. borivilianum* on MS medium augmented with 2.0 mg L⁻¹ mT after 8 weeks of culture. (C) Shoot multiplication in MS medium enhanced with 2.0 mg L⁻¹ mT + 1.0 mg L⁻¹ BAP, after 8 weeks of culture. (D, E) *In vitro* rooting on MS medium fortified with 1.0 mg L⁻¹ IBA after 4 weeks of culture. (F) Acclimatization of *in vitro* rooted plant.

4.6.6 Phytochemical analysis

Evaluations of phytochemical homogeneity in mother plants and *in vitro* developed plantlets are crucial to the success of any recommended tissue culture process. The therapeutic qualities of plants are due to the secondary metabolites found in them. Diosgenin and stigmasterol was quantitatively analysed by HPTLC in the current work. Based on the peak area, the concentration of diosgenin and stigmasterol in methanol extracts of *in vivo* plants (mother plant) and *in vitro* regenerants of *C. borivilianum* was determined. The content of bioactive compounds revealed by HPTLC analysis is presented in Table 6 (Supplementary File 1). In mother plant 0.66% stigmasterol and 0.71% diosgenin was found. In *In vitro* plantlets the stigmasterol content was observed in the range of 0.62% - 0.71% and diosgenin (0.72% - 0.77%). Presence of both compounds in mother and *in vitro* plants confirmed phytochemical homogeneity though variations were found in the content of these two compounds. Similar

investigations regarding the assessment of phytochemical fidelity in diverse medicinal plants using HPTLC have been documented (Nazir et al., 2021a; Sasidharan et al., 2017; Behera et al., 2018). The physiological and environmental factors influence the accumulation of secondary metabolites in plants (Verpoorte et al., 2002). For the production of novel substances with pharmacological value, plant tissue culture offers a biotechnological option for the retrieval of secondary metabolites from plants cultured *in vitro*. Therefore, it gives the opportunity to generate standardized material regardless of the environmental conditions (Matkowski, 2008).

Table 4.6 Stigmasterol and diosgenin content in *in vivo* and *in vitro* regenerated plants of *C. borivilianum*

Plant sample	Stigmasterol (%)	Diosgenin (%)
<i>In vivo</i> plant (Mother plant)	0.66 ± 0.15^c	0.71 ± 0.01^d
<i>In vitro</i> plant (P1)	0.62 ± 0.09^d	0.74 ± 0.05^b
<i>In vitro</i> plant (P2)	0.70 ± 0.11^b	0.72 ± 0.09^c
<i>In vitro</i> plant (P3)	0.71 ± 0.07^a	0.77 ± 0.03^a

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

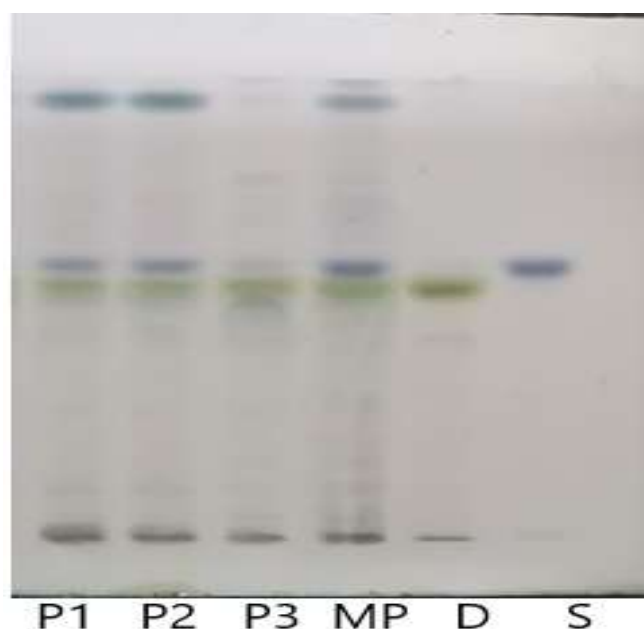


Fig. 4.2 HPTLC fingerprinting of *in vitro* regenerants (P1, P2, P3) and mother plant (MP) compared with standard biomarker diosgenin (D) and stigmasterol (S)

4.6.7 Genetic fidelity of *in vitro* plantlets

Although somaclonal variance is unlikely to appear in micropropagated plants, it is possible owing to the conditions of cultivation and the effects of growth hormones applied in the course of the *in vitro* multiplication phase and should not be disregarded (Larkin and Scowcroft, 1981; Gupta and Roy, 2002). Hence, evaluation of genetic homogeneity is required to confirm the genetic consistency amidst the regenerated plants. SCoT and ISSR markers were employed to confirm the validity of the present clonal production procedure due to their affordability, non-radioactive, have better reproducibility, and require no previous sequence knowledge for DNA amplification (Lakshmanan et al., 2007; Behera et al., 2018). On average, 6 of the 10 primers that were tested amplified genomic DNA with the most consistent bands in ISSR 6 (Table 7). The primer generated 14 unique, monomorphic bands with sizes varying from 400 to 2000 bp (Fig. 3). The quantity of bands used for each ISSR and SCoT primer varied from 2 to 6. The *in vitro* growth conditions did not cause any epigenetic variance in the developed regenerated plantlets, confirming their genetic homogeneity. Similar findings have also been documented in tissue cultured regenerants of different species, including *Eleusine coracana*, *Cornus alba*, *Dendrocalamus strictus*, *Dioscorea deltoidea* (Goyal et al., 2015; Illiczuk and Jacygard, 2016; Babu et al., 2018; Nazir et al., 2021a; 2022).

Table 4.7 Response of different ISSR and SCoT primers on assessment of genetic homogeneity among *in vitro* regenerates *C. borivilianum*

S. No	Primer code	Primer sequence (5'-3')	Number of bands	Range of amplified bands (bp)
1	ISSR6	CAACAATGGCTACCAC GC	6	600-2000
2	ISSR7	CAACAATGGCTACCAC GG	3	600-1700
3	ISSR10	CCATGGCTACCACCGC AG	5	400-1600
4	SCOT-1	(AG)8C	2	400-500
5	SCOT-2	(AG)8G	2	800-1100
6	SCOT-4	(TC)8C	2	400-500

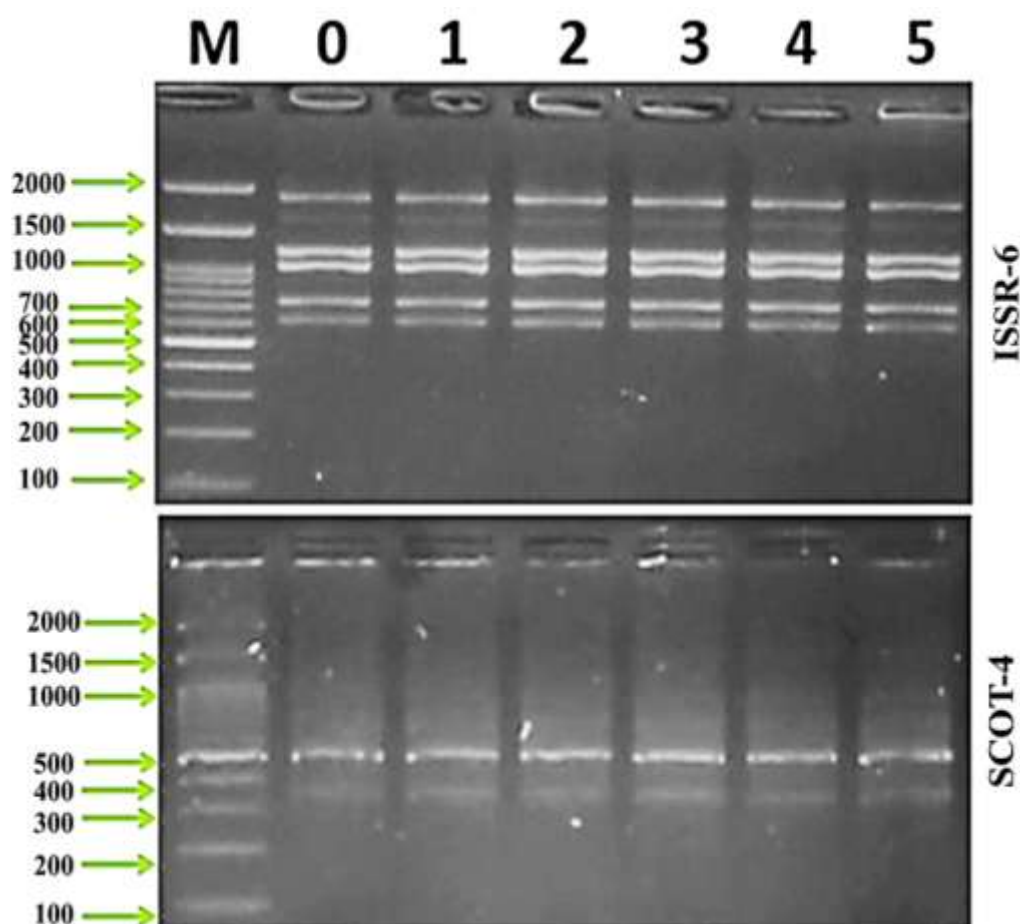


Fig. 4.3 Comparison of DNA fingerprinting pattern among *in vitro* raised plants with mother plant by ISSR and SCoT markers. M DNA Ladder (100 bp); 0 denotes mother plant; 1 to 5 of 5 randomly selected *in vitro* plants.

4.7 Conclusion

The enhancement of *in vitro* culture techniques enabled the establishment of a feasible, simple, efficient, or acceptable procedure for the mass production of the *C. borivillianum* and secondary metabolite synthesis. In this study, mT and other cytokinins interactions and combinations led to improved shoot proliferation and auxins promote rooting. Our findings indicate that mT, both alone and in combination with BAP, effectively induced and multiplied shoots. Furthermore, among the plant growth hormones tested, IBA exhibited the most successful rooting. These results underscore the potential of mT and IBA for enhancing shoot and root formation, respectively, in the cultivation of *C. borivillianum*. The procedure will prove useful in sustainable utilization of the species while preserving its native habitat. The *in vitro* cultured plants coped well in the field conditions, and according to ISSR and SCOT markers, no somaclonal variants were found among regenerants. Therefore, it would seem

that plants grown *in vitro* are better able to harvest secondary metabolites because they displayed a better content of phytoconstituents. The results could be used to develop an efficient micropropagation method for large-scale plant production with a good quantity of bioactive compounds without introducing any variability.

CHAPTER 5

Biotic and Abiotic factors influencing saponins content in *Chlorophytum borivillianum* L.: A response surface methodology approach

5.1 Introduction

Chlorophytum borivillianum Sant & Fernand (commonly known as “Safed Musli”) is a medicinal herb of the Liliaceae family, valued in traditional Indian medicine for its adaptogenic, aphrodisiac, anti-inflammatory, and nutritive properties (Guo et al., 2010; Goossens et al., 2003). The roots/tubers of *C. borivillianum* are rich in steroidal and furostanol saponins, which are believed to underlie many of these therapeutic effects. Given increasing demand for Safed Musli, both for medicinal and commercial uses, there is interest in enhancing its bioactive compounds, especially saponins, through cultivation, biological elicitors, and stress-induced manipulation. Abiotic stresses such as drought are known to affect plant physiology, often resulting in reduced growth. However, a number of studies show that under controlled stress, plants may increase production of secondary metabolites-defensive or adaptive compounds including saponins, alkaloids, phenolics, etc. as part of stress response. The use of polyethylene glycol (PEG) to simulate drought/osmotic stress in vitro or in growth media is well-established, allowing researchers to modulate water potential without soil drying (Ramirez-Estrada et al., 2016). Such PEG-induced stress has been shown to induce accumulation of compounds such as alkaloids and phenolics, and to alter gene expression related to secondary metabolite biosynthesis in several species (Ali et al., 2007; Zao et al., 2005).

Various elicitation strategies have also been tried in *C. borivillianum*. Mycorrhizal inoculation has significantly increased saponin content in tubers up to 25-fold at early growth stages and 4-fold at harvest when inoculated with *Glomus mosseae* compared to non-inoculated plants. Also, in vitro tuber formation experiments show that cultured tubers produce saponin levels comparable to *in vivo* tubers, suggesting tissue culture / *in vitro* elicitation could be viable.

Despite these advances, there remains a gap in studies that combine drought (or osmotic) stress via PEG with elicitation to specifically enhance saponin production in *C. borivillianum*. Understanding how PEG-induced stress affects saponin biosynthesis, both at the physiological level and in gene expression/metabolic network regulation, could help develop strategies for maximizing yield of these valuable compounds under cultivation or in vitro system.

This work is on the drought stress and elicitation by chitosan to enabling improvement better optimized for saponin yield.

5.2 Materials and methods

5.2.1 Chemicals and reagents

Compounds of reference and appropriate solvents have been detailed in detail in Chapter 3. (Section 3.2 5.2.2 Sterilization of explants. The explants apical buds collected from *C. borivilianum* mother plant were washed with tap water for 15 min, then treated with tween-20 for 5 min and then rinsed with distilled water. Finally, surface sterilized for 3-5 min with mercuric chloride 0.1% (Merck Specialities Pvt. Ltd, Mumbai, India) and washed again 3- 4 times with distilled water.

5.2.2 Inoculation of explants

Sterilised explants inoculated on MS (Murashige and Skoog, 1962) fortified with different plant growth hormones. Medium pH was attuned to 5.8 and autoclaved 121 °C for 15 min at 105 kPa. Cultures were maintained in a growth chamber having 16/8 h photoperiod altering regime of 45-50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ intensity provided by cool-white fluorescent lamps at incubation temperature $25 \pm 1^\circ\text{C}$ and relative humidity 40-50%.

5.2.3 Direct Organogenesis

For all types of culture, media were made of MS medium enhanced with 3% sucrose, 0.7% agar and different plant growth regulators (PGRs) (Sigma-Aldrich, India). Different plant hormones with various concentrations were used individually or in combinations to check their effect on shoot induction, multiplication and root formation as presented in Table 1, 2. The explants were placed in Erlenmeyer flasks (250 cm³) with 50 cm³ experimental media. The initiated plants from the medium that demonstrated the best initiation were sub-cultured in shoot multiplication media, and after 4 weeks of culture, the number and length of shoots were recorded based on the response percentage (%) of explants. The fully developed shoots were transferred to root formation medium augmented with different concentrations of Naphthalene acetic acid (NAA), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) for 4 weeks (Table 2) and after 4 weeks, culture response (%), root length and number per shoot were recorded. After proper root formation, the *in vitro* rooted plants were transferred to tuber media supplemented with NAA (0.5 mg L⁻¹), IBA (0.75 mg L⁻¹) and different concentrations of sucrose (20-60 g L⁻¹) for 6 weeks. Culture media devoid of any plant hormone were set as controls.

5.2.4 Hardening and Acclimitisation:

In vitro grown healthy plantlets with well-developed roots were rinsed thoroughly to eradicate agar traces and placed in plastic pots containing different mixture of sand, Fym, loam, perlite and vermicompost in various ratios as presented in Table 4 and kept in growth chamber for 15-20 days. After required time period the plantlets were shifted to experimental plots and survival percentage was recorded.

5.2.5 *In vitro* drought induction

In vitro screening was conducted using different concentrations of polyethylene glycol 6000 (PEG 6000) with varying concentrations viz. 0, 5, 10 and 15 % to study induced drought responses in plants. Observations were recorded at 30 DAI for shoot induction whereas root induction was recorded at 30 days after culturing microshoots in rooting media.

5.2.6 Modeling and optimization studies

For elicitors, CCD (FCC) was applied for the analysis of different types and concentrations of elicitors. Experimental layout is generated using Minitab.

Central Composite Design (CCD)

Table 4.2 shows 13 experiments, with each variable tested at three levels: -1, 0 and +1. All variables were coded using the following equation (Maran and Manikandan, 2012):

$$xi = Xi - X0/\Delta Xi \dots \dots \dots (2)$$

$$i = 1, 2, 3, \dots \dots \dots k$$

Where xi stands for the coded value's predictor variable, Xi for the actual value's predictor variable, X0 for the actual value at the midpoint, and ΔXi for the step change value's predictor variable. Each experiment was performed in triplicate, and the mean values were used for statistical analysis. The optimal levels were then determined using the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \dots \dots \dots (3)$$

Predicted response is denoted by Y, the modal constant by β_0 , significant factors by X_1 , and X_2 , linear coefficients by β_1 , and β_2 quadratic coefficients by β_{11} , and β_{22} , and interactive coefficients by β_{12} .

Similarly, for optimization of elicitors and their concentrations CCD was employed. Table 4.3 shows 13 experiments, with each variable tested at three levels: -1, 0 and +1. All variables were coded using the following equation (Maran and Manikandan, 2012):

$$xi = Xi - X0/\Delta Xi \dots \dots \dots (4)$$

i = 1, 2, 3..... k

Where xi stands for the coded value's predictor variable, Xi for the actual value's predictor variable, X0 for the actual value at the midpoint, and ΔXi for the step change value's predictor variable. Each experiment was performed in triplicate, and the mean values were used for statistical analysis. The optimal levels were then determined using the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \dots \dots \dots (5)$$

Predicted response is denoted by Y, the modal constant by 0, significant factors by X₁, X₂, linear coefficients by β_1 , β_2 quadratic coefficients by β_{11} , β_{22} , and interactive coefficients by β_{12} . The data were analyzed using regression and One- way analysis of variance (ANOVA) in Minitab statistical software, and a quadratic polynomial model was subsequently developed. ANOVA was performed, and then Tukey's test was performed for post-hoc comparisons. Minitab 15 was used for statistical analysis, and significance levels of p < 0.05 were evaluated.

5.2.7 Elicitors preparation and treatment

In this study, CS as elicitors, was used in the concentration of (100, 150, and 200 μ M). Stock solutions of 10 mM were prepared separately. 250 μ L CH in 1% acetic acid in 99% ethanol. After preparation, all stock solutions were filter sterilized through a 0.22 μ M syringe filter into autoclaved amber bottles (50ml) inside the laminar airflow chamber. The *in vitro* regenerants on 9th week of culture so were treated with elicitors on the 9th week of culture for 4 days. After elicitor treatment, the *in vitro* plantlets were harvested for the production of diosgenin and stigmasterol. All the treatments were carried out in triplicates.

5.2.8 HPTLC methodology

HPTLC analysis was carried out using winCATS Planar Chromatography Manager (V1.4.6) with a CAMAG TLC Scanner (S/N 180710). Samples and standards (Diosgenin, Stigmasterol, each 0.01 mg/spot) were applied on silica gel 60 F254 plates (20 × 10 cm, Merck, batch COM 6) using a CAMAG applicator. The mobile phase consisted of *n*-hexane: ethyl acetate (9:1, v/v). Plates were developed in a twin-trough glass chamber (CAMAG) pre-saturated with mobile phase vapors for 30 min at room temperature (25 ± 2 °C, 60 ± 5% RH). The development distance was set to 75 mm. The application position was 8 mm, solvent front was developed to 75 mm, and bands were scanned at 515 nm under absorption mode with D2 and W lamp, slit dimension 4.0 × 0.3 mm, scanning speed 20 mm/s, and resolution 100 µm/step. Data were processed using Savitsky–Golay 7 filter with baseline correction at lowest slope.



Figure 5.1 Multiple shoot induction from apical buds of *C. borivillianum*. (A) Apical segments regeneration after 2 weeks, (B) Axillary bud proliferation from apical segments after 3 weeks of culture; (C, D) Shoot multiplication on MS medium supplemented with 2.0 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA; (E, F) *In vitro* shoot multiplication using additives MS medium supplemented with 2.0 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA (H) *In vitro* rooting of shoots after weeks (I, J) Tuber formation after 8 weeks of culture (G) rooted plant growing in the plastic cup with soil, sand, vermicompost (1:1:1)

5.3. Results and Discussion

5.3.1 HPTLC fingerprinting

HPTLC fingerprinting of the plant extracts revealed the presence of multiple bands corresponding to different phytoconstituents (Figure 5.2). The bands observed were well resolved and comparable with standard diosgenin and stigmasterol, confirming their presence in the samples.

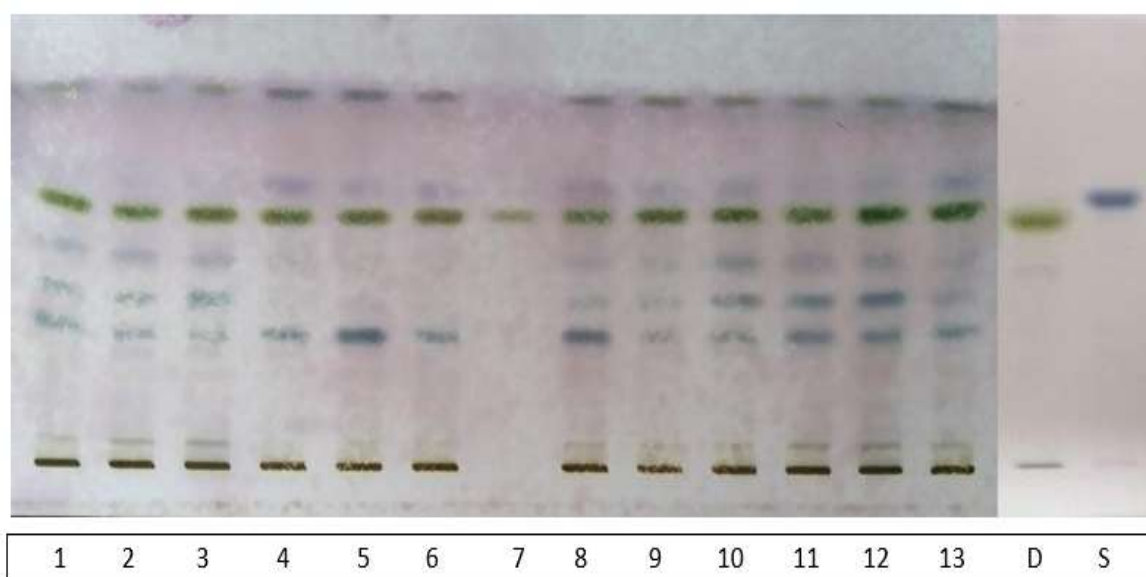


Fig. 5.2 HPTLC chromatograms

The overlay UV spectral scans of all tracks showed sharp and distinct peaks corresponding to diosgenin and stigmasterol standards. Both compounds exhibited characteristic absorbance maxima at λ_{max} 515 nm, which matched with the test samples, further validating their identification (Figure 5.4). Densitometric scanning at 515 nm clearly resolved diosgenin and stigmasterol at R_f values of 0.67 and 0.72, respectively (Figure 5.3). The peaks were well separated from other matrix components, demonstrating method specificity. The sharpness and intensity of the peaks indicated high sensitivity and suitability of the developed method for quantitative analysis

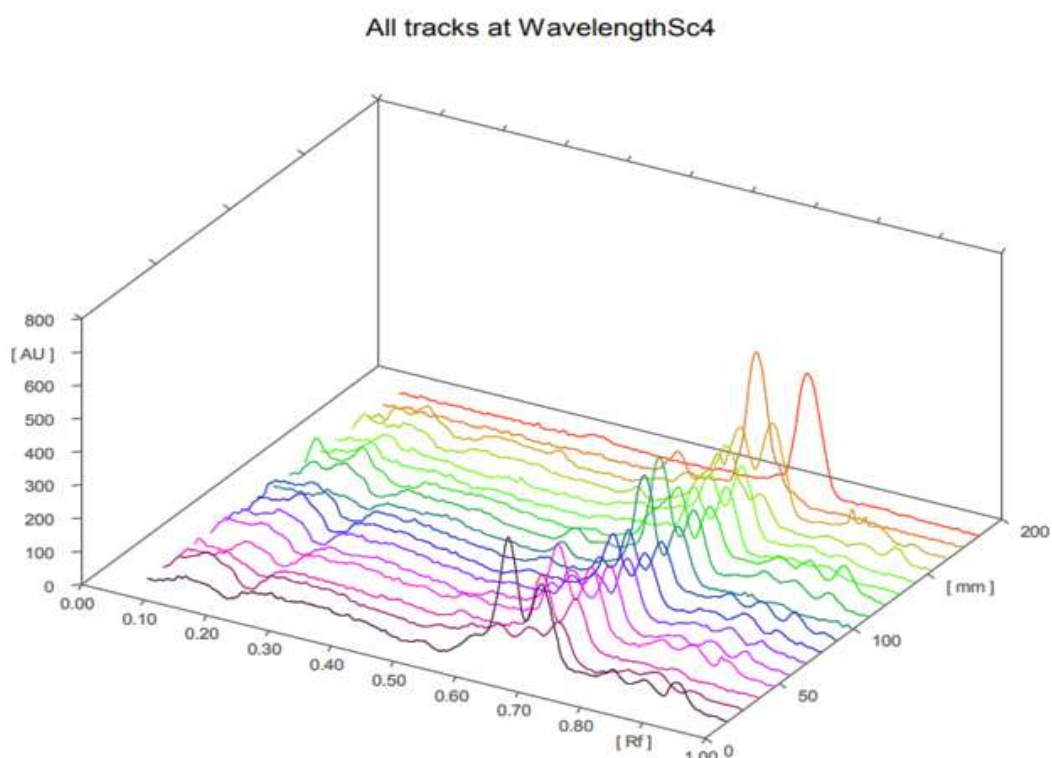


Figure 5.3 Overlay UV spectra scanning of samples with standards diosgenin and stigmasterol

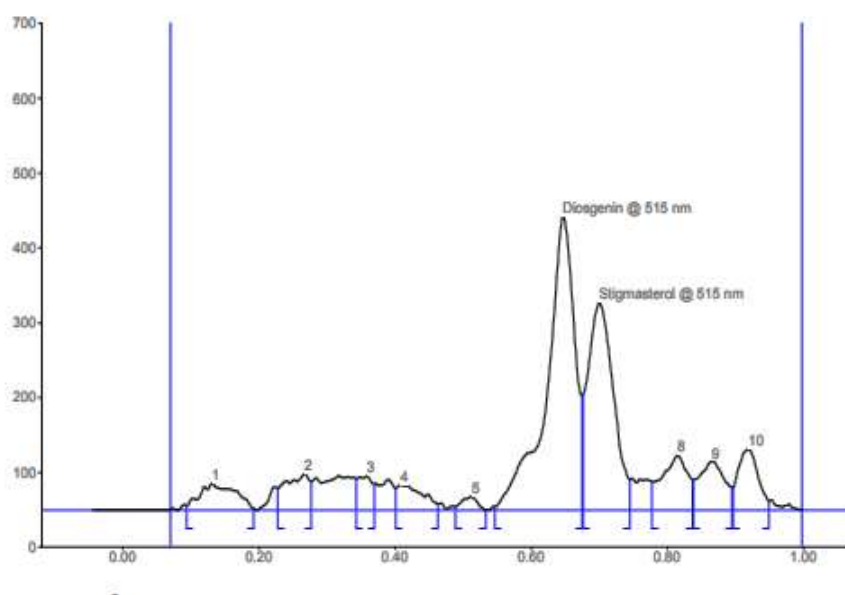


Figure 5.4 Peak for diosgenin and stigmasterol



Fig. 5.5 Multiple shoot induction from apical buds of *C. borivilianum* used in the CCD with tuber formation.

5.3.2 Diosgenin Accumulation

The response surface methodology (RSM) was used to optimize PEG 6000 and chitosan concentrations for diosgenin production in *C. borivilianum*. Diosgenin content ranged from 0.55% to 0.88%, with the highest accumulation observed at 7.5 g/L PEG and 150 mg/L chitosan. ANOVA confirmed the statistical significance of the model ($p < 0.01$), with a high coefficient of determination ($R^2 = 96.23\%$, adj. $R^2 = 93.53\%$). Both PEG and chitosan exerted significant positive linear effects, while quadratic effects were negative, indicating reduced diosgenin accumulation at higher concentrations. The PEG $\times$ chitosan interaction was marginally significant ($p = 0.051$), suggesting limited synergy between the two elicitors. Diagnostic plots confirmed the adequacy of the model, with observed values closely matching predicted responses. Only one experimental run (StdOrder 12) showed mild deviation. Overall, the fitted model effectively explained the variation in diosgenin content.

5.3.3 Stigmasterol Accumulation

Stigmasterol levels ranged from 0.52% to 0.81%, with the maximum yield again observed at 7.5 g/L PEG and 150 mg/L chitosan. The model exhibited excellent fit ($R^2 = 97.56\%$, adj. $R^2 = 95.82\%$), with significant positive linear effects of both PEG and chitosan ($p < 0.01$). Unlike diosgenin, the PEG $\times$ chitosan interaction was statistically significant ($p = 0.009$), suggesting a synergistic effect on stigmasterol biosynthesis. Both quadratic terms were strongly negative, highlighting the requirement for optimized concentrations to prevent inhibition. Observed and predicted values were closely aligned, with no significant lack-of-fit ($p > 0.05$), indicating strong model reliability for process optimization.

Table 5.1 Central Composite Design (CCD) matrix showing the effects of polyethylene glycol (PEG 6000) and chitosan concentrations on diosgenin and stigmasterol production in *C. borivilianum*

Std Order	Run Order	Polyethylene glycol 6000	Chitosan	% Diosgenin	Predicted (% Diosgenin)	% Stigmasterol	Predicted (% Stigmasterol)
1	1	5.0	100	0.55	0.546	0.52	0.515
2	2	10.0	100	0.68	0.671	0.64	0.649
3	3	5.0	200	0.71	0.704	0.68	0.679
4	4	10.0	200	0.73	0.719	0.68	0.692
5	5	5.0	150	0.73	0.738	0.71	0.714
6	6	10.0	150	0.79	0.808	0.81	0.788
7	7	7.5	100	0.66	0.671	0.61	0.604
8	8	7.5	200	0.76	0.775	0.72	0.708
9	9	7.5	150	0.83	0.836	0.79	0.773
10	10	7.5	150	0.85	0.836	0.78	0.773
11	11	7.5	150	0.81	0.836	0.76	0.773
12	12	7.5	150	0.88	0.836	0.77	0.773
13	13	7.5	150	0.84	0.836	0.75	0.773

Table 5.2 Estimated regression coefficients, standard errors, t-values, and significance levels (p-values) for diosgenin accumulation as influenced by PEG 6000 and chitosan, including linear, quadratic, and interaction terms

Term	Coef	SE Coef	T	P
Constant	0.83655	0.009724	86.025	0.000
Polyethylene glycol 6000	0.03500	0.009561	3.661	0.008
Chitosan	0.05167	0.009561	5.404	0.001
(Polyethylene glycol 6000) ²	-0.06293	0.014092	-4.466	0.003
(Chitosan) ²	-0.11293	0.014092	-8.014	0.000
Polyethylene glycol 6000 × Chitosan	-0.02750	0.011710	-2.348	0.051

Table 5.3 Analysis of variance (ANOVA) for diosgenin accumulation under PEG 6000 and chitosan treatments

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.097	0.097	0.019	35.69	0.000
Linear	2	0.023	0.023	0.011	21.30	0.001
Square	2	0.071	0.071	0.035	65.17	0.000
Interaction	1	0.003	0.003	0.003	5.52	0.051
Residual Error	7	0.0038	0.0038	0.0005		
Lack-of-Fit	3	0.0012	0.0012	0.0003	0.58	0.660
Pure Error	4	0.0027	0.0027	0.0007		

Table 5.4 Observed and predicted values of diosgenin content with associated standard errors, residuals, and standardized residuals from the fitted regression model

Obs	StdOrder	% Diosgenin	Fit	SE Fit	Residual	St Resid
1	1	0.550	0.547	0.021	0.003	0.32
2	2	0.680	0.672	0.021	0.008	0.79
3	3	0.710	0.705	0.021	0.005	0.48
4	4	0.730	0.720	0.021	0.010	0.95
5	5	0.730	0.739	0.016	-0.009	-0.52
6	6	0.790	0.809	0.016	-0.019	-1.12
7	7	0.660	0.672	0.016	-0.012	-0.72
8	8	0.760	0.775	0.016	-0.015	-0.92
9	9	0.830	0.837	0.010	-0.007	-0.31
10	10	0.850	0.837	0.010	0.013	0.63
11	11	0.810	0.837	0.010	-0.027	-1.25
12	12	0.880	0.837	0.010	0.043	2.04 R
13	13	0.840	0.837	0.010	0.003	0.16

Table 5.5 Estimated regression coefficients for stigmasterol accumulation

Term	Coef	SE Coef	T	P
Constant	0.77345	0.007015	110.263	0.000
Polyethylene glycol 6000	0.03667	0.006897	5.317	0.001
Chitosan	0.05167	0.006897	7.492	0.000
(PEG 6000) ²	-0.02207	0.010165	-2.171	0.067
(Chitosan) ²	-0.11707	0.010165	-11.517	0.000
PEG 6000 × Chitosan	-0.03000	0.008447	-3.552	0.009

Table 5.6 Analysis of variance (ANOVA) for stigmasterol accumulation under PEG 6000 and chitosan treatments. Model significance, lack-of-fit test, and contribution of linear, quadratic, and interaction terms are summarized

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	0.079895	0.079895	0.015979	55.99	0.000
Linear	2	0.024083	0.024083	0.012042	42.19	0.000
Square	2	0.052211	0.052211	0.026106	91.47	0.000
Interaction	1	0.003600	0.003600	0.003600	12.61	0.009
Residual Error	7	0.001998	0.001998	0.000285		
Lack-of-Fit	3	0.000998	0.000998	0.000333	1.33	0.382
Pure Error	4	0.001000	0.001000	0.000250		

Table 5.7 Observed and predicted values of stigmasterol content with associated standard errors, residuals, and standardized residuals from the fitted regression model

Obs	Std order	Observed	Fit	SE Fit	Residual	St Resid
1	1	0.520	0.516	0.015	0.004	0.52
2	2	0.640	0.649	0.015	-0.009	-1.20
3	3	0.680	0.679	0.015	0.001	0.09
4	4	0.680	0.693	0.015	-0.013	-1.63
5	5	0.710	0.715	0.012	-0.005	-0.39
6	6	0.810	0.788	0.012	0.022	1.83
7	7	0.610	0.605	0.012	0.005	0.44
8	8	0.720	0.708	0.012	0.012	1.00
9	9	0.790	0.773	0.007	0.017	1.08

5.4 Comparative Insights

Both diosgenin and stigmasterol biosynthesis were enhanced by PEG and chitosan elicitation, with maximum accumulation at optimized concentrations. However, stigmasterol biosynthesis showed higher sensitivity to the PEG $\times$ chitosan interaction, while diosgenin was primarily influenced by individual elicitor concentrations. This indicates that although the two metabolites share common precursors, their regulatory mechanisms differ, an important consideration for metabolic engineering and large-scale production strategies. Stigmasterol, both PEG 6000 (linear, $p = 0.001$) and chitosan ($p < 0.001$) had significant positive effects, while their squared terms and interaction showed negative coefficients, confirming that excess levels reduce yield. diosgenin, a similar pattern was observed, with both PEG 6000 and chitosan strongly influencing production, but excessive concentrations again led to decreased metabolite yield due to negative quadratic effects. The contour plots (Figures 1-4) revealed that intermediate concentrations of PEG 6000 and chitosan favored maximum metabolite accumulation.

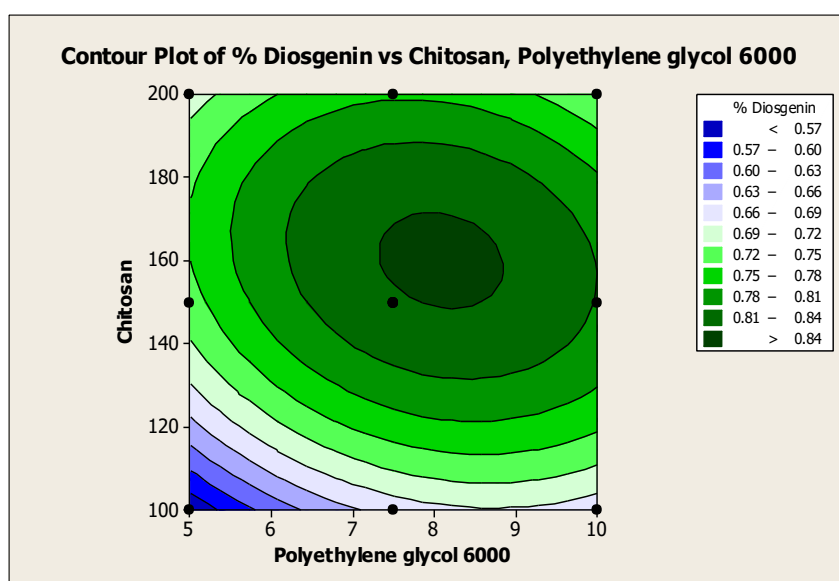
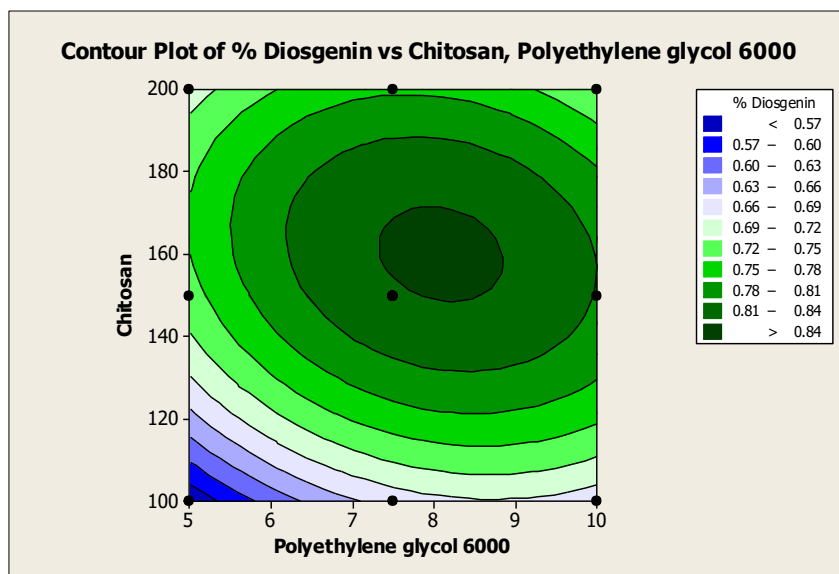


Fig. 5.6 Contour plot of % stigmasterol as a function of polyethylene glycol 6000 and chitosan concentration.

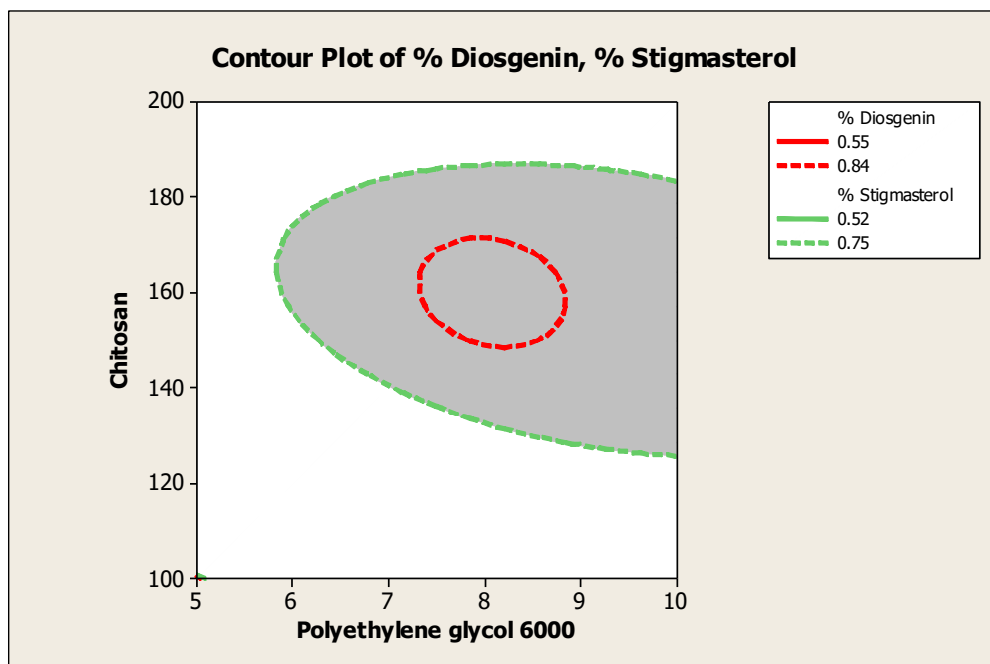


Fig. 5.7 Overlay contour plot representing the interaction of diosgenin and stigmasterol accumulation.

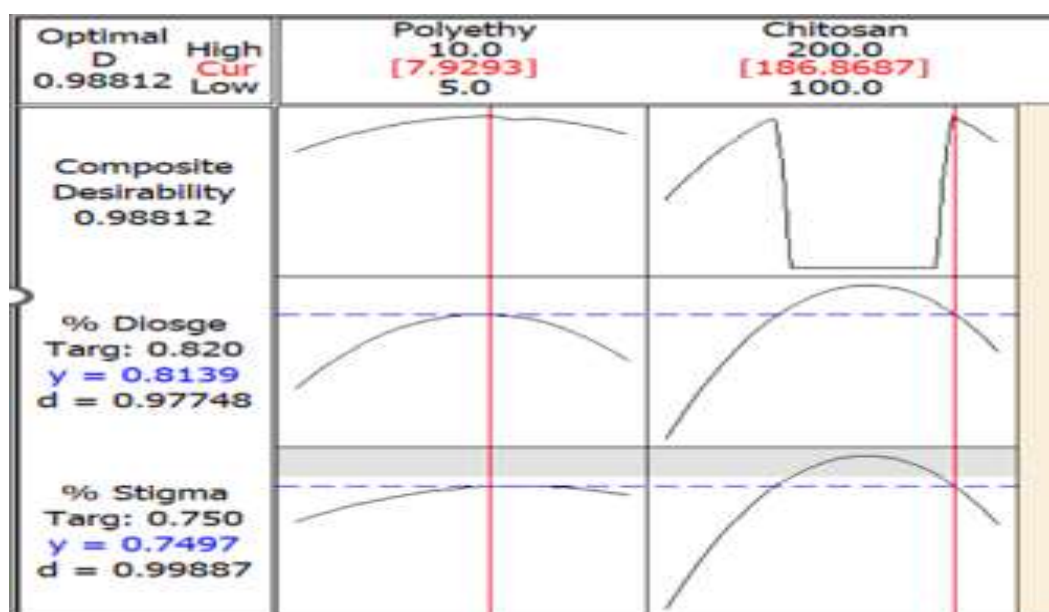


Figure 5.8 Numerical optimization plot obtained through response surface methodology (RSM).

5.5 Conclusion

This study successfully established and validated an HPTLC method for the simultaneous detection and quantification of diosgenin and stigmasterol in *C. borivilianum*. Well-resolved peaks at R_f values 0.67 and 0.72 confirmed the identity of the biomarkers, and the developed method showed excellent linearity, low LOD and LOQ, and high recovery rates, thereby validating its robustness for routine analysis. TLC fingerprinting further established the phytochemical profile of the plant extracts, while overlay UV spectra and densitometric scanning confirmed the presence of target compounds. The optimized formulation demonstrated high composite desirability, indicating the effectiveness of the experimental design approach. The validated HPTLC method, combined with statistical optimization, provides a reliable analytical and formulation strategy for standardization and quality control of herbal preparations containing diosgenin and stigmasterol. The integration of validated HPTLC analysis with RSM-based optimization provides a powerful framework for phytochemical standardization, quality control, and formulation development. These findings contribute to the broader goal of advancing phytopharmaceutical research and open avenues for scale-up and metabolic engineering strategies targeting steroidal metabolites in medicinal plants.

CHAPTER 6

Biostimulant-driven enhancement of saponins in drought-stressed *Chlorophytum borivilianum* L.: A full factorial design approach

6.1 Introduction

Global demand for medicinal plants for the health maintenance through disease prevention and treatment is increasing by 7.22 % annual growth rate. Among all nations, Indian and China plays an important role for the export of medicinal plants due to their rich traditional medical system, i.e. Ayurveda and traditional Chinese medicine (TCM) (Zamani et al., 2025). As the medicinal plant market grows, it is critical to focus on finding and selecting high throughput elite populations, cultivation and authenticating significant medicinal plants and characterizing their bioactive chemicals, which have enormous therapeutic potential and commercial relevance. Among all the medicinal plants *Chlorophytum borivilianum* (Family: Anthericaceae), or Safed Musli (meaning “white tubers”) is an annual herb well-known for its high content of steroidal saponins, which have notable therapeutic properties. Its roots are particularly valued for their diverse therapeutic properties (Visavadiya & Narasimhacharya 2007; Thakur et al. 2011). *C. borivilianum* used globally as natural alternative to “Viagra” (Kaushik, 2005) due to its activity against many sexual disorders (Das et al. 2016; Mararajah et al. 2024). The plant is renowned for its broad spectrum of biological activities, including aphrodisiac (Mararajah et al., 2024), immunomodulatory (Thakur et al., 2011), antitumor (Deore and Khadabadi, 2010), antidiabetic (Vikhe et al., 2024), antioxidant (Vyas et al., 2022), antimicrobial (Sharma et al., 2021), and anti-inflammatory (Panda et al., 2011) properties. *C. borivilianum* has been utilized as functional food since it is rich in carbohydrates (35-45%), proteins (5-10%), and so on (Verma et al., 2020). Besides this, *C. borivilianum* contains many other important health promoting secondary metabolites such as steroidal saponins like takarogenin, Tokorogenin, diosgenin, sarsasapogenin, hecogenin, and stigmasterol (Acharya et al., 2008; Basu et al., 2011; Kaushik, 2005; Khedkar et al., 2024). This plant is mainly found in central Indian states in subtropical and tropical climates up to an altitude of 1500 m. Traditionally, this plant is used to manage male impotency, oligospermia, increasing lactation etc. (Haque et al., 2011). According to IUCN *C.*

borivilianum listed as a critically endangered plant due to overexploitation, lack of conservation methods, ecological and habitat degradation (Pawar et al., 2023). Despite its vast therapeutic potential and commercial significance, *C. borivilianum* presents serious challenges for long-term cultivation.

Several abiotic stresses, notably drought, is a major consequence due to climate conditions change and leads to significant impact on species productivity and quality (Dobson et al., 2021). Therefore, it is an urgent need to find effective methods for increasing saponin yields and conserving *C. borivilianum*. Steroidal saponins like diosgenin act as a precursor for the synthesis of corticosteroids, sex hormones, and contraceptives, underlining the urgency of securing consistent and enhanced production (Herráiz, 2017). Whereas, stigmasterol is an important intermediate in the biosynthesis of progesterone and other steroid compounds (Lindemann, 2015). Looking into the challenges of climate-induced stresses and the necessity of enhancing saponins content of *C. borivilianum* because of high market demand, employing bio stimulants for combating climate induced stresses while simultaneously enhancing metabolites could be a promising approach. Biostimulants are organic substances that increase crop resistance to abiotic stresses (Das et al., 2022; du Jardin, 2015; Rouphael and Colla, 2020). In a study on enhancement of diosgenin, through *in vitro* cultures using different elicitors have been investigated by (Chauhan et al., 2018). They found that 2.1-fold higher production of diosgenin in the micro-tubers of *C. borivilianum*, highlighting the use of biotic elicitation strategies to enhance valuable secondary metabolites (Chauhan et al., 2018). Several studies have demonstrated the efficacy of biostimulants in reducing drought and other abiotic stresses while simultaneously enhancing the biosynthesis of bioactive compounds (Rafiee et al., 2016; Tadele and Zerssa, 2023). Thus, integrating biostimulants into the cultivation of *C. borivilianum* offers a viable strategy to improve its saponin content and secure its production against environmental challenges. Moreover, selection of biostimulants is an extremely important step for the enhancement of particular bioactive compounds in plants. Commonly use biostimulants are *Azotobacter*, *Bacillus* spp and arbuscular mycorrhizal fungi (AMF) etc (Fusco et al., 2022; Sun and Shahrajabian, 2025) for the sustainable development of plants. Full factorial design analysis (FFD) is a multi-factor group analysis to test the differences between various levels and interaction between each factor (Cheng, 2016). In this study we included FFD as a systematically evaluation tool for individual and interactive effects of different biostimulants to determine the optimal conditions for targeted responses. This study focuses on the use of the microbial consortium

method with full factorial optimization which improves both the growth and saponin contents in *C. borivilianum*. This research also optimizes the saponin content, under water stressed conditions. Results of our study shown perspectives on altering drought stressed with elicitor treatments for effective growth of *C. borivilianum*. To the best of our knowledge, no prior studies have documented in this approach. Thus, this research aims to optimize growth and saponin content of the *C. borivilianum* in a drought condition.

6.2. Materials and method

6.2.1 Plant materials

Healthy *C. borivilianum* plants were collected from Haryana, India, morphologically identified, and voucher specimens deposited in the Herbarium of Lovely Professional University (Voucher No. 35698) (Karwasra et al., 2024). The bioinoculants i.e *Glomus spp.*, *Azotobacter spp.* And *Bacillus spp.* used for the study were collected from Department of Microbiology, Harayana Agriculture University, Hisar, Haryana, India

6.2.2 Full factorial design methodology

The full two-level factorial design with k factors (FD, 2k) is an experimental design that reduces the number of experiments to be carried out without compromising the quality of the results sought in order to achieve the best overall optimization of the process. This technique involves analyzing all data simultaneously in order to determine the influence of each component and their relationships. Full factorial design entails testing all possible combinations of the levels of the factors, where each combination represents a treatment. This design enhances the accuracy of estimating main factor effects and interactions. By including every setting of each factor with every other factor, it allows for evaluation of both the independent influence of each factor on the response and the variation in factor effects due to changes in other factors. The design assumes that responses, such as growth, diosgenin content, and stigmasterol content, are linear functions of all factors.

$$Y = b_0 + \sum b_i X_i + \sum \sum b_{ij} X_i X_j (i \neq j) \quad (1)$$

Whereas, Y is the predicted response; b_0 the model constant; b_i the main effects of factors X_i ; b_{ij} the effects of second-order interactions between factors X_i and X_j .

6.2.3 Selection of parameters and experimental domain

In our study on *C. borivilianum* we employed three factors in two levels. For this study we analysed growth, diosgenin, stigmasterol content with different combinations of treatments

which are *Azotobacter* spp., *Bacillus* spp and AMF. Here we choose (-, +) i.e. (absent, present) as qualitative factor which maybe influence the response or outcome. All the factors are selected based on their roles. *Azotobacter* spp. is a nitrogen-fixing bacterium which promotes plant growth (Sumbul et al., 2020); whereas *Bacillus* spp. facilitates nutrient solubilization (Yadav et al., 2023). AMF is a symbiotic fungus, helps in phosphorus uptake (Ravnskov et al., 1999). Experimental domain relays on different range of factors with their different variation for the precise model establishment. In this domain, each component has a low and high level, denoted by (-1) and (+1) in the coded variable, representing the factors' possible values. Table 1 outlines the parameters and experimental domains in our investigation. MINITAB software was used to determine the experiment matrix, experiment table and effect values. When R^2 exceeds 0.80 and the coefficient of variation (C.V) is less than 5%, the model is considered validate. The MINITAB software calculates R^2 .

6.3 Phytochemical analysis

6.3.1 Sample preparation

Plant samples of *C. borivilianum* first collected and dried under shade. After that dried samples finely powdered using electric mixer grinder. For the extraction of samples from each soil type plants, 1 g of powdered samples were used to maceration with 30 mL ethanol (50%) for 24 h followed by sonication for 10 min. After that each extracted samples in solvent strained through Whatman filter paper No.1 followed by dehydrated in rotary evaporator (Buchi, R-300, India) at 40 °C. The dried residues of each sample were then mixed with hydrochloric acid (20 mL of 10%) (HPLC grade, E. Merck, India). These samples were subjected to acid hydrolysis using water bath for 1 hr at 98 °C to isolate the saponins (Nazir et al., 2022). After the acid hydrolysis was completed, Chloroform was added to each sample after acid hydrolysis, for the development of distinct layers. Whereas upper layer was removed. After that lower layer was rewashed with chloroform afterward drying with rotary evaporator. Residue from rotary evaporator than dissolved in methanol and utilized for HPTLC analysis.

6.3.2 HPTLC analysis of diosgenin and stigmasterol

The analysis was performed using HPTLC (Linomat5 applicator CAMAG; Switzerland), a 100 ml syringe, and a TLC scanner-3, as well as Win CATS software (Scanner_180710" S/N 180710 (2.01.02)) with 8 tracks, application position 15.0 mm, solvent front 75.0 mm, track distance 7.5 mm, slit dimensions 4.00 × 0.30 mm (Micro Light), and scanning speed 20 mm/s. Mobile phase used here is toluene: ethyl acetate (7:3 v/v) with precoated silica gel 60 F₂₅₄ 8 × 10 cm TLC plates as stationary phase. Dissolved samples of *C. borivilianum* and marker

compounds (diosgenin and stigmasterol) of 3 μ l were administered to the plates. For derivatization, anisaldehyde reagent (1 mL anisaldehyde, 20 mL glacial acetic acid, 170 mL methanol, and 10 mL conc. sulphuric acid) was sprayed on the plates (Nazir et al., 2021b). Plates were derivatized and then dried in a hot air oven (Bionics Scientific, India) for five minutes at 100 °C. Camag TLC scanner 3 and Win Cats software were scanned the plates at 515 nm wavelength. The concentration was calculated from the calibration curves by comparing the retardation factor (Rf) of the extract peak to that of the marker chemicals.

6.4 Method validation for HPTLC

The method was validated as per ICH guidelines for accuracy, precision, linearity, LOD, LOQ, repeatability, specificity, and recovery; precision, detection, and quantification limits were evaluated using replicate analyses and signal-to-noise ratios.

6.5 Analysis and interpretation of data

FFD analysis was used to optimize each experiment using three factors AMF, *Bacillus* species, and *Azotobacter* species and two levels. A variety of statistical indices were used to examine all experimental data. The statistical program MINITAB 15 was used for the statistical analysis, and the created model was used to generate an optimization surface map to forecast the relationships between independent and dependent variables. The mean $\pm$ standard deviation was used to display the response data.

6.6 Results and discussion

6.6.1 Growth Responses under Different Treatments

For the study of growth response in *C. borivillianum*, different biotic and abiotic treatments were used (Table 2). Among the different treatments, T7 which is a combination of *Bacillus* spp. (phosphate solubilizer), *Glomus* spp (plant mycorrhiza) and *Azotobacter* spp. (nitrogen fixers), resulted in the highest growth, with 28 leaves and 9 roots per plant, with improved diosgenin content as 0.67%. However, if these three biotic elicitors were used along, moderate improvements like AMF (T4) produces 20 leaves and 7 roots followed by *Azotobacter* (T2) (16 leaves, 6 roots) and PSB (T3) (18 leaves, 7 roots) were observed (figure 1). Under abiotic stress treatments i.e. cadmium (TB), lead (TC), Light (TE), salinity (TF), and darkness (TG) resulted in reduced growth of the plant. One notable result was seen from the drought treatment (TD), which caused a complete suppression of plant growth, as seen by the absence of detectable leaves and roots. Despite the growth limitation, diosgenin levels were determined to be relatively high i.e. 0.68%. This suggests that under water stress conditions,

the plant may have initiated a stress-induced metabolic response, resulting in increased diosgenin accumulation even in the absence of considerable vegetative development.

6.6.2 Optimizations of treatments with full factorial analysis

Based on these preliminary results, T7 and T11D were selected for further optimization using a full factorial experimental design. Our study used a full two-level factorial design (2^3) to investigate the impact of Phosphate Solubilizers (PS), Plant Mycorrhiza (PM), and Nitrogen Fixers (NF) on growth, diosgenin, and stigmasterol levels during drought stress. In this experiment, each element was examined on two levels. In this experiment, each factor was examined on two levels. The experimental matrix included eight different treatment combinations. The observed growth rate (leaves per plant) varied from 9 (Run 1: PS1, PM1, NF1) to 23 (Run 8: PS2, PM2, NF2). Diosgenin content ranged from 0.70-0.96 % and stigmasterol content ranged from 0.60-0.78% (Table 2, 3). The highest values for all three reactions were consistently found when all three biostimulant factors were at their peak levels (PS2, PM2, and NF2).

HPTLC analysis results of samples with standard compound shown in figure 2. Main plots and interaction plots for factors are presented in figure 3,4,5,6. Main plots for Growth, diosgenin, stigmasterol shown positive effects suggesting biostimulants enhancing growth, diosgenin and stigmasterol accumulation. Interactions are mostly additive, indicating low to moderate synergy among biostimulants. Table 4 summarizes the main and interaction effects of three PS, PM, and NF. Interaction analysis shown synergistic effects, whereas Main effects analysis revealed PS significantly enhanced all traits. PS exhibited the highest values for both growth and diosgenin content (14.83 ± 0.17 cm to 18.83 ± 0.17 cm; and $0.802 \pm 0.011\%$ to $0.917 \pm 0.011\%$).

6.6.3 Method validation by HPTLC

In the present study, HPTLC analysis was employed for the quantification of diosgenin and stigmasterol in plant samples. Well-resolved spots were observed at R_f values of 0.65 and 0.70 with λ_{max} of 436 nm and 312 nm, respectively. The calibration curve showed good linearity in the range of 300–1200 ng/spot with correlation coefficients (r^2) of 0.992 for diosgenin and 0.997 for stigmasterol, and regression equations $Y = 10.21X + 1250$ and $Y = 9.381X + 97$. The LOD and LOQ were 35 ng and 105 ng for diosgenin, and 30 ng and 90 ng

for stigmasterol, respectively. The method exhibited high precision, with intra-day and inter-day RSD values below 2%, and recovery studies showed mean recovery percentages of 99.79% for diosgenin and 99.15% for stigmasterol, confirming accuracy and reliability of the developed method (Table 5; figure 7).

6.6.4 Statistical Validation of the Factorial Model

Analyses of Variance (ANOVA) were conducted for all three factors and found highly significant effects on plant growth ($P < 0.001$). All two-way (PS*PM, PS*Nf, PM*Nf) and three-way (PS*PM*Nf) interaction were also statistically significant indicates a complex and interdependent relationship between biostimulants. PS, Nf, PM showed strong significant effects for diosgenin. For stigmasterol content, PS had a significant effect ($P = 0.003$). Interaction terms were not statistically significant ($P > 0.05$) for stigmasterol. The robustness of the statistical models was assessed using R^2 and adjusted R^2 values. These values confirm that the factorial model is robust and reliable for the prediction of plant growth and diosgenin accumulation, while the moderate fit for stigmasterol suggests the need for further investigation into other influencing factors (Table 6,7,8,9).

6.7 Discussion

In this research we aimed to increase *C. borivillianum*'s growth and saponin content during drought by using the microbial consortium. *C. borivillianum* is well known for its many medicinal uses and high steroidal saponin content (Visavadiya & Narasimhacharya 2007; Thakur et al. 2011). There is a strong market demand for saponins like diosgenin and stigmasterol-rich plant sterols. By 2033, the diosgenin market is projected to grow to a size of USD 75 million (<https://www.verifiedmarketreports.com/product/diosgenin-market/>). Whereas, stigmasterol-rich plant sterols market is expected to reach 1,234.6 million, by the end of 2035 (<https://www.futuremarketinsights.com/reports/stigmasterol-rich-plant-sterols-market>).

The growth of *C. borivillianum* is significantly challenged by abiotic stress, like drought, darkness, salinity, etc (Table 2). In our study we found that abiotic stresses reduce the leaves and root development in *C. borivillianum*. However, during drought condition, diosgenin contains was increased. This has made it necessary to investigate sustainable methods that can reduce stress while preserving or increasing the yields of phytochemicals. Many recent studies reported drought stress can enhance the production of secondary metabolites. Drought conditions increased sesquiterpene production in *Salvia dolomitica* (Caser et al., 2019).

According to one study on *Paris podophylla*, using AMF and plant growth-promoting endophytes inoculum in drought, decreased saponin accumulation and improved soil quality (Qian et al., 2024).

The most noticeable increase in biomass and diosgenin content was observed in the application of beneficial microbial inoculants, specifically the consortium treatment consisting of Azo+PSB+AMF. The highest number of leaves (28), maximum root development (9 roots/plant), and diosgenin output 0.67 % were all displayed by plants under T7, suggesting the supportive function of microbial biostimulants in fostering growth and the synthesis of secondary metabolites. This indicates the combine benefits of microbial interactions in plant growth under stress conditions. Abiotic stresses like cadmium, light, salinity, and darkness, had a suppressive effect on vegetative growth but not in diosgenin content. Other studies have also observed that abiotic stresses effects on growth and metabolites of the plants (Gengmao et al., 2015; Zhang et al., 2021). Kumar et al 2022; shown that PGPRs can reduce the effect of such factors and further improved the growth of the plants (Kumar et al., 2022). Treatment T11 showed negative to overall biomass accumulation and root development with highest diosgenin content 0.68 %.

The FFD analysis provided valuable insights into optimizing the effects of biotic elicitors under drought stress conditions. Through this experiment, we observed by increasing the levels of PS, PM, and NF led to enhanced plant growth and production of diosgenin, and stigmasterol content. Under optimal conditions, the diosgenin content reached up to 96% and stigmasterol up to 78% under optimal conditions, falls within or even exceeds some previously reported ranges for *C. borivilianum*. In various studies *in vitro* hairy root cultures of *C. borivilianum* have shown high accumulation of stigmasterol (up to 83.952 ± 0.01) (Bathoju, 2018), and 46.4 mg/g DCW. Chauhan et al. 2018, demonstrated that diosgenin accumulation in these micro-tubers can be significantly influenced by the use jasmonic acid and salicylic acid (Chauhan et al., 2018). While previous *in vitro* studies demonstrated successful enhancement of saponin accumulation, they lacked the ability to mimic the multifaceted environmental interactions that occur in natural or field conditions. Our study presents a novel integrative approach that combines the use of different biotic elicitors PS, PM, NF in drought stress under more physiologically relevant conditions. This dual treatment not only promotes plant growth but also significantly raises metabolite accumulation by activating both growth and defense pathways. FFD study emphasizes the synergistic effect of these coupled elements by revealing the optimal conditions for maximal phytochemical

production. To our knowledge, this is one of the first research to investigate such an integrative technique in *C. borivillianum* linking the gap between laboratory-scale metabolite increase and field-applicable sustainability for this important ethnomedicinal plant.

6.8 Conclusion

This study demonstrates that the use of microbial biostimulants, particularly the consortium of *Azotobacter*, *Bacillus*, and AMF, effectively enhances the growth and saponin content of *C. borivillianum* under drought stress. While drought alone suppressed vegetative development, it induced higher diosgenin accumulation, indicating a stress-triggered metabolic shift. The FFD further highlighted the significant main and interaction effects of the three microbial factors, with phosphate solubilizers showing the strongest influence on growth and secondary metabolite enhancement. Our findings underscore the dual role of microbial inoculants in alleviating drought-induced growth limitations while simultaneously boosting key bioactive compounds such as diosgenin and stigmasterol. This integrative strategy offers a sustainable pathway to improve both the resilience and phytochemical productivity of *C. borivillianum*, with implications for its conservation, commercial cultivation, and pharmaceutical applications.

Table 6.1 Factors and experimental domain of FD, 2^k

Coded variables (X _i)	Factors (U _i)	Low level (-1)	High level (+1)
X ₁	<i>Azotobacter</i> spp.	Absent 1	Present 2
X ₂	<i>Bacillus</i> Spp.	Absent 1	Present 2
X ₃	AM Fungi (AMF)	Absent 1	Present 2

Table 6.2 Effect of Biotic and Abiotic factors on growth and diosgenin content in *C. borivilianum*

Code	Treatment	Leaves/plant	Roots/plant	Diosgenin (%)
T1	Soil (control)	13	5	0.43
T2	Azotobacter	16	6	0.52
T3	PSB	18	7	0.55
T4	AMF	20	7	0.59
T5	Azo + PSB	22	8	0.62
T6	Azo + AMF	24	8	0.64
T7	Azo + PSB + AMF	28	9	0.67
TB	Cadmium	10	4	0.54
TC	Lead	11	4	0.57
TD	Drought	—	—	0.68
TE	Light	14	5	0.54
TF	Saline	10	4	0.51
TG	Dark	10	4	0.43

Table 6.3 Full factorial design: effect of biotic elicitors under drought stress on growth and secondary metabolites

Run	PS	PM	NF	Growth	Diosgenin (%)	Stigmasterol (%)
1	1	1	1	9	0.70	0.60
2	1	1	2	15	0.81	0.71
3	1	2	1	14	0.75	0.66
4	1	2	2	17	0.83	0.68
5	2	1	1	16	0.87	0.69
6	2	1	2	18	0.89	0.72
7	2	2	1	20	0.91	0.74
8	2	2	2	23	0.96	0.78

Table 6.4 Least squares mean for main effects and interactions

Factor	Level	Growth ($\pm$ SE)	Diosgenin ($\pm$ SE)	Stigmasterol ($\pm$ SE)
PS	1	14.08 $\pm$ 0.17	0.802 $\pm$ 0.011	0.689 $\pm$ 0.016
	2	19.58 $\pm$ 0.17	0.917 $\pm$ 0.011	0.766 $\pm$ 0.016
PM	1	14.83 $\pm$ 0.17	0.839 $\pm$ 0.011	0.708 $\pm$ 0.016
	2	18.83 $\pm$ 0.17	0.879 $\pm$ 0.011	0.747 $\pm$ 0.016
NF	1	15.08 $\pm$ 0.17	0.830 $\pm$ 0.011	0.707 $\pm$ 0.016
	2	18.58 $\pm$ 0.17	0.888 $\pm$ 0.011	0.748 $\pm$ 0.016

Table 6.5 Method validation for diosgenin and β -sitosterol quantification

S/N	Parameters	Diosgenin	Stigmasterol
1	Linearity range (ng/spot)	300-1200	300-1200
2	Correlation coefficient (r^2)	0.992	0.997
3	Regression equation	$Y = 10.21X + 1250$	$Y = 9.381X + 97$
4	Calculated SD value	1.43	1.35
5	^b Limit of detection (LOD) (ng) [3 $\times$ SD/S]	35	30
6	^b Limit of quantification (LOQ) (ng) [10 $\times$ SD/S]	105	90
7	R_f	0.65	0.70
8.	λ_{max} (nm)	436	312
Precision and accuracy			
8	Intra-day RSD (%), n = 5	1.21	1.36
9	Inter-day RSD (%), n = 5	1.62	1.71
Recovery			
10	Amount of standard in plant samples ($\mu\text{g mg}^{-1}$) having maximum bioactive compounds	9.6	7.8
11	Amount of standards added in plant samples ($\mu\text{g mg}^{-1}$)	9, 18, 27	7, 14, 21
12	Amount of standard found ($\mu\text{g mg}^{-1}$)	18.62, 27.53, 36.43	14.61, 21.62, 28.68
13	Recovery (%)	100.10, 99.74, 99.53	98.71, 99.17, 99.58
14	Mean Recovery (%)	99.79	99.15

^a Four concentration levels in triplicate. ^bSD is the standard deviation of the blank response, and S is the slope of the calibration plot

Table 6.6 ANOVA for growth

Source	DF	Adj SS	Adj MS	F	P
PS	1	181.500	181.500	544.50	0.000
PM	1	96.000	96.000	288.00	0.000
NF	1	73.500	73.500	220.50	0.000
PS × PM	1	1.500	1.500	4.50	0.050
PS × NF	1	6.000	6.000	18.00	0.001
PM × NF	1	1.500	1.500	4.50	0.050
PS × PM × NF	1	6.000	6.000	18.00	0.001
Error	16	5.333	0.333		
Total	23	371.333			

Table 6.7 ANOVA for diosgenin content

Source	DF	Adj SS	Adj MS	F	P
PS	1	0.07935	0.07935	54.10	0.000
PM	1	0.00960	0.00960	6.55	0.021
NF	1	0.02042	0.02042	13.92	0.002
PS × PM	1	0.00060	0.00060	0.41	0.531
PS × NF	1	0.00482	0.00482	3.28	0.089
PM × NF	1	0.00007	0.00007	0.05	0.834
PS × PM × NF	1	0.00167	0.00167	1.14	0.302
Error	16	0.02347	0.00147		
Total	23	0.13998			

Table 6.8 ANOVA for stigmasterol content

Source	DF	Adj SS	Adj MS	F	P
PS	1	0.03527	0.03527	12.20	0.003
PM	1	0.00882	0.00882	3.05	0.100
NF	1	0.01042	0.01042	3.60	0.076
PS × PM	1	0.00167	0.00167	0.58	0.459
PS × NF	1	0.00027	0.00027	0.09	0.765
PM × NF	1	0.00135	0.00135	0.47	0.504
PS × PM × NF	1	0.00240	0.00240	0.83	0.376
Error	16	0.04627	0.00289		
Total	23	0.10645			

Table 6.9 Model Strength Based on R² Values

Response Variable	R-squared (R ²)	Adjusted R ²
Growth	98.56%	97.94%
Diosgenin	83.24%	75.90%
Stigmasterol	56.54%	37.52%

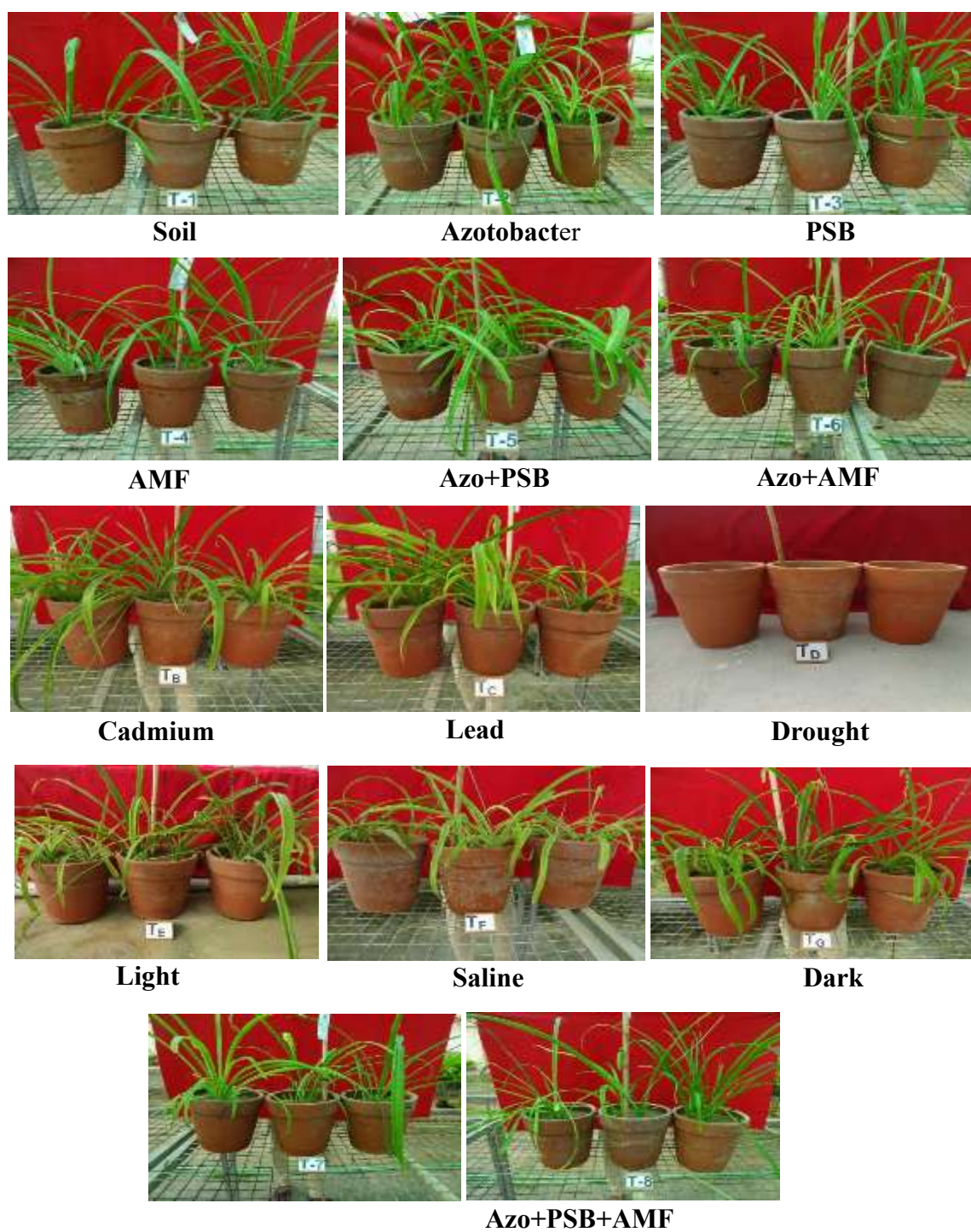


Fig. 6.1A Effect of individual biotic elicitors (AMF, Azotobacter, and PSB) on leaf and root development



Fig. 6.1B The *C. borivilianum* with tubers.

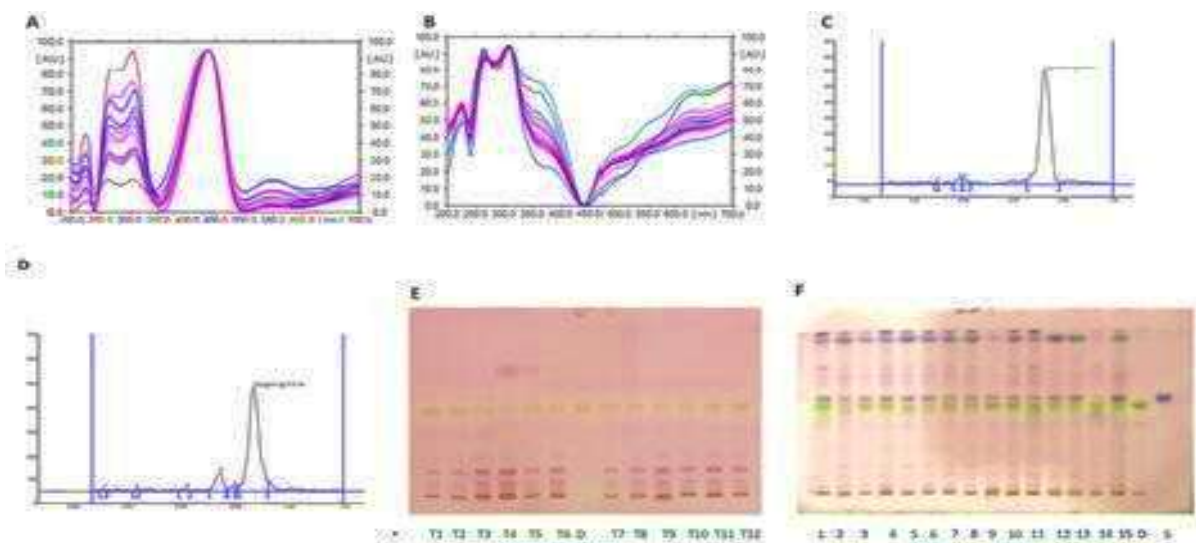


Fig. 6.2 (A) Overlay UV spectra scanning of samples with standards diosgenin from 200 to 700 nm; B) Overlay UV spectra scanning of samples with standards stigmasterol from 200 to 700 nm; (C) Peak for diosgenin; (D) Peak for Stigmasterol; (E) HPTLC chromatograms of samples and standard diosgenin; (F) HPTLC chromatograms of samples, standard diosgenin and stigmasterol.

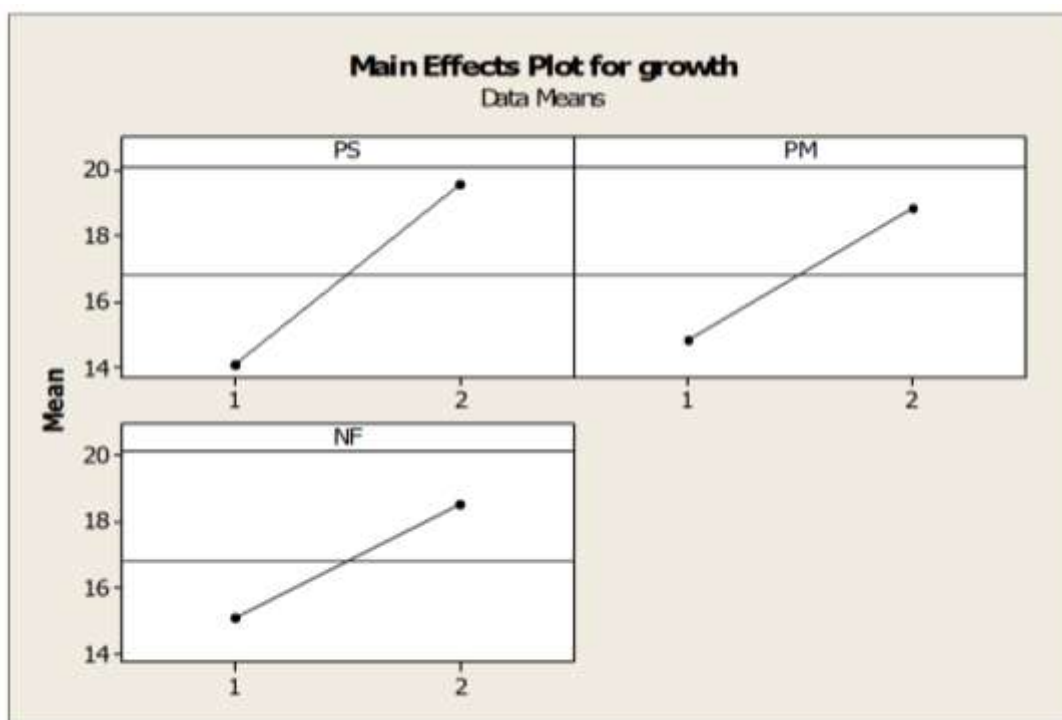


Fig. 6.3 Main effects plot for growth. Figure demonstrated the individual effects of three factors on the mean growth of the plant.

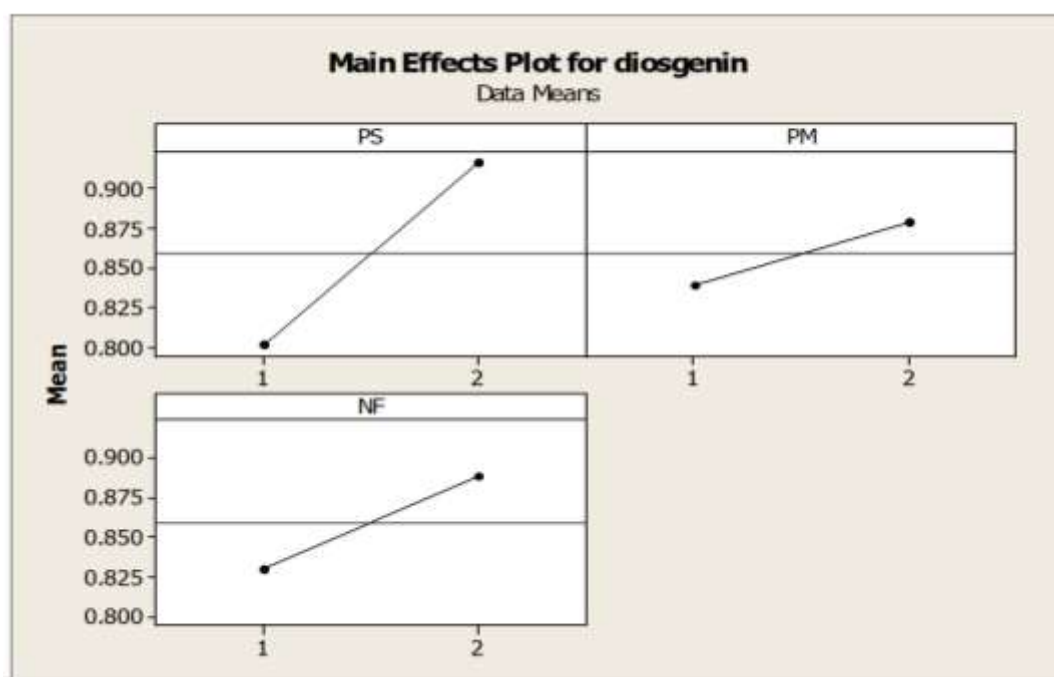


Fig. 6.4 Main effects plot for Diosgenin content. Figure demonstrated the individual effects of three factors on the mean diosgenin content

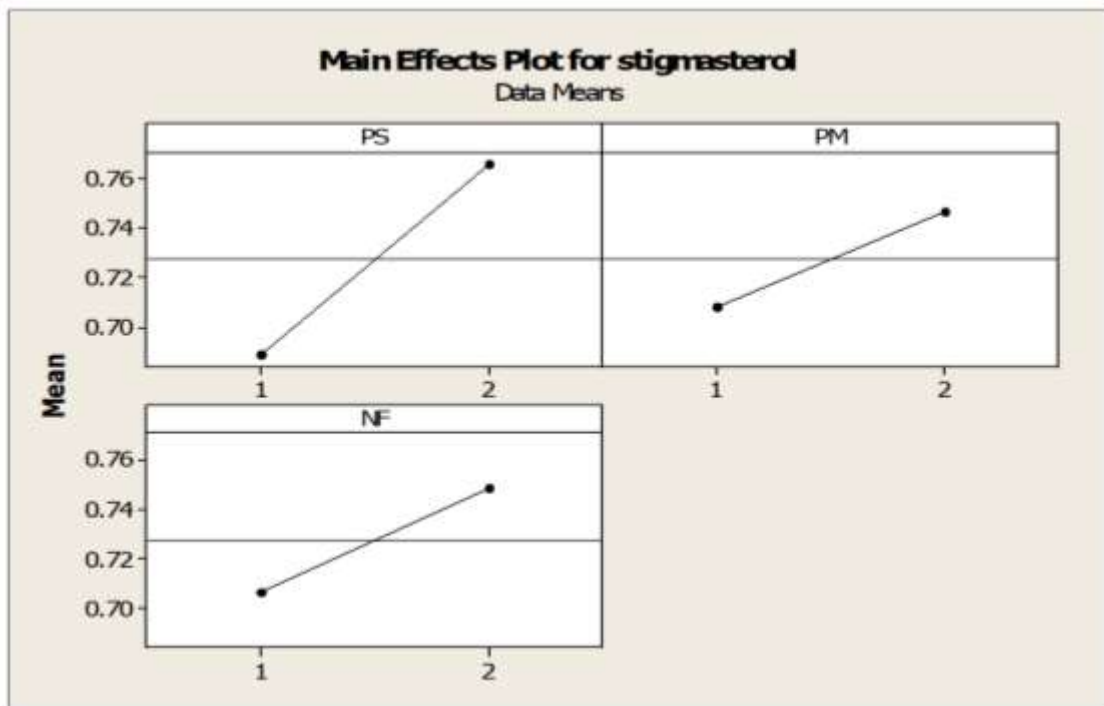


Fig 6.5 Main effects plot for stigmasterol. Figure demonstrated the individual effects of three factors on the mean stigmasterol content.

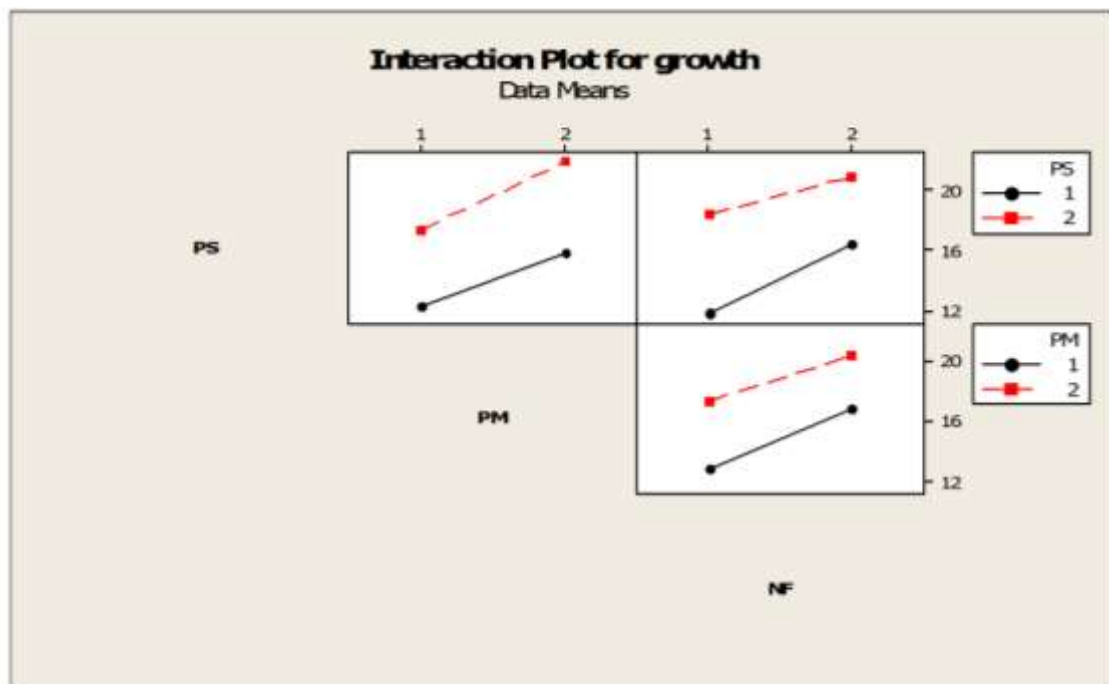


Fig. 6.6 Interaction effects plot for growth of the plant.

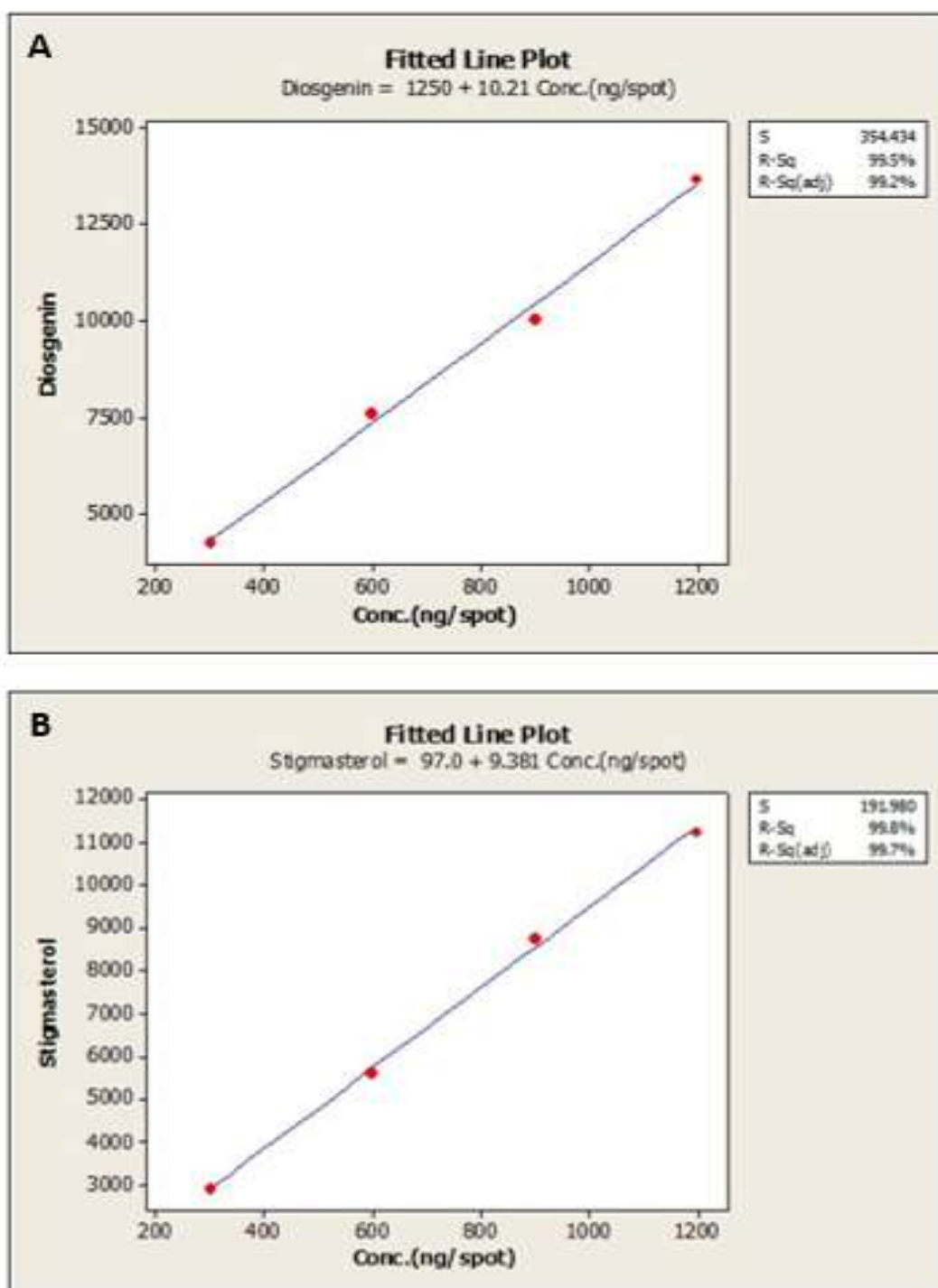


Fig. 6.7 Calibration curve of (A) Diosgenin; (B) Stigmasterol

CHAPTER 7

Summary and Conclusion

7.1 Summary and conclusion

Chlorophytum species were collected from various regions to identify the elite species based on the presence of saponins. The plants were collected from the regions of Punjab, Haryana and Maharashtra. The plant samples collected were screened by HPTLC method of quantification. All samples collected contained saponins but the content varied according to the geographical region. In the case of *Chlorophytum borivillianum*, the highest amount of saponins (Diosgenin and Stigmasterol) was observed in plant samples collected from Akola, Maharashtra.

The enhancement of *in vitro* culture techniques enabled the establishment of a feasible, simple, efficient, or acceptable procedure for the mass production of the *C. borivillianum* and secondary metabolite synthesis. In this study, mT and other cytokinins interactions and combinations led to improved shoot proliferation and auxins promote rooting. Our findings indicate that mT, both alone and in combination with BAP, effectively induced and multiplied shoots. Furthermore, among the plant growth hormones tested, IBA exhibited the most successful rooting. These results underscore the potential of mT and IBA for enhancing shoot and root formation, respectively, in the cultivation of *C. borivillianum*. The procedure will prove useful in sustainable utilization of the species while preserving its native habitat. The *in vitro* cultured plants coped well in the field conditions, and according to ISSR and SCOT markers, no somaclonal variants were found among regenerants. Therefore, it would seem that plants grown *in vitro* are better able to harvest secondary metabolites because they displayed a better content of phytoconstituents. The results could be used to develop an efficient micropropagation method for large-scale plant production with a good quantity of bioactive compounds without introducing any variability.

Our study presents a novel integrative approach that combines the use of different biotic elicitors PS, PM, NF in drought stress under more physiologically relevant conditions. This dual treatment not only promotes plant growth but also significantly raises metabolite accumulation by activating both growth and defence pathways. FFD study emphasizes the synergistic effect of these coupled elements by revealing the optimal conditions for maximal phytochemical production. To our knowledge, this is one of the first research to investigate such an integrative technique in *C. borivillianum* linking the gap between laboratory-scale

metabolite increase and field-applicable sustainability for this important ethnomedicinal plant.

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

The protocols and techniques developed during the present investigation may serve as useful tools for researchers, pharmaceutical industries, and cultivators associated with the production and commercial utilization of saponins. The integration of germplasm screening, micropropagation, HPTLC-based phytochemical analysis, and elicitation strategies provides an effective framework for sustainable production and quality improvement of *Chlorophytum borivilianum*.

Further studies are required to investigate the influence of additional biotic and abiotic elicitors, precursor-feeding strategies, and environmental factors for enhanced secondary metabolite production. Advanced molecular approaches including gene expression analysis, metabolic pathway elucidation, molecular marker-assisted selection, and genetic transformation techniques may also be explored to achieve further improvement in saponin biosynthesis and development of superior elite lines of *Chlorophytum borivilianum*.

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RESEARCH PUBLICATIONS

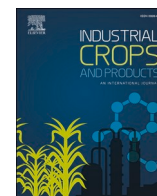
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Paper communicated

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Meta-topolin mediated improved micropropagation, phytochemical profiling, and assessment of genetic fidelity in *Chlorophytum borivilium* Sant. et Fernand., an endangered medicinal herb

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ABSTRACT

A highly effective method has been developed to examine the impact of different combination of meta-topolin and various cytokinins on *Chlorophytum borivilium* Sant. et Fernand. using apical bud explants. The effects of benzyl adenine purine (BAP), kinetin (Kin), meta-topolin (mT), and thidiazuron (TDZ) were explored separately and in different concentrations (ranging from 0.5 to 3.0 mg L⁻¹) on shoot proliferation, as well as the influence of auxins like Indole-3-acetic acid (IAA), α -Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) on rooting. When grown on MS medium supplemented with mT, highest growth rates were observed followed by BAP. Within the permutations tested, 2.0 mg L⁻¹ of mT and 1.0 mg L⁻¹ of BAP produced the best results with 96.3% success rate and average of 19.2 ± 1.92 shoots measuring 6.72 ± 0.49 cm in length after 8 weeks of culture. Among the various auxins used for *in vitro* root formation, 95.5% of multiple shoots effectively established roots on MS medium supplemented with 1.0 mg L⁻¹ of IBA resulting in an average of 14.0 ± 1.39 root numbers and 3.36 ± 0.27 cm in length after 4 weeks of culture. Furthermore, 94% of the acclimated plants not only survived but also thrived when grown in a mixture of loam, sand and fym (in 1:2:1 ratio) with an average weight of 16 g. High-performance thin layer chromatography (HPTLC) analysis revealed the presence of diosgenin and stigmasterol in both *in vitro* regenerants and mother plant, although there were variations in their content. To ensure genetic fidelity among regenerants, ISSR and SCOT markers were used, but no somatic differences were detected. Therefore, the established procedure seems to be easy and consistent for large scale development of genetically identical clones with a greater content of bioactive compounds.

1. Introduction

Throughout prehistoric times, therapeutic plants have served as a valuable reservoir of natural and man-made drugs to treat illnesses. There are 20,000 species that have been used for healing. China and India have long been pioneers in the use of herbal medicines for therapeutic and prophylactic medicinal procedures. Many eminent and famous scientists have developed herbal medicines, and because they are still widely available, traditional medicine practitioners continue to use them (Devendra et al., 2012). Traditional and modern medicinal products are produced using medicinal plants as natural resources and therapeutic agents. A significant quantity of foreign currency can be

generated by commercializing the extraction of pharmaceuticals from herbal plants, in addition to exporting medicinal plants. This then gives indigenous herbal species an economic perspective, encouraging the conservation and development of the plantation regions as well as contributing to the local economy.

Chlorophytum borivilium Sant. et Fernand is an uncommon aphrodisiac plant valued for its desiccated fasciculated roots and is in risk of extinction. Earlier it was a member of family Liliaceae but currently it is categorized under the Asparagaceae family (Shinde et al., 2016). In India it is recognized as Safed musli, in England and the United States referred as Indian spider plant, spider plant, and white musale in France, and Shaqaule in Saudi Arabia (Devendra et al., 2012). In terms of

Abbreviations: IAA, Indole-3-acetic acid; BAP, Benzyl adenine Purine; IBA, Indole-3-butyric acid; NAA, α -Naphthalene acetic acid; Kin, Kinetin; MT, Meta-topolin; TDZ, Thidiazuron; MS, Murashige and Skoogs medium; ISSR, Inter-simple sequence repeat; HPTLC, High Performance Thin Layer Chromatography.

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pharmacological properties, *C. borivilianum* (Root tubers) demonstrated a wide range, including antimicrobial, larvicidal activities, antioxidant hypolipidemic, anthelmintic, analgesic, anticancer, antipyretic, anti-inflammatory, hepatoprotective, antiarthritic, antidiabetic and immunomodulatory (Khanam et al., 2013). The safed musli roots are widely employed in the Ayurveda and Unani medical system for a range of remedial purposes due to the presence of saponins being one of the key phytochemical components (Basu and Jha, 2014). Besides saponins, the roots of *Chlorophytum* plants represents a substantial reservoir of carbohydrates, amino acids, polysaccharides, steroids, alkaloids, vitamins, resins, and phenol (Thakur et al., 2009). *C. borivilianum* is a highly valuable and well-known medical shrub. In the Indian market, the desiccated fasciculated roots sell for an alluring price of \$30 USD per kg (Dave et al., 2003). Overharvesting of the species from the natural has resulted from constantly rising requirement in domestic (35 000 t) and international (300–700 t) marketplaces (Khanam et al., 2013). Safed musli rarely propagates through seeds, but rather vegetatively through its thick roots. The gap between supply and demand has been made wider by the rapid loss of habitat, destructive harvesting methods, extended quiescence (Kaur et al., 2009), and limited seed germination (Rizvi et al., 2007). Therefore, high-quality planting material is needed in large quantities for the marketing of this crop, which calls for the development of an efficient, rapid, and profitable propagation technique.

The use of biotechnological techniques can provide the perfect way to get around the current issues. The reports that describe tissue culture-based propagation of *C. borivilianum* are mostly focused on proliferation of shoots derived from shoot base explants or stem disc (Dave et al., 2003; Kumar et al., 2010; Samantaray and Maiti, 2010a,2010b) or somatic embryogenesis arising from callus tissue derived from shoot meristemoid or stem disc (Arora et al., 1999). Several studies have contributed significantly to the understanding and advancement of tissue culture techniques in *Chlorophytum* species, particularly *Chlorophytum borivilianum*, an endangered medicinal herb. Rizvi et al. (2010) demonstrated successful micropropagation of *C. borivilianum*, providing a foundation for further research in this area. Samantaray and Maiti, (2010a),(2010b) expanded upon this work by not only propagating the plant *in vitro* but also assessing the genetic fidelity of the regenerated plants, ensuring their authenticity. Joshi and Purohit (2012) optimized factors that *in vitro* propagation of *C. borivilianum*, further refining the tissue culture techniques for this species. Additionally, Khatri et al. (2019) focused on the effect of additives on enhanced micropropagation aspect, providing insights into the optimal conditions for mass multiplication of this valuable medicinal herb. These studies collectively highlight the diversity of approaches employed in tissue culture research of *Chlorophytum* species and underscore their significance for the conservation and sustainable utilization of these valuable plants. However, there are very few reports available on direct organogenesis-based *in vitro* growth of *C. borivilianum*.

It is crucial to emphasize that, even though roots of *C. borivilianum*'s abundant in saponin are the main ingredient in numerous herbal preparations and are commonly employed in their raw form, comprehensive data regarding the quantification of sapogenins, saponins, and other bioactive compounds specific to root remain undisclosed for various populations growing in distinct agroclimatic regions across India. To keep consistency, guarantee quality, and maximise the effectiveness of the production of herbal drugs, *in vitro* clonal multiplication of highly productive varieties or populations holds significant appeal from an economic and pharmacological standpoint. Thus, in the current investigation, efforts were made to establish a dependable procedure for direct *in vitro* regeneration of safed musli along with genetic and phytochemical assessment of the *in vitro* regenerants, taking into consideration the gaps and inadequacies of the prior research.

2. Materials and methods

2.1. Plant material and culture conditions

In the September, 2018, *C. borivilianum* plants were collected from Haryana, India. On the basis of morphology, identification was done by Taxonomist and specimens were deposited in Herbarium of Lovely Professional University, Punjab, (Voucher Specimen No.35698).

The explants apical buds taken from the mother plant were extensively cleaned in distilled water after being thoroughly washed in flowing tap water for 20 min, cleansed for 10 min with tween-20 (Sisco Research Laboratories Pvt Ltd, India). Finally, externally disinfected for 3–5 min with mercuric chloride 0.1% (Merck Specialties Pvt. Ltd, Mumbai, India) and rinsed repeatedly in Laminar Air Flow Chamber with distilled water 3–4 times and inoculated on MS (Murashige and Skoog, 1962) supplemented with 0.7% agar and 3% sucrose and different plant growth regulators. Medium pH was attuned to 5.8 and autoclaved 121 °C for 15 min at 105 kPa. The cultures were sustained in a growth chamber having 16 h light and 8 h dark cycle receiving light from cool-white fluorescent lamps with intensity 45–50 $\mu\text{mol m}^{-2}\text{s}^{-1}$ at incubation temperature $25 \pm 1^\circ\text{C}$ and relative humidity 40–50%.

2.2. Shoot bud induction and multiplication

The morphogenic response of the apical bud explants was assessed using MS medium that had been enriched with various growth hormones such as BAP, Kin, TDZ or mT (Sigma-Aldrich and Himedia India) at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg L^{-1}) either separately or in conjunction for axillary shoot induction and multiplication (Table 1). Five explants were placed in each Erlenmeyer flasks (250 cm^3) with 50 cm^3 experimental media. Plantlets that had been successfully initiated from the most effective medium were transferred to the same medium for shoot multiplication, and after 4 weeks of culture, the number and length of shoots were recorded based on the response percentage (%) of explants.

2.3. Rooting, tuberization and acclimatization

The 8 week old fully developed shoots of average length 5.5 cm were shifted to rooting media augmented with varying concentrations of IAA, NAA and IBA for 4 weeks (Table 2) and after 4 weeks, culture response (%), root number per shoot and length were recorded. After proper root formation, the *in vitro* rooted plants were transplanted to tuber media enriched with IBA (0.75 mg L^{-1}), NAA (0.5 mg L^{-1}) and varying amount of sucrose (20 – 60 g L^{-1}) for 6 weeks. Culture media devoid of any plant hormone were set as controls.

In vitro grown healthy plantlets of 14 weeks old with well-developed roots were rinsed thoroughly to completely remove residues of agar and planted in plastic pots containing different combination of sand, Fym, loam, perlite and vermicompost in various ratios as presented in Table 4 and kept in growth chamber for 15–20 days. After required time period the plantlets were shifted to experimental plots and the percentage of their survival was recorded.

2.4. Phytochemical analysis

2.4.1. Sample preparation

C. borivilianum sample was dried under shade and finely powdered using electric mixer grinder (Philips JMG4-HL7581/00). 1 g sample of each soil type plants were subjected to maceration with 30 mL ethanol (50%) for 24 h and then sonicated for 10 min. The extracts of each sample were strained through Whatman filter paper No.1 and dehydrated in rotary evaporator (Buchi, R-300, India) at 40 °C. Following this, 20 mL of 10% hydrochloric acid (HPLC grade, E. Merck, India) were added to the dried remains of each sample. These samples were subjected to acid hydrolysis using water bath for 1 hr at 98 °C to isolate the

Table 1
Effect of different cytokinins on *in vitro* shoot induction and multiplication of *Chlorophytum borivilianum* after 8 weeks of culture.

MS + growth regulators (mg L ⁻¹)				Culture response (%)	Mean no of shoots/explant produced	Mean shoot length (cm)
BAP	Kin	TDZ	mT			
0	0	0	0	00.0	0.0 ± 0.0	0.0 ± 0.0
0.5				28.3 ^{s,t}	3.8 ± 1.30 ⁿ	2.40 ± 1.14 ^m
1.0				38.7 ^p	5.6 ± 0.89 ^j	2.60 ± 0.89 ⁱ
1.5				64.2 ^{e,f}	6.7 ± 0.89 ^f	3.32 ± 0.45 ^e
2.0				72.6 ^c	8.8 ± 1.30 ^d	3.22 ± 0.31 ^{e,f}
2.5				69.1 ^{d,e}	8.1 ± 1.22 ^e	2.92 ± 0.19 ^f
3.0				61.8 ^g	6.0 ± 0.70 ^{g,h}	1.88 ± 0.51 ^r
	0.5			23.0 ^t	2.4 ± 1.14 ^s	1.96 ± 0.91 ^q
	1.0			29.5 ^s	2.8 ± 1.48 ^r	2.14 ± 0.87 ⁿ
	1.5			47.3 ^k	3.5 ± 1.14 ^p	2.26 ± 0.56 ⁿ
	2.0			62.9 ^f	5.8 ± 2.58 ⁱ	2.70 ± 0.48 ^{h,i}
	2.5			56.0 ^h	5.6 ± 1.14 ^j	2.50 ± 0.45 ^k
	3.0			45.2 ^l	3.6 ± 3.05 ^o	1.96 ± 0.30 ^q
		0.5		19.8 ^u	2.2 ± 1.64 ^t	1.68 ± 0.26 ^s
		1.0		30.5 ^r	3.1 ± 0.83 ^q	2.56 ± 0.35 ^j
		1.5		39.3 ^o	3.7 ± 0.83 ^{n,o}	2.71 ± 0.45 ^h
		2.0		52.9 ⁱ	6.1 ± 0.83 ^g	2.78 ± 0.68 ^g
		2.5		50.4 ^j	5.9 ± 1.58 ^h	2.46 ± 0.49 ⁱ
		3.0		44.7 ^m	4.4 ± 0.83 ^l	2.06 ± 0.72 ^p
			0.5	33.3 ^q	3.9 ± 1.14 ^m	2.56 ± 0.47 ^j
			1.0	41.7 ⁿ	4.8 ± 1.30 ^k	3.76 ± 0.43 ^d
			1.5	69.2 ^d	9.1 ± 2.0 ^c	4.08 ± 0.13 ^c
			2.0	87.6 ^a	12.4 ± 1.14 ^a	4.76 ± 0.95 ^a
			2.5	82.1 ^b	11.8 ± 1.92 ^b	4.14 ± 0.66 ^b
			3.0	64.5 ^e	8.0 ± 2.23 ^{e,f}	2.74 ± 0.86 ^{g,h}

Means within each column followed by the same letter are not significantly different at *P* < 0.05 using DMRT

saponins (Nazir et al., 2022). Chloroform was added to each sample after the completion of acid hydrolysis resulting in the formation of two distinct layers, upper layer was eliminated and lower layer was washed again with chloroform and subsequent drying via a rotary evaporator. The dried residue was then treated with methanol, subjected to syringe filtration, and subsequently utilized for quantitative analysis through HPTLC.

2.4.2. HPTLC analysis of diosgenin and stigmasterol

This work made use of an HPTLC system utilizing a Linomat5 applicator CAMAG (Switzerland) equipped with a 100 mL syringe, a TLC scanner-3 run, in conjunction with Win CATS software (version: 1.4.6.2002). HPTLC was performed using toluene: ethyl acetate (7:3 v/v) as the mobile phase on precoated silica gel 60 F254 20 × 10 cm TLC plates. Using a Linomat-5 applicator CAMAG fitted with a 100 mL syringe adjusted to the specified settings: band length 6 mm, application rate 10 sec/mL, 8 mm gap between bands, distance from the side edge of the plate 1.5 cm, and distance from the plate's bottom 2 cm, the 3 mL of each sample of *C. borivilianum* and marker compounds (diosgenin and stigmasterol) solution was administered to the plates in the form of bands measuring 6 mm in width. Anisaldehyde reagent (1 mL anisaldehyde, 20 mL glacial acetic acid, 170 methanol, and 10 mL conc. sulphuric acid) was sprayed on the plates to post-derivatize them (Nazir et al., 2021b). The plates were then subjected to a 5 min drying process

Table 2
Effect of optimized level of mT (2.0 mg L⁻¹) with cytokinins after 8 weeks of culture.

MS + growth regulators (mg L ⁻¹)				Culture response (%)	Mean no of shoots/explant produced	Mean shoot length (cm)
mT	Kin	TDZ	BAP			
0	0	0	0	00.0	0.0 ± 0.0	0.0 ± 0.0
2.0	0.5			78.3 ⁱ	11.6 ± 1.51 ^h	3.41 ± 1.14 ^k
	1.0			88.7 ^d	13.7 ± 1.34 ^{e,f}	4.62 ± 0.89 ^d
	1.5			83.2 ^e	10.2 ± 1.30 ⁱ	3.32 ± 0.45 ^l
	2.0			70.6 ^m	9.8 ± 1.30 ^k	3.22 ± 0.31 ^m
	2.5			64.1 ^p	6.5 ± 1.18 ⁿ	2.92 ± 0.19 ^o
	3.0			60.8 ^q	5.6 ± 1.34 ^p	2.38 ± 0.51 ^q
2.0		0.5		78.7 ⁱ	11.8 ± 0.83 ^g	3.65 ± 0.91 ⁱ
		1.0		82.6 ^f	12.0 ± 1.58 ^f	3.96 ± 0.87 ^g
		1.5		80.3 ^g	10.1 ± 0.83 ^{i,j}	3.78 ± 0.56 ^h
		2.0		72.9 ^k	8.8 ± 0.83 ^l	2.71 ± 0.48 ^p
		2.5		66.0 ⁿ	7.8 ± 0.83 ^m	2.13 ± 0.45 ^r
		3.0		65.2 ^o	6.2 ± 1.64 ^{n,o}	2.96 ± 0.30 ⁿ
2.0			0.5	89.8 ^c	17.0 ± 2.70 ^c	6.06 ± 0.11 ^b
			1.0	96.3 ^a	19.2 ± 1.92 ^a	6.72 ± 0.49 ^a
			1.5	94.5 ^b	18.6 ± 1.34 ^b	5.18 ± 0.26 ^c
			2.0	79.9 ^h	16.6 ± 1.14 ^d	4.54 ± 0.42 ^e
			2.5	75.4 ^j	13.8 ± 1.30 ^e	4.28 ± 0.44 ^f
			3.0	71.7 ^l	10.0 ± 1.58 ^j	3.51 ± 0.41 ^j

Means within each column followed by the same letter are not significantly different at *P* < 0.05 using DMRT

at 100 °C in a hot air oven (Bionics Scientific, India). Using the Camag TLC scanner 3 and Win Cats software, the plates were scanned at 433 nm reflectance wavelength after the formation of the bands. By comparing the extract peak's retardation factor (R_f) to that of the marker compounds, the concentration was determined from the calibration curves.

2.5. Clonal fidelity of *in vitro* regenerants

ISSR and SCOT markers were used to examine the genetic integrity of plants cultured under *in vitro* condition. Young, healthy leaves (100–200 mg) of both source plants and cultured plantlets were harvested and total genomic DNA was extracted using the CTAB technique (Doyle and Doyle, 1990). Additionally, the amount and purity of DNA were assessed using a 0.8% agarose gel. After that, TE buffer (Tris-EDTA; 25 ng µl⁻¹) was added, and the samples were preserved at 4 °C before being used in subsequent studies. In order to assess the genetic stability of the plants produced from tissue culture, 5 ISSR primers and 5 SCOT primers (Merck Biosciences, Germany) were chosen, three of these primers of ISSR and SCOT (Table 3) displayed distinct and reproducible bands. In a Gradient Thermal Cycler (iGene Labserve or G-storm), polymerase chain reactions (PCR) were performed. The amplification cycle consisted of an initial denaturation step at 94 °C for 3 min, 35 cycles of 94 °C for 20 sec (denaturation), 58 °C for 30 sec (annealing), 72 °C for 2 min (extension) and 1 cycle of 72 °C for 10 min (final extension). With the aid of TAE 1x solution and agarose (2.5%) gel electrophoresis, amplification products were separated, and the sizes of the amplicons

Table 3Effect of different auxins on *in vitro* rooting of *Chlorophytum borivilianum* after 4 weeks of culture.

MS + growth regulators (mg L ⁻¹)			% culture response	Roots/explant	Root length (cm)
NAA	IAA	IBA			
0	0	0			
0.1			42.6 ^q	5.4 ± 0.39 ^m	1.35 ± 0.16 ^q
0.2			58.1 ^p	7.8 ± 1.14 ^j	1.91 ± 0.20 ⁿ
0.5			85.3 ^f	10.6 ± 0.72 ^e	2.16 ± 0.14 ^k
1.0			80.2 ^h	9.4 ± 1.32 ^h	2.36 ± 0.21 ⁱ
	0.5		66.8 ^m	4.8 ± 0.23 ^o	1.56 ± 0.11 ^p
	1.0		61.4 ⁿ	6.6 ± 0.98 ^l	2.52 ± 0.15 ^h
	1.5		77.1 ⁱ	8.6 ± 0.91 ⁱ	1.64 ± 1.41 ^o
0.5	0.5		72.7 ^k	7.8 ± 1.15 ^j	2.10 ± 1.59 ^l
	1.0		83.3 ^g	9.9 ± 0.74 ^f	2.04 ± 0.57 ^m
	1.5		91.2 ^c	11.0 ± 1.39 ^d	2.86 ± 0.27 ^e
		0.25	60.3 ^o	5.0 ± 0.91 ⁿ	2.64 ± 1.41 ^g
		0.5	73.1 ^j	6.8 ± 1.15 ^k	2.80 ± 1.59 ^f
		0.75	88.7 ^d	12.4 ± 0.74 ^{c,d}	3.14 ± 0.57 ^d
		1.0	95.5 ^a	14.0 ± 1.39 ^a	3.36 ± 0.27 ^a
0.5	0.5		68.9 ^l	9.5 ± 1.15 ^g	2.20 ± 1.59 ^j
	0.75		87.5 ^e	12.8 ± 0.74 ^c	3.16 ± 0.57 ^c
	1.0		93.1 ^b	13.2 ± 1.39 ^b	3.31 ± 0.27 ^b

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

were measured and compared using a 100 bp DNA ladder (GeneRuler SM0321, Thermo Scientific; USA).

2.6. Statistical analysis

Each treatment included 10 explants, and all tests were carried out in

triplicate. Using the SPSS 16 (SPSS Inc.; Chicago, USA) statistical analysis programme, observations of shoot induction, root development and tuber formation were periodically assessed and analysed using one-way analyses of variance (ANOVA). Mean differences were successfully achieved through the application of Duncan's multiple range test (DMRT) at a confidence level of 95%.

3. Result and discussion

3.1. Effect of individual cytokinins on *in vitro* shoot initiation and multiplication

Explants grown in control (MS medium) produced no morphogenic response. However, after a week of inoculation, emergence of shoot was observed in all examined doses of BAP, Kin, mT, or TDZ. In regards to promoting shoot buds per explant, mT exhibited a more robust response compared to BAP, Kin, or TDZ of the cytokinins that were tested individually. Following 8 weeks of inoculation, it was determined that the MS medium fortified with 2.0 mg L⁻¹ mT produced the best (87.6%) morphogenic response with a maximum average shoot number (12.4 ± 1.14) and shoot length (4.76 ± 0.95) (Fig. 1a, b). The number of shoots reach its peak level at mT (2.0 mg L⁻¹), after which a decline in growth metrics was noticed (Table 1). The sequence of response in the shoot initiation and multiplication was noticed as mT > BAP > Kin > TDZ. Culture response (28.3–72.6%) with 3.8 ± 1.30 – 8.8 ± 1.30 mean shoot number/explant was recorded in the media fortified with BAP. Kin supplemented media produced 2.4 ± 1.14 – 5.8 ± 2.58 mean number of shoots having average shoot length 1.96 ± 0.91 – 2.7 ± 0.48 cm. TDZ containing media showed response in the range of 19.8–52.9% producing 2.2 ± 1.64 – 6.1 ± 0.83 shoots. Previous studies have indicated

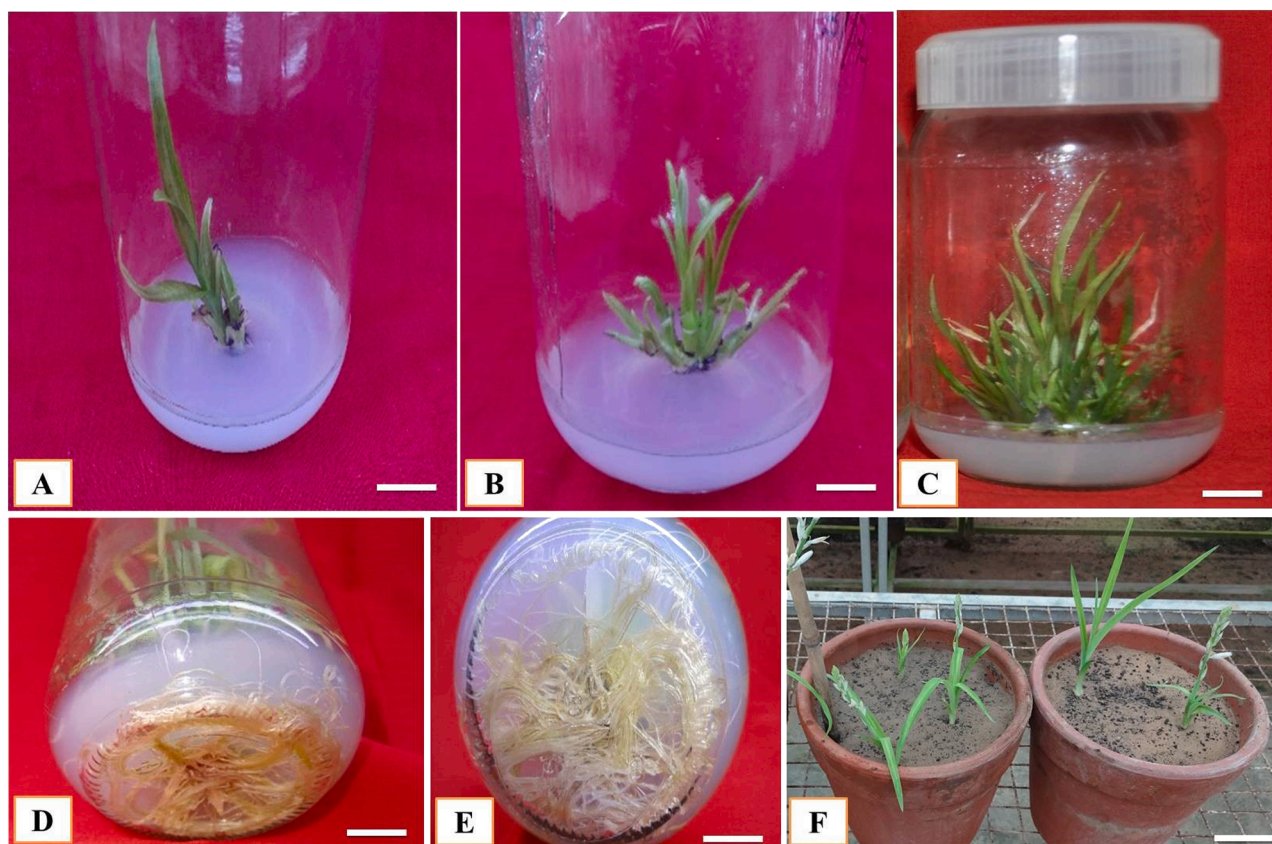


Fig. 1. (A) Shoot induction in *C. borivilianum* on MS medium augmented with 2.0 mg L⁻¹ mT after 3 weeks. (B) Shoot induction in *C. borivilianum* on MS medium augmented with 2.0 mg L⁻¹ mT after 8 weeks of culture. (C) Shoot multiplication in MS medium enhanced with 2.0 mg L⁻¹ mT + 1.0 mg L⁻¹ BAP, after 8 weeks of culture. (D, E) *In vitro* rooting on MS medium fortified with 1.0 mg L⁻¹ IBA after 4 weeks of culture. (F) Acclimatization of *in vitro* rooted plant.

that the optimal medium for shoot proliferation of Safed musli includes supplementation with 3.0 mg L^{-1} BAP, resulting in an average shoot count of 18.90 after 28 days of inoculation (Kemat et al., 2010). This finding is consistent with reports from various other studies (Ashraf et al., 2014; Khatri et al., 2019), suggesting that BAP outperforms other cytokinins in initiating and proliferating shoots. In our current investigation, BAP demonstrated superiority over Kin and TDZ, with mean shoot counts and lengths of 8.8 shoots and 3.22 cm, respectively (Fig. 2).

The cytokinin mT has been discovered to exhibit efficacy in enhancing morphogenic processes across various species, including *Curcuma longa* (Salvi et al., 2002), *Musa* species (Escalona et al., 2003), *Aloe* spp. (Bairu et al., 2007), *Pelargonium* varieties (Wojtania, 2010), *Pyrus communis* (Dimitrova et al., 2016), *Allamanda cathartica* (Khanam et al., 2019), *Ribes grossularia* (Kucharska et al., 2020), *Scaevola taccada* (Shekhawat et al., 2021), *Iris* × *hollandica* (Verma et al., 2022) and *Caralluma umbellata* (Jayaprakash et al., 2023). The efficacy of mT for promoting maximum shoot initiation is due to the existence of hydroxyl group on its side chain in its fine chemical structure, which aids in the production of non-toxic and readily interchangeable forms of cytokinin storage known as O-glucoside conjugate forms. Additionally, it moves quickly and readily within plant tissues (Werbrouck et al., 1996; Amoo et al., 2011; Kaminek et al., 1987). Cultures stimulated by mT were robust and exhibited no aberrations, such as hyperhydricity or shoot tip necrosis, over the course of extended cultures, as was seen with other cytokinins. There have been reports of Tang et al. (2002), Petri and Scorza (2010), Aremu et al. (2012), Mala et al. (2013), Naaz et al. (2019), Hussain et al. (2019), Singh and Kumaria, (2020), Manokari et al. (2022) reporting comparable morphogenic reactions induced by mT on cultures grown *in vitro*. Anis et al. (2023) found that mT at $5.0 \text{ }\mu\text{M}$ significantly enhanced shoot induction and proliferation in *Feronia limonia*, outperforming BA. In a study by Kaur et al. (2022), mT proved superior in promoting shoot regeneration in *Crinum brachynema* cultures, with $5.0 \text{ }\mu\text{M}$ mT resulting in the highest shoot regeneration frequency (11.10) compared to the control. No reports on the application of topolins in the micropropagation of *Chlorophytum* species exist as of yet. This study represents the first investigation on the use of mT on *C. borivilianum* that we are aware of.

3.2. Effect of optimized concentration with other cytokinins

The establishment of a successful procedure for the large-scale multiplication and preservation of elite genotypes depends critically on the choice of cytokinin type, concentration, and combination with other cytokinins. The combination of mT with BAP was determined to be



Fig. 2. HPTLC fingerprinting of *in vitro* regenerants (P1, P2, P3) and mother plant (MP) compared with standard biomarker diosgenin (D) and stigmasterol (S).

more proficient in shoot elongation and proliferation than any other combinations examined. After 8 weeks of culture, the MS medium enriched with 2.0 mg L^{-1} mT and 1.0 mg L^{-1} BAP exhibited the best response (96.3%) with a mean of shoots 19.2 ± 1.92 and $6.72 \pm 0.49 \text{ cm}$ in length (Table 2, Fig. 1 C). The sequence of response in the shoot initiation and multiplication was noticed as $\text{mT} + \text{BAP} > \text{mT} + \text{Kin} > \text{mT} + \text{TDZ}$. In the mT and Kin combination, 88.7% response was observed at 1 mg L^{-1} Kin with 13.7 ± 1.34 mean shoot numbers having $4.62 \pm 0.89 \text{ cm}$ average length. With TDZ, 82.6% response was noticed at 1 mg L^{-1} TDZ concentration with 12.0 ± 1.58 mean number of shoots having $3.96 \pm 0.87 \text{ cm}$ average length. With increase in the concentration of BAP, Kin, TDZ decline in the response was observed. The adventitious shoot regeneration from apical bud explants in *C. borivilianum* must therefore be maximized by cytokinin combinations. The morphogenic response in apical bud explants was recorded in Table 2 for the mT and other cytokinin combinations, whereas Kin and TDZ combinations with mT exhibited less shoots as compared to BAP.

Research on the synergistic effects of meta-topolin (mT) with other cytokinins in *in vitro* shoot induction and multiplication has provided valuable insights into their combined efficacy. When combined with other cytokinins/auxins, the mT has been shown to produce the highest shoot regeneration rates in a variety of plant species, including *Pterocarpus marsupium* (Ahmad and Anis, 2019); *Coleonema album* (Fajinmi et al., 2014). Nowakowska and Pacholczak (2020) investigated the effect of mT in combination with BAP on *Daphne mezereum* cultures, finding that at a concentration of 1 mg L^{-1} showed superior effects on shoot regeneration explants compared to BA at the same concentration, indicating its potential as an alternative cytokinin. The cytokinin profiling study by Novák et al. (2008) provided further evidence of the importance of cytokinins, including meta-topolin, in plant tissues, highlighting their diverse roles in growth and development. Anis et al. (2023) observed that combining $5.0 \text{ }\mu\text{M}$ mT with $0.50 \text{ }\mu\text{M}$ NAA resulted in the highest mean shoot numbers and average shoot length after 12 weeks of culture. These studies highlight the potential synergistic effects of mT with other cytokinins, emphasizing the importance of optimizing their concentrations for enhanced shoot proliferation and rooting in tissue culture systems. Furthermore, cytokinins engage with each other at different amounts in diverse species to produce the best possible physiological reaction (Coenen and Lomax, 1997).

3.3. In vitro rooting of regenerated microshoots

Three auxins, NAA, IAA and IBA were tested for *in vitro* rooting proficiency in regenerated shoots. Microshoots failed to develop roots on the control medium even after four weeks of culture, but formation of root was aided by the introduction of various auxins to the MS medium. The most successful *in vitro* root initiation (95.5%) was accomplished by incorporating 1.0 mg L^{-1} IBA on MS liquid medium, which after 4 weeks of culture caused maximal (14.0 ± 1.39) average root number and ($3.36 \pm 0.27 \text{ cm}$) average root length (Table 3, Fig. 1D, E). Compared to IAA and NAA, IBA produced more roots (Fig. 1c). NAA at 0.5 mg L^{-1} showed 85.3% culture response producing 10.6 ± 0.72 mean root numbers with average root length $2.16 \pm 0.14 \text{ cm}$. Culture response ranges from 66.8% to 77.1% in media enriched with different concentrations of IAA with mean root numbers 4.8 ± 0.23 – 8.6 ± 0.91 . The optimized concentration of NAA (0.5 mg L^{-1}) was used in conjunction with IAA and IBA and in this combination, IBA at 1.0 mg L^{-1} showed maximum response (93.1%) producing 13.2 ± 1.39 mean shoot number with mean shoot length $3.31 \pm 0.27 \text{ cm}$. Integration of IAA and NAA (0.5 mg L^{-1}) showed culture response in the range of 72.7–91.2% producing 7.8 ± 1.15 – 11.0 ± 1.39 mean number of shoots.

Auxins are essential for rooting. IBA is the most prevalent natural auxin and is reported to be more stable in culture media than IAA (Pacurar et al., 2014). Different species of plants have documented similar effects of IBA on *in vitro* induction of roots in micro shoots (Spethmann and Hamzah, 1988; Ludwig-Müller, 2000; Perveen et al.,

2013; Hussain et al., 2018, 2019). IBA's fine structure, stability, and ease of translocation within plant tissues give it an advantage over other growth hormones in promoting robust root growth (Ludwig-Müller, 2000; Hussain et al., 2018). Another explanation why IBA gives the greatest promotional response might be because it is kept in free auxin conjugate forms, which are generated through hydrolysis and released as needed during the culture process (Nordstrom et al., 1991; Yang and Davies, 1999). Recent research has demonstrated the effectiveness of IBA in promoting root formation in tissue-cultured medicinal plants. Thorat et al. (2022) found that IBA significantly promoted root formation in tissue-cultured *Withania somnifera*. Similarly, Ahmed (2022) reported that IBA was more effective than NAA in inducing rooting in tissue-cultured *Lycium barbarum*. Additionally, Tadhani et al. (2006) and Patel and Shah (2009) observed that IBA was the most effective auxin for root induction in tissue-cultured *Stevia rebaudiana*. Anis et al. (2023) achieved the highest efficiency of *in vitro* root induction (90%) on $\frac{1}{2}$ MS medium supplemented with 5.0 μ M IBA, resulting in a mean root number of 3.20 ± 0.20 and mean root length of 4.52 ± 0.10 cm after four weeks of culture. These studies highlight the continued relevance of IBA as a rooting hormone in tissue culture systems for medicinal plants.

3.4. *In vitro* tuberization of *C. borivilianum*

For the *in vitro* tuberization of *C. borivilianum*, the best rooting media NAA 0.5 mg L⁻¹ and IBA 0.75 mg L⁻¹ that produced the best quantity of roots and length were further modified with five concentrations of sucrose (20–60 g L⁻¹). The findings showed that the treatments had a substantial impact on the weight, and number of tubers. In comparison to the tuber count obtained through the other treatments, the maximum number of tubers (8 ± 0.69) with a weight of 6.6 ± 0.77 g was produced on 50 g L⁻¹ sucrose (Table 4). Sucrose at 40 and 60 g L⁻¹ almost showed same no of tubers 7 ± 0.67 and 7 ± 0.68 with 6.3 g and 6.5 g fresh average weight. Very less difference was observed in media fortified with 40 and 60 g L⁻¹ sucrose in terms of tuberization. However, optimal concentration of sucrose for tuber formation of *C. borivilianum* was found as 50 g L⁻¹. Minimum tubers were produced in media fortified with 20 g L⁻¹ sucrose producing 4 ± 0.37 tubers with 4 ± 0.34 g average weight. These results align with the research conducted by Nazir et al. (2022), where impact of varying sucrose concentrations was investigated (40, 60 and 80 g L⁻¹) with BAP 0.9 mg L⁻¹ in *D. deltoidea* *in vitro* culture and documented 60 g L⁻¹ sucrose concentration optimum for tuber production as compared to 40 and 60 g L⁻¹. Moreover, in another study led by Kohmura et al. (1995) also reported 60 g L⁻¹ sucrose to be more efficient than 30 g L⁻¹ concentration of sucrose for tuber formation. Enzymatic activity may get possibly induced at high sucrose supplementations for the starch production and its accrual in storage tissues (Staikidou et al., 2005). According to the current research, sucrose played a vital part in the *in vitro* tuberization process because the number and lengths of *in vitro* tubers obtained at 50 g L⁻¹ sucrose were optimal. A comparable finding was also observed by Chauhan et al. (2018) during *in vitro* tuber formation of *C. borivilianum* and

Table 4
Effect of different sucrose concentration on *in vitro* tuberization of *Chlorophytum borivilianum* after 6 weeks.

$\frac{1}{2}$ MS + PGR (mg L ⁻¹)			No of rooted plants showing tuber induction	Average tuber fresh weight per plant (g)
NAA	IBA	Sucrose (g L ⁻¹)		
0.5	0.75	20	4.0 ± 0.37^d	4.0 ± 0.34^e
0.5	0.75	30	6.0 ± 0.57^c	5.8 ± 0.47^d
0.5	0.75	40	7.0 ± 0.67^b	6.3 ± 0.57^c
0.5	0.75	50	8.0 ± 0.69^a	6.6 ± 0.77^a
0.5	0.75	60	7.0 ± 0.68^b	6.5 ± 0.67^b

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

microrhizome formation in *Acorus calamus* (Quraishi et al., 2017). (Table 5)

3.5. Acclimatization and hardening of *in vitro* plants

Any micropropagated protocol developed will be successful or unsuccessful based on the capacity of *in vitro* developed plantlets to endure in natural environmental settings (Anis and Ahmad, 2016; Naaz et al., 2019). Plantlets have to shift from a protective to a non-protective environment in the open at this crucial stage, which could be harmful to their survival. As a result, it might not be feasible to move *in vitro* developed plantlets directly to field conditions (Kant et al., 2010; Hussain et al., 2018). Plantlets with well-developed root system having three to five completely formed leaves were delicately placed in thermocol containers filled with sterilized soilrite. The plants were moved to clay pots containing different soil types with various ratios to check their effect on survival and yield potential of *in vitro* regenerated plantlets of *C. borivilianum*. Highest survival rate 94% was observed in Loam: sand: Fym (1:2:1) with 16 g average weight and 12 g fresh weight of plants survived. Sand: vermicompost (1:1) also showed good survival percentage 92% with 12 g average weight of tubers per plant and 9.4 g fresh weight. Sand: Fym: perlite (2:1:1) showed 93% survival rate with 11 g average and 8 g fresh weight of tubers per plant. Lowest survival rate 86% was observed in the soil type comprising of Sand: Fym: perlite (1:1:1) with 13 g average weight and 8.7 g fresh weight of tubers. In this study, Loam: sand: Fym (1:2:1) soil type was found best for hardening of *in vitro* regenerated plants of *C. borivilianum*. The pots were then moved to a greenhouse, where they flourished with a regular development cycle with no morphological variance (Fig. 1 F).

3.6. Phytochemical analysis

Evaluations of phytochemical homogeneity in mother plants and *in vitro* developed plantlets are crucial to the success of any recommended tissue culture process. The therapeutic qualities of plants are due to the secondary metabolites found in them. Diosgenin and stigmasterol was quantitatively analysed by HPTLC in the current work. Based on the peak area, the concentration of diosgenin and stigmasterol in methanol extracts of *in vivo* plants (mother plant) and *in vitro* regenerants of *C. borivilianum* was determined. The content of bioactive compounds revealed by HPTLC analysis is presented in Table 6 (Supplementary File 1). In mother plant 0.66% stigmasterol and 0.71% diosgenin was found. In *In vitro* plantlets the stigmasterol content was observed in the range of

Table 5
Effect of soil type on survival and yield potential on regenerated plantlets of *Chlorophytum borivilianum*.

Soil Type	Percent plants survived in green house (%)	Average weight of tubers per plant	Fresh weight of tubers per plant (g)
Loam: sand: Fym (2:1:1)	88	14.0 ± 0.22^b	10.0 ± 0.33^b
Loam: sand: Fym (1:1:1)	89	12.0 ± 0.25^d	9.0 ± 0.39^d
Loam: sand: Fym (1:2:1)	94	16.0 ± 0.19^a	12.0 ± 0.31^a
Sand: Fym: perlite (2:1:1)	93	11.0 ± 0.15^e	8.0 ± 0.42^g
Sand: Fym: perlite (1:1:1)	86	13.0 ± 0.17^c	8.7 ± 0.32^e
Sand: vermicompost (2:1)	89	$11.0 \pm 0.23^{e,f}$	8.4 ± 0.45^f
Sand: vermicompost (1:1)	92	12.0 ± 0.29^d	9.4 ± 0.47^c

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

Table 6

Stigmasterol and diosgenin content in *in vivo* and *in vitro* regenerated plants of *C. borivilianum*.

Plant sample	Stigmasterol (%)	Diosgenin (%)
<i>In vivo</i> plant (Mother plant)	0.66 ± 0.15 ^c	0.71 ± 0.01 ^d
<i>In vitro</i> plant (P1)	0.62 ± 0.09 ^d	0.74 ± 0.05 ^b
<i>In vitro</i> plant (P2)	0.70 ± 0.11 ^b	0.72 ± 0.09 ^c
<i>In vitro</i> plant (P3)	0.71 ± 0.07 ^a	0.77 ± 0.03 ^a

Means within each column followed by the same letter are not significantly different at $P < 0.05$ using DMRT

0.62% - 0.71% and diosgenin (0.72% - 0.77%). Presence of both compounds in mother and *in vitro* plants confirmed phytochemical homogeneity though variations were found in the content of these two compounds. Similar investigations regarding the assessment of phytochemical fidelity in diverse medicinal plants using HPTLC have been documented (Nazir et al., 2021a; Sasidharan et al., 2017; Behera et al., 2018). The physiological and environmental factors influence the accumulation of secondary metabolites in plants (Verpoorte et al., 2002). For the production of novel substances with pharmacological value, plant tissue culture offers a biotechnological option for the retrieval of secondary metabolites from plants cultured *in vitro*. Therefore, it gives the opportunity to generate standardized material regardless of the environmental conditions (Matkowski, 2008).

3.7. Genetic fidelity of *in vitro* plantlets

Although somaclonal variance is unlikely to appear in micro-propagated plants, it is possible owing to the conditions of cultivation and the effects of growth hormones applied in the course of the *in vitro* multiplication phase and should not be disregarded (Larkin and Scowcroft, 1981; Gupta and Roy, 2002). Hence, evaluation of genetic homogeneity is required to confirm the genetic consistency amidst the regenerated plants. SCOT and ISSR markers were employed to confirm the validity of the present clonal production procedure due to their affordability, non-radioactive, have better reproducibility, and require no previous sequence knowledge for DNA amplification (Lakshmanan et al., 2007; Behera et al., 2018). On average, 6 of the 10 primers that were tested amplified genomic DNA with the most consistent bands in ISSR 6 (Table 7). The primer generated 14 unique, monomorphic bands with sizes varying from 400 to 2000 bp (Fig. 3). The quantity of bands used for each ISSR and SCOT primer varied from 2 to 6. The *in vitro* growth conditions did not cause any epigenetic variance in the developed regenerated plantlets, confirming their genetic homogeneity. Similar findings have also been documented in tissue cultured regenerants of different species, including *Eleusine coracana*, *Cornus alba*, *Dendrocalamus strictus*, *Dioscorea deltoidea* (Goyal et al., 2015; Ilicuk and Jacygard, 2016; Babu et al., 2018; Nazir et al., 2021a; 2022).

4. Conclusion

The enhancement of *in vitro* culture techniques enabled the establishment of a feasible, simple, efficient, or acceptable procedure for the mass production of the *C. borivilianum* and secondary metabolite synthesis. In this study, mT and other cytokinins interactions and combinations led to improved shoot proliferation and auxins promote rooting. Our findings indicate that mT, both alone and in combination with BAP, effectively induced and multiplied shoots. Furthermore, among the plant growth hormones tested, IBA exhibited the most successful rooting. These results underscore the potential of mT and IBA for enhancing shoot and root formation, respectively, in the cultivation of *C. borivilianum*. The procedure will prove useful in sustainable utilization of the species while preserving its native habitat. The *in vitro* cultured plants coped well in the field conditions, and according to ISSR and SCOT markers, no somaclonal variants were found among

Table 7

Response of different ISSR and SCOT primers on assessment of genetic homogeneity among *in vitro* regenerates *C. borivilianum*.

(AG)				
S. No	Primer code	Primer sequence (5'-3')	Number of bands	Range of amplified bands (bp)
1	ISSR6	CAACAATGGCTACCACGC	6	600–2000
2	ISSR7	CAACAATGGCTACCACGG	3	600–1700
3	ISSR10	CCATGGCTACCACGCAG	5	400–1600
4	SCOT-1	(AG) ₈ C	2	400–500
5	SCOT-2	(AG) ₈ G	2	800–1100
6	SCOT-4	(TC) ₈ C	2	400–500

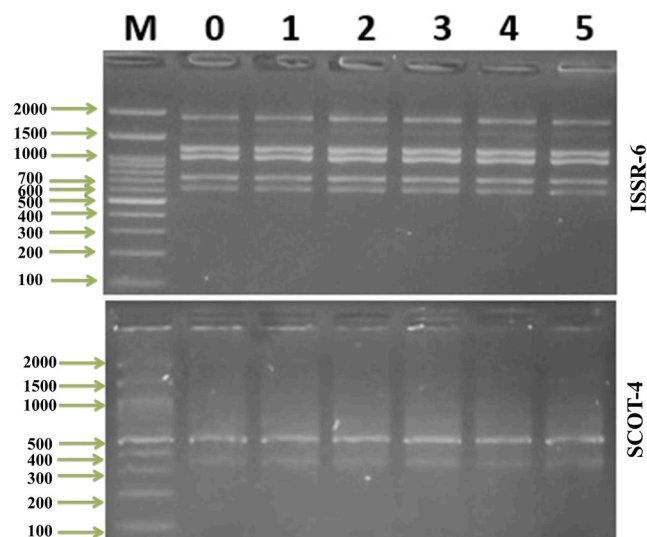


Fig. 3. Comparison of DNA fingerprinting pattern among *in vitro* raised plants with mother plant by ISSR and SCOT markers. M DNA Ladder (100 bp); 0 denotes mother plant; 1–5 of 5 randomly selected *in vitro* plants.

regenerants. Therefore, it would seem that plants grown *in vitro* are better able to harvest secondary metabolites because they displayed a better content of phytoconstituents. The results could be used to develop an efficient micropropagation method for large-scale plant production with a good quantity of bioactive compounds without introducing any variability.

CRediT authorship contribution statement

Rohtas Kumar Karwasra: Experiment, Writing – original draft. **Romaan Nazir:** Methodology, Formal analysis. **Devendra Kumar Pandey:** Validation, Supervision, Software, Project administration, Conceptualization.

Declaration of Competing Interest

All authors declare that they have no conflict of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indcrop.2024.118463](https://doi.org/10.1016/j.indcrop.2024.118463).

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